INFLAMMATION DURING PREGNANCY AND ITS ASSOCIATION WITH PRETERM BIRTH IN MEXICO CITY

by

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DEDICATION

This dissertation is dedicated to the memory of my father, Joseph A. Buxton, who from the very early stages of my educational journey encouraged academic excellence and let me take charge of my education, and in so doing, gave me the confidence to aim high.

And

In honor of my mother, Louise Reeves Buxton, who continues to share with me her love of reading; she is the wisest woman I know and "the wind beneath my wing"*.

And

To the many families around the world who have been affected by adverse pregnancy outcomes, especially preterm birth.

*Bette Midler, "The Wing Beneath My Wing" 1988

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PREFACE

The risks, morbidity and in some cases, subsequent mortality associated with pregnancy (especially in certain parts of the world) are unacceptable for a basic life event that should come with great expectations and boundless excitement. I have therefore decided to focus my dissertation on this topic and join others before me on a journey that has the potential to impact countless number of lives due to the preventable nature of a number of these deaths. It is my hope that my research on this topic (as well as the tireless efforts of other researchers and health care workers in this field), will help us understand and find solutions to the major drivers of adverse pregnancy outcomes such as maternal mortality and preterm birth, and provide information needed to reduce adverse pregnancy outcomes for both mother and child.

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LIST OF ABBREVIATIONS

BMI	Body Mass Index
CI	Confidence Interval
CO	Carbon monoxide
ECDF	Empirical Cumulative Distribution Function
ICC	Intraclass correlation coefficient
IFNγ	Interferon gamma
IL-10	Interleukin – 10
IL-12P40	Interleukin-12p40
IL-12P70	Interleukin-12p70
IL-17	Interleukin -17
IL-1RA	Interleukin-1 receptor antagonist
IL-1α	Interleukin - 1 alpha
IL-1β	Interleukin - 1 beta
IL-2	Interleukin – 2
IL-4	Interleukin -4
IL-6	Interleukin-6
IL-8	Interleukin-8
IP-10	Interferon gamma inducible protein -10
LMP	Last menstrual period
LOD	Limit of Detection
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
NADA	Nondetects And Data Analysis
NLMixed	Non-linear mixed models
OR	Odds ratio
PM10	Particulate matter less than 10 μm in aerodynamic diameter
PPM	Parts per million
PPROM	Preterm premature rupture of membranes
PROM	Premature rupture of membranes
sIL-2Rα	Soluble IL-2 receptor alpha
SIMAT	Sistema de Monitoreo Atmosférico
TNFα	Tumor necrosis factor - alpha
UNAM	National Autonomous University of Mexico
VEGF	Vascular endothelial growth factor

WHO	World Health Organization

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ABSTRACT

Preterm birth (PTB) is a global public health problem with significant short and long term effects. Preterm babies are at increased risk of neonatal mortality and long term morbidity. While the etiology of PTB is poorly understood, inflammation occurs frequently in the pathway between several identified risk factors and PTB. Inflammation is also a normal part of pregnancy. Therefore, longitudinal measures are required to evaluate when and what markers of inflammation are normal and what predict PTB. The objectives of this dissertation were to evaluate the expression of inflammatory biomarkers (specifically cytokines) during pregnancy, and to explore whether patterns of cytokine secretion can identify women at risk of PTB.

Using longitudinal data from a pregnancy cohort in Mexico City, this dissertation 1) characterized 20 cervico-vaginal cytokines obtained during gestation and described cytokine patterns among 181 term births; 2) evaluated differences in concurrently measured systemic and reproductive tract cytokines, and evaluated the associations between air pollution exposure and serum and cervico-vaginal cytokines among 104 term births; and 3) compared cytokine expression between women who delivered term (n=78) and preterm (n=12); and evaluated associations between individual and patterns of cervico-vaginal cytokines at points in pregnancy and PTB.

Among term births, mean cervico-vaginal cytokine concentrations were stable during gestation within cytokines but varied across cytokines; inflammation severity varied. Cervico-

vaginal cytokines were uncorrelated with serum cytokines; air pollution was positively associated with serum but inversely associated with cervico-vaginal cytokines. Finally, cervico-vaginal cytokine concentrations differed between term and PTBs, and the first trimester was most predictive of PTB.

This dissertation increases understanding of the role of inflammation and PTB. The range of cytokines in normal pregnancy is wide, such that deviation from 'mean' concentrations is an insufficient marker of risk; the effects of air pollution on these levels may be specific to serum or the cervico-vaginal cavity. Finally, early differences in cytokine levels between term and preterm births may be useful for risk identification. Future studies should use other cytokine categories, particularly ratios and inflammatory milieu, to assess for deviations from homeostasis, especially during the first trimester, to evaluate for preterm risk.

CHAPTER 1

Dissertation Introduction

1.1 Introduction

In 2000, the General Assembly of the United Nations adopted the United Nations Millennium Declaration, in which the global community was called to work together to "uphold the principles of human dignity, equality and equity". To achieve its objectives, the Millennium Declaration was further defined to highlight eight specific goals along with associated targets (commonly known as the Millennium Development Goals).[1] Among the eight goals identified, two – "Improve maternal health" and "Reduce child mortality" – are especially affected by preterm birth, which is the focus of this dissertation.

1.2 Background and Public Health Significance

Preterm birth is an outcome of pregnancy in which delivery of a live infant or infants occurs before 259 days or 37 completed weeks of gestation.[2] Preterm birth is a public health problem of global significance with far reaching implications. It is a major cause of morbidity and mortality among infants, and preterm babies that survive often experience lifelong medical complications which can be taxing on families and health care systems.[3] In some rural or resource limited settings where health facilities are not only few and far away but also under-

equipped to handle complications, preterm birth may play a role in maternal morbidity and or mortality.[4] Furthermore, the burden of preterm birth extends to economic challenges encountered by families through the loss of income due to maternal deaths[5] and may even extend to infant/child morbidity and mortality due to the loss of a primary caregiver for surviving children, particularly in the first year of life.[6]

Even in settings where advanced and specialized medical services are available, e.g. the United States, preterm birth remains a major public health problem;[7] although outcomes differ substantially in such settings (with a further widening in the difference gap the earlier a child is born) compared to settings lacking advanced medical services.[3] For example, advances made in treating preterm babies – such as the administration of surfactant therapy to treat respiratory distress syndrome - have contributed to differences in survival between preterm babies born in low income countries compared to high income countries.[8, 9]

Etiologically, preterm birth may result from spontaneous onset of labor or premature rupture of membranes (PROM), or be medically indicated, which is when a baby is delivered preterm (regardless of delivery type) due to medical complications affecting mother and or child.[10] Spontaneous onset of labor and PROM preterm births are usually grouped together and referred to as spontaneous preterm birth.[11] Blencowe and colleagues reported that global estimates of the percent of spontaneous and indicated preterm births are not made due to unavailability of data;[12] but reports from previous studies conducted in either black or white populations in the United States indicate that spontaneous preterm births may account for approximately 75% of preterm births occurring among singleton pregnancies.[13]

According to the World Health Organization (WHO), preterm birth may be classified into three categories based on gestational age, namely: extremely preterm (delivery before 28 weeks of gestation), very preterm (28 and before 32 weeks) and moderate to late preterm (32 and before 37 weeks).[14] Extremely preterm births account for the smallest number of cases, estimated at 5.2 percent, while the majority of preterm birth cases occur in the moderate to late preterm birth category, estimated at 84.3 percent. [12] However, the most severe outcomes of preterm birth, regardless of setting, occur more frequently among extremely preterm babies.[11]

Globally, preterm birth rates range from 5-18 percent;[3] the highest regional preterm rates in 2010 were in Southeastern Asia, South Asia, and sub-Saharan Africa.[12] Preterm birth rates have increased for a number of regions in the preceding decades and are projected to continue to increase. [3] Factors such as improvement in ascertainment of gestational age, and increase in rates of risk factors among childbearing women such as advanced maternal age, chronic medical conditions, use of fertility treatments, and increased use of caesarean section have been implicated in the increase in preterm birth rates.[14]

1.3 Risk Factors

A number of risk factors have been associated with preterm birth but the specific causes of preterm birth are not completely understood. Maternal factors including age, infection (especially reproductive tract infection), smoking, high and low body mass index (BMI), race, chronic conditions and obstetric characteristics such as previous preterm birth and short cervical length are established risk factors for preterm birth.(Reviewed in [7]) Socio-economic

factors, (reviewed in [15]) and air pollution [16-18] have been associated with preterm birth as well. Inflammation has been implicated in the mechanistic pathway between a number of these risk factors and preterm birth. [11]

1.4 Inflammation and Preterm Birth

Inflammation plays important roles in normal physiologic processes, but it is also associated with pathology. The inflammatory pathway is reported to be influenced by reproductive tract and systemic infections, [19] as well as other factors such as the direct [20]) and indirect effects of environmental pollution.[21] Pregnancy, independent of lower reproductive tract infection, is also associated with inflammation.[22, 23] Pathologic expression of inflammatory markers released in response to exogenous stimulants (particularly reproductive tract infection) has the potential to initiate preterm labor.[24] Released inflammatory markers (including cytokines) can activate the fetal and maternal membranes to produce prostaglandin and other substances.[25] Prostaglandin subsequently induces labor through its roles in cervical ripening, initiating uterine contractions, and the rupture of membranes.[26]

Although reproductive tract infections play a major role in preterm birth etiology, and treatments have been shown to be effective in clearing lower genital tract infection during pregnancy, [27] interventions involving medically indicated and even prophylactic treatments for the prevention of preterm birth have had mixed results. Results have ranged from beneficial, [28, 29] to no effect, [30, 31] and in some cases treatment even appeared to be associated with adverse outcomes, [32, 33]. Reviewing the literature as a whole, antibiotic administration for the treatment of genital tract infection has not had an overall impact in

reducing preterm birth rates.[34, 35] The lack of success in preventing preterm birth has been attributed to differences in the mechanisms of action of antibiotics administered, timing, [36] and route of administration of antibiotics[37]. However, in some instances, eradication of bacteria may be insufficient to reverse the damage caused by the initial infection.[38] What is not clear is the mechanism by which the inflammatory effects of infection are perpetuated following antibiotic treatment. Perpetuation of effects may be due to an inflammatory cascade set in place at the time of infection; currently, it remains unclear whether this cascade is modifiable after initiation.

The involvement of inflammation in both physiologic and pathologic processes has made it difficult to isolate the particular characteristic(s) of inflammation that define(s) the transition from physiologic to pathologic. This highlights a need to further understand inflammation during pregnancy, and its role in pregnancy duration.

1.5 Cytokines During Pregnancy

The physiological processes associated with pregnancy may lead to changes in the expression of pro and anti-inflammatory cytokines at different points during gestation. [22, 39, 40] Inflammatory biomarkers such as cytokines are increasingly evaluated, albeit cross sectionally, as indicators of the state of inflammation; they may be used as potential candidates for predicting preterm birth in part because of their ability to reflect immunologic response to both infectious and non-infectious activities.[22] Amniotic fluid presents an opportunity to directly assess the inflammatory state to which the fetus is exposed. However, the invasive nature of obtaining amniotic fluid samples (amniocentesis) makes this option not

feasible for routine research purposes. Many research studies have therefore focused on the use of peripheral blood or cervico-vaginal fluid as proxies of inflammatory processes that occur during pregnancy.[41, 42] However, few of these studies evaluate cytokine concentrations over the course of gestation.[43] Cytokine quantification can be expensive [44] and this cost is assumed to play a major role in the number of cytokines evaluated in any given study.

1.6 Research Setting and Data

Mexico City is a densely populated[45] mega-city where distinctive geographical features and manmade combustion sources contribute to high air pollution levels.[46] Because air pollution reportedly influences inflammation levels, [47, 48] Mexico City provides an appropriate setting to examine if and how air pollution influences systemic and lower reproductive tract inflammation, and the association between cervical inflammation and preterm birth.

This research uses data from a longitudinal study conducted among pregnant women in Mexico City, the *Air Pollution and Birth Outcomes Study*.[49] The study has approximately monthly data on 20 inflammatory markers obtained from cervico-vaginal exudates and peripheral blood, repeated measures of clinical, microbiological, and behavioral data, and data on birth outcomes The combination of 20 cytokines and monthly repeated measurements is rare in prenatal research on inflammation. Therefore this study provides a unique opportunity to describe cytokine concentrations over the course of pregnancy and to evaluate how longitudinal data on several cytokines may be used to predict preterm birth.

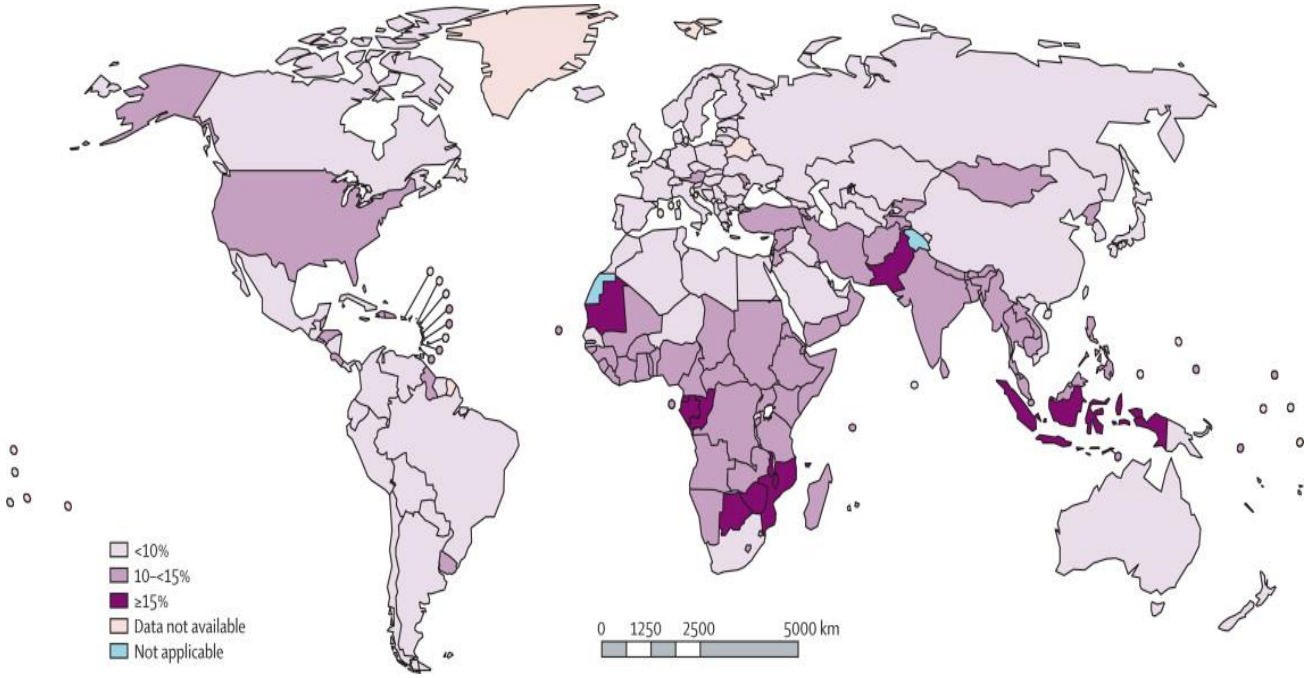
1.7 Summary and Specific Aims

Although survival prognosis for preterm infants has improved [26] - more so in some areas compared to others[3] - efforts aimed at reducing the incidence of preterm birth have not had a major impact on preterm rates. The goal of this dissertation is to provide additional information that will improve our understanding of the role that inflammation plays during pregnancy, and how this information may be used to help identify those at risk for preterm labor/delivery. Due to the richness of the cytokine data, the findings will inform how reproductive tract cytokine levels can be used to predict preterm birth in a population exposed to higher than normal levels of air pollution, and provide information about how longitudinal information can be used to inform cross sectional studies.

The specific aims of this dissertation are:

1. To characterize cervico-vaginal cytokines obtained longitudinally during the course of gestation and describe cytokine patterns among women who delivered term babies.
2. A) To evaluate differences in concurrently measured systemic and reproductive tract cytokines among women who delivered at term. B) To evaluate the associations between air pollution on serum, and cervico-vaginal cytokines among term births.
3. To compare cytokine expression between women who subsequently delivered term and preterm babies. Also, to simultaneously evaluate the association between cervico-vaginal cytokines measured at different points in pregnancy and preterm birth.

Figure 1.1. Estimated preterm birth rates by country for the year 2010. [12]



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Chapter 2

Inflammation During Pregnancy: Characterization of Cervico-Vaginal Cytokines in Term Births Using Longitudinal Data

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2.1 Abstract

Inadequately regulated inflammation during pregnancy is associated with preterm birth, but what is a pathologic departure remains to be defined. We used repeated measures of 20 cervico-vaginal cytokines among 181 term pregnancies in Mexico City (2009-2014) to describe the range of cytokine concentrations obtained during the course of term pregnancy.

Concentration distributions differed more between than within cytokines. In every trimester, cytokines IL-1RA, IL-1α, and IL-8 consistently had high concentrations. Cytokine intra-class correlation coefficients ranged from 0.48 – 0.83, with IL-1RA, IL-1α, and IL-8 levels varying by point in pregnancy. Spearman correlation coefficients among cytokine pairs varied but correlation directions were stable; 95.3% of the 190 correlation pairs remained either negative or positive at three points in pregnancy. Mean longitudinal patterns of log-transformed cytokines from Tobit regression varied across cytokines but less within cytokines.

Overall, mean concentrations of cervico-vaginal cytokines among term pregnancies were largely stable over time. The high cytokine concentrations among term pregnancies corroborate that pregnancy leads to an inflammatory state, and suggest that high versus low

cytokine categories, versus metrics of inflammation that incorporate ratios and functional properties, may be insufficiently specific for predicting preterm birth.

2.2 Introduction

Preterm birth is a major cause of neonatal morbidity and mortality and can have lifelong sequelae.[1] Infection is a known risk factor,[2] and inadequately regulated inflammation during pregnancy has been associated with preterm birth,[3, 4] but what constitutes a pathologic departure remains to be defined. Particularly lacking is a characterization of the range of normal variation in inflammation biomarkers during a 'healthy' pregnancy. Inflammation is an important and normal part of pregnancy. [5] For example, implantation and parturition are associated with inflammatory states similar to those found in pathological conditions[6, 7] while other points in normal pregnancy are marked by controlled systemic inflammation [8] not influenced by infection. [9] However, inadequately regulated inflammation can lead to pathological states, including adverse pregnancy outcomes.[4, 10-13] Cytokines are proteins that regulate host response inflammatory response to infection and injury, both locally and systemically.[14] Cytokines may be measured from multiple sources, including cervico-vaginal exudates, and these sources may reflect systemic or local processes. Cytokine actions are usually paracrine or autocrine but some cytokines (including interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNF-α)) exhibit endocrine or systemic effects.[11, 15]

Cytokines have multiple functions (including the regulation of inflammation) and redundant properties. Cytokines interact to produce effects, making the study of cytokines particularly challenging.[11, 16, 17] Understanding the effects of these complex processes on pregnancy outcomes - such as modulations in immune activity during pregnancy to allow for acceptance

and subsequent development of the “non-self” fetus while simultaneously working to stave off infection - requires measuring multiple cytokines.[7, 18]

Moreover, cytokine levels change with time corresponding to specific processes occurring during pregnancy. [6, 11, 19] Therefore, cytokines measured only once during gestation are unlikely to reflect variability in cytokine levels over time. [20] Most previous studies are cross-sectional.[21]

To fill these gaps, our objective was to characterize trajectories and concentrations of 20 cervico-vaginal cytokines during pregnancy among women delivering term babies. Cytokines were selected because they represent three important components of the inflammatory process, namely: pro-inflammatory, anti-inflammatory and chemotaxis; in addition the selected cytokines reflect those with previously reported associations with preterm births.

2.3 Methods

Study population

This study uses data collected as part of an ongoing cohort of 907 pregnant women in Mexico City. [22] The aim of the original study was to evaluate the association between air pollution and birth outcomes. The study was approved by the Institutional Review Board from the University of Michigan and ethics committees from Secretaría de Salud del Gobierno de la Ciudad de México (Mexico), and the School of Medicine of the National Autonomous University of Mexico (UNAM).

Pregnant women 18 years or older who lived and/or worked in metropolitan Mexico City (Mexico City and surrounding areas) who presented before 18 weeks of gestation (this

requirement was required for a specific study objective relating to confirmation of gestational age by ultrasound) and provided written informed consent were enrolled in the study between 2009 and 2014. All participants agreed to attend monthly prenatal visits and all had singleton pregnancies. Clinical samples (blood, urine, cervico-vaginal exudates) and demographic information were collected during monthly visits.

Gestational age was calculated using baby's birth date and the first day of last menstrual period (LMP) based on reliable and accurate recall from the earliest available visit record or screening. This analysis was limited to the 181 participants who had term births on whom data from at least one visit was available at the time of analyses.

Collection and processing of biological samples

Cervico-vaginal samples collected at each visit were used for quantification of cytokines. The samples were collected using a Dacron swab which was rotated for 10 seconds in the cervico-vaginal section of the lower reproductive tract; samples were subsequently frozen at -20^{o} C. Infection status was determined by clinical exam and microbiological data.

Cytokine quantification

A total of 20 cervico-vaginal cytokines (Eotaxin, Interferon gamma (IFNγ), IL-10, IL-12p40, IL-12p70, IL-17, IL-1rα, soluble IL-2 receptor alpha (sIL-2rα), IL-1α, IL-1β, IL-2, IL-4,Il-6, IL-8, Interferon gamma inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), TNFα, and vascular endothelial growth factor (VEGF)) were quantified in monthly

cervico-vaginal samples using the Millipore MILLIPLEX® MAP human cytokine/chemokine magnetic bead panel kit (Millipore Corporation, Billerica, MA, USA) . The Millipore MILLIPLEX® MAP human cytokine/chemokine kit works by simultaneously quantifying several cytokines from a single sample. Per the manufacturer's manual, the Milliplex MAP system uses the Luminex® xMAP® technology to color code microspheres with two fluorescent dyes to create distinctly colored bead sets, which are then coated with specific capture antibody.[23]

This system generates a standard curve against which concentrations of cytokines in biological samples can be quantified. Analyses were done in the laboratories of the National Autonomous University of Mexico or its affiliate, the National Institute of Genomic Medicine using previously frozen samples following the published protocol of the manufacturer.[23] Analyses were done in duplicate for every cervico-vaginal exudate sample, and standard controls run as a basis for comparison.

Thawed samples were briefly vortexed and centrifuged for 20-30 seconds and 25 µL of thawed cervico-vaginal exudate samples were added to each well. 25 µL of previously mixed beads was added to each well and 200 µL of wash buffer was added to all wells before the addition of 25µL of detection antibodies to each well. 25 µL streptavidin-phycoerythtin was subsequently added to the wells, washed twice and then run on MAGPIX® with XPONENT software for analysis.

Cytokine concentrations are reported in picograms per milliliter (pg/ml). Per the manufacturer's protocol, the minimum detectable concentration in pg/ml, intra-assay coefficient of variation (CV) %, and inter-assay CV % respectively for each cytokine are shown in Supplemental Table S2.1 in the appendix.

The percent of observations below the limit of detection (LOD) varied across cytokines and these observations were reported by the equipment as "<LOD value", which differed by cytokine. These values were transformed as LOD/√2 in order to have a numerical value for subsequent analyses.

