Etiology of sterile intra-amniotic inflammation: An exploratory study

Zoe M. Taylor  
*University of South Florida*

Follow this and additional works at: [https://digitalcommons.usf.edu/etd](https://digitalcommons.usf.edu/etd)  
Part of the [Genetics Commons](https://digitalcommons.usf.edu/etd)

**Scholar Commons Citation**  
https://digitalcommons.usf.edu/etd/9478

This Thesis is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact scholarcommons@usf.edu.
Etiology of sterile intra-amniotic inflammation: an exploratory study

by

Zoe M. Taylor

A dissertation submitted in partial fulfillment of the requirements for the degree of Master’s of Science in Public Health with a concentration in Genomics
College of Public Health
University of South Florida

Major Professor: Derek Wildman, PhD.
Monic Uddin, PhD.
Jamie Corvin, PhD.
Chengqi Wang, PhD.

Date of Approval:
May 26, 2022

Keywords: preterm birth, gene expression, epigenetic, hypothalamic-pituitary-adrenal axis

Copyright © 2022, Zoe M. Taylor
Acknowledgements

I would like to thank Derek Wildman, PhD., for his continuous support, affirmation, and guidance in the development of this project. Dr. Wildman has vast knowledge of both pregnancy evolution and epigenetic modulation from stress response, which he utilized in supporting and facilitating my academic growth. I hope to model his inquisitive nature as I progress in my career. I am grateful for the opportunities provided by both he and Monica Uddin, PhD., who allowed me to grow within their laboratory. My deepest thanks to Dr. Uddin who opened the door, allowing me into the lab to deepen my understanding of epigenetics and its relation to public health. She has been instrumental in shaping my time within the genomics core. I would also like to extend my appreciation and admiration to Jamie Corvin, PhD., who helped solidify my foundations of public health practice. Dr. Corvin’s teachings and background in maternal and child health have allowed me to root my efforts in forward thinking. I provided my sincerest thank you to Chengqi Wang, PhD., contributed enormously to my efforts in this project with his computational knowledge and support. Without Dr. Wang’s support and guidance this project would not be where it is today. Lastly, I would like to thank the other members of the Uddin-Wildman lab to include Agaz Wani, PhD., Zachary Graham, and Jan Dahrendorff for their support. I have great respect, appreciation, and admiration for each of you and thank you for our guidance and support on this journey.
# Table of Contents

List of Tables .................................................................................................................. ii

List of Figures .................................................................................................................. iii

Abstract ............................................................................................................................ iv

Introduction ....................................................................................................................... 1
  Understanding the drivers of PTB .................................................................................. 1
  Pathogen Born Intra-Amniotic Inflammation ............................................................... 4
  Sterile Intra-Amniotic Inflammation ........................................................................... 6
  Stress and Inflammation ............................................................................................... 7
  Objective ......................................................................................................................... 8

Materials and Methods .................................................................................................... 9
  Data Source .................................................................................................................... 9
  Demographic Collection .............................................................................................. 10
  Identification of Genes of Interest ................................................................................. 10
  Data Sourcing ................................................................................................................ 11
  Gene Table Creation ..................................................................................................... 11

Results ............................................................................................................................. 13

Discussion ....................................................................................................................... 19
  Cytokine Genes ............................................................................................................ 19
  Glucocorticoid Receptor Regulatory Genes ............................................................... 20
  Limitations .................................................................................................................... 22
  Practical Implications ................................................................................................. 23
  Future Directions ......................................................................................................... 24

References ....................................................................................................................... 25
List of Tables

Table 1: Demographic information of participants modified from Kashima et 2021 ...............13
Table 2: Contextualizing findings utilizing gene information from the University of California
Santa Cruz Genome Browser.................................................................................................17
List of Figures

Figure 1: PubMed literature search.................................................................3
Figure 2: Cytokine CpG sites with padj <0.1 for differential methylation...............14
Figure 3: GRRN CpG sites with padj <0.1 for differential methylation ...............15
Abstract

Preterm birth is the event of spontaneous birth prior to 37 weeks of gestation. In the United States, 1 in 10 babies are born prematurely. Low gestational age has been linked with increased morbidity and mortality. Preterm birth is multifactorial, where a myriad of contributors have been identified to include inflammation, chorioamnionitis, and inflammatory conditions to include hypertension and diabetes. To further explore the role inflammation plays in low gestational age, a literature review was done using PubMed. Two classes of inflammation quickly emerged: Pathogen born intra-amniotic inflammation and Sterile intra-amniotic inflammation. The latter form of inflammation has no identifiable source. Hypothalamic-pituitary-adrenal axis [HPA-axis] dysregulation was explored as a possible source to sterile intra-amniotic inflammation. Data was obtained from an epigenetic wide association study that analyzed 157 peripheral blood samples from infants using a 450k array to output β-values. Two gene lists were created, the glucocorticoid receptor regulatory network that details 82 genes that are pertinent to HPA-axis function and a “regulation of cytokine production involved in inflammatory response” gene list was obtained from Gene Ontology. Gene tables were created to identify CpG sites from the 450k array that are associated with the genes of interest. A Wilcoxon sum rank test was used to evaluate differential methylation between infants who did and did not experience chorioamnionitis with p-adjusted values < 0.1 were considered significant. Nine genes had CpG sites with differential methylation including FKB5, EP300, GATA3, GSK3B, IL6R, NFATC1, NR3C1, SAMARCA4, HIF1AN.
Introduction