In addition to values below the LOD, some samples had high concentrations of cytokines that exceeded the upper limit of detection of the equipment, and could only be quantified as greater than a given value, e.g. " > 10,000 pg/ml", or extrapolated beyond the standard curve, without further sample dilution. For statistical analyses, these very high values were transformed by adding 10 pg/ml. Data sets which have continuous values but on the lower end of the distribution take on constant values (e.g. LOD/√2) are referred to as 'left-censored'; similarly, 'right-censored' is a term used to describe constant values (e.g. 10,010 pg/ml) at the upper end of the distribution. Appropriate statistical methods must be chosen that account for such censored data; this censoring is a common characteristic of cytokine data. [24]

Statistical Analysis

Statistical analyses were performed using SAS Statistical Software version 9.3 (SAS Institute Inc. Cary, NC, USA) and R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics on demographic and obstetric/gynecologic characteristics were generated to describe the sample of study participants. Probability of infection for each trimester was determined by calculating the mean across all participants of (number of infections per trimester for each participant/number of visits per trimester for each participant). The distribution of each cytokine was examined using histograms and q-q plots.

For descriptive purposes and to gain insights into how cervico-vaginal cytokine concentrations varied across time, we calculated concentrations of cytokines at given percentiles of all observations for each cytokine by trimester. In addition to being sometimes left and right censored, some of the cytokine concentrations were not normally distributed. Therefore, we used empirical cumulative distribution functions (ECDF) to graphically represent the distributions for each cytokine by select months of gestation. The *cenfit* function from the NADA package in R was used to generate ECDF plots. Cenfit uses the Kaplan-Meier method on left censored data that have been automatically "flipped" by the NADA package to compute ECDFs and appropriately displays the part of the distribution where information is lacking due to values below the LOD.[25]

Spearman correlations were calculated for pairs of cytokines for each trimester, using the LOD/√2 substitution for values below the LOD, and the upper LOD plus 10 pg/mL substitution for values above the upper LOD. The correlations were graphically illustrated by the use of heat maps. [26, 27] A correlation heat map is a visual presentation of data from a correlation matrix wherein darker colors reflect stronger (closer to 1 or -1) positive or negative coefficients and lighter shades reflect coefficients of lesser magnitude.[28] To separate the influence of correlation within women due to repeated measurements, Spearman correlation coefficients were computed using cytokine concentrations from the gestation month with the highest number of observations for each trimester. To enhance interpretability of the heat maps, the ordering of cytokines on the axes was based on functional groups. P-values for correlations were adjusted to control the false discovery rate.[29]

We also calculated intraclass correlation coefficients (ICCs) for each cytokine. ICCs are calculated to quantify reproducibility (stability) of cytokine concentrations within participants across the duration of pregnancy. ICCs are commonly obtained by fitting a random intercept model to the repeated measures from participants, assuming normally distributed data; log-normality is often assumed for highly skewed variables and log transformations are taken prior to ICC calculations. These conventional analyses are not fully appropriate for our data for two reasons: the cytokine distributions were right- and left-censored as described previously, and a number of the cytokines were not normally distributed even after log transformation. To address lack of normality, we applied the Box-Cox transformation [30] technique to the cytokine with the lowest percent of observations below the LOD, and identified that an inverse fourth root transformation yielded distributions closer to the normal distribution; this transformation was applied to all cytokines. To address the censoring, we fitted models for repeated measures subject in SAS NLMixed; this procedure uses maximum likelihood estimation to fit mixed effects models and accounts for left censoring of log-normally distributed data.[31] The NLMixed log transformed procedure was modified to account for the inverse fourth root transformation. Because the distribution of the transformed data still did not appear completely normal, two ICCs were calculated for each cytokine using a combination of one statistical procedure and two data transformations to allow assessment of how robust the ICCs were to techniques for transforming data. The following techniques were used to calculate ICCs: NLMixed using log transformed,[31] and NLMixed using inverse fourth root transformed data. The resulting mean from the ICCs and standard deviation were reported for each cytokine.

Finally, we characterized cytokine patterns over time in women who delivered at term using Tobit regression (Survival package in R).[32] Tobit regression is a parametric regression technique which properly accounts for both left and right censoring [33] and may be used for data with repeated measures.[34] This kind of model is also called a censored regression model, and the purpose in this case is to estimate and plot the form of the relationship between time (gestational age in weeks in the x-axis) and mean cytokine concentrations (y-axis), with their 95% confidence intervals. The Tobit model takes the following form:

$$E(\ln(\text{cytokine}_{ij})) = \alpha + \beta_{gw}\, gw_{ij}$$

where α is the average cytokine level at the time of LMP (i.e., gestational week=0) and gw_{ij} is a set of variables representing a natural cubic spline parameterization of the gestational weeks at the time of the ith observation in the jth woman. A working independence assumption is used to fit the model, but robust standard errors of model coefficients are computed using the sandwich formula. We used the estimated parameters α and β_{gw} to construct plots that illustrate mean cytokine patterns during the course of pregnancy by calculating

$$\ln(cytokineij \mid t) = \{\widehat{a}\} + \{\widehat{\beta gw}\}t \text{ for a grid of values of t.}$$

Plots from Tobit regression models illustrate mean cytokine patterns during the course of gestation while the ECDFs as described above allow the generation of percentiles which can subsequently be plotted. ECDF plots graphically display the complete distribution of each cytokine concentrations for a given time point. Once the proper linear or non-linear form of the function between time and the cytokine concentrations for each cytokine is established in the

model, the Tobit model assumes that the model residuals are normally distributed. This assumption was assessed using residual plots.

2.4 Results

The median gestational age at enrollment for the 181 participants was 12.4 weeks (range 7 – 25.4 weeks of gestation) and the median number of visits over the course of pregnancy was 4 (range 1-8). Women were primarily in the 20-35 age group (70%) and most reported having had at least one previous child (54.7%). Most participants were either normal weight (33.2%) or overweight (32.6%) prior to pregnancy. The probability of clinician diagnosed reproductive tract infection was highest in the second trimester (0.37) and decreased in the third trimester (0.28) (Table 2.1).

The percent of observations below the LOD, and above the upper LOD for each cytokine varied across pregnancy with no consistent pattern or trend in the monthly percentages. (Data not shown) For the lower LOD, most cytokines had an overall percent below the LOD between 10%-30%. The majority of cytokines had less than 5% above the upper LOD, including five cytokines (IL-2, TNFα, IL-12p70, IL-17, and IFN-γ) with no observations above the upper LOD. The overall percent below the LOD and above the upper LOD as well as range in percent above or below for each cytokine, derived from gestational months, are reported in table 2.2. Cytokine concentrations were not normally distributed so log transformed values were used in analyses that required a normal distribution and indicated when this was the case; all other analyses used non-transformed cytokine values.

Percentiles of non-transformed cytokine values are presented in table 2.3. Concentrations differed across cytokines; three cytokines (one anti-inflammatory (IL-1RA) and two pro-inflammatory (IL-1α, and IL-8)) consistently had high concentrations for the median, 75^{th}, and 95^{th} percentiles for all trimesters, while IP-10, and VEGF consistently had high concentrations at the 95^{th} percentile across pregnancy but substantially lower concentrations at the 50^{th}, and 75^{th} percentiles. Similarly, plots for all 20 cytokines for select gestational months (3, 5 and 7) presented in supplemental figures S2.1-S2.5, showed differences in ECDFs across cytokines but ECDFs did not differ across time for most cytokines.

Using average intraclass correlation coefficients (ICCs) obtained from log transformed data and inverse fourth root data, many of the cytokines exhibited good reproducibility (ICC between 0.60-0.74) over the course of pregnancy (Table 2.4). Across all cytokines, ICCs ranged from 0.48 – 0.83 (fair to excellent reproducibility).[35] Although the ICCs estimated from the log transformed data differed numerically from the ICCs from inverse fourth root transformed data, in most instances, both estimates for each cytokine were within the same category except for 3 cytokines MIP-1β, MIP-1α and IL-1RA which moved across categories based on the data transformation type.

Spearman correlations among the 20 cytokines illustrated in heat maps (figures 2.1-2.3) showed that correlation coefficients across pairs of cytokines varied over time but the direction of correlation was stable over the course of pregnancy. 95.3% of the 190 correlation pairs remained either negative or positive at the three points evaluated in pregnancy. The strongest positive correlation at month 3 was 0.84 for pro-inflammatory pairs IL-12p40 and IL-2 (same for month 7) and INF-γ and VEGF, and the strongest positive correlation at month 5 was 0.81 for

pro-inflammatory pairs IL-1β and IL-1α. The strongest negative correlation at month 3 was -0.43 for anti -inflammatory cytokine IL-1RA and pro-inflammatory cytokine TNFα (month 7 correlation coefficient was -0.42) and the strongest negative correlation at month 5 was -0.52 for anti-inflammatory cytokine IL-1RA and pro-inflammatory cytokine MIP-1β. A total of 570 Spearman correlation coefficients (190 for each time point) for months 3, 5, and 7, p-values and number of observations for each pair of cytokines are reported in supplemental Table S2.2 in the appendix.

The mean longitudinal patterns of log transformed cytokines adjusted for lower reproductive tract infection status varied across cytokines, but little variability was observed within cytokines (Figures 2.3-2.8). Lower reproductive tract infection did not appear to substantially influence mean cytokine patterns as unadjusted patterns (figures not shown) did not differ from patterns adjusted for infection for most cytokines. Among the few cytokines that did change, subtle shifts in mean levels occurred for: IP-10* – slight decrease (*indicates borderline significance), and sIL-2Rα*, IL-10, TNFα, IL-17, and IL-1β – slight increase. (Data not shown) Mean levels were high throughout pregnancy for IL-1RA, IL-1α, IL-8 and these 3 cytokines were the most variable compared to the other cytokines. They exhibited a pattern of slight decrease towards the end of the first trimester of pregnancy followed by a slight increase late in the second trimester (around week 25) and a decrease by the end of pregnancy. INF-γ also exhibited some variability during the course of gestation but mean levels were lower compared to the other cytokines that were variable. IL-4 and IL-1β shared a similar pattern, slightly decreasing by mid pregnancy and increasing by the end of pregnancy, but overall IL-1β levels were higher than IL-4. The mean levels of most other cytokine including TNFα, IP-10, IL-

10, sIL-2Rα, and MCP-1 remained stable across pregnancy; however, a few stable cytokines including Eotaxin, IL-2, IL-12p40, IL-12p70, IL-17, and IL-6 showed a slight increase toward the end of pregnancy. MIP-1α and MIP-1β were also stable but showed slight (MIP-1α) and very slight (MIP-1β) decreases at the end of pregnancy.

2.5 Discussion

This research used longitudinal data to describe individual characteristics, as well as joint relationships between pairs, of 20 cervico-vaginal cytokines among women who delivered at term in order to better understand the normal baseline of the trajectory of inflammatory processes during pregnancy, and how key biomarkers of these processes relate to one another. One critical finding was that lower reproductive tract infection adjusted mean concentrations varied by cytokine, but within individual cytokines, little to no variability in mean levels over time was observed. Even among cytokines that exhibited some variability in mean levels, the changes were gradual over time. This suggests that cytokines measured at a single point in time during pregnancy may be representative of overall pregnancy levels.

This study was conducted in a population of middle income women residing in Mexico City, so use of similar methods in other populations would be useful to characterize the inflammatory 'baseline' among term pregnancies and determine if the patterns/findings from this population are generalizable. Even though cytokine concentrations in our study were within range of previously reported studies, [36-38] concentrations in this study were generally higher than the ranges frequently reported. Infection is a known stimulant of immune activity so the finding that mean cervico-vaginal patterns were not substantially affected by infection was

unexpected. However, previous studies have had inconsistent results with findings of increased, no difference, or decreased levels of cytokines in the context of bacterial vaginosis. (Reviewed in [39]) It may be because pregnancy encompasses periods of varying severity of inflammation [5], infection did not further influence inflammation levels in this population. This may also partly explain a lack of change in cytokine levels when adjusted for infection, as concentrations were already reflective of high levels that would otherwise (i.e. outside of pregnancy) indicate pathology.

In contrast to the largely stable patterns over time within individual cytokines, the association between cytokines varied across pairs and the strength of correlation changed over the course of pregnancy for some cytokines. However, the direction of the Spearman correlation coefficients remained stable for most cytokine pairs. Due to the variability in the Spearman correlations across cytokines, summary statements about correlation by functional groups (i.e. pro vs anti-inflammatory) cannot be easily made, but correlations that were significant and remained stable over time can potentially be used to inform associations between cytokine pairs across trimesters without having to obtain data for each time point.

Additionally, cytokines in this study exhibited fair to excellent reproducibility over time, regardless of the type of data transformation used. Of note is that 4 of the 6 cytokines with mean ICCs indicating fair reproducibility were chemokines IL-8, IP-10, MCP-1 and MIP-1α (the two exceptions were IL-6 and VEGF). A cutoff category of fair indicates that these cytokines exhibited some degree of variability within participants across the study sample and may be reflective of frequent expression of chemokines for the many different roles in which they are reportedly involved. Chemokines are multifunctional cytokines that are involved in activating

and directing the movement of cells of the immune system in response to infection and injury; they are also reported to be involved in cellular differentiation, placental development, and the balance between immune-efficiency and immune-tolerance in the presence of the developing fetus. [40-42] Since both up and down regulation of chemokines are needed for these processes to occur, this provides some insights regarding why reproducibility for each of these chemokines is lower compared to other cytokines. Interestingly, although not a chemokine, IL-6 essentially acts as a chemokine due to reported chemotactic activities [43] on T-cells [44] and monocytes [45]. This also may in part explain the lower ICCs for IL-6 due to frequent upregulation or least changes in expression due to its dual involvement in proinflammatory and chemotactic activities.

The role of pro-inflammatory and anti-inflammatory cytokines in pregnancy - especially their potential influence on the length of pregnancy - is an important topic which is being increasingly evaluated in studies. However, a dearth of longitudinal studies describing cervico-vaginal cytokine patterns over the course of pregnancy [21] highlights a need for comprehensive studies describing what may be regarded as "normal" immunologic responses to pregnancy. A search of the literature found very limited information on longitudinal cervico-vaginal cytokine patterns among persons who delivered at term. Donders et al. evaluated - among 92 women in Leuven, Belgium - whether six vaginal cytokines (IL-6, IL-8, IL-1β, IL-IRA, LIF (Leukemia inhibitory factor), and TNFα) varied during the course of uncomplicated pregnancy (n=30) and further compared trimester specific cytokine distributions to non-pregnant participants (n=62). Cytokine concentration ranges were different in this study compared to ours so comparison between the two studies will focus on patterns. Similar to our results,

Donders et al. reported a slight decrease in mean concentration of IL-1β between the first and second trimesters followed by a slight increase during the third trimester. IL-6 levels were low with an increase at the end of pregnancy in both studies, but Donders et al. showed a steeper increase. Although greatly different numerically in terms of concentrations, patterns were similar for IL-8 until the very end of pregnancy where we showed a decrease not seen in Donders at al. This may reflect true differences between the studies or might be influenced by the availability of data points up to 40 weeks in our study whereas Donders et al. collected data up to 38 weeks of gestation. Finally, mean levels of TNFα were low, and IL-1Ra showed variability over time in both studies, but IL-1RA patterns were different.

Heng et al. reported IL-1β and IL-1RA profiles among 153 women in Heidelberg, Victoria, Australia. In analysis of the 65 term pregnancies at 24-35 weeks of gestation, no linear trend in IL-1β levels as gestational age increased was seen, but a weak increasing trend was noted for IL-1RA. Although we did not specifically evaluate for trend, our findings are similar in that, overall both cytokines were reported to have a consistent expression during the second half of pregnancy (prior to labor). IL-1β and IL-1RA were significantly correlated in Heng et al. and our study. By contrast, in a study of 119 women, Tanaka et al. reported significant increases in IL-1β and IL-8 as gestational age increased among women in the second and third trimesters who were not in labor (n=65).[46] The reasons for these differences are unclear and warrant further investigation.

Information on correlations among cervico-vaginal cytokines in term births was limited in previously published work; the few studies identified primarily focused on correlations between cytokines, but in the context of infection and/or race; [47] or other pregnancy

characteristics such as age, [36] cervico-vaginal fetal fibronectin,[48] and body mass index stratified by lower reproductive tract infection status.[49] However, in rare instances, correlations between cytokines for select points in pregnancy were identified. [46] Therefore, this work fills a specific gap about the relationship between pairs of cytokines over the course of pregnancy.

From the results presented here, it appears that "normal" immunologic response to pregnancy encompasses a range of inflammation levels (not necessarily indicative of pathology) and that the more important aspect of inflammation in terms of adverse pregnancy outcomes may be deviations from a balanced state/homeostasis.

Although the ability to evaluate pairwise correlations at multiple points in gestation is a strength of our study, but the complexity and temporal cascades of the inflammatory process and the multiple overlapping functions that cytokines play are important considerations for future studies. Much of the literature addressing the possibility of using biomarkers as potential predictors of preterm birth relies on concentrations in biological samples taken at one or more discrete time points in gestation. Thus, evaluating these concentrations over time and within and between cytokines exploits available data to provide insights into patterns and relationships that signify non-pathological inflammatory processes and could be later complemented by additional analytic approaches, data permitting.

Our study was somewhat limited as the percent of cytokines below the limit of detection was high – although comparable to other studies evaluating inflammatory markers where the percent below the LOD for a number of cytokines was as high or higher. [50, 51] When evaluated using monthly data, no consistent trend over time was observed, and this

indicates that lack of observed cytokine values was not systematically linked to the stage of gestation.

Another limitation is that our results represent a characterization of cytokines from the third month to the ninth month of pregnancy, as only two participants were enrolled in the prior to month three. Because the first trimester is in essence represented by data from month three of gestation (data are "left-censored with a limit of detection at month 3"); if early first trimester (especially months 1 and 2) represents a period of substantial variability in cytokines, our conclusions would need to be re-evaluated. Finally, evaluation of the role of infection on mean cytokine concentrations over the course of gestation was limited due to the lack of information on the type of lower reproductive tract infection.

Among the strengths of this study is the use of statistical methods that appropriately account for data characteristics (i.e. observations below the limit of detection). Evaluating relations between pairs of cytokines with Spearman rank correlations properly accounts for left censored observations. Also, use of percentiles (50^{th}, 75^{th}, and 95^{th}) to describe cytokine concentrations over time accurately reflects observed values with given substantial numbers of observations above the LOD (overall data). Additionally, methods used to calculate ICCs (modified mixed effects models),[31] longitudinal patterns (Tobit regression),[33] and ECDF (Cenfit which uses Reverse Kaplan Meier)[25] all appropriately accounted for left censoring. However, Tobit regression was the only method that accounted for right censoring, and this represents a limitation of the study.

In conclusion, variability in mean concentrations of cervico-vaginal cytokines obtained over the course of pregnancy was minimal among women who subsequently delivered at term.

These findings further understanding of cytokine patterns during the course of pregnancy and suggest that, even after accounting for lower reproductive tract infection status, information on cytokine concentrations from any given point in pregnancy is informative about concentrations over the course of pregnancy. These results are potentially useful for informing future studies evaluating cytokine levels during pregnancy, implying that multiple repeated measures are not necessary in all cases. Additionally, the finding of very high cytokine levels in term pregnancies further corroborates that pregnancy- independent of pathology/infection - leads to an inflammatory state. This is especially useful information to put in context for studies evaluating the role of inflammatory markers on pregnancy duration- that categorizing cytokines as high versus low cytokine may not be sufficient markers for preterm birth risk. Thus, other evaluations of inflammatory markers that incorporate ratios, and functional properties should be considered.

Table 2.1. Demographic and Obstetric Characteristics of Mexican Women Delivering Term Births, 2009-2014 (N=181).	
Age	**N (%)**
<20	34 (18.8)
20-35	127 (70.2)
>35	20 (11.1)
Pre-pregnancy BMI	**N (%)**
<18.5 kg/m^2	9 (5.0)
18.5-24.9 kg/m^2	60 (33.2)
25-29.9 kg/m^2	59 (32. 6)
≥30 kg/m^2	31 (17.1)
Missing	22 (12.2)
Parity	**N (%)**
Nulliparous	51 (28.2)
Parous	99 (54.7)
Missing	31 (17.1)
Reproductive Tract Infection	**Probability of infection***
Trimester 1	0.31
Trimester 2	0.37
Trimester 3	0.28

* Mean of (number of infection per trimester/number of visits for each participant per trimester)

Table 2.2. Percent of cervico-vaginal cytokine concentrations below the lower limit of detection (LOD) and above the upper LOD† based on the manufacturer's manual, among 181* pregnant women in Mexico City, over entire pregnancy and range by month.

Cytokine	Lower LOD (pg/mL)	Percent < Lower LOD		Percent > Upper LOD	
		Overall	Range	Overall	Range
Anti-inflammatory					
IL-1RA	8.3	19.7	15.1 - 28.6	19.6	15.8 - 25.0
IL-4**	4.5	41.9	35.4 - 50.7	0.8	1.4 - 2.1
IL-10	1.1	15.9	11.9 - 18.6	1.4	1.7 - 2.5
sIL-2rα	11.2	33.6	26.3 - 44.1	0.9	0.8 - 1.7
IL-12p40	7.4	40.7	33.9 - 57.1	2.9	2.3 - 4.2
Pro-inflammatory					
IL-1α	9.4	13.0	9.7 - 42.9	9.8	5.8 - 11.5
IL-1β	0.8	13.0	5.9 - 16.4	5.9	4.2 - 7.6
IL-2	1	26.5	20.3 - 30.6	0	--
IL-6	0.9	11.6	6.7 - 14.8	3.2	2.3 - 4.2
IL-8**	0.4	0	--	25.3	19.2 - 34.0
TNF-α	0.7	17.1	13.3 - 19.7	0	--
IL-12p70**	0.6	21.5	12.5 - 32.3	0	--
IL-17	0.7	28.3	19.2 - 32.9	0	--
IP-10	8.6	3.8	0.8 - 5.7	6.9	5.7 - 8.2
IFN-γ**	0.8	29.2	10.2 - 51.6	0	--
MCP-1	1.9	2.1	0.8 - 3.0	3.2	1.2 - 5.1
MIP-1α	2.9	16.3	10.8 - 28.6	2.5	1.7 - 3.4
MIP-1β	3	4.4	1.7 - 6.4	1.3	0.6 - 2.5
Eotaxin	4	11.9	7.6 - 16.3	0.4	0.6 -0.8
VEGF	26.3	22.4	14.3 - 26.1	5.9	4.0 - 8.2

†All cytokine values greater than 10,000 (pg/mL), the upper limit of detection, were assigned a value of 10,010

*Sample size varied monthly and small Ns at the end of pregnancy influenced the wide ranges. Number of visits ranged from 1-8.

**More than 50% of observations missing

Table 2.3. Distribution of Cervico-vaginal Cytokine Concentrations (in pg/mL) in Term Births by Trimester (Number of samples = 902)

	First Trimester (N†=88)			Second Trimester (N=443)			Third Trimester (N=371)		
Cytokine	**P50**	**P75**	**P95**	**P50**	**P75**	**P95**	**P50**	**P75**	**P95**
Anti-inflammatory									
sIL-2Rα	14	44	1,840	22	51	921	20	54	1,436
IL-4*	10	57	1,941	8	54	1,699	10	122	1,746
IL-10	5	14	98	5	17	412	5	18	382
IL-12p40	9	24	111	10	25	634	11	29	988
IL-1RA	4,219	7,043	10,010	4,087	7,654	10,010	4,674	9,178	10,010
Pro-inflammatory									
IL-1α	1,162	2,799	10,010	784	2,310	10,010	690	2,486	10,010
IL-1β	82	1,011	4,285	47	871	9,750	44	538	10,010
IL-2	3	10	41	3	12	163	3	14	230
IL-6	11	31	344	11	28	534	10	22	1,905
IL-8*	2,440	10,010	10,010	1,442	7,189	10,010	1,408	10,010	10,010
TNF-α	3	10	321	4	11	312	4	12	498
IL-12p70*	1	5	249	1	6	278	1	8	233
IL-17	1	4	80	2	6	165	2	6	186
IP-10	154	612	10,010	248	648	10,010	254	619	10,010
IFN-γ*	1	11	408	3	13	358	3	15	575
MCP-1	102	402	4,563	135	428	7,363	143	568	8,371
MIP-1α	14	44	1,886	10	45	3,455	8	48	2,861
MIP-1β	25	67	1,106	26	67	1,754	28	74	1,945
Eotaxin	16	30	401	21	37	1,255	22	39	2,001
VEGF	145	318	10,010	133	251	10,010	128	254	10,010

†N= number of samples

* More than 50% of observations missing

Table 2.4. Intraclass Correlation Coefficients of Cervico-vaginal Cytokine Concentrations in Term Births (N=181), Mexican Cohort, 2009-2014.