Preterm birth [PTB] is the event of a live infant being born before reaching 37 weeks of gestation. Within this broad term, there are three levels to describe severity: extremely preterm (<28 weeks), very preterm (28 to 32 weeks), and moderate to late preterm (32 to 37 weeks) (Boyle et al., 2017; The World Health Organization [WHO], 2018). In 2020, the rate of preterm birth in the United States was 1 in 10 infants, which reflected the global rate reported in 2018 (The Center for Disease Control and Prevention [CDC], 2021b; WHO, 2018). With constrained gestational age, crucial developmental time in-utero is lost. On an individual level, Infants born prematurely are at increased risk for morbidity—including bronchopulmonary dysplasia [BPD], intracerebral hemorrhage, feeding difficulties, necrotizing enterocolitis [NEC], cerebral palsy, developmental delay, retinopathy of prematurity [ROP], and risk of chronic disease later in life—as well as mortality (Boyle et al., 2017; CDC, 2021a; Humberg et al., 2020). Inflated PTB rates impact both societal function and economic wellbeing. In a 2007 report the Institute of Medicine reported that PTB in the United States was associated with a $26.2 billion cost annually (March of Dimes, 2015). Low birth weight, a common result of PTB, was reported to cost taxpayers between $94,000 to $274,000 in medical bills that accumulate in the first year of life only (National Healthy Start Program, 2021). Interestingly, each half pound of weight gained was associated with a $16,000 savings per infant (National Healthy Start Program, 2021).

Understanding the Drivers of PTB

Preterm birth is a multifactorial condition that can occur spontaneously or through induction (WHO, 2018). The risk factors for delivering preterm are being heavily studied.
Common causes include multiple pregnancies, a personal history of PTB, low socioeconomic status, substance use, short time interval between pregnancies, infections, and chronic conditions including diabetes and hypertension (Boyle et al., 2017; CDC, 2021b; WHO, 2018). In many cases, there is no one identifiable cause for why preterm birth has occurred. Along with individual risk factors, there are observed disparities where some groups of birthing people [BP] are found to shoulder more burden than others. Non-Hispanic black BP have the highest reported rate of PTB (14.4%), while non-Hispanic white BP have the lowest rate (9.1%) (CDC, 2021b). This risk of PTB is also reflected in the racial disparities of Infant Mortality [IM]. In 2018, the rate of IM was 10.8 per 1,000 live births for non-Hispanic black BP, and 4.6 per 1,000 for non-Hispanic white BP (CDC, 2021b).

Inflammation and infection have been asserted to account for 30- 50% of all preterm labor (Lu & Claud, 2018; Migale et al., 2016). Inflammation, commonly mediated by pro and anti-inflammatory cytokines, is one of the drivers that allows labor to progress through initial uterine contractions to cervical ripening and ultimately membrane rupturing (Boyle et al., 2017; Hantsoo et al., 2019; Migale et al., 2016). However, during pregnancy the body must carefully balance immune response and tolerance to the fetus (Valeff et al., 2020). Activation of the stress response is an inherently inflammatory process, which leads to a rise in proinflammatory cytokines as is seen in labor (Boyle et al., 2017; Hantsoo et al., 2019; Vohr et al., 2017). To further investigate the role of inflammation in PTB, including inflammation from dysregulation of stress and immune response, a literature search was preformed utilizing PubMed. Search Terms included: Prenatal maternal stress AND Inflammation, Inflammation AND preterm birth. Records were first assessed for relevance by reviewing the title and abstract. Most papers initially excluded were centered in
infection and malaria. Further assessment was performed in reviewal of methods and results sections, resulting in an additional 73 to be excluded with two being duplicate papers.

Figure 1 PubMed literature search to identify foundational information on inflammation and preterm birth and its relation to HPA-axis engagement.

As previously described by the CDC, infection is one of the common identifiable contributors to PTB (CDC, 2021b; Yoshikawa et al., 2020). The fetus is semi-allogenic as it expresses both maternal and paternal genes which encode for proteins that may read as foreign to the BP (Valeff et al., 2020). This warrants a balanced immune response strong enough to ward off infection, while delicate enough to not trigger inflammatory cascade relating to labor or rejection (Valeff et al., 2020). The literature review uncovered many studies, both human and animal, that investigated key biological players to sterile and pathogen born intra-amniotic
inflammation. The most common inflammatory cytokines included IL-1β, IL-6, IL-23, TNF-α, IL-10, IL-8, and MCP-1 (Gomez-Lopez et al., 2019; Lu & Claud, 2018; Rodriguez-Benitez et al., 2020; Sullivan et al., 2021; Zein et al., 2021) In birthing people, high plasma levels of IL-6 were identified to be moderately predictive of intra-amniotic infection and preterm labor and has been tested for its efficacy in predicting PTB (Chaemsaiithong et al., 2016; Park et al., 2018; Ragsdale et al., 2019; Ross et al., 2019). Some studies identified general inflammatory patterns throughout labor characterizing the first and third trimester as broadly proinflammatory, with significant increases in IL-6, TNF-α, and IL-1β were observed from early to late pregnancies (Boyle et al., 2017; Gillespie et al., 2016; Hantsoo et al., 2019). Interestingly, one study found a spectrum of association where the relationship of IL-6 to IL-10 was correlated with birthweight: when IL-6 was greater than IL-10 low birth weight was observed—when IL-6 was less than IL-10 greater birth weight was observed (Ragsdale et al., 2019). This study indicated the impact of a dysregulated immune balance between pro and anti-inflammatory cytokines.

Pathogen Born Intra-Amniotic Inflammation

Chorioamnionitis—characterized by bacterial infection or inflammation of amniotic membranes and fluid—was found to lead to lower gestational age, spontaneous vaginal delivery, and premature rupture of membranes (Sullivan et al., 2021). Sullivan (2021) identified differences in plasma levels of BDNF, C3, C5a, C9, IL-8, MCP-1, and MMP-9 between preterm infants with and without chorioamnionitis five days post birth. This difference indicated sustained fetal immune response and possible dysregulation. Other genetic studies attempted to characterize the modulation of chorioamnionitis though DNA methylation. Konwar (2018) assessed methylation in chorionic villi and identified 66 sites of differential methylation with the majority located in immune related genes; however, with statistical testing only three CpG sites
were found to be significant. Another study sought to investigate the relationship of inflammation and PTB by targeting retinopathy of prematurity [ROP], specifically researching methylation of inflammatory genes. Increases in methylation in the body region of \textit{TNFRSF1A} was associated with an increased risk of ROP development, while increase methylation in the body region of \textit{MPO} was seen to be protective against ROP (Bulka et al., 2019). Interestingly, Bulka (2019) reported higher levels of circulating BDNF in the first month of life to be associated with an 80% reduction in ROP development. ROP is one of many diseases that is seen to be comorbid with low gestational age, research stating the protective role of inflammatory control lends further support to the role inflammation may play in morbidity and mortality in infants born prematurely.

In addition to evaluating cytokine profiles in relation to preterm birth, studies involving other immune related proteins were identified. In a 2020 study, the role of ASK1, a crucial protein in the innate immune response that triggers inflammation via activation of JNK and p38, was studied using ASK1 deficient pregnant mice (Yoshikawa et al., 2020). Their study design compared labor in wild type [WT] and ASK1 \textit{-/-} mice, where both received lipopolysaccharides [LPS] injection to stimulate infection. Mice that were ASK1 \textit{-/-} showed a significantly lower rate of PTB 48 hours post injection, as well as suppression of inflammatory cytokines IL-1\textbeta, IL-6, TNF-\textalpha compared to wild type mice (Yoshikawa et al., 2020). This study contributes to the understanding that infection born inflammation is tied to preterm birth, where the suppression of proinflammatory response was linked to lower rates of PTB. Another study investigated the role of Nrf2, a transcription factor that plays a regulatory role in oxidative and inflammatory stress. In this study, WT and Nrf2 deficient pregnant mice were injected with LPS to induce inflammation (Sussan et al., 2017). Using a dose response method, WT mice followed a standard dose
response curve where the greater amounts of LPS were associated with earlier delivery. However, Nrf2 -/- mice showed no dose response and had consistent accelerated labor due to infection indicating the impacts of their inability to regulate oxidative stress and inflammation. This study further supports that unregulated inflammation plays a key role in initiation of preterm labor and therefore preterm birth.

**Sterile Intra-Amniotic Inflammation**

Sterile intra-amniotic inflammation, characterized by inflammation with no detectable microorganisms or infection, is a relatively new concept. To better understand the condition, Gomez-Lopez (2019) utilized flow cytometry to characterize immune responses between PTB infants with sterile and non-sterile intra-amniotic inflammation. The results showed that sterile inflammation yielded similar progression of upregulated neutrophils, monocytes, macrophages, CD4+ T cells and corresponding inflammatory cytokines as infection born inflammation just to a lesser degree (Gomez-Lopez et al., 2019). Another study looked at four different placental types—normal, presence of vasculopathy, inflammation, and vasculopathy + Inflammation—of extremely PTB infants (23-28 weeks) and their relation to inflammatory cytokines. Samples were collected at three time points (time of birth, 1-3 days old, 21-28 days old), and found difference between placental groups. IFN-Y, IL-4, and MCP-1 were significantly different between groups at time points one and two; however, no significant difference was found at time point three for any cytokines (Zein et al., 2021). This study excluded any participants who experienced infection and is important in the contextualization of how sterile inflammation relates to PTB, as well as the longevity of fetal inflammatory response.