Cytokine	Log	Inverse Fourth Root	Mean	Standard Deviation
Anti-inflammatory				
IL-1RA	0.82	0.67	0.74	0.11
IL-4	**0.76**	0.75	0.76	0.01
IL-10	0.69	**0.64**	0.67	0.04
sIL-2Rα	0.68	**0.71**	0.70	0.02
IL-12p40	0.76	**0.79**	0.78	0.02
Pro-inflammatory				
IL-1α	0.74	0.68	0.71	0.04
IL-1β	0.69	0.64	0.67	0.04
IL-2	0.63	**0.62**	0.62	0.01
IL-6	0.59	**0.50**	0.55	0.06
IL-8	0.56	0.41	0.48	0.10
TNFα	0.62	**0.62**	0.62	0.00
IL-12p70	0.86	0.79	0.83	0.05
IL-17	0.71	**0.65**	0.68	0.04
IP-10	**0.51**	0.50	0.51	0.01
IFN-γ	0.78	0.78	0.78	0.00
MCP-1	**0.51**	0.45	0.48	0.05
MIP-1α	0.61	0.56	0.58	0.04
MIP-1β	0.68	**0.58**	0.63	0.07
Eotaxin	0.83	**0.82**	0.83	0.01
VEGF	**0.58**	0.45	0.52	0.09

Blue font indicates the log transformed cytokine distribution closer to normal.Red font indicates the inverse 4th root transformed cytokine distribution closer to normal.

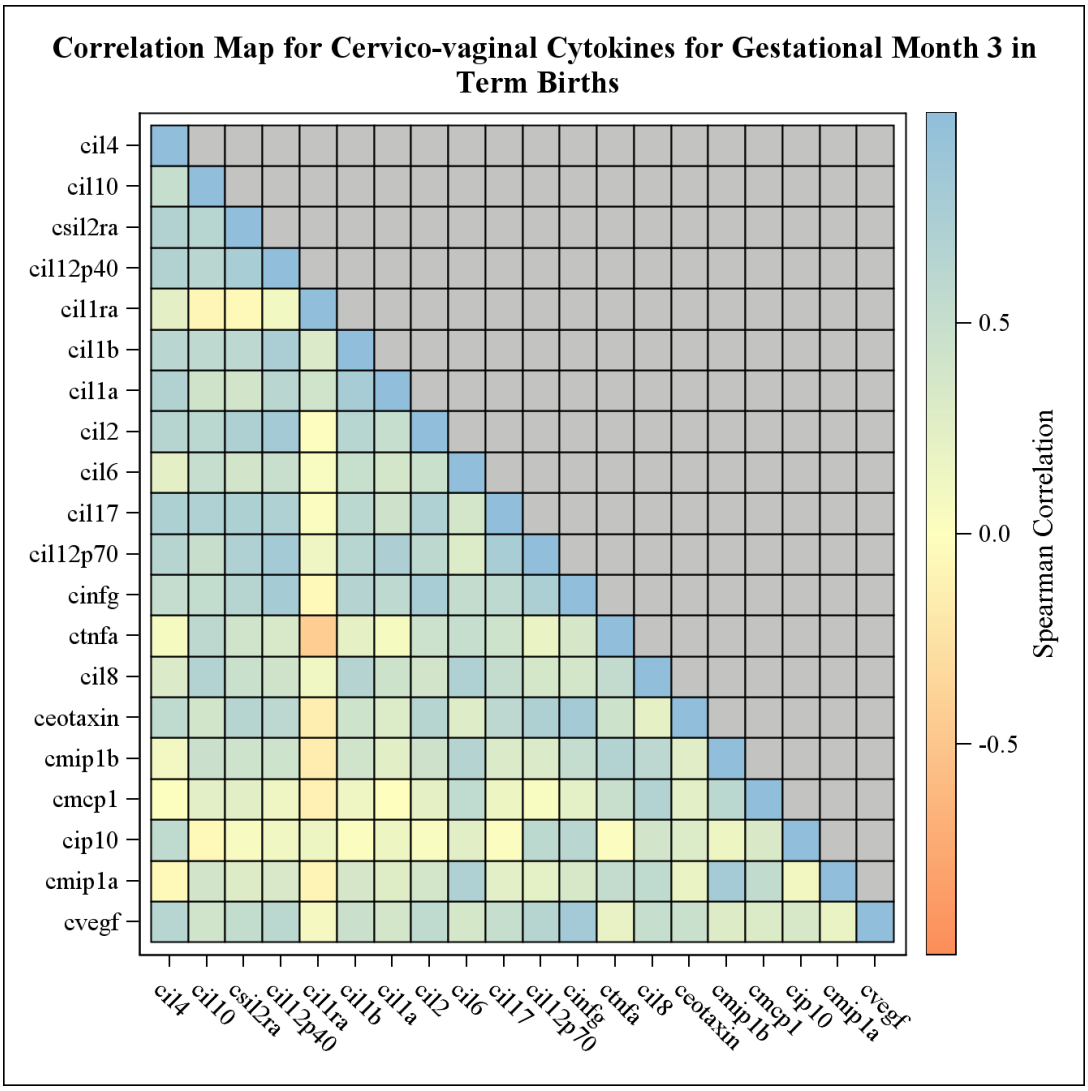

Figure 2.1. Correlation heat map based on Spearman correlation coefficients for cervico-vaginal cytokines at month 3 of gestation. Darker colors indicate stronger negative (tan) or positive (blue) correlations.

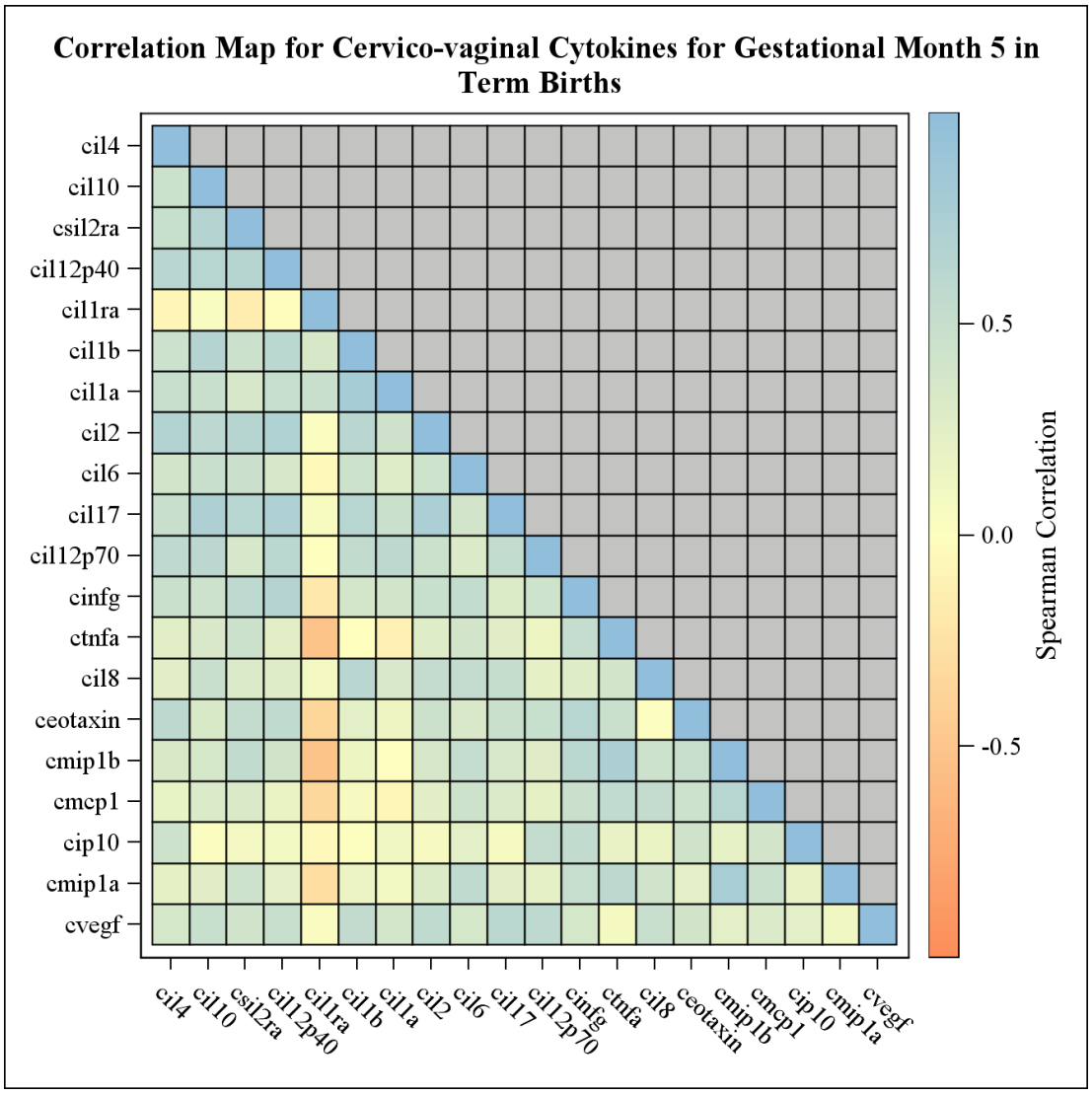

Figure 2.2. Correlation heat map based on Spearman correlation coefficients for cervico-vaginal cytokines at month 5 of gestation. Darker colors indicate stronger negative (tan) or positive (blue) correlations.

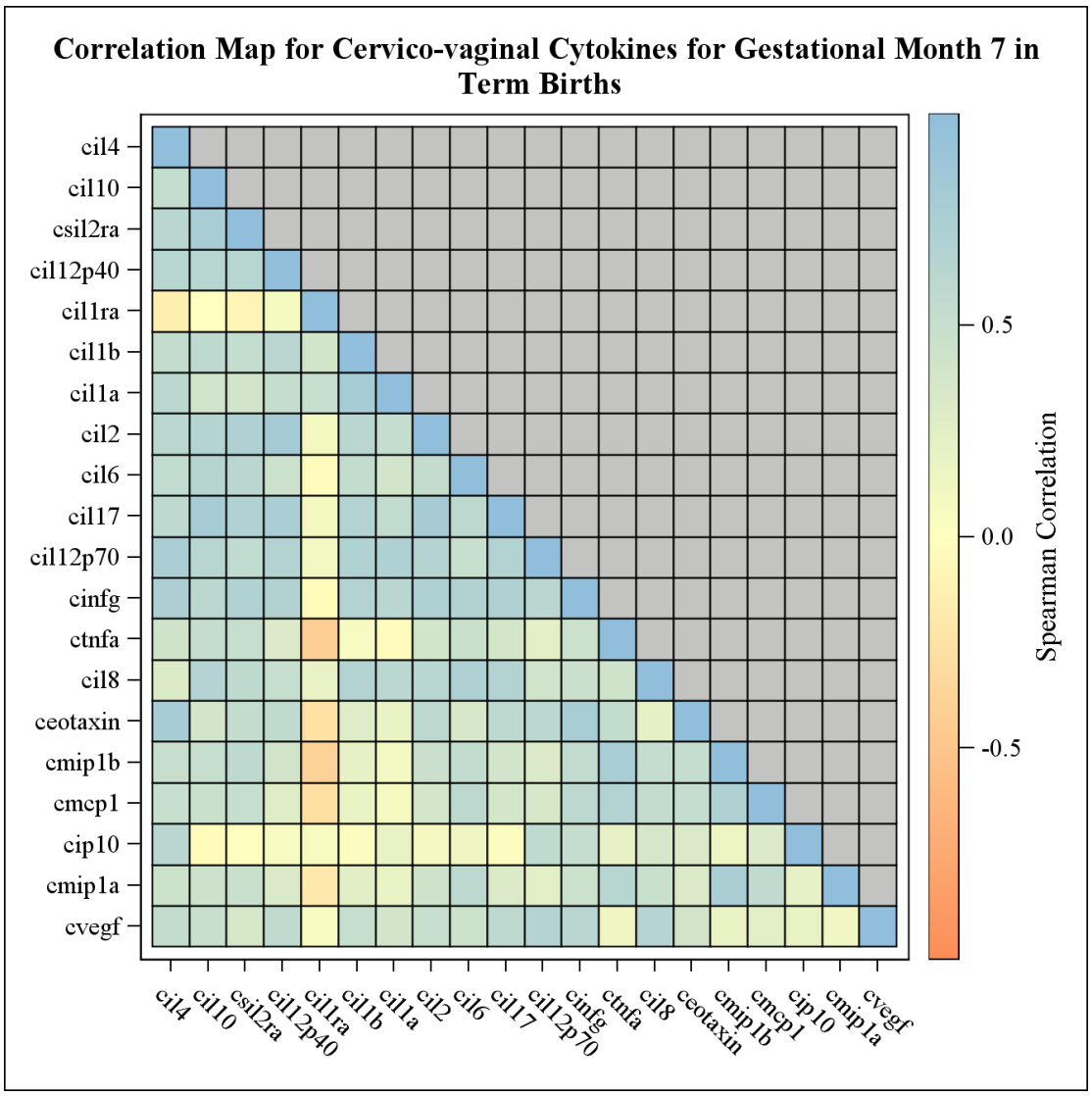

Figure 2.3. Correlation heat map based on Spearman correlation coefficients for cervico-vaginal cytokines at month 7 of gestation. Darker colors indicate stronger negative (tan) or positive (blue) correlations.

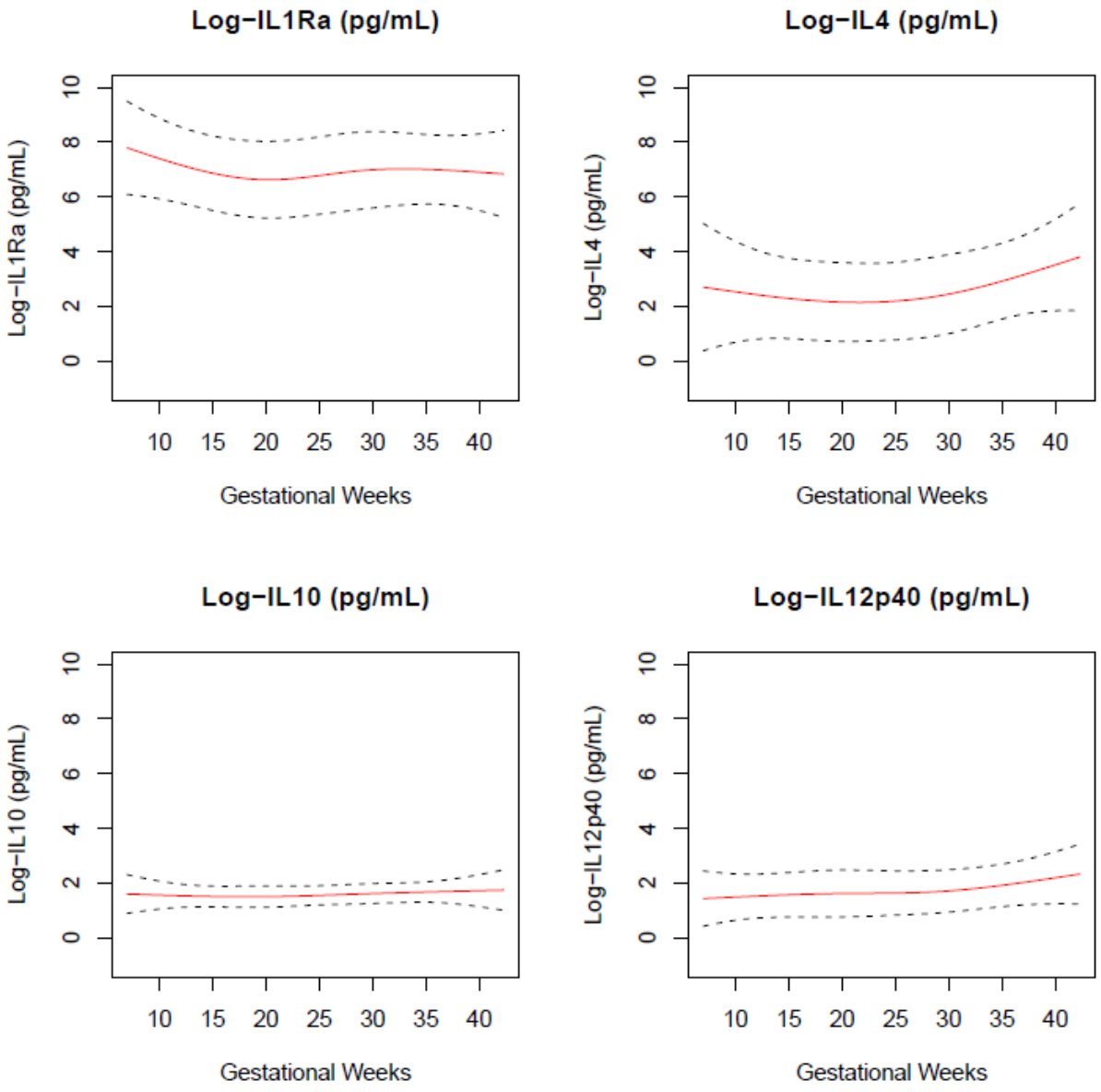

Figure 2.4. Longitudinal Cytokine Patterns from Tobit Regression models adjusted for infection

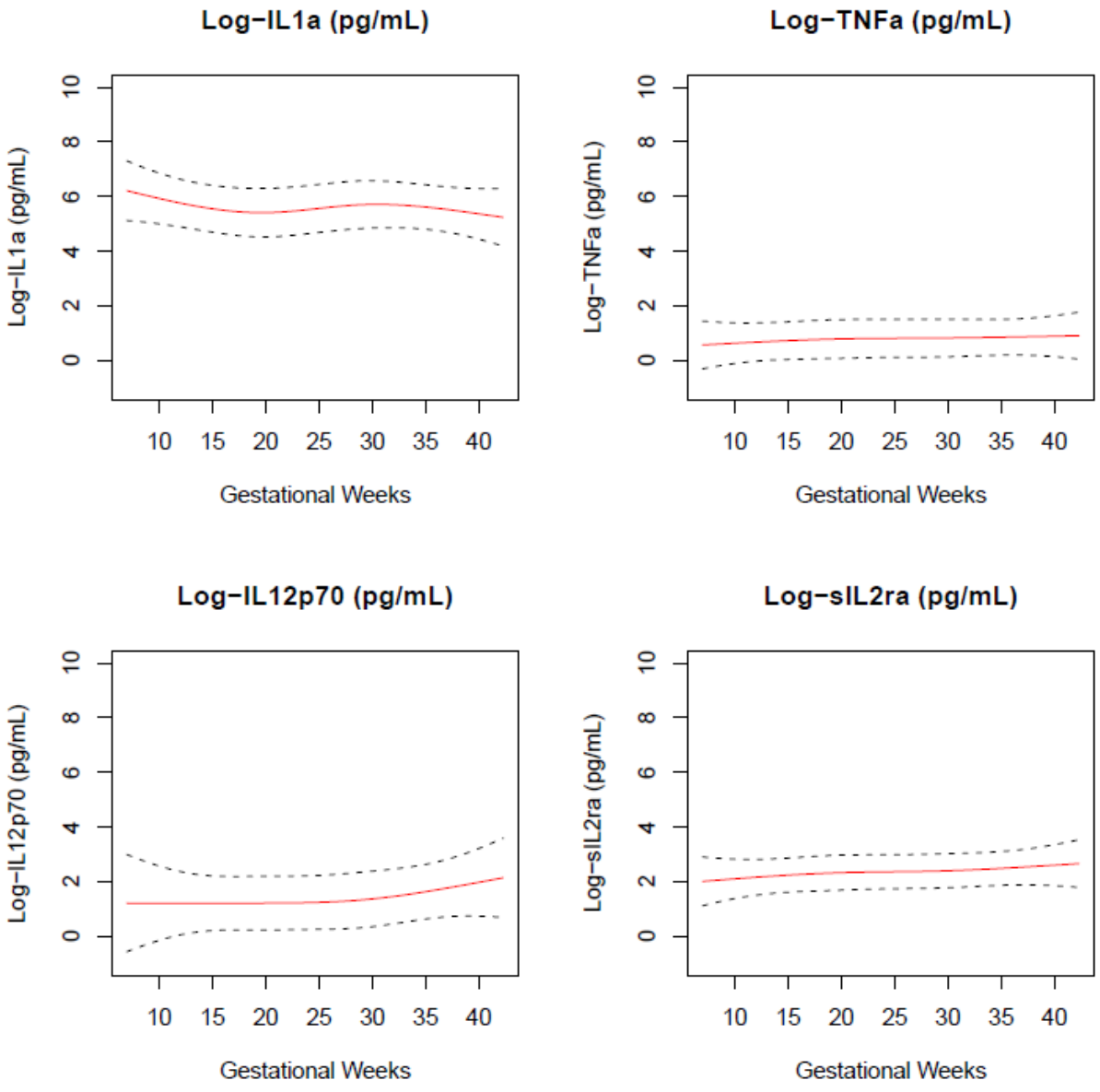

Figure 2.5. Longitudinal Cytokine Patterns from Tobit Regression models adjusted for infection

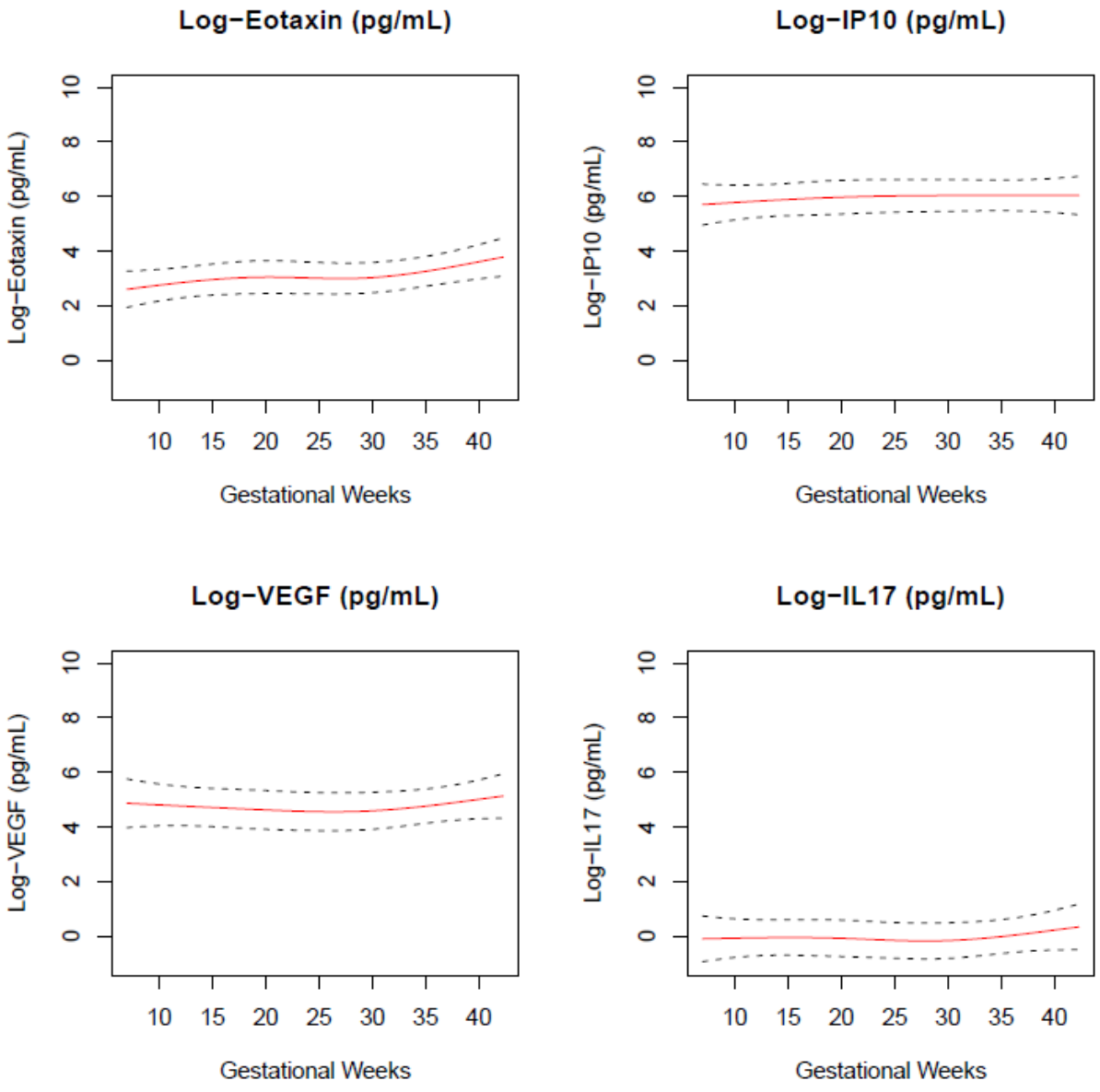

Figure 2.6. Longitudinal Cytokine Patterns from Tobit Regression models adjusted for infection

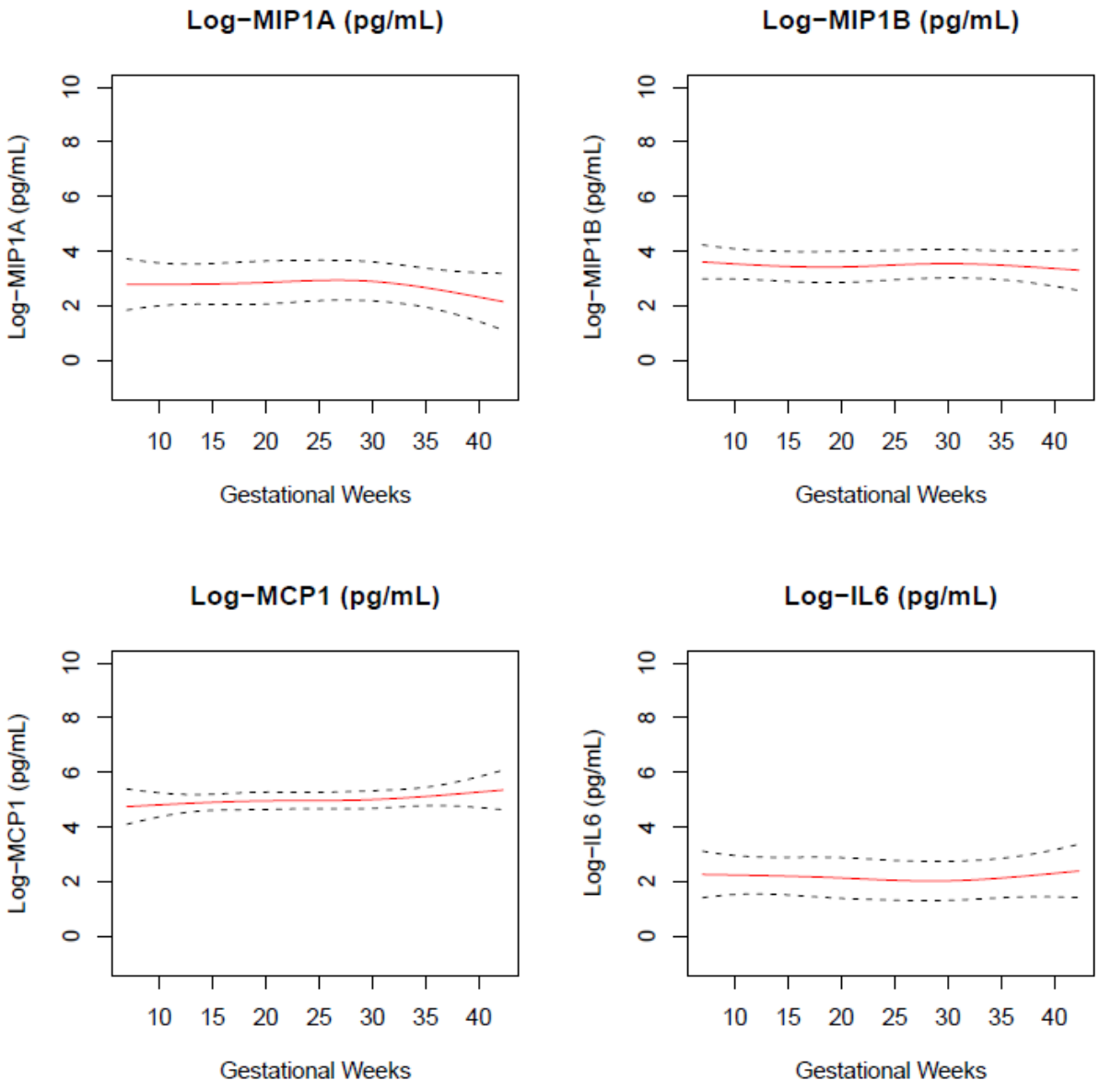

Figure 2.7. Longitudinal Cytokine Patterns from Tobit Regression models adjusted for infection

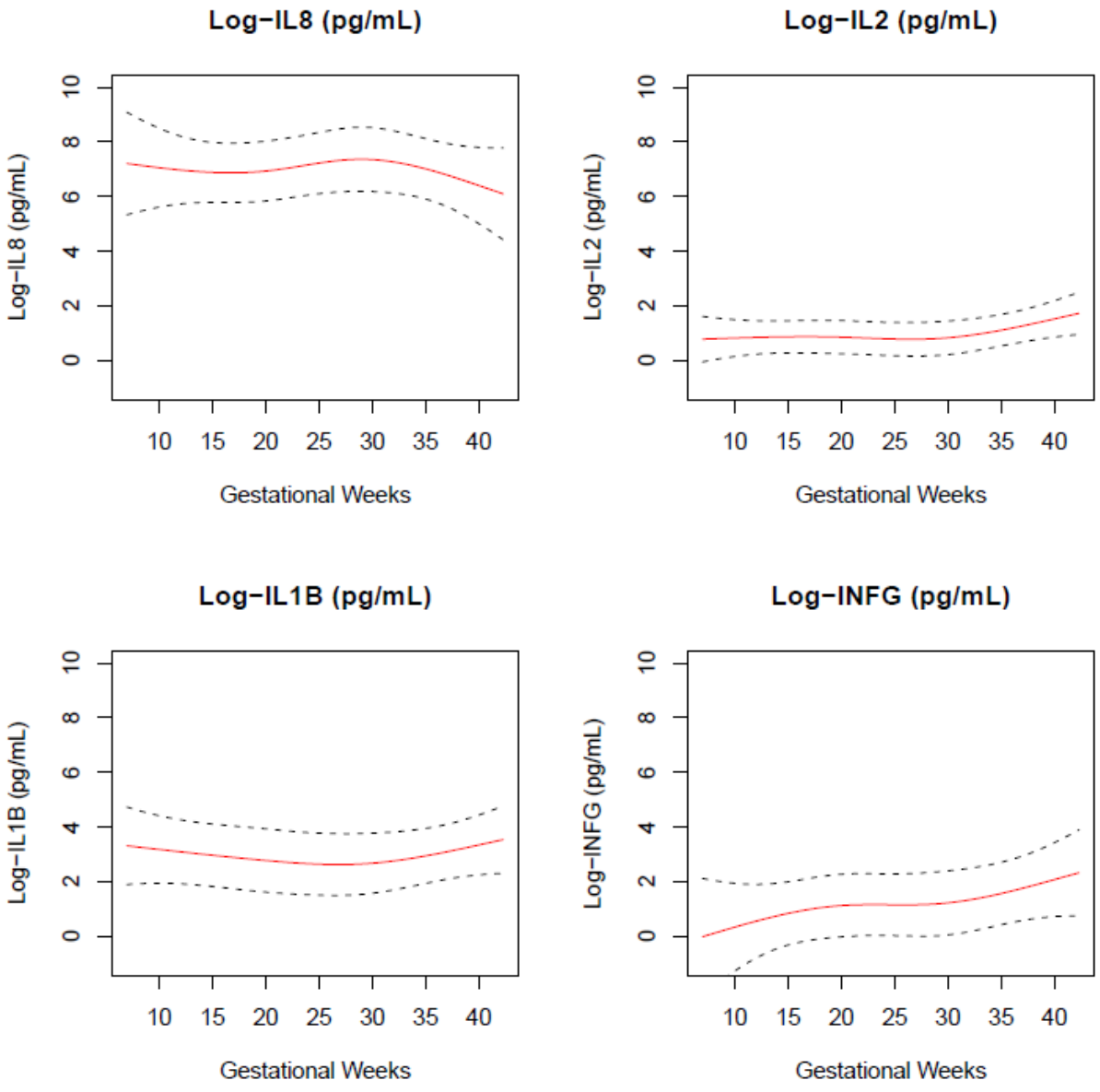

Figure 2.8. Longitudinal Cytokine Patterns from Tobit Regression models adjusted for infection

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Chapter 3

Concurrent Repeated Measures of Systemic and Reproductive Tract Cytokines During Term Pregnancy: Implications for Evaluating Environmental Contributors to Inflammation

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3.1 Abstract

Inflammation is associated with preterm birth. Reproductive tract infection and environmental toxicant exposures can stimulate inflammation. However, few studies have evaluated the associations between longitudinal measures of systemic and reproductive tract inflammation and environmental exposures.

We quantified cytokines from cervico-vaginal exudates and serum obtained concurrently among 178 women with term pregnancies and estimated PM_{10} and carbon monoxide (CO) exposure. Serum and cervico-vaginal cytokines were compared using the Wilcoxon signed-ranks test and Spearman rank correlations for select gestational months. We used intraclass correlation coefficients (ICCs) to quantify reproducibility of cytokine measurements, and Tobit regression to estimate associations between air pollution and cytokines.

Median cervico-vaginal levels of IL-6, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, and TNFα were higher than corresponding serum cytokines, significantly so for IL-6 and IP-10. Cervico-

vaginal and serum cytokines were not correlated, but cytokines from the same fluid were correlated. ICCs for most serum cytokines were ≤ 0.40, while ICCs were higher in cervico-vaginal cytokines (range 0.52-0.83). IP-10 and Eotaxin had the highest ICCs for both cytokine sources. Adjusted for age and infection, PM_{10} was positively associated with serum cytokines lL-6 and MIP-1β but inversely associated with cervico-vaginal cytokine TNFα. CO was inversely associated with cervico-vaginal TNFα, MIP-1β and MCP-1.

Inflammatory processes are compartment-specific. Systemic inflammatory markers may provide information regarding immunologic processes and response to environmental exposures, but are not proxies of lower reproductive tract inflammation.

3.2 Introduction

Pregnant women are exposed to environmental toxicants in the air, water, and diet, but few studies have evaluated the effect of these agents on pregnancy duration.[1] Air pollution is associated with systemic inflammation [2, 3] and oxidative stress [4, 5]. Alveolar macrophages are believed to be one of the links between inhaled pollutants that lead to inflammation in the lungs, and the subsequent systemic inflammatory response [6] that may cross the placental barrier through hematogenous dissemination.[7] Additionally, air pollution has been posited to play a role in affecting maternal susceptibility to infection.[8-10] Air pollution has been associated with premature rupture of membranes (PROM) – rupture of membranes at least an hour before the start of contractions, [11] and an increase in preterm premature rupture of membranes (PPROM) – PROM which occurs before 37 weeks of gestation.[12] Reproductive tract infection is an established risk factor for PROM, PPROM and preterm birth.[13]

The degree to which exposure to air pollution affects inflammatory markers is poorly understood. A systematic review and meta-analysis of studies up to 2010 on inflammatory markers and preterm birth in asymptomatic women found that elevated levels of IL-6 obtained from amniotic and cervico-vaginal fluids, and C-reactive protein from amniotic fluid but not plasma, were strongly associated with spontaneous preterm birth among asymptomatic women.[14] However, a current limitation is identifying the relevant tissue for measurement, as it is uncertain whether systemic inflammatory markers and lower reproductive tract inflammatory markers in pregnancy reflect the same or different pathways. This is important for understanding the role of air pollution in local and systemic inflammation, and how air pollution might affect pregnancy outcomes through one or both inflammatory pathways.

Mexico City, a mega-city where distinctive geographic features and manmade sources contribute to high pollution levels,[15] represents an ideal setting to examine the effects of air pollution on inflammatory markers such as cytokines. The primary aim of this study was to compare serum and cervico-vaginal cytokine concentrations throughout pregnancy among women who delivered at term. A secondary aim was to evaluate whether air pollution concentrations were associated with observed cytokine concentrations.

3.3 Methods

Study participants

Study participants were selected from women participating in the *Air Pollution and Birth Outcomes Study,* an ongoing longitudinal study based in Mexico City.[16] Participants were women with singleton pregnancies who were followed at approximately monthly intervals. In addition to other enrollment requirements [16], all participants signed an informed consent form; they were 18 years or older, and lived and or worked in Mexico City. Women in the study agreed to provide clinical, microbiological, and behavioral data at each visit. The University of Michigan Institution Review Board and the Ethics Committees from the Secretaría de Salud del Gobierno de la Ciudad de México (Mexico), and the School of Medicine of the National Autonomous University of Mexico (UNAM) granted approval for the study.

To directly compare serum and cervico-vaginal cytokines, we included all women for whom we had cervico-vaginal and serum samples collected on the same date (n=104). This dataset was used for all analyses. The median number of visits was 3 for this dataset.

Air pollution

We used air pollution data collected by the Mexico City Atmospheric Monitoring System known as SIMAT. [17] SIMAT routinely collects air pollution information on carbon monoxide (CO), nitrogen dioxide (NO_2), ozone (O_3), particulate matter less than ten microns in aerodynamic diameter (PM)-$_{10}$, PM $_{2.5}$, and sulfur dioxide (SO_2). Data are collected hourly by 34 automatic monitors located throughout Mexico City and surrounding areas.

We chose to analyze PM_{10} in micrograms per cubic meter ($\mu g/m^3$) and CO in parts-per-million (ppm), because each one represents a different type (particle versus gaseous) of air pollution and have been previously evaluated during pregnancy. (Reviewed in [18]) Additionally, PM_{10} is a more spatially homogeneous pollutant whereas CO varies more in proximity to combustion sources. We used daily averages for PM_{10} and CO estimated from the monitor nearest each participant's address for the day before each clinic visit. Because all monitors did not collect information on every pollutant, when data from the nearest monitor were not available, data from either the second or third nearest monitor were used.[19] The selection of an exposure window for air pollution estimates for the day prior was based on previous studies on the effects of air pollution on systemic inflammation which used the same day or the preceding 24-hour period prior to collection of blood.[3, 20] We used average air pollution estimates for the day prior instead of the day of visit because the exact time of sample collection from study participants was not known.

Cytokine Quantification

The Millipore MILLIPLEX® MAP human cytokine/chemokine magnetic bead system (Millipore Corporation, Billerica, MA, USA) was used to measure 15 pro-inflammatory, and 5 anti-inflammatory cytokines obtained from monthly serum and cervico-vaginal samples.[21] The Millipore MILLIPLEX® MAP system simultaneously quantify various cytokines from a single sample.[22] We measured 20 cervico-vaginal cytokines (Eotaxin, Interferon gamma (IFNγ), IL-10, IL-12p40, IL-12p70, IL-17, IL-1rα, soluble IL-2 receptor alpha (sIL-2rα), IL-1α, IL-1β, IL-2, IL-4,Il-6, IL-8, Interferon gamma inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), TNFα, and vascular endothelial growth factor (VEGF). If no value was detected for a cytokine, we used in the analysis a value less than the limit of detection (LOD) as determined by the manufacturer. The LODs varied for each cytokine, and undetected observations were substituted with LOD/√2 in all analyses. Observations at the upper limit of detection (> 10,000 pg/mL) were assigned a value of 10,010 for analysis.

Statistical analysis

The study population was first characterized using standard descriptive statistics. The trimester specific probability of clinician diagnosed infection was estimated by taking an overall average of the number of infections per participant for each trimester divided by the number of visits the participant made during that trimester. The Wilcoxon signed-ranks test, which evaluates whether the median difference between groups is zero, was used to compare cervico-vaginal and serum cytokine concentrations obtained from the same set of participants for gestational months 3, 5 and 7. Because of the very high percent of observations below the

LOD, especially among serum cytokines, only pairs of cytokines with 50% or more values observed were evaluated, as was done in a previous study by Vogel et al. [23]

To determine the relationships between pairs of cervico-vaginal and serum cytokines across pregnancy we used Spearman rank correlations for select gestational months. The Spearman correlation coefficients were illustrated in heat maps with a color gradient to indicate the strength and direction of the association between cytokine pairs.[24] Intraclass correlation coefficients (ICCs) divide the between-person variance by the summed between- and within- and -person variances to measure reproducibility of cytokine concentrations within participants across the duration of pregnancy (i.e. a summary measure across participants of how similar cytokine concentrations are within each participant). Values of the ICC < 0.40; 0.40–0.75; and ≥ 0.75 indicate poor; fair to good and excellent reproducibility, respectively.[25] The ICCs were calculated for each serum and cervico-vaginal cytokine using log transformed and inverse fourth root transformation. These transformations were previously identified using a Box-Cox procedure to accommodate the fact that our cytokine data was not normally distributed, a key assumption for ICCs (Buxton et al, unpublished data (Aim 1)). To calculate the ICCs, we used a maximum likelihood estimation for lognormally distributed data which accounted for values below the limit of detection. [26]

Finally, we used Tobit regression to estimate associations of the air pollutants PM_{10} and CO with select cervical and serum cytokine levels.[27] Tobit regression accounts for both undetected values at the lower and upper ends of the data and can be used for repeated measures.[28] False discovery rate (FDR) was used to adjust the p-values from Tobit regression

models to account for multiple testing; FDR addresses the concern that multiple comparisons could yield statistically significant associations 'by chance' .[29]

3.4 Results

The majority of participants were between 20-35 years of age and were either normal weight or overweight before the start of pregnancy (Table 3.1). Most participants had previously given birth to at least one child. The probability of infection increased from the first trimester to the second trimester and then decreased in the third trimester. The median gestational age at enrollment was around 13 weeks of gestation.

The percent of observations below the limit of detection (LOD) varied across cytokines and the variation was even greater across cytokine source (i.e. serum versus cervico-vaginal samples) (Data not shown). Due to this variability, especially among the serum cytokines where the percent below LOD ranged from 0-97.8%, (data not shown) further analyses were restricted to cytokines with at least 50% of observations above the LOD in both cervico-vaginal and serum samples. The seven cytokines that met this criterion were: IL-6, Eotaxin, IP-10, MCP-1, MIP-1α, MIP-1β, and TNFα.

All cervico-vaginal cytokine levels were substantially higher than the corresponding serum cytokines, but significant median differences between cervico-vaginal and serum cytokines were found only for IL-6 and IP-10 across all months evaluated (gestational months 3, 5, and 7) (Table 3.2).

Serum cytokines showed poor to good reproducibility over the course of pregnancy. (Table 3.3a) Average ICCs for most serum cytokines were less than 0.40 indicating poor reproducibility, except IP-10 and Eotaxin which exhibited fair and good reproducibility,

respectively.[25] Reproducibility was better for the corresponding cervico-vaginal cytokines; ICCs values were higher and reproducibility ranged from fair to excellent (average ICCs 0.52-0.83) Similar to serum ICCs, IP-10 and Eotaxin had the highest ICC values. (Table 3.3b) Overall and individually, and at every time point, cervico-vaginal and serum cytokines were not correlated, even between matching pairs (for example, serum IL-6 vs. cervico-vaginal IL-6). Heat maps are presented in figures 3.1-3.3. Even for the few serum/ cervico-vaginal pairs with a statistically significant association, correlations were very weak. By contrast, cytokines from the same biological compartment tended to correlate. The strongest correlations were among cervico-vaginal cytokines TNFα and MIP-1β for months 3 (rho=0.86), 5 (rho=0.89), and 7(rho=0.92). (Data not shown) MIP-1α and MIP-1β had the strongest correlations among serum cytokines for all time points evaluated (rho = 0.76, 0.84, 0.77 for month 3, 5, and 7 respectively).

PM_{10} averaged 56.0 $\mu g/m^3$ (SD=21.8) and CO averaged 1.5 ppm (SD=0.5) across the 582 days of observation. In Tobit models adjusted for age and infection status, air pollutants were positively associated with serum cytokine concentrations (table 3.4). PM_{10} was significantly associated (p-value < 0.05) with serum cytokines TNFα, IL-6, IP-10, MIP-1β, and Eotaxin; and CO was marginally associated with serum cytokine Eotaxin. On the other hand, PM_{10} and CO were negatively associated with cervico-vaginal cytokines. PM_{10} was significantly associated with TNFα, IP-10 and MIP-1β, while CO was significantly associated with all cytokines evaluated except IP-10 and Eotaxin. However, after controlling for the false discovery rate, PM_{10} was significantly associated with only IL-6 and MIP-1β from serum, and significantly associated with cervico-vaginal cytokines TNFα, while CO remained associated with TNFα, MIP-1β and MCP-1.

3.5 Discussion

We compared cervico-vaginal and serum cytokines obtained over the course of term pregnancy and evaluated their associations with particulate and carbon monoxide levels measured on the day prior to specimen collection. The relationship between inflammatory markers from these two compartments is of fundamental interest in perinatal epidemiology because of important role inflammation plays throughout healthy pregnancy, and because dysregulated inflammation, in response to infection, oxidative stress, and other factors, is suspected to be involved in the pathology of preterm birth.

Our first key finding was that serum and cervico-vaginal cytokines were not correlated, and cervico-vaginal cytokines concentrations were substantially higher than for the corresponding serum cytokines. These findings indicate that inflammatory processes are compartment specific, with little to no crossover between compartments, at least when evaluated at the same point in time. Vogel et al. also found no correlation between cervico-vaginal and serum cytokines in a study of 57 pregnant women in Birmingham, Alabama with a previous preterm birth.[23] Similarly, cervico-vaginal and serum cytokines were not correlated among 26 post-menopausal women with and without vaginal symptoms.[30] This suggests that compartment-specific immunologic response is not an anomaly of pregnancy but rather a normal physiologic process across conditions and/or stages of life.