It is well reported that increased prenatal maternal stress is associated with higher circulating levels of proinflammatory cytokines (Gillespie et al., 2016; Hantsoo et al., 2019; Ross
Stress occurring prior to conception was not found to have any statistical association to pro-inflammatory gene expression in later pregnancy, elevated antiviral, or differential activity of NF-KB or AP-1 transcriptional factors (Ross et al., 2019). However, stress during pregnancy was related to higher expression of pro-inflammatory genes when comparing groups of high and low reported stress. The levels of antivirals were not associated with stress during pregnancy, as sterile inflammation does not involve microorganisms. This study assessed transcriptomic profiles from peripheral blood samples and identified 387 gene transcripts that had greater than a 1.5-fold difference in expression between the high and low stress report group which indicates the impact of increased proinflammatory efforts (Ross et al., 2019). This was amplified in another study that assessed both fetal chorionic villi and umbilical blood, and identified 344 transcripts showing a greater than or equal to 1.25-fold change following a range of reported maternal socioeconomic disadvantage (Miller, Borders, et al., 2017).

**Stress and Inflammation**

During the pregnancy, the stress response system communicates needed information on the external environmental to the fetus (Vohr et al., 2017). A dysregulated stress response may have compromised efficacy. A few publications investigated the relation of stress and inflammation to maternal and fetal health. Maternal childhood disadvantage was found to be associated with higher levels of IL-6, which was previously stated to be a predictor for preterm birth (Boyle et al., 2017; Miller, Culhane, et al., 2017). The same study found that BP who experienced stressful childhoods were more likely to deliver preterm and have longer hospital stays for both themselves and their offspring (Miller, Culhane, et al., 2017). However, one study did show IL-6 to be lower in black BP when comparing white BP who reported similar stress levels (Ekeke et al., 2020).
As mentioned earlier, the balance of pro- and anti-inflammatory cytokines is crucial to preserve the semi-allogenic fetus. A study assessed immune dysregulation and glucocorticoid resistance in socioeconomically disadvantaged women, finding a negative relationship between cortisol levels and pro and anti-inflammatory cytokine ratios in the low-risk study population (Corwin et al., 2013). However, this correlation was not observed in high-risk women who reported social and economic disadvantages, which was interpreted as suggestive of maternal stress response dysregulation with glucocorticoid resistance (Corwin et al., 2013). Slopen (2015) investigated the long-term impacts of prenatal and childhood social adversity on systemic inflammation—finding that high prenatal adversity was associated with a level of C-reactive protein [CRP] in offspring that classified as high risk for cardiovascular disease (Slopen et al., 2015). Other studies have shown results relating maternal immune dysregulation measured by inflammation to offspring health outcomes including neurological, lung, heart, kidney, and gut microbiome development (Arenas-Hernandez et al., 2019; Gilman et al., 2017; Humber et al., 2020; Miller, Borders, et al., 2017)

Objective

Sterile intra-amniotic inflammation is a relatively new area of study, where many etiologies are being explored. This study seeks to further explore the etiology of sterile intra-amniotic inflammation through a lens of HPA-axis engagement.
Materials and Methods

Data source

Data for this study was obtained from a Japanese study (GEO accession number CSE110829), where birthing-person infant pairs were recruited from the University of Tokyo Hospital or the Tokyo Metropolitan Bokutoh Hospital (Kashima et al., 2021). 144 BP provided written informed consent to be enrolled into the study at or around time of delivery from October 2014 to July of 2016. Biological samples were collected from the infants at two time points: the first from umbilical cord blood at time of delivery (cord blood mononuclear cells) and the second a peripheral sample via venipuncture via heel prick for the infant two weeks post birth (peripheral blood mononuclear cells). However, 51.4% of the participants did not provide the second sample. The main cited for the 74 participates not engaging in the secondary sample collection was hospital discharge occurring prior to two weeks post birth.

The original study sought to investigate epigenetic changes associated with PTB and small for gestational age using DNA methylation and gene expression microarrays. An effort for longitudinal data analysis was made; however, large amounts of attrition constrained these efforts. The study design began with extracting genomic DNA from blood samples and preforming bisulfite conversion prior to being processed with an Illumina Human Methylation 450 k array. This assessment output methylation values for over 485,000 CpG sites. The level of methylation is represented by a $\beta$-value, which ranges from zero (completely unmethylated) to one (completely methylated). $\beta$-values are calculated using the formula: (intensity of methylated allele) / ((intensity of unmethylated allele) + (Intensity of methylated allele) + 100). Quality
control testing was then preformed and low quality samples from bisulfite conversion and non-CpG probes were excluded.

**Demographic collection**

Demographics were collected through both medical records and questionnaires. Data including method of delivery, gestational age, birth weight, maternal age, pre-pregnancy body mass index (BMI), use of assisted reproductive technology, smoking status, pregnancy complications, and maternal age were sourced from hospital medical records. Pregnancy complications measures included gestational diabetes mellitus, preeclampsia, placenta previa, idiopathic premature rupture of membranes and chorioamnionitis. Data on paternal BMI, height, and age were obtained through a questionnaire.