Our second key finding was cervico-vaginal and serum cytokines had different associations with particulates and carbon monoxide, suggesting that the biological mechanisms associated with systemic versus localized immunologic response to air pollution and other environmental exposures leading to oxidative stress/inflammation may differ. Our finding

reinforces the importance of a recommendation by Ferguson and colleagues. In a repeated measures study evaluating the association between exposure to six phthalates (estimated from urinary metabolites) and five systemic inflammatory markers during pregnancy (in 30 individual models), found only one significant and three marginally significant associations. Ferguson et al. concluded that inflammatory markers from more specific sources might be important to evaluate the association between phthalate exposure and the subsequent inflammatory response that can lead to preterm birth.[31]

These results have implications for studies using biomarkers to evaluate the effects of inflammation on pregnancy outcomes: namely, that inflammation measured from a source contiguous to the site of the developing fetus might be more representative of the environment to which the fetus is exposed, compared to inflammation measured in the systemic circulation. Georgiou and colleagues, in a recent review of advances made in the use of biomarkers to predict preterm birth, also concluded that because of its compositional characteristic of being a mixture of secretions from gestational and reproductive tissues, cervico-vaginal fluid is ideal for consideration of biomarkers related to labor.[32] By contrast, biomarkers obtained from blood components (plasma and serum) have been reported to be non-specific due to peripheral blood availability across body tissues and the potential that these biomarkers are either diluted or lack information about the exact source from which inflammation originated. Conversely, cervico-vaginal fluid has been reported to better represent immunologic processes occurring in the genital tract and may be more specific and sensitive compared to systemic biomarkers. (Reviewed in [33]). Ideally, amniotic fluid would be a more specific source of the inflammatory milieu that the fetus is exposed to; but the invasive

nature of obtaining amniotic fluid precludes routine collection of samples for research purposes. Cervico-vaginal cytokine IL-6 was found by Jun and colleagues to significantly correlate with amniotic fluid IL-6 in women with PPROM, as such cervico-vaginal cytokines may serve as a low risk proxy for assessing upper reproductive tract inflammation.[34]

The idea that cytokines obtained from serum/plasma are non-specific is supported by the ICCs obtained for serum cytokines in this study. Serum cytokines exhibited more variability compared to cervico-vaginal samples and this may be an indication of the myriad factors capable of influencing cytokine levels in the peripheral circulation. Interestingly, ICCs for the corresponding cervico-vaginal cytokines were higher for the same participants for samples collected at the same time, again suggesting different processes occurring during these same time points and that these processes are compartment specific.

Increases in serum cytokines linked with air pollution were expected but the negative association with cervico-vaginal cytokines was initially surprising. However, considered in the context of the relationship between air pollution and preterm birth - which has been hypothesized to be through its effects on maternal susceptibility to infection [8-10] -insights into this mechanistic pathway begin to unfold. Air pollution may act similarly to the posited role of cigarette smoke on lower reproductive tract infection, by suppressing immunologic response. This lowered state of inflammatory response could permit the ascent of pathogens from the lower to the upper reproductive tract. Although inconsistent across studies and not well defined, the immunosuppressive effects of smoking have been associated with a decrease in response by lymphocytes to mitogen (a substance needed for the start of cell division[35]) and also with impaired chemotactic activities of polymorphonuclear leukocytes. (Reviewed in

[36]) Leukocytes are producers of proinflammatory chemokines such as MIP-1a, MCP-1, and IP-10; these cytokines are involved in recruitment of monocytes and macrophages which are producers of other pro-inflammatory cytokines.[37] Thus, it appears that cigarette smoke interferes with a major positive feedback loop system that involves activation and recruitment of cytokine producing cells; and this interference might be similar to the process occurring between air pollution and reproductive tract immunologic response in our study. Although the mechanisms associated with the opposite expression of cytokines from systemic and lower reproductive tract and air pollution are unclear and need to be further evaluated, one possible explanation is that immunologic response may vary by pollutant type and part of the body being evaluated, and may be categorized in two ways: direct or indirect. For example, response to physical coarse particles in the lungs is direct and is associated with the release of pro-inflammatory cytokines [3] while remote locations such as the reproductive tract might operate through other reported “sequelae pathways” such as the suppression of certain cell types.[36]

Finally, the use of inflammatory markers collected longitudinally on the same participants on the same dates to evaluate median differences, reproducibility, and correlation between local and systemic sources is a major strength of this study. However, some limitations should be considered. The data used in the study contained values below and above the LOD. Except for the Tobit regression models which properly accounted for all censored observations, (ICCs calculation only accounted for observations below the LOD) censored values were replaced with common substitution methods. Simple replacement methods are commonly used for censored data but are known to have limitations especially when the percent below the LOD

reaches 25%.[38] Another limitation is that other sources of inflammation and anti-inflammatory medication use were not accounted for.

In summary, in a sample of pregnant women based in Mexico City, longitudinal measures of reproductive tract and systemic cytokines behaved dissimilarly, and that their associations with estimated short-term air pollution exposure differed. Not only were these inflammatory associations with air pollution compartment specific, they were in the opposite direction. Although systemic inflammation may provide information regarding immunologic processes, inflammatory markers obtained from peripheral blood are not specific and may represent different as well as remote processes and should not be used as proxies of lower reproductive tract inflammation.

Table 3.1. Demographic and Obstetric Characteristics of Pregnant Women Delivering at Term (N=104). Mexico City, 2009-2014

Age	**N (%)**
<20	18(17.3)
20-35	75(72.1)
>35	11(10.6)
Pre-pregnancy BMI	**N (%)**
<18.5 kg/m^2	7(6.7)
18.5-24.9 kg/m^2	36(34.6)
25-29.9 kg/m^2	37(35.6)
≥30 kg/m^2	17(16.4)
Missing	7(6.7)
Parity	**N (%)**
Nulliparous	30(28.9)
Parous	58(55.8)
Missing	16(15.4)
Reproductive Tract Infection	**Probability of infection***
Trimester 1	0.3
Trimester 2	0.37
Trimester 3	0.31

* Mean of (number of infection per trimester/number of visits for each participant per trimester)

Table 3.2. Lower limits of detection (LOD) and descriptive statistics for concurrently measured cervico-vaginal exudate (CVE) and serum cytokine concentrations (in pg/mL), during overall pregnancy and specific months in gestation, among 104 pregnant women in Mexico City, 2009-2014.

		Overall		Overall		Month 3		Month 5		Month 7	
	Lower LOD	% < LOD		Range		Median		Median		Median	
Cytokine	(in pg/mL)	CVE	Serum	CVE	Serum	CVE	Serum	CVE	Serum	CVE	Serum
IL-6	0.9	11.4	40.6	<LOD - 10,010	<LOD - 259.8	**11.0**	**0.6**	**8.5**	**3.0**	**12.3**	**1.9**
Eotaxin	4	16.3	9.2	<LOD - 10,010	<LOD - 286.8	19.7	29.6	**23.1**	**23.8**	24.8	24.4
IP-10	8.6	4.9	0	<LOD - 10,010	23.5 - 1,334	**323.4**	**257.1**	**347.7**	**228.3**	**352.8**	**256.7**
MCP-1	1.9	1.7	0	<LOD - 10,010	3.2 - 2,277	174.3	347.0	222.8	322.1	226.5	276.5
MIP-1α	2.9	12.4	11	<LOD - 10,010	<LOD - 1,166	13.1	29.1	16.5	76.3	7.6	53.2
MIP-1β	3	4	0.3	<LOD - 10,010	<LOD - 952.7	39.4	60.0	45.4	73.8	31.4	67.9
TNFα	0.7	11.9	1	<LOD - 6,405	<LOD - 278.9	9.6	11.2	9.2	16.1	6.6	15.3

LOD/√2 was substituted for values below the limit of detection for each cytokine

Bold text: p-values ≤ 0.05 from the Wilcoxon Signed Rank Test. Median difference was tested only for cytokines in which both sources had 50% or more of observations above the LOD.

Values greater than 10,000 (in pg/mL) were assigned a value of 10,010

Red font indicates borderline significance

Table 3.3a. Intraclass Correlation Coefficients of Serum Cytokine Concentrations in Term Births

Cytokine	Log	Inverse Fourth Root	Mean	Standard Deviation
sIL6	0.28	0.13	0.21	0.11
sTNFα	0.20	0.23	0.22	0.03
sIP-10	0.57	0.59	0.58	0.01
sMCP-1	0.38	0.33	0.36	0.04
sMIP-1α	0.32	0.25	0.29	0.05
sMIP-1β	0.24	0.24	0.24	0.00
sEotaxin	0.71	0.69	0.70	0.01

s prefix indicates serum cytokine

Table 3.3b. Intraclass Correlation Coefficients of Cervico-vaginal Cytokine Concentrations in Term Births

Cytokine	Log	Inverse Fourth Root	Mean	Standard Deviation
cIL6	0.58	0.55	0.57	0.02
cTNFα	0.61	0.54	0.58	0.05
cIP-10	0.64	0.69	0.67	0.03
cMCP-1	0.53	0.50	0.52	0.02
cMIP-1α	0.57	0.54	0.55	0.02
cMIP-1β	0.64	0.56	0.60	0.05
cEotaxin	0.84	0.81	0.83	0.02

c prefix indicates cervico-vaginal cytokine

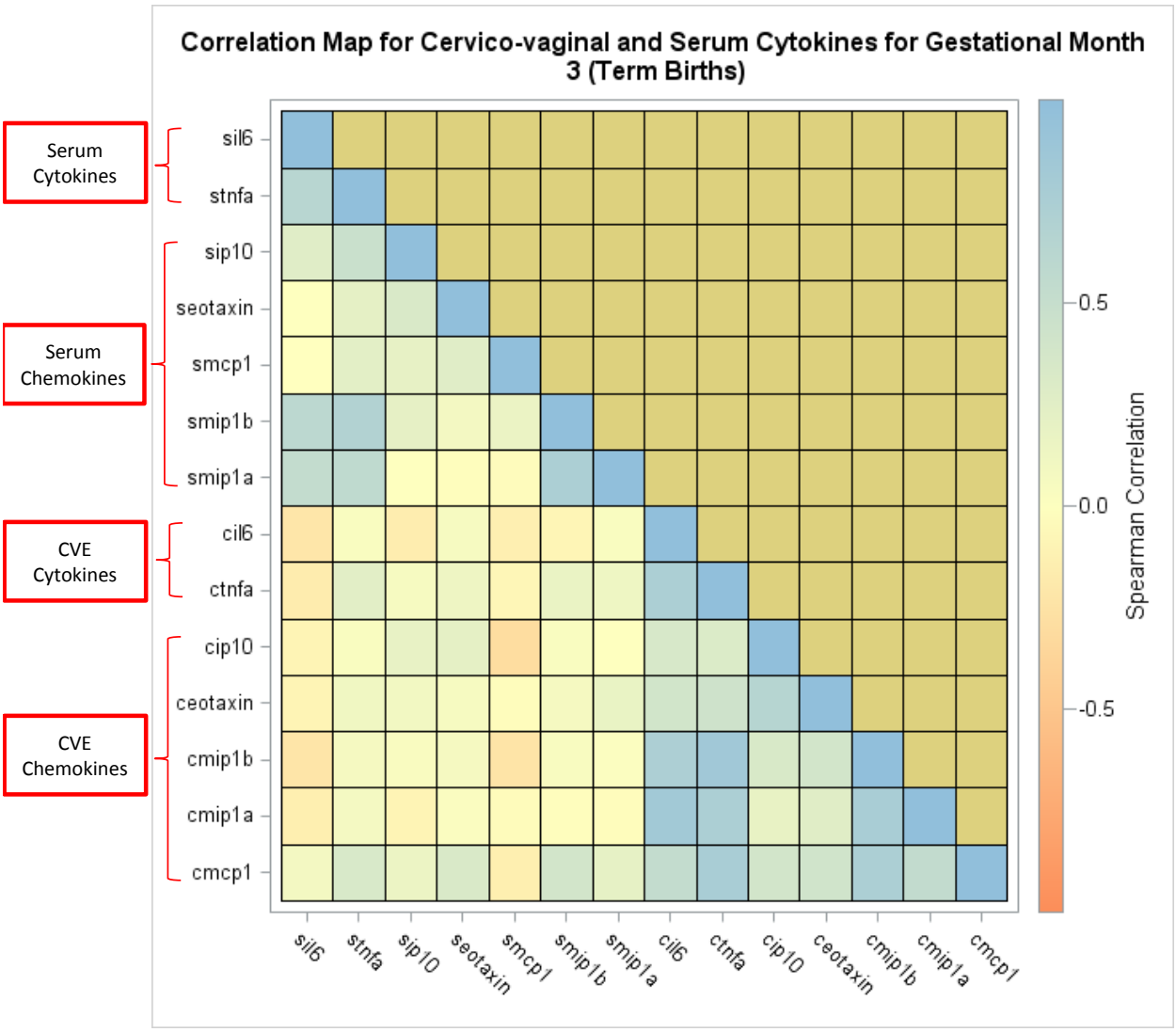

Figure 3.1: Correlation map based on Spearman coefficients for cervico-vaginal and serum cytokines for women who delivered at term using data from gestation month 3

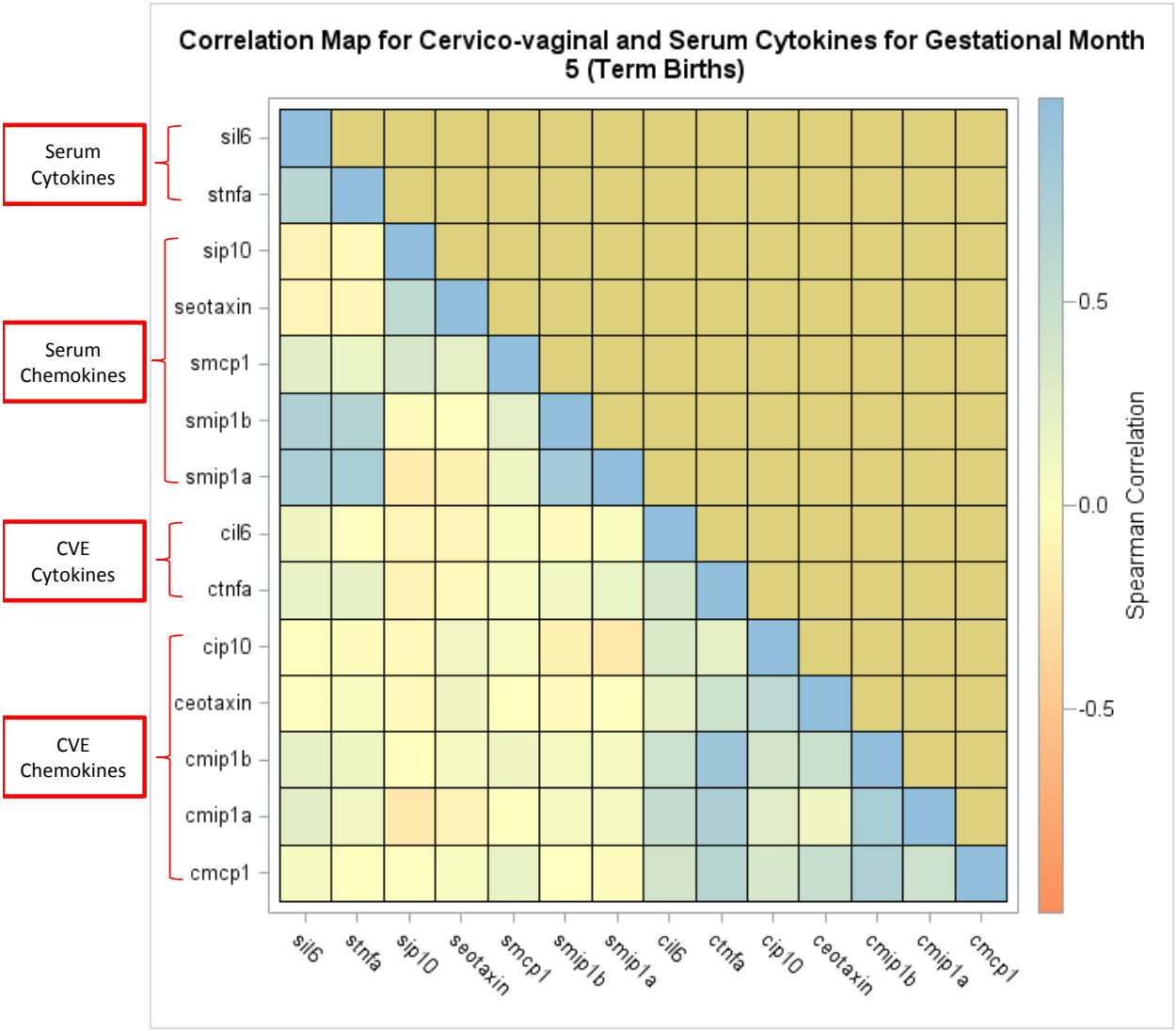

Figure 3.2: Correlation map based on Spearman coefficients for cervico-vaginal and serum cytokines for women who delivered at term using data from gestation month 5

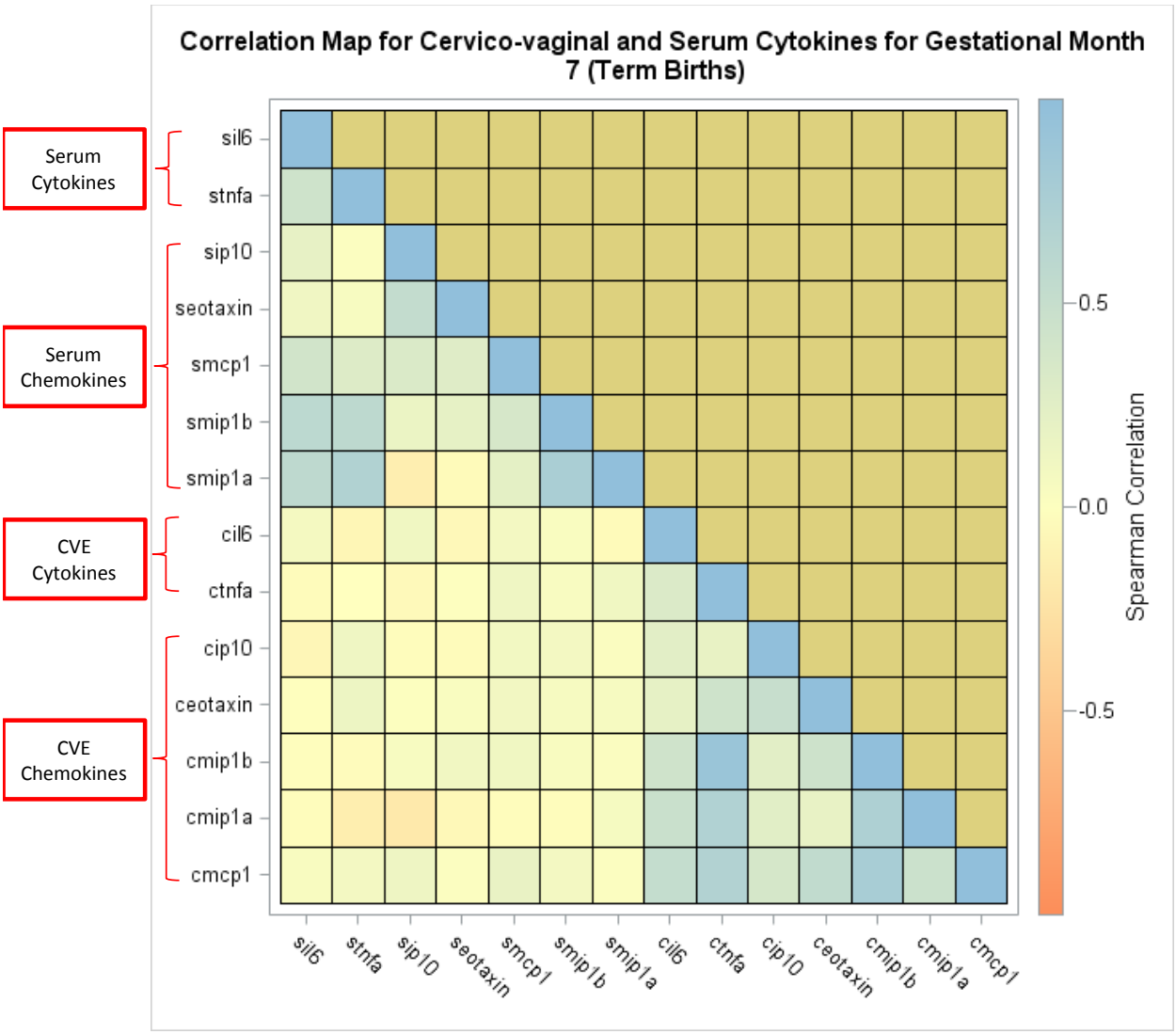

Figure 3.3: Correlation map based on Spearman coefficients for cervico-vaginal and serum cytokines for women who delivered at term using data from gestation month 7

Table 3.4. Standard deviation (s.d.) changes in seven log-transformed pro-inflammatory cytokines from cervico-vaginal exudate (CVE) and serum and corresponding standard errors (s.e.) per 10 unit higher ambient air pollutant concentration near residence, 1 day prior to clinic visit. Results are from infection and age-adjusted Tobit regression models that account for censored cytokine concentrations and repeated measures within 104 pregnant women residing in Mexico City, Mexico, 2009-2014.

	Cervico-vaginal						
	TNFα	**IL-6**	**IP-10**	**MIP-1α**	**MIP-1β**	**MCP-1**	**Eotaxin**
Overall s.d. of distribution	2.15	2.38	1.78	2.48	1.96	2.22	2.02
Betas and s.e. by pollutant							
PM_{10} (per 10 μg/m^3)	**-0.18 (0.07)**	-0.09 (0.09)	-0.11 (0.06)	-0.13 (0.08)	-0.12 (0.06)	-0.08 (0.08)	-0.11 (0.07)
CO (per 10 ppm)	**-0.92 (0.27)**	-0.56 (0.34)	-0.01 (0.25)	-0.64 (0.33)	**-0.59 (0.26)**	**-0.74 (0.26)**	-0.32 (0.29)
	Serum						
Overall s.d. of distribution	1.12	1.7	0.71	1.72	0.92	0.59	1.02
PM_{10} (per 10 μg/m^3)	0.06 (0.03)	**0.24 (0.07)**	0.05 (0.02)	0.10 (0.06)	**0.08 (0.02)**	0.01 (0.01)	0.07 (0.03)
CO (per 10 ppm)	-0.05 (0.11)	0.19 (0.30)	0.12 (0.08)	-0.36 (0.24)	-0.07 (0.08)	-0.08 (0.05)	0.20 (0.12)

Given the wide range of the cytokines, we have interpreted them on the log scale.

Bold font = p-value ≤ 0.05 after adjusting for false discovery rate (FDR)

Bold and blue - marginally significant after adjustig for FDR

Air pollution data from nearest monitor

SD for serum and CVE calculated in SAS

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Chapter 4

Cervico-Vaginal Cytokine Concentrations During Pregnancy and the Risk of Preterm Birth: A Comparative Analysis

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4.1 Abstract

Preterm birth remains a major cause of perinatal morbidity and mortality worldwide; inflammation is considered an important component in the disease pathway. However, results from cross-sectional studies on the association between inflammatory cytokines and preterm birth are inconsistent. We fill this gap by characterizing expression of 20 cervico-vaginal cytokines at multiple time points among 12 preterm and 78 term births.

Empirical cumulative distribution and Spearman correlations for select months were computed for each birth outcome. Categories of inflammatory state were compared by birth outcome using Fisher's exact test. Finally, we used logistic regression to estimate the association between preterm birth and five types of cytokine measures collected during pregnancy.

Inflammatory state was associated with birth outcome. Individual cytokines in the first trimester had the strongest associations with preterm birth. IL-1α was the only cytokine with a protective association across pregnancy. IL-1RA was positively correlated with other IL-1 cytokines among term births but negatively correlated among preterm births: Rho for term vs.

preterm for months 3, 5, and 7 were: 0.37 vs. -0.49; 0.38 vs. -0.62; and 0.34 vs. -0.67, respectively.

Differences in cytokine expression, and dysregulation of anti and pro-inflammatory cytokines among preterm births as early as the first trimester indicate deviations from immune-regulatory homeostasis. Processes leading to preterm birth can occur early in pregnancy, facilitating effective screening. Future studies should consider homeostasis in cytokine expression when evaluating risk factors for preterm birth.

4.2 Introduction

Preterm birth or delivery before 37 completed weeks of gestation is a major cause of perinatal morbidity and mortality. [1] Despite decades of research, a clear understanding of the causes of preterm birth has yet to emerge [2]. The burden of preterm birth is especially high in low- and middle-income countries where resources and specialized health services are limited. For example, a major difference in survival (10% versus 90%) exists between extremely preterm babies (born before 28 weeks of gestation) in low income countries compared to high income countries. [3] Moreover, in some rural and resource-limited settings where the nearest health facility may be a considerable distance away, preterm labor may lead to delivery at home or in facilities under-equipped to handle obstetric complications, and may also contribute to maternal mortality.[4] Even in the United States where advanced and specialized medical services are available, preterm birth is the leading cause of infant mortality, and has annual rates at approximately 12 percent. [5, 6]

A number of maternal risk factors - including behavioral, medical and socio-demographic factors – are associated with preterm birth. [7] However, interventions targeting some of these risk factors such as nutritional supplementation, prophylactic and medically indicated antibiotic treatments have had mixed results, and overall have failed to have a significant impact in reducing preterm birth rates. [6] Consequently, much remains to be understood regarding how these risk factors interact to influence preterm risk.
Inflammation is one of four major pathways implicated in decidual activation that may subsequently lead to preterm birth. [6] Pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor-alpha (TNF-α) or anti-inflammatory (e.g. IL- 1Ra, IL-4, IL-10), are

proteins involved in the body's response to infection and injury.[8] They also are important indicators of infectious and non-infectious injury to the body even among persons who are asymptomatic. [9] The physiological processes associated with pregnancy can moderate changes pro and anti-inflammatory cytokine expression; [9-11] but deviations from expected patterns at a particular point in pregnancy may indicate pathologies that might lead to preterm birth. [12-16]

However, available evidence on the association of cytokines with preterm birth is inconsistent; perhaps because studies are small, and vary in biomarker selection, sampling timeframe, and tissue assayed. For example, Simhan et al found that women with high anti-inflammatory/low pro-inflammatory first trimester cervical cytokines were at higher risk of early spontaneous preterm birth compared to women who had low anti-inflammatory/high pro-inflammatory or balanced levels. [17] Similarly, Smith et al. found that levels of pro-inflammatory cytokine IL-6 obtained from vaginal samples were lower in women who delivered preterm compared to women who delivered full term. [18] By contrast, Goepfert et al reported higher mean concentrations of IL-6 in women who delivered preterm compared to term.[19] Similar inconsistencies have been noted in studies using serum/plasma samples. [20-22]

Our study addresses some of the limitations in previous studies. We used longitudinal data on 20 cytokines obtained from monthly cervico-vaginal samples to compare cytokine expression between women who subsequently delivered term and preterm babies. We further used comparative analyses to evaluate how well cytokine measures from different points in pregnancy predict preterm birth.

4.3 Methods

This study used data from a longitudinal study (enrollment 2009-2014) based in Mexico City whose primary objective was to evaluate the association between air pollution and birth outcomes.[23] The research study received approval from the University of Michigan Institutional Review Board, and the ethics committees from the Secretaría de Salud del Gobierno de la Ciudad de México (Mexico City) and the School of Medicine of the National Autonomous University of Mexico (UNAM).

Participants in the study were pregnant women with singleton pregnancies who resided and/or worked in Mexico City and surrounding areas. Participants were 18 years or older, enrolled before 18 weeks of gestation, agreed to attend approximately monthly prenatal visits, and provided written informed consent. Additional data such as clinical samples (blood, urine, cervico-vaginal exudates), behavioral, and demographic information were collected during monthly visits. Participants in this study included two groups, namely: group 1 included participants that had at least one visit, along with the corresponding cytokine data, for each trimester; and group two included all participants regardless of the frequency of cytokine data.

Gestational age calculation

Monthly gestational age of the fetus was calculated using the date of visit and the reported first day of the last menstrual period (LMP); gestational age at birth was calculated based on the infant's date of birth and the first day of the LMP. Both calculations were based on reliable and accurate recall of LMP from data collected to screen for eligibility.

Collection of biological samples

Cytokines were quantified from cervico-vaginal samples collected at each visit. Samples were obtained using a Dacron swab rotated for 10 seconds in the cervico-vaginal section of the reproductive tract and frozen at -20^{o} C for later processing.

Quantification of cytokines

The following cytokines were obtained from cervico-vaginal samples: Eotaxin, Interferon gamma (IFNγ), IL-10, IL-12p40, IL-12p70, IL-17, IL-1rα, soluble IL-2 receptor alpha (sIL-2rα), IL-1a, IL-1β, IL-2, IL-4,Il-6, IL-8, Interferon gamma inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), macrophage inflammatory protein-1 beta (MIP-1β), TNFα, and vascular endothelial growth factor (VEGF).

Cervico-vaginal cytokines were selected instead of serum cytokines because we previously demonstrated that cervico-vaginal and serum cytokines were not correlated. Further, cervico-vaginal cytokine concentrations were higher than serum concentrations (Buxton et al, Aim 2).

Cytokine quantification was done using the Millipore MILLIPLEX® MAP human cytokine/chemokine magnetic bead panel kit (Millipore Corporation, Billerica, MA, USA) based on the published protocol of the manufacturer. An advantage of the Millipore MILLIPLEX® MAP system is the concurrent quantification of several cytokines from a single sample.[24] Analyses were done in the laboratories of the National Autonomous University of Mexico or its affiliate, the National Institute of Genomic Medicine, using previously frozen samples. All analyses were

done in duplicate for cervico-vaginal exudate samples, and standard controls were run to serve as a basis for comparison.

Data were left and right censored as the MILLIPLEX system is relatively insensitive at the lower and upper ends of the range of values. LOD/√2 (left censored) and a value of 10,010 (right censored) were substituted for these observations in all analyses.

Statistical analysis

Trimester specific probability of infection was computed by averaging the number of infection diagnosed during a given trimester divided by the number of visits for that trimester. Fisher's exact test and T-test were used to evaluate differences between term and preterm births. Cytokine distributions were evaluated using histograms to evaluate whether cytokine data needed to be transformed. Empirical cumulative distribution functions (ECDF) were calculated to illustrate month specific cytokine distributions by outcome. Plots were created using the *cenfit* and *cendiff* functions in NADA package in R. Cenfit utilizes the Kaplan-Meier method for left censored data to compute ECDFs, and cendiff uses a Log-rank or Mantel-Haenszel test for left censored data to evaluate the difference between two or more ECDFs.[25] Spearman Rank correlations for select months were calculated for each outcome group to separately evaluate the relationship between pairs of cytokines. Heat maps were generated to illustrate correlations between cytokines.[26] The p-values from the Spearman correlations were adjusted using Benjamini-Hochberg corrections for multiple testing.[27] We used Fisher's Exact Test to evaluate if the ratio of pro-inflammatory to anti-inflammatory cytokines was associated with preterm birth, applying the method used by Simhan et al to data

groups 1(participants with at least one measure per trimester, N=90) and 2 (participants with varying degrees of follow up data).[17] The application of the same analysis on data groups 1 and 2 was done to separate the effects of changing sample size (i.e. different subgroups of participants) by trimester on results. Specifically, data group 2 analyses were conducted to evaluate replicability of the results among participants that may be more representative of cohort data resulting from repeated measures studies.

Trimester specific averages of each cytokine for each participant were computed, and each cytokine value was standardized by dividing by the interquartile range value of the cytokine due to the wide range of cytokine concentrations in this study. (Simhan et al used the medians of the cytokines). The resulting values were summed and divided by the number of cytokines for each cytokine category (pro. vs. anti-inflammatory) to generate a score. The scores were divided into quartiles; those appearing in quartiles 1-3 were classified as low and quartile 4 as high. Women were then categorized into 3 groups, namely: high pro-inflammatory/low anti-inflammatory, low pro-inflammatory/high anti-inflammatory, and the two other combinations were classified as balanced.

Finally, comparative analyses were conducted using logistic regression to evaluate the association between preterm birth and cytokines measured at select points in pregnancy. Logistic regression models were fit for individual cytokine models for data group 1 using the following cytokine data as predictors of preterm birth: i. first trimester average, ii. second trimester average, iii. third trimester average, iv. first, second, and third trimester averages combined in one model, and v. overall average. P-values from the logistic regression models were adjusted using Benjamini-Hochberg corrections for multiple testing.[27] All analyses

included data up to month eight of gestation. All analyses were conducted in SAS 9.3 (SAS Institute, Cary, North Carolina) and R version 3.2.2 (R Foundation for Statistical Computing; Vienna, Austria).

4.4 Results

The median gestational age at enrollment was higher among term births than preterm births (11.2 weeks vs. 10.3 weeks); the median number of visits from enrollment to month 8 was 3 for both groups. Other than finding those delivering preterm were more likely to be nulliparous ($p<0.05$), we observed no statistically significant associations between demographic characteristics and preterm birth. (Table 4.1)

Among term births, cytokine distributions varied little over time, but some variations over time occurred among preterm births (figures 1-10). For the majority of cytokines, women who delivered preterm had higher concentrations compared to the corresponding percentiles for women who delivered at term. IL-1α was the only cytokine with observations up to the 60^{th} percentile that were statistically lower among preterm births compared to term births at all points evaluated. IL-1RA concentrations did not differ between groups but within the preterm group, shape of the distributions for IL-1RA and IL-1α appeared different.

Patterns of correlations among cytokines were similar for months 3 and 7 for term and preterm births, but correlations were stronger among women who delivered preterm (figures 11-16). Except for IL-1RA, IL-6 was positively correlated with all other cytokines at each time point with the exception at month 5 where among preterm, but not term births, IL-6 was negatively correlated with all cytokines. IL-1RA was positively correlated with other IL-1

cytokines (IL-1α and IL-1β) at all points among term births but negatively correlated among preterm births.

By pro and anti-inflammatory ratios, the majority of participants had balanced cytokine levels, i.e. both pro-inflammatory and anti-inflammatory scores were high or both scores were low; but birth outcome was associated with inflammatory state. This was true when limited to women with data at all time points (table 4.2a) and when all participants, regardless of number of samples, were included (table 4.2b).

In logistic regression models, individual cytokines averaged across the first trimester had the strongest associations with preterm birth; 14 cytokines were either statistically significant or marginally significant after adjusting for multiple testing. Overall average models had 11 estimates that were either statistically or marginally significant compared to 8 for second trimester concentrations. First trimester VEGF concentrations had the strongest association with preterm birth (odds ratio = 2.11; 95% CI 1.43, 3.10) and remained the strongest statistically significant predictor at other points during pregnancy (except for the 2^{nd} and 3^{rd} trimesters in the combined models). Although not statistically significant at every window evaluated, IL-1α was the only cytokine that exhibited a protective association across pregnancy. These analyses were conducted using the 16 cytokines where data were sufficient. Measures of cytokines IL-4, IL-8, INFγ, and IL-12p70 fell below the level of detection for >50% of samples.(Table 4.3) Complete information on all models including combined trimester models are presented in supplemental table S4.1.

4.5 Discussion

Ours is the first study that evaluated the associations of individual and groups of cytokines at multiple time points during pregnancy and risk of pre-term birth. Patterns of cytokines were statistically significant different between term and preterm births. Cytokine distributions appeared relatively stable throughout pregnancy among term births, compared to a more variable pattern for some cytokines among preterm births, notably, for preterm birth the production of cytokine IL-1α was low compared to IL-Ra.

The IL-1 family of cytokines represents a partially closed system that can potentially be used as a proxy for pro- and anti-inflammatory processes. IL-1α and IL-1β are pro-inflammatory members of the IL-1 family while IL-1ra, which acts as an anti-inflammatory cytokine, inhibits the actions of both pro-inflammatory cytokines IL-1α and IL-1β by binding to cell receptors without activating cells.[13] In our previous analysis of term births, these cytokines appeared highly regulated, maintaining stable average levels throughout pregnancy, even after adjusting for infection. (Buxton et al, unpublished data (Aim 1)). An unbalanced production of IL-1α and IL-1β, and IL-1Ra is associated with diseased states. For example, Simhan et al reported strong associations (odds ratio 7.7 (95% CI, 4.9-9.1) between women with high anti-inflammatory and low pro-inflammatory first trimester scores and subsequent delivery before 34 weeks of gestation. Even studies that did not specifically evaluate if IL-1α or IL-1β and IL-1RA concentrations were balanced have reported similar findings when pro-inflammatory cytokine levels were low. Kalinka et al reported strong associations between low levels of midgestation pro-inflammatory cytokines IL-1α and IL-1β and preterm birth among persons with lower genital tract infection.[28]

The hypothesized mechanism is that an impaired pro-inflammatory response permits lower reproductive tract pathogens to ascend to the upper reproductive tract, which might increase susceptibility to chorioamnionitis.[28, 29] Chorioamnionitis, or inflammation of the membranes (chorion, amnion and placenta), is a known risk factor for preterm birth and is associated with several maternal and neonatal adverse outcomes.[30]

In contrast, high pro-inflammatory response with a corresponding low anti-inflammatory response has also been associated with preterm birth. Genc et al reported that low concentrations of IL-1RA and or high concentrations of IL-1β in response to colonization of the lower reproductive tract by select pathogens was associated with spontaneous preterm birth.[15] These studies and our study suggest that deviations from a balanced inflammatory state, rather than categories of high versus low cytokine concentrations might be potentially more specific in identifying pathologies that may lead to preterm birth.

Supporting this idea, correlations between IL-1RA and IL-1α differed between term and preterm births. We found that IL-1RA was positively correlated with IL-1α at months 3, 5, and 7 among term births in contrast to negative correlations seen among preterm births. The expression of these two cytokines in opposite directions between term and preterm births further supports the idea that dysregulation of the anti-inflammatory cytokine IL-1RA and pro-inflammatory IL-1α is important in the pathology leading to preterm birth.

The fact that several first trimester cytokines were predictors of preterm birth in this study is exciting. This suggests that processes occurring early on in pregnancy are potentially useful in identifying women at risk of delivering before 37 completed weeks of gestation and

may represent an opportunity for early intervention to possibly reduce the risk of preterm birth.

Finally, several studies have previously evaluated associations between preterm birth and a small number of cytokines obtained from a single point during pregnancy. Although some of these studies found associations between cytokine levels and preterm birth, results from these studies have been inconsistent with results ranging from null, associations with low levels, and high levels. [2, 18, 19, 31] The reason for these inconsistencies is not entirely clear but has been previously reported to be associated with factors such as biomarker selection, and sampling timeframe.[2] Therefore, the simultaneous evaluation of up to 20 cytokines obtained monthly over the course of gestation from women who delivered at term and preterm is a main strength of this study.

However, several limitations need to be considered; first, data for the first trimester are represented by month 3 data only due to almost all participants (except two) being enrolled at three months of gestation or later. Therefore, the first trimester estimates are actually estimates for the third month of pregnancy. One could only speculate how the month 3 estimates would change when combined with data from months 1 and 2. Second, the small number of preterm births limited our ability to adjust for known confounders. Although data were collected on infection status, parity, and body mass index, etc. all logistic regression models were adjusted only for age. Moreover, the small number of preterm birth precluded the evaluation of preterm birth by etiology. Third, cytokine concentrations included left and right censored observations, and these were substituted with LOD/√2 and 10,010 respectively. Although substitution methods are commonly used in the analysis of data containing censored

observations, limitations have been reported when the percent above the LOD exceeds 25%.[32]

In conclusion, cytokine expression during the course of gestation differed between women who delivered at term compared to those who delivered before 37 weeks of gestation. Differences in cytokine expression and dysregulation of the anti and pro-inflammatory cytokines among women who delivered preterm were observed during the first trimester and indicate that problems or processes that potentially lead to preterm birth occur early in pregnancy. This implies that early dysregulation of cytokines could play a role in identifying women who may be at risk of preterm birth and may allow time for interventions to reduce preterm risks. Future studies should therefore consider balance in cytokine expression when evaluating factors that are associated with preterm birth.

Table 4.1. Demographic and obstetric characteristics of Mexican women who delivered term (N=78) and preterm babies (N=12), Mexico City, 2009-2014.

Age	**Term N(%)**	**Preterm N(%)**
<20	13(16.7)	4(33.3)
20-35	52(66.7)	8(66.7)
>35	13(16.7)	-
Pre-pregnancy BMI		
<18.5 kg/m^2	2(2.6)	-
18.5-24.9 kg/m^2	23(29.5)	6(50.0)
25-29.9 kg/m^2	27(34.6)	3(25.0)
≥30 kg/m^2	11(14.1)	1(8.3)
Missing	15(19.2)	2(16.7)
Parity[†]		
Nulliparous	19(24.4)	8(66.7)
Parous	45(57.7)	4(33.3)
Missing	14(18.0)	-
Reproductive Tract Infection	**Probability of infection***	
Trimester 1	0.32	0.33
Trimester 2	0.33	0.44
Trimester 3	0.22	0.38

* Average of (number of infection per trimester/number of visits for each participant per trimester)

[†] Fisher's exact test < 0.05

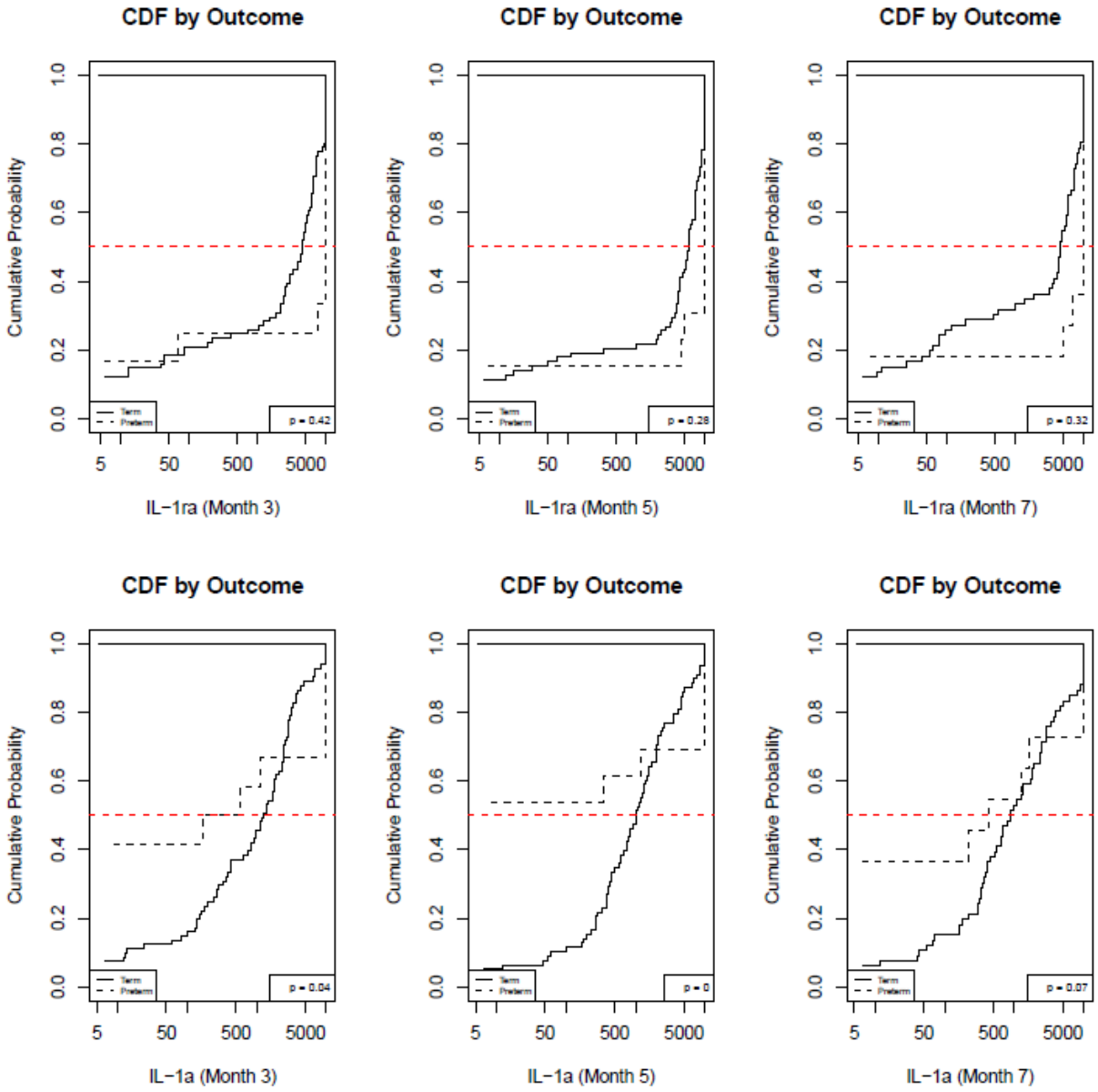

Figure 4.1: Cumulative distribution functions of cytokines (IL-1RA and IL-1α) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

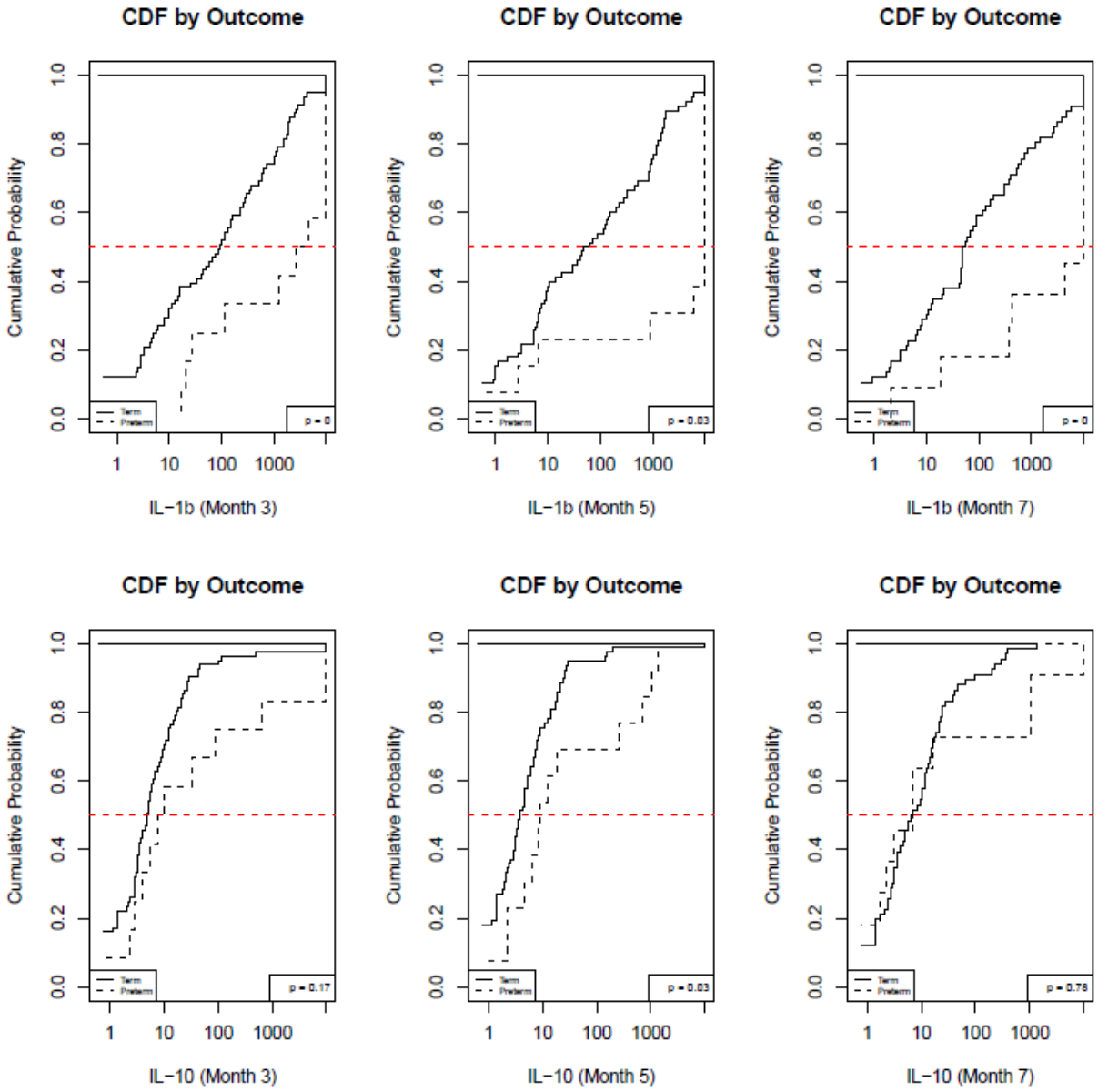

Figure 4.2: Cumulative distribution functions of cytokines (IL-1β and IL-10) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