**Identification of genes of interest**

To assess the relationship of hypothalamic-pituitary-adrenal axis [HPA-axis] engagement to sterile intra-amniotic inflammation, two subsets of candidate genes were identified. The glucocorticoid receptor regulatory network [GRRN] gene list comprising of 82 genes was obtained through the University of South Florida Uddin-Wildman lab, but was created by Kira Anthony and is listed on the NCI interaction database (Rouillard et al., 2016). The GRRN gene list acts as a representation for HPA-axis activity. The gene list for inflammatory cytokines was created utilizing gene ontology “GO” terms. Starting with the parent term “inflammatory response”, child terms were assessed for relevancy. The child term “regulation of cytokine production involved in inflammatory response” was selected as genes listed under the term were represented in the literature sources. Once the GO term was identified, the associated gene list was input into DAVID bioinformatic resource (Sherman et al., 2021). Utilizing the conversion
tool, the gene list was cleaned from 86 to 54 to exclude genes that are not well established and backed by literature findings.

**Data sourcing**

Data from this study was obtained through the National Center for Biotechnology Information [NCBI], specifically through the Gene Expression Omnibus [GEO] CSE110829. The database provides a file for each sample listing the $\beta$-values obtained post sequencing. All 157 sample files were manually downloaded and assessed to ensure the order of CpG sites were consistent with all samples prior to a merger. This was done by utilizing a random number generator to select 10% of samples. Because CpG sites are bountiful, a random number generator was used again to select 15 row numbers to compare CpGs among all samples. Once all samples were assessed to have the same ordering of CpG sites, they were manually merged into an excel file.

GEO was also utilized to obtain data on non-normal pregnancy conditions to include smoking, gestational diabetes, chorioamnionitis, idiopathic PROM, preeclampsia, previa, and utilization of assisted reproduction technology like invitro fertilization. Dichotomous tables were created for each individual non-normal pregnancy condition where 0= did not have condition and 1=had condition. Once this was specified for all conditions, a matrix was created to show clustering of conditions. Using R statistical software, a Wilcoxon Rank Sum test was performed on all non-normal pregnancy conditions individually to compare $\beta$-values groups with and without the condition.

**Gene Table Creation**

Utilizing data from the 450k platform array file, CpG sites that were related to genes of interest were manually obtained as well as their region of location on the gene. Two separate
Utilizing data from the 450k platform array file, CpG sites that were related to genes of interest were manually obtained as well as their region of location on the gene. Two separate gene tables were made for each grouping of candidate genes, totaling 55 cytokine candidate genes with 994 CpG sites of interest and 82 GRRN genes with 1935 CpG sites of interest. Regions included the gene Body, 3’UTR, TSS1500, 5’UTR, 1st Exon, TS200. Obtaining information on where the CpG site exists within the gene is crucial to teasing out expression of epigenetic modulations. Once gene tables were complete, R 2021.09.2+382 was utilized to identify CpG sites of interest that yielded a p-adjusted value < 0.1 from the Wilcox testing among samples that did and did not experience chorioamnionitis.
## Results

Table 1. Demographic information of sample participants modified from (Kashima et al., 2021).