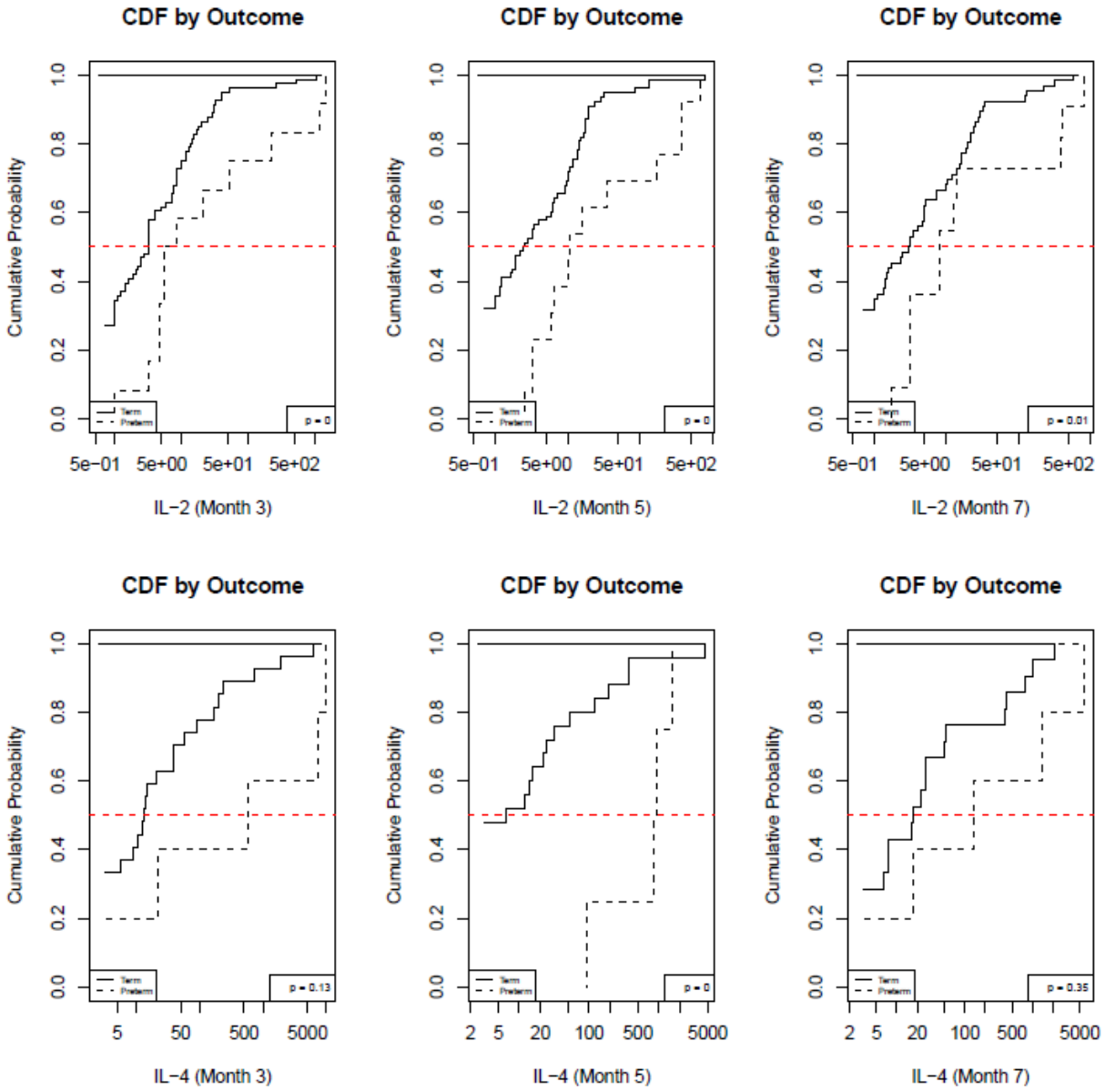

Figure 4.3: Cumulative distribution functions of cytokines (IL-2 and IL-4) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

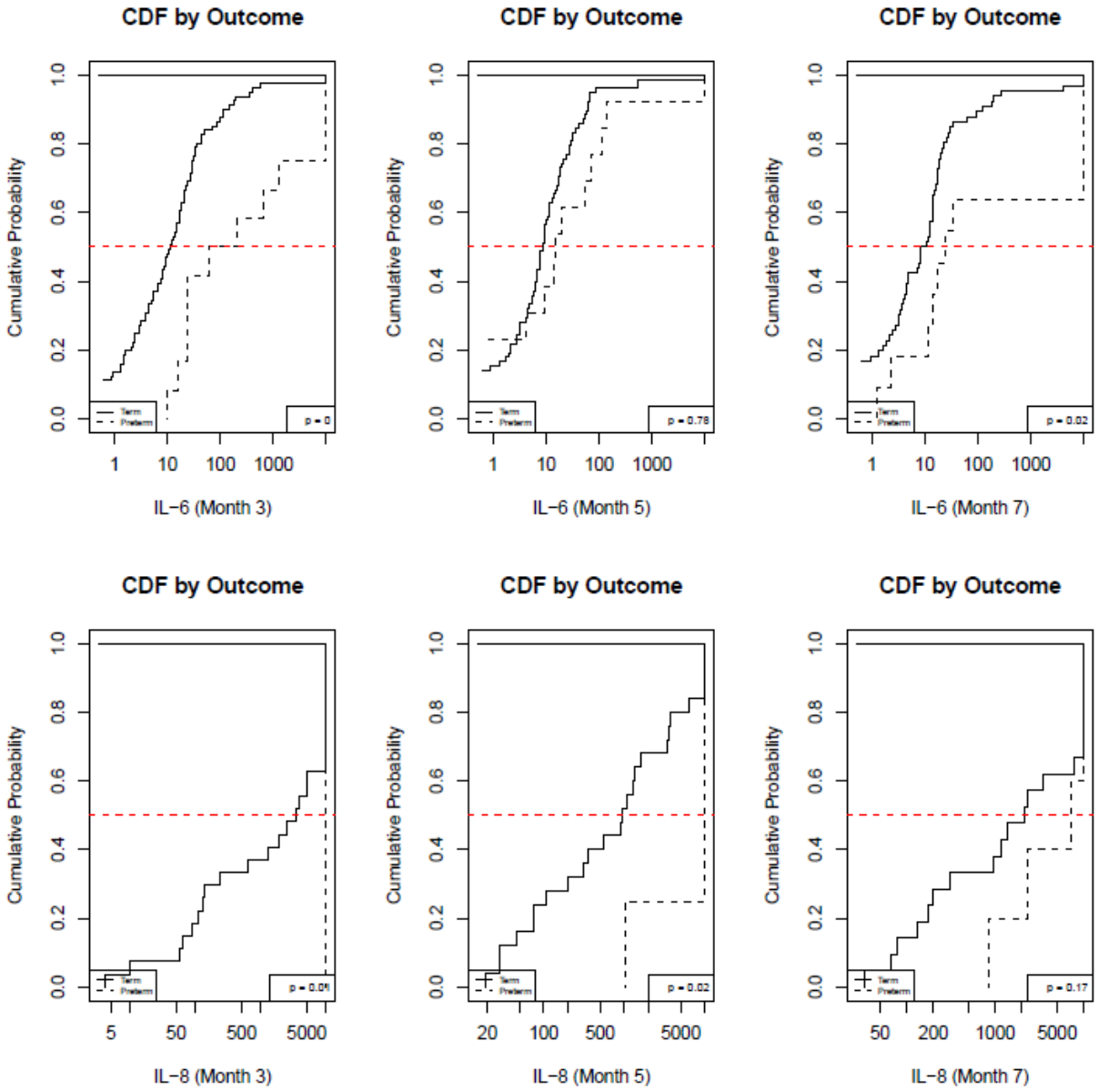

Figure 4.4: Cumulative distribution functions of cytokines (IL-6 and IL-8) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

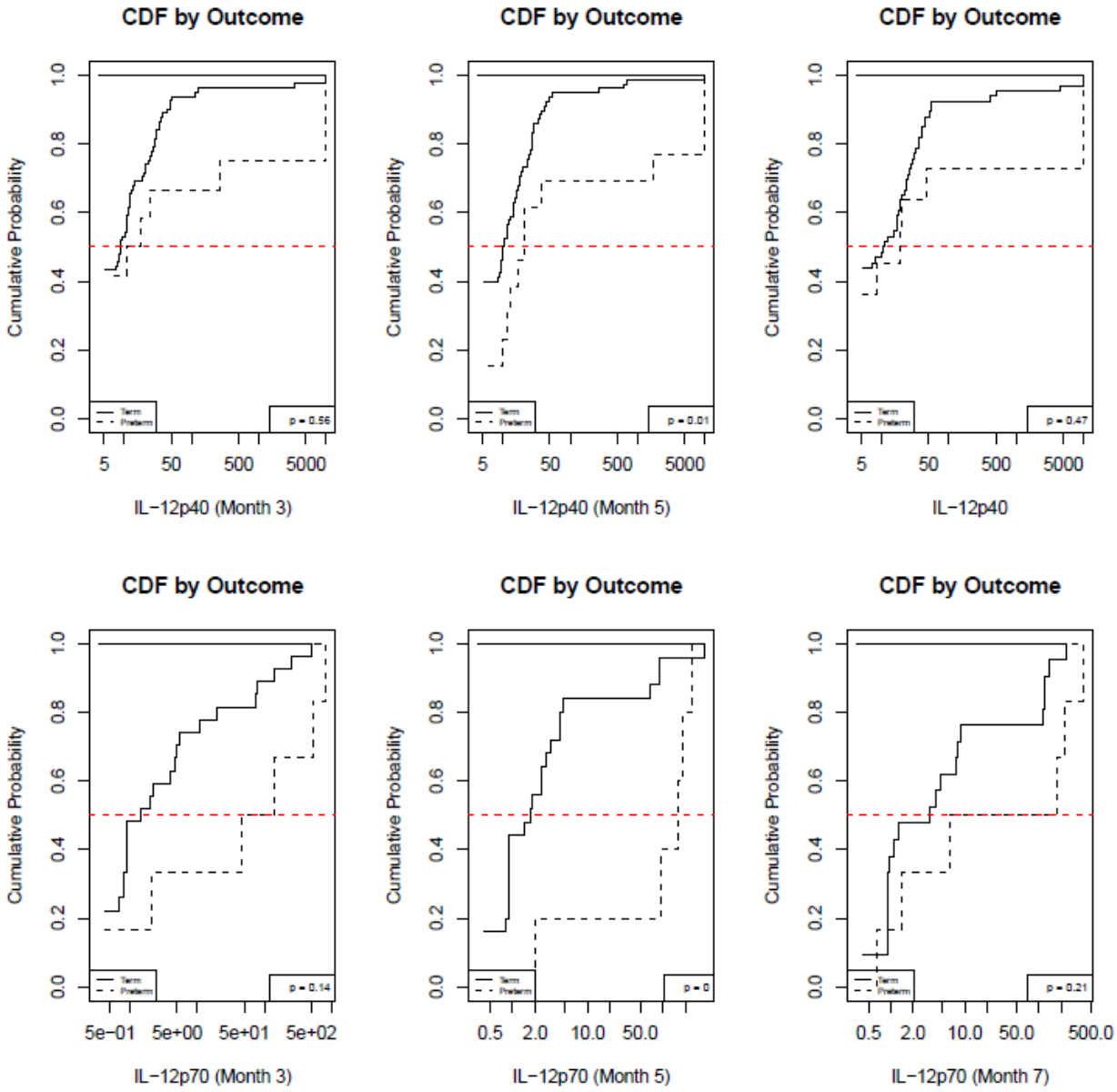

Figure 4.5: Cumulative distribution functions of cytokines (IL-12p40 and IL-12p70) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

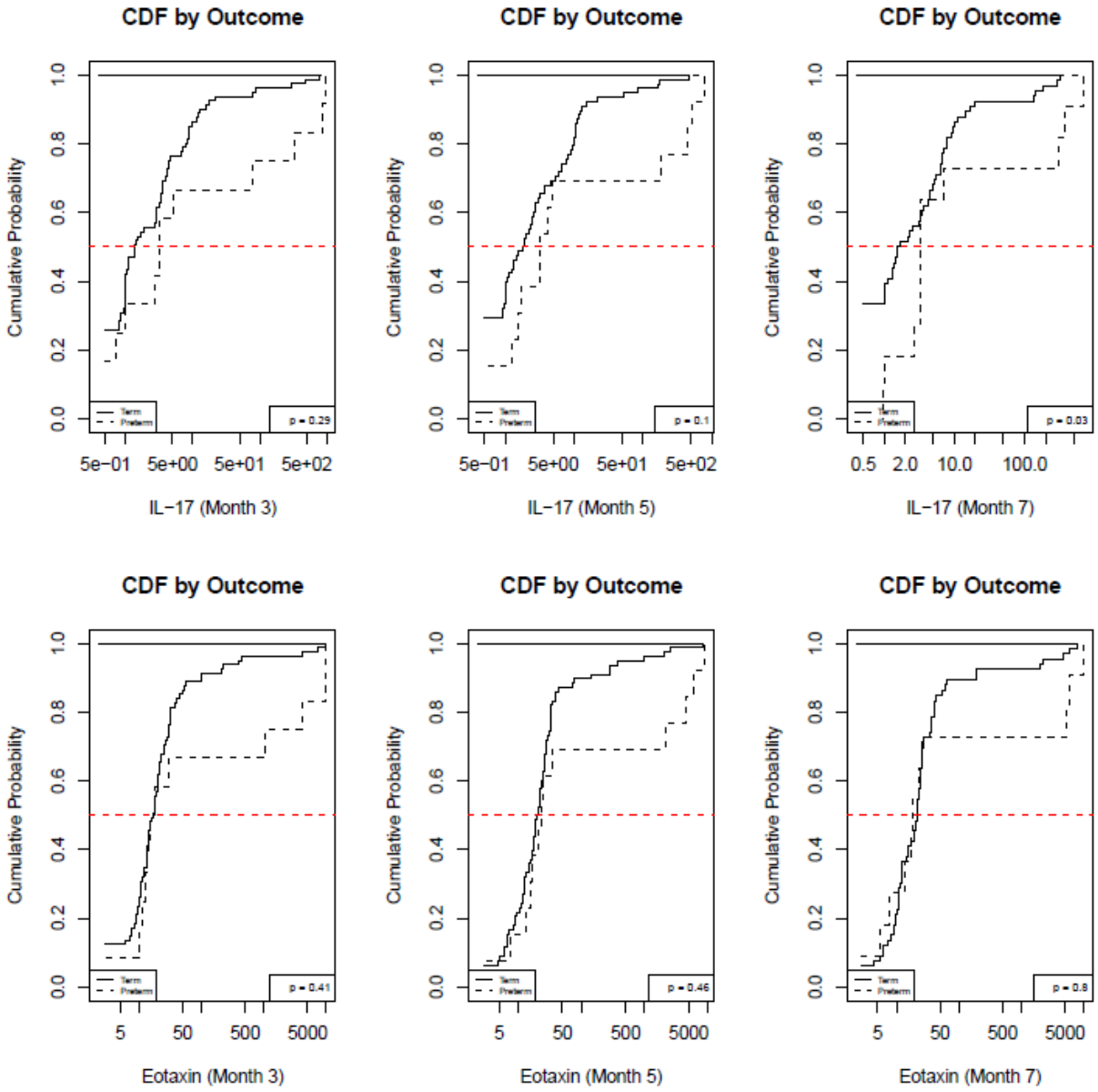

Figure 4.6: Cumulative distribution functions of cytokines (IL-17 and Eotaxin) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

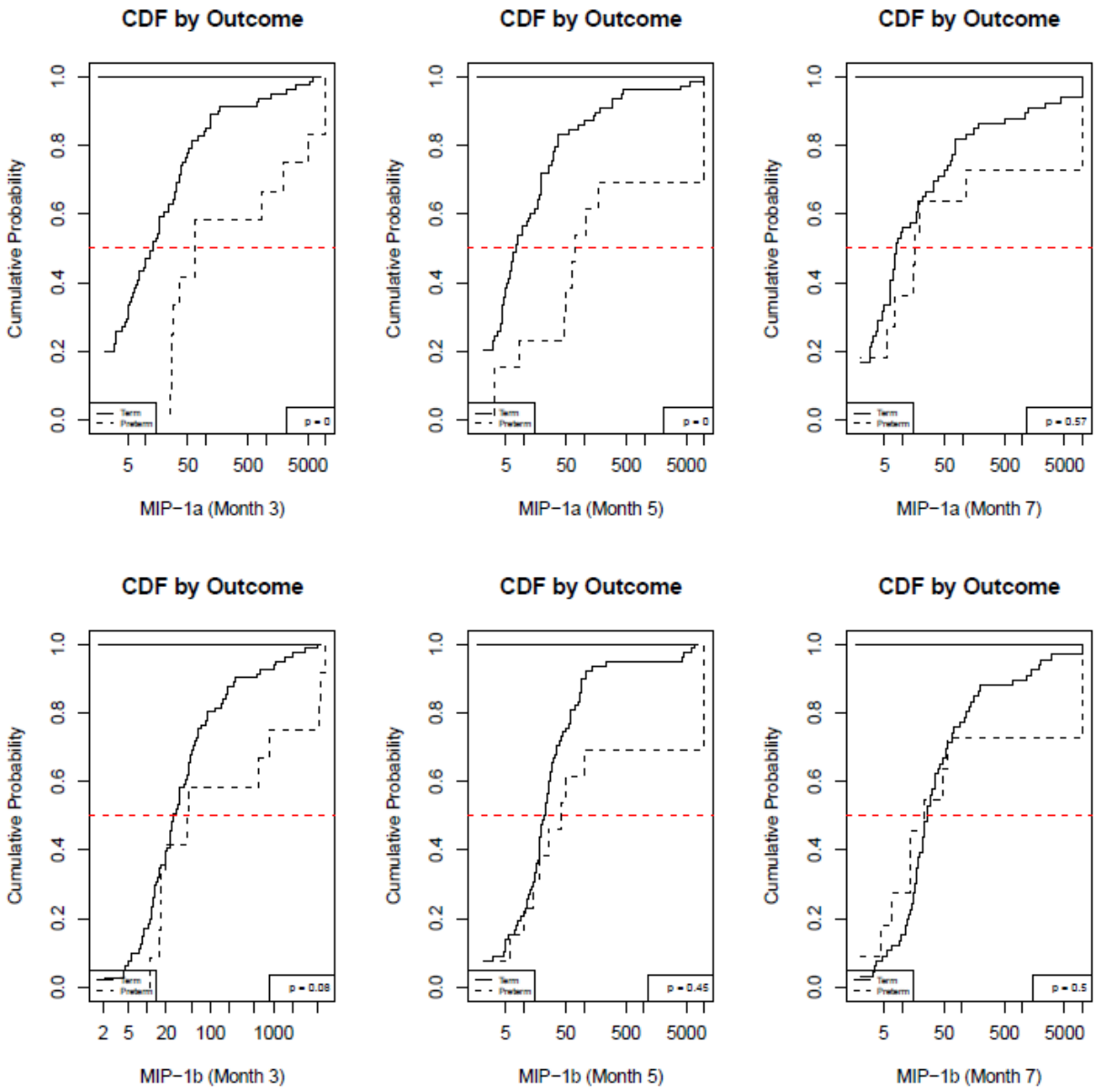

Figure 4.7: Cumulative distribution functions of cytokines (MIP-1α and MIP-1β) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

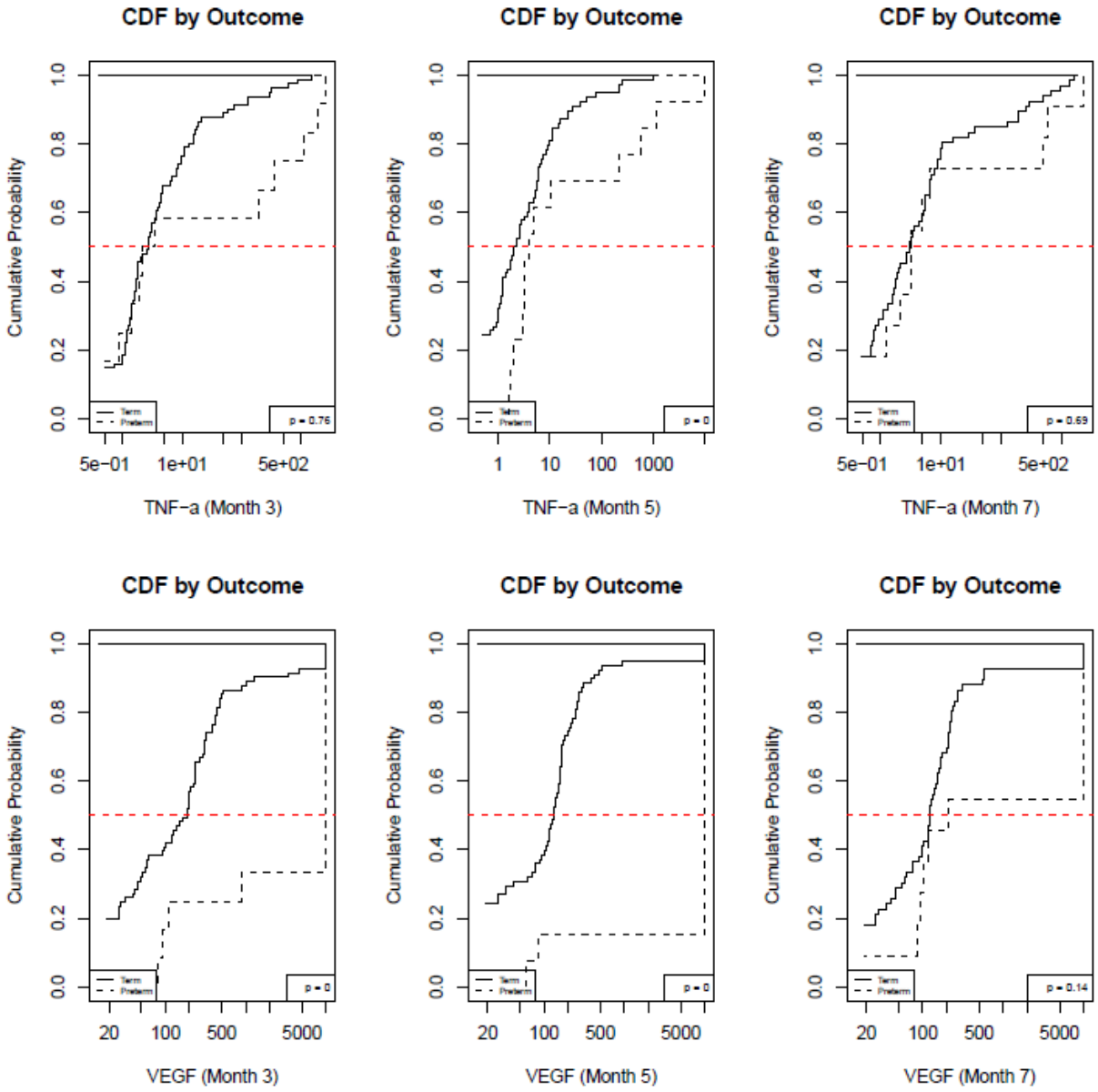

Figure 4.8: Cumulative distribution functions of cytokines (TNFα and VEGF) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