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Mean</th>
<th>Median</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>53</td>
<td>47</td>
<td>(48.2%)</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td>(51.8%)</td>
</tr>
<tr>
<td>BP Age</td>
<td>33.8</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>1</td>
<td>0.9%</td>
<td></td>
</tr>
<tr>
<td>25-30 years</td>
<td>20</td>
<td>18.2%</td>
<td></td>
</tr>
<tr>
<td>30-35 years</td>
<td>41</td>
<td>37.3%</td>
<td></td>
</tr>
<tr>
<td>35-40 years</td>
<td>34</td>
<td>30.9%</td>
<td></td>
</tr>
<tr>
<td>&gt;40 years</td>
<td>14</td>
<td>12.7%</td>
<td></td>
</tr>
<tr>
<td>BP BMI (pre-pregnancy)</td>
<td>21.1</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>&lt;18.5 kg/m²</td>
<td>23</td>
<td>20.9%</td>
<td></td>
</tr>
<tr>
<td>18.5-25 kg/m²</td>
<td>77</td>
<td>70.0%</td>
<td></td>
</tr>
<tr>
<td>25-30 kg/m²</td>
<td>4</td>
<td>3.6%</td>
<td></td>
</tr>
<tr>
<td>&gt;30 kg/m²</td>
<td>6</td>
<td>5.5%</td>
<td></td>
</tr>
<tr>
<td>Parity &gt;0</td>
<td>43</td>
<td>39.1%</td>
<td></td>
</tr>
<tr>
<td>Smoked before pregnancy</td>
<td>2</td>
<td>1.8%</td>
<td></td>
</tr>
<tr>
<td>Smoked during pregnancy</td>
<td>7</td>
<td>6.4%</td>
<td></td>
</tr>
<tr>
<td>Utilized assistive reproductive technology</td>
<td>25</td>
<td>22.7%</td>
<td></td>
</tr>
<tr>
<td>Gestational Diabetes (yes)</td>
<td>4</td>
<td>3.6%</td>
<td></td>
</tr>
<tr>
<td>Preeclampsia (yes)</td>
<td>20</td>
<td>18.2%</td>
<td></td>
</tr>
<tr>
<td>Placenta previa (yes)</td>
<td>10</td>
<td>9.1%</td>
<td></td>
</tr>
<tr>
<td>Chorioamnionitis (yes)</td>
<td>25</td>
<td>22.7%</td>
<td></td>
</tr>
<tr>
<td>Idiopathic PROM</td>
<td>11</td>
<td>10.0%</td>
<td></td>
</tr>
<tr>
<td>Cesarean section (yes)</td>
<td>89</td>
<td>80.9%</td>
<td></td>
</tr>
<tr>
<td>Gestational age at birth</td>
<td>34</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>&lt;28 weeks (Extremely preterm)</td>
<td>17</td>
<td>15.5%</td>
<td></td>
</tr>
</tbody>
</table>
The eight CpG sites where the difference in methylation between those who experienced chorioamnionitis and those who did not was below \( \text{p-adjusted} < 0.1 \) can be seen in Figure 2. The methylation values for CpG sites residing within the \( FKB5 \) gene are the only ones that are more methylated in those without chorioamnionitis. The remaining six methylation sites were increased in those who were born to individuals with diagnosed intra-amniotic infection. Figure 3 details the two genes from the cytokine list that showed differential methylation between groups. \( HIF1AN \) was seen to be more methylated in those who did not experience chorioamnionitis.
Figure 3. CpG sites aligned with GRRN gene lists that held padj < 0.1 when tested for methylation difference between groups with and without chorioamnionitis diagnosis.
Table 2 details the region of the gene that houses the CpG site as well as what is cited by the University of California Santa Cruz as the expected range of methylation. The specific region where the CpG resides is vital in contextualizing the impact of epigenetic modulation. Also, a baseline understanding of expected methylation values derived from University of California Santa Cruz Genome Browser will further ground results in existing knowledge of the general methylation status for a CpG site. The methylation data reported in this table is sourced from the International HapMap project, which aims to characterized genetic variation within the human genome. Peripheral blood collections were preformed, with specific cell type reported as B-lymphocytes, to obtain sample then 450k array testing was preformed to obtain the β-values presented from UCSC genome browser.
Table 2 (Continued).

Table 2. Contextualizing findings utilizing gene information from University of California Santa Cruz Genome Browser.

<table>
<thead>
<tr>
<th>Gene</th>
<th>CpG</th>
<th>Region</th>
<th>Coordinates</th>
<th>Methylation (β-values)</th>
<th>Tissue Type</th>
<th>Lineage (Male or Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKBP5</td>
<td>Cg00130530</td>
<td>3’UTR</td>
<td>Chr6:35657203-35657203</td>
<td>&lt;0.2</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>FKBP5</td>
<td>Cg20813374</td>
<td>TSS1500</td>
<td>Chr6:35657181-35657181</td>
<td>&lt;0.2</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>EP300</td>
<td>Cg00187244</td>
<td>Body</td>
<td>Chr22:41492007-41492007</td>
<td>&lt;0.2</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>GATA3</td>
<td>Cg03935183</td>
<td>Body</td>
<td>Chr10:8100563-8100563</td>
<td>&gt;0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>GSK3B</td>
<td>Cg13250001</td>
<td>Body</td>
<td>Chr3:119810598-119810598</td>
<td>&lt;0.2</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>IL6R</td>
<td>Cg21262032</td>
<td>Body</td>
<td>Chr1:154437694-154437694</td>
<td>&gt;0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>NFATC1</td>
<td>Cg06751398</td>
<td>Body</td>
<td>Chr18:77162457-77162457</td>
<td>&gt;0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Cg18998365</td>
<td>5’ UTR</td>
<td>Chr5:142781532-142781532</td>
<td>0.2 &lt; β &lt; 0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>SAMARCA4</td>
<td>Cg15001636</td>
<td>Body</td>
<td>Chr19:11144178-11144178</td>
<td>&gt;0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
<tr>
<td>HIF1AN</td>
<td>Cg18133905</td>
<td>Body</td>
<td>Chr10:102296115-102296115</td>
<td>0.2 &lt; β &lt; 0.6</td>
<td>Blood</td>
<td>Mesoderm (F)</td>
</tr>
</tbody>
</table>
Discussion

Inflammation is a main contributor to preterm birth, which is associated with morbidity, mortality, and economic strain (Boyle et al., 2016; CDC, 2021b; Lu, L., & Claud, C., 2018; Migale et al., 2016; WHO, 2018). Inflammation during pregnancy has been categorized in two main ways: intra-amniotic inflammation and sterile intra-amniotic inflammation, where the latter does not root in detectable infection or presence of microorganisms (Gomez-Lopez et al., 2019). To better understand the etiologies of sterile-intra amniotic inflammation, a candidate gene study was preformed using data from a Japan epigenomic wide association study to identify epigenetic differences in genes associated with inflammation and HPA-axis engagement in infants born to a BP with and without a diagnosis of intra-amniotic infection—chorioamnionitis (Kashima et al., 2021). This exploratory study identified 10 significantly different CpG regions (p-adjusted values <0.1).

Cytokine Genes

Of the 55 cytokine-related genes assessed, two CpG sites within two genes were identified to be significantly different between those born to BP with and without diagnosed chorioamnionitis. One of the CpG sites were found within the interleukin-6 receptor gene (IL6R). Within the literature search, many papers mentioned the role of the protein IL-6 in inflammatory action (Chaemsaithong et al., 2016; Park et al., 2018; Ragsdale et al., 2019; Ross et al., 2019). One paper specifically detailed that infants not exposed to chorioamnionitis had higher levels of IL-6 protein than those exposed (Sullivan, et al., 2021). Other papers linked BP self-report of a stressful childhood to be associated with higher levels of IL-6 highlighting its association with
preterm birth (Boyle et al., 2017; Miller, Culhane, et al., 2017). While contextualizing the results from our study, it is important to touch on the region of methylation, as this impacts function of the gene (Jjingo, et al., 2012). In the 450k array, cg21262032 was stated to be within the body of gene \textit{IL6R}. Methylation within the body region is generally associated with increased expression of the gene (Jjingo et al., 2012). With this, our results posit that infants who did experience chorioamnionitis have greater methylation in the body region, therefore greater expression of \textit{IL6R} than those who did not experience intra-amniotic infection.

The second cytokine related gene is \textit{HIF1AN} (Hypoxia Inducible Factor 1 Subunit Alpha Inhibitor), which has many functions but is most notably known for its negative regulation in the notch signaling pathway (UniProt, 2022c). The notch signaling pathway plays role in cell proliferation and differentiation (Kopan, 2012). Increased methylation in the promoter region is associated with gene silencing (Jjingo et al., 2012). With this knowledge, infants in our sample that did not experience chorioamnionitis displayed higher methylation rates in the promoter associated CpG site within \textit{HIF1AN}. The potential silencing of this gene may have impacts in the ability to regulate this notch pathway and have impacts on cell differentiation and proliferation which is crucial in the developmentally intensive period that is infancy.

\textbf{Glucocorticoid Receptor Regulatory Network Genes}

Genes identified in the glucocorticoid receptor response network are used in this study to represent HPA-axis engagement. From the list, CpG sites belonging to seven genes were found to be statistically significant. For both CpG sites associated with \textit{FKBP5}, methylation was found to be higher in those who did not exhibit chorioamnionitis. This gene is a key player in the function of the HPA-axis and has been suggested to be an indicator for glucocorticoid receptor sensitivity (Zannas et al., 2016). Increased methylation in these CpG sites located in the
promoter region indicate potential silencing of the gene, which would impact the negative feedback loop for glucocorticoid receptor signaling (Jjingo et al., 2012; Zannas et al., 2016). However, it is important to notate that expression of FKB5, and other genes, vary depending on the tissue (Zannas et al., 2016). Another CpG identified was within the promoter region of NR3C1, where there was more methylation in those who did experience chorioamnionitis. This gene encodes for a glucocorticoid receptor, which is vital to stress response functionality (National Library of Medicine, 2022). When comparing our results to University of California Santa Cruz Genome Browser, the methylation value for those who did not experience chorioamnionitis is still elevated when compared to the expected range reported for the CpG site (see Table 2).

The other five genes within the GRRN all showed elevated levels of methylation among those who experienced chorioamnionitis. This is understandable as GATA3 encodes for a transcriptional activator that interacts with immune related T-cells post immune activation, which would fit into the narrative of experiencing infection via chorioamnionitis (UniProt, 2022e). NFATC1 produces protein that plays role in expression of T-cells as well as cytokines, most notably IL-2 and IL-4 (UniProt, 2022d). SMARCA4 has many functions, one of which being the control—both activation and repression—of genes that play role in neural differentiation and proliferation through chromatin remodeling (UniProt, 2022f). Interestingly, EP300 had elevated methylation for both groups when compared to UCSC expectations (see Table 2). EP300 encodes for Histone acetyltransferase p300, which functions to regulate transcription in a multitude of processes, to include DNA damage response through p53/TP53 transcription and apoptosis, through remodeling of chromatin (UniProt, 2022b). GSK3B also showed elevated methylation in both groups than what was expected from UCSC. This gene
encodes for a protein kinase that exudes negative regulation of glucose homeostasis and impacts cellular response to interleukin-3 (UniProt, 2022a).

**Limitations**

Limitations of this study include smaller sample size, low understanding of the infant’s role in preterm birth and lack of indication of stress through self-reporting measures. The data utilized in this study is peripheral blood samples obtained at two time points from the infant, where a large number of participants did not provide the second sample. Due to the missing data for the second time point, all samples were grouped regardless of collection method and analyzed. There is also a gap in the literature, where exploration is still needed of the role the fetus plays in initiation of birth and preterm birth. The results of this study do indicate differences in HPA-axis related gene between groups; however, assessing these differences in infant groups is novel so there is little grounding for results. The addition of psychological indications in the form of self-reporting questionnaire of perceived stress could be used to further ground the results.

Another important limitation is that rates of preterm birth and infant mortality are markedly lower in Japan than other countries, to include the United States. According to the United Nations Children’s Fund [UNICEF], the rate of infant mortality in recent years is 2.5 per 1,000 live births in Japan, which is compared to 6.3 per 1,000 in the United States (UNICEF, 2020). A 2019 study reported preterm birth rate in Japan as 5.7% compared to the United States who showed the highest of all countries assessed reporting 9.6% (Isayama, T., 2019). Given the disproportionate rates, caution should be taken when contextualize the knowledge gained. Also, it is vital to consider the demographic and cultural differences.
While the US has disproportionately high rates compared to other countries, different groups within the US also experience disparities in these rates. For example, the Center for CDC released a 2018 report detailing disparity in infant mortality rates per race and ethnicity in the country (CDC, 2021a). In this report, Non-Hispanic Black BP had over twice the rate of infant mortality (10.8) when compared to Non-Hispanic White BP (4.6). A 2021 report states this disparity persists when comparing Non-Hispanic Black rates of Preterm Birth (14.4%) to Non-Hispanic white (9.1%) (CDC, 2021). There are many hypotheses that center the concept of weathering, a process of degradation to bodily systems due to sustained stress response, as a contributor to these disparities (Corwin et al., 2013).

**Practical Implications**

Study results support difference within the HPA-axis functionality between those who did and did not experience chorioamnionitis, even more so when comparing methylation levels to the International HapMap project. Specifically, CpG sites within genes FKB5, EP300, GSK3B, and NR3C1 were found to have higher β-values in PTB compared to full-length gestation. The impact of this increased methylation is dependent on which region houses the CpG Site. In one example, the highly methylated CpG sites within FKB5 in PTB were both within the promoter region, where increased methylation indicates gene silencing. This could be interpreted as an indication of HPA-axis dysfunction as FKB5 plays a crucial role in the glucocorticoid sensitivity to cortisol. These results were obtained using data from a Japanese study, where crude disparities in birth outcomes do not exist among various racial and ethnic groups. This study calls for further investigation to see if the results are amplified in locations where these disparities and high stress are reported among racial and ethnic groups.
Future directions

This study sought to be exploratory for the etiology of sterile-intra amniotic inflammation. Now with more information on the possible relevance of the stress response—mediated by the HPA-axis—to non-pathogen born inflammation during pregnancy, further steps should be taken to contextualize this information in its relation to preterm birth and possibly the disparities seen within the US. Future studies could be conducted utilizing US cohorts where both the birthing person and offspring provide genomic data and administer addition testing to further gauge the BP perceived stress. These studies should also include facets to investigate racial and ethnic disparities perceived in rates of preterm birth and mortality in the context of sterile intra-amniotic inflammation.
References


https://doi.org/10.1038/s41598-021-83016-3

https://doi.org/10.1101/cshperspect.a011213

https://doi.org/10.1007/s40139-018-0159-9


https://doi.org/10.1186/s12916-016-0632-4


https://doi.org/10.1016/j.bbi.2017.04.014


   http://www.nationalhealthystart.org/healthy_start_initiative

NR3C1 [Internet]. Bethesda (MD): National Library of Medicine (US). National Center for Biotechnology Information: 2004-[2022 May, 12]. Available from:


   https://doi.org/10.1002/ajhb.23245


   https://doi.org/10.1016/j.bbi.2018.11.009

Andrew D. Rouillard, Gregory W. Gundersen, Nicolas F. Fernandez, Zichen Wang, Caroline D. Monteiro, Michael G. McDermott, Avi Ma’ayan, The harmonizome: a collection of
processed datasets gathered to serve and mine knowledge about genes and
proteins, *Database*, Volume 2016, 2016,
baw100, https://doi.org/10.1093/database/baw100

annotation of gene lists (2021 update). *Nucleic acids research*, gkac194.Advance online
publication. https://doi.org/10.1093/nar/gkac194


Gilman, S. E. (2015). Early origins of inflammation: An examination of prenatal and
childhood social adversity in a prospective cohort study. *Psychoneuroendocrinology*, 51,
403-413. https://doi.org/10.1016/j.psyneuen.2014.10.016


Persists in Infants Exposed to Histologic Chorioamnionitis. *Front Immunol*, 12, 722489.
https://doi.org/10.3389/fimmu.2021.722489

Sussan, T. E., Sudini, K., Talbot, C. C., Jr., Wang, X., Wills-Karp, M., Burd, I., & Biswal, S.
(2017). Nrf2 regulates gene-environment interactions in an animal model of intrauterine
https://doi.org/10.1038/srep40194

https://data.unicef.org/country/jpn/

UniProt ConsortiumEuropean Bioinformatics InstituteProtein Information ResourceSIB Swiss