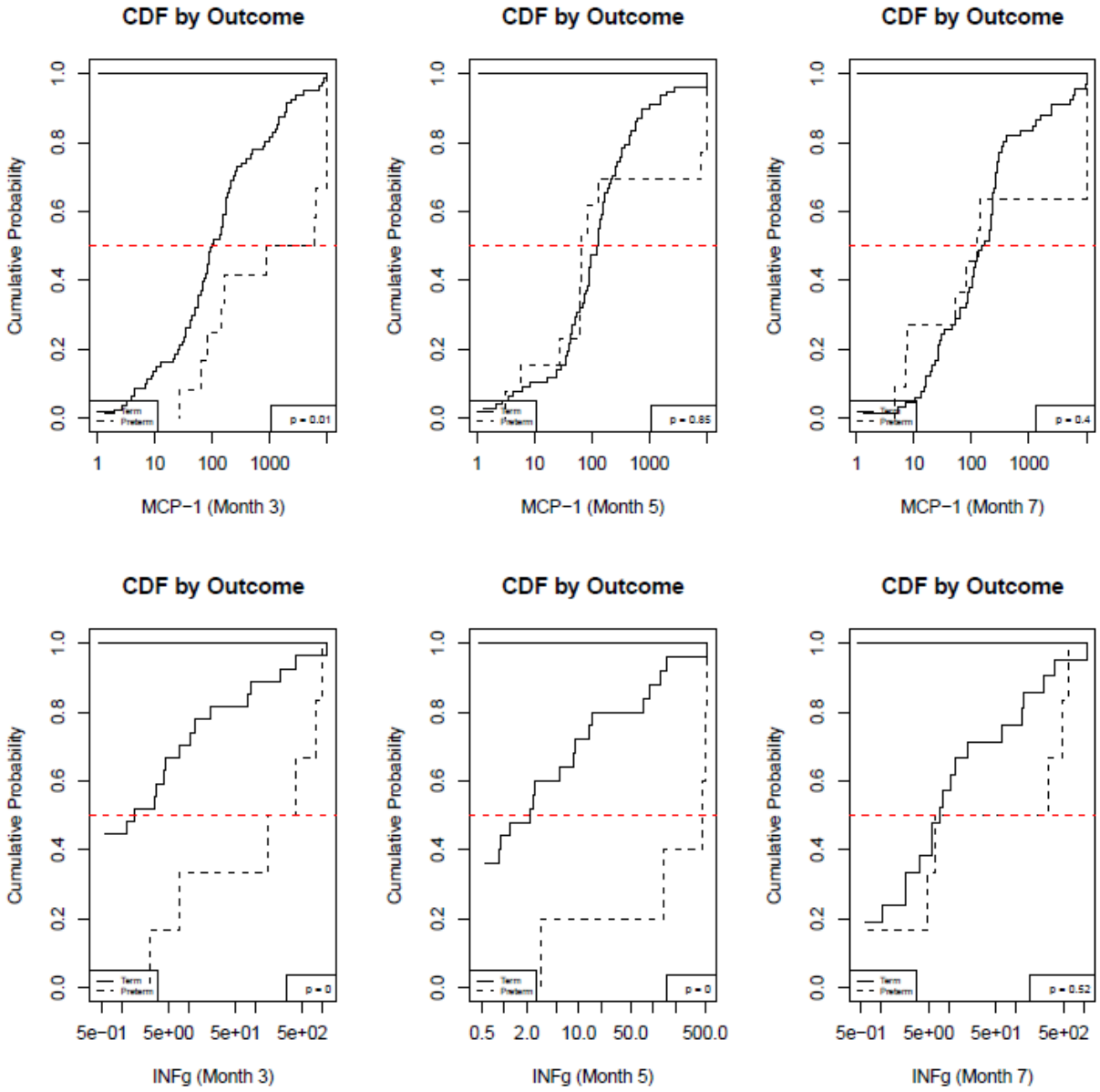

Figure 4.9: Cumulative distribution functions of cytokines (MCP-1 and INFγ) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

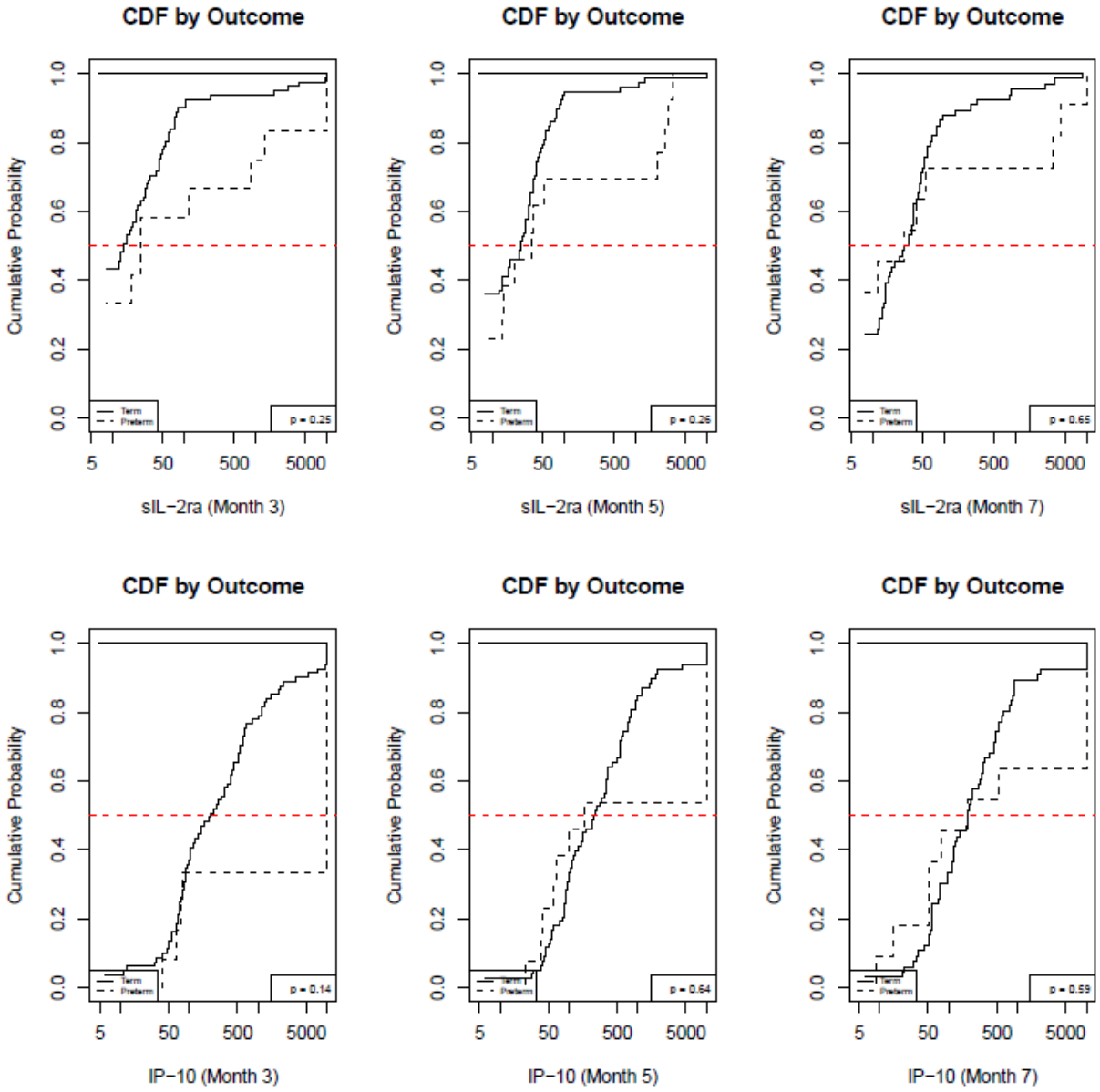

Figure 4.10: Cumulative distribution functions of cytokines (sIL-2Rα and IP-10) evaluated among term and preterm births at three time points during pregnancy among 90 Mexico City-based pregnant women, 2009-2014.

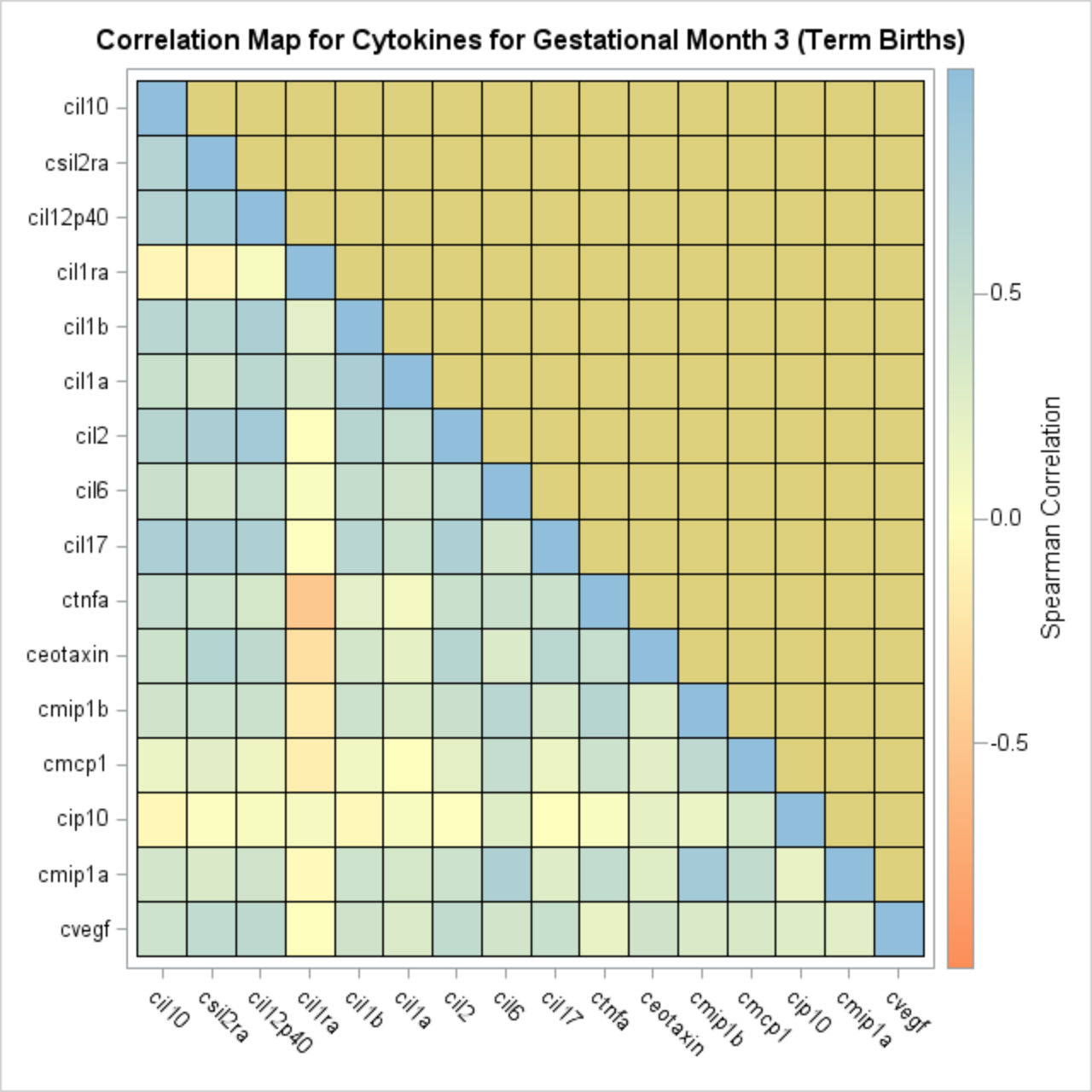

Figure 4.11: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered at term using data from gestation month 3.

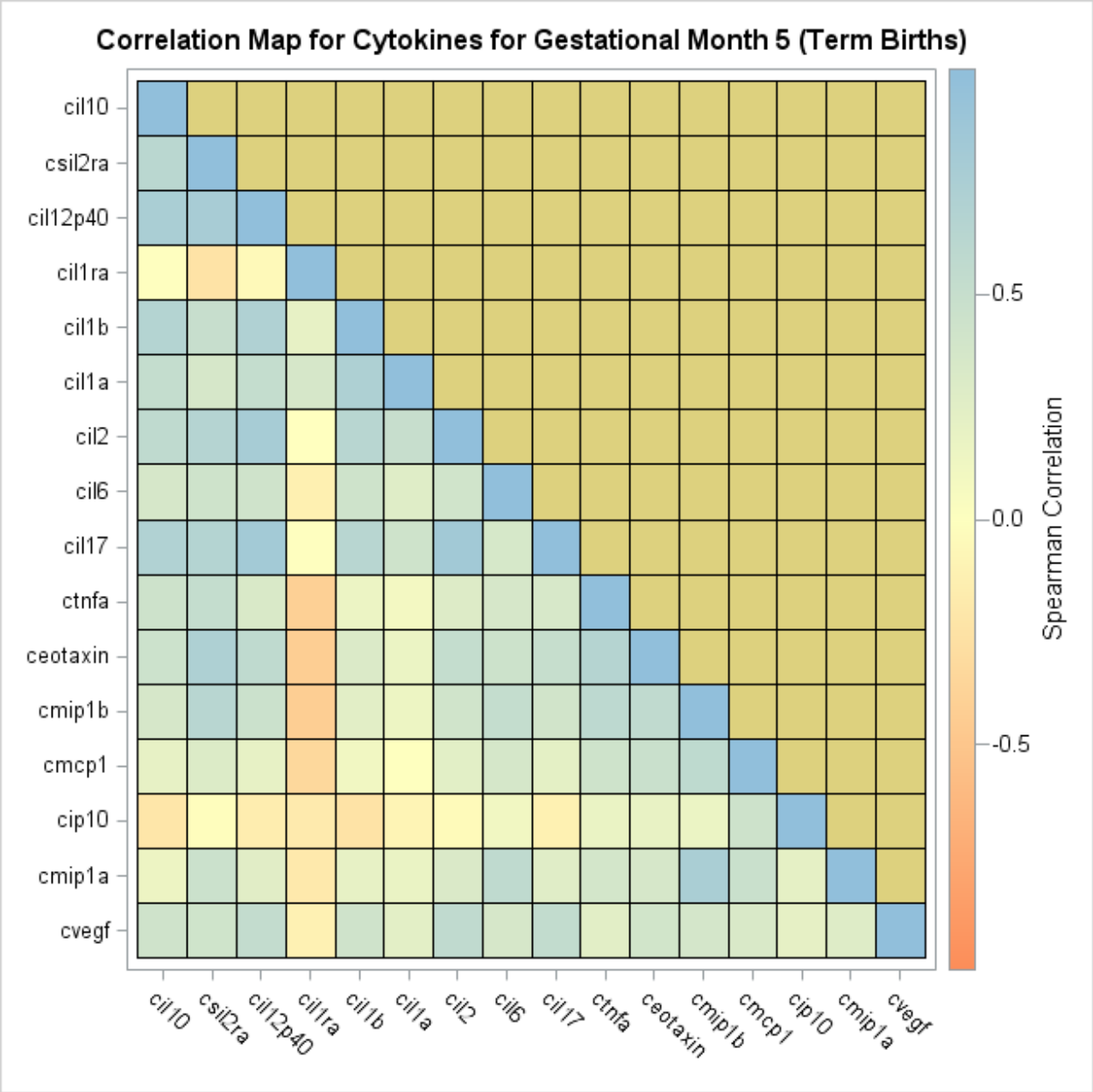

Figure 4.12: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered at term using data from gestation month 5.

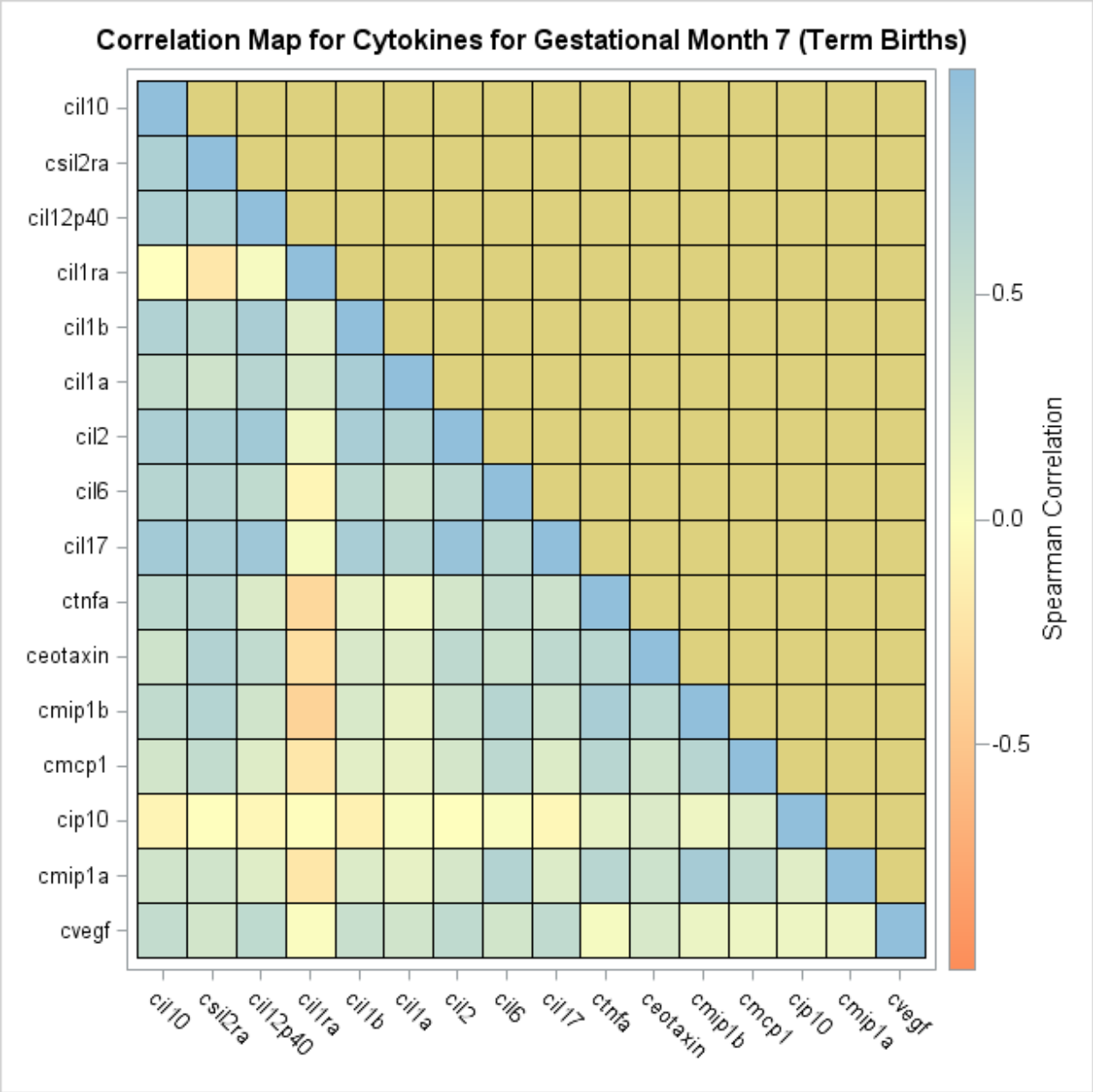

Figure 4.13: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered at term using data from gestation month 7.

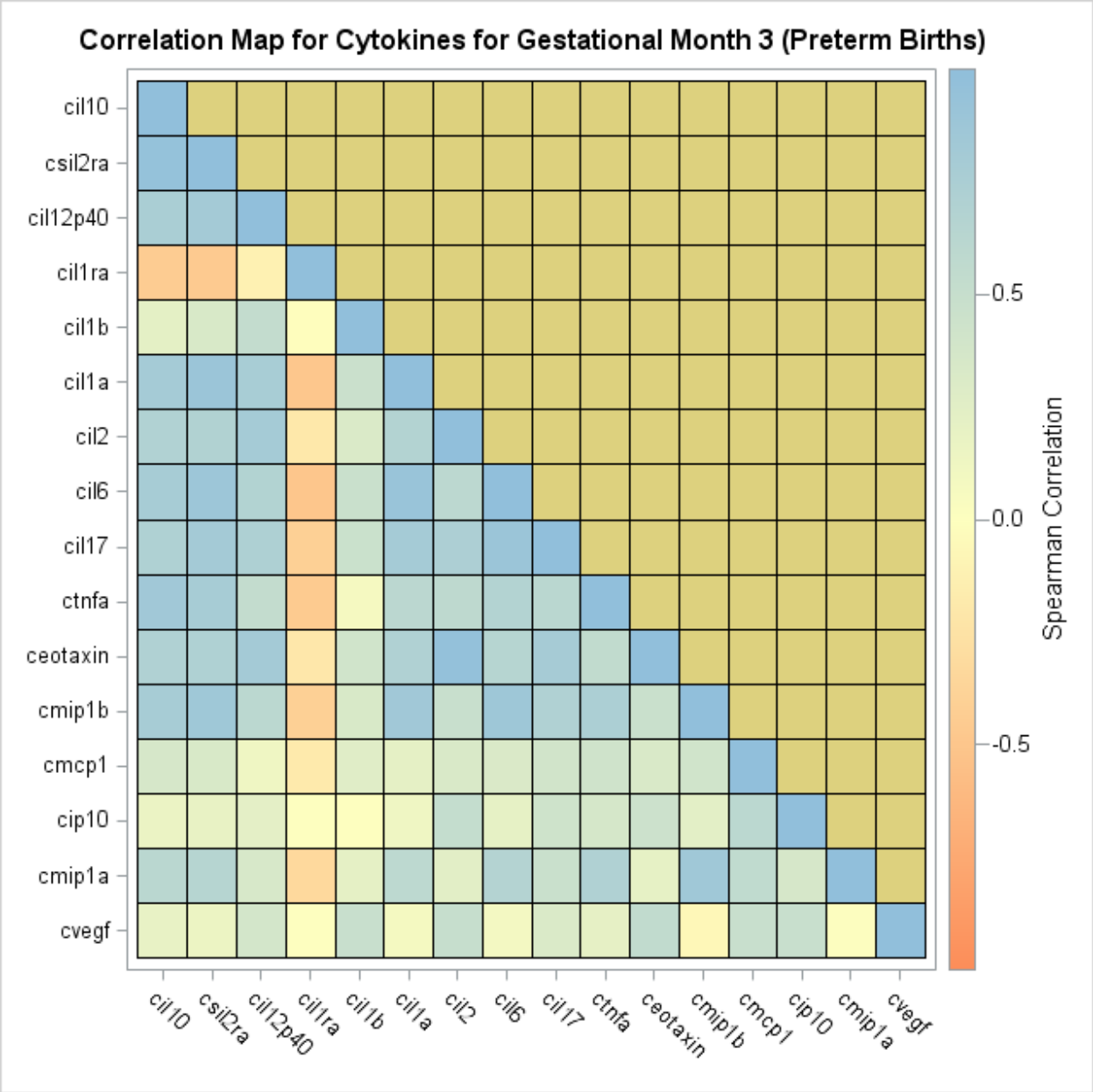

Figure 4.14: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered preterm using data from gestation month 3.

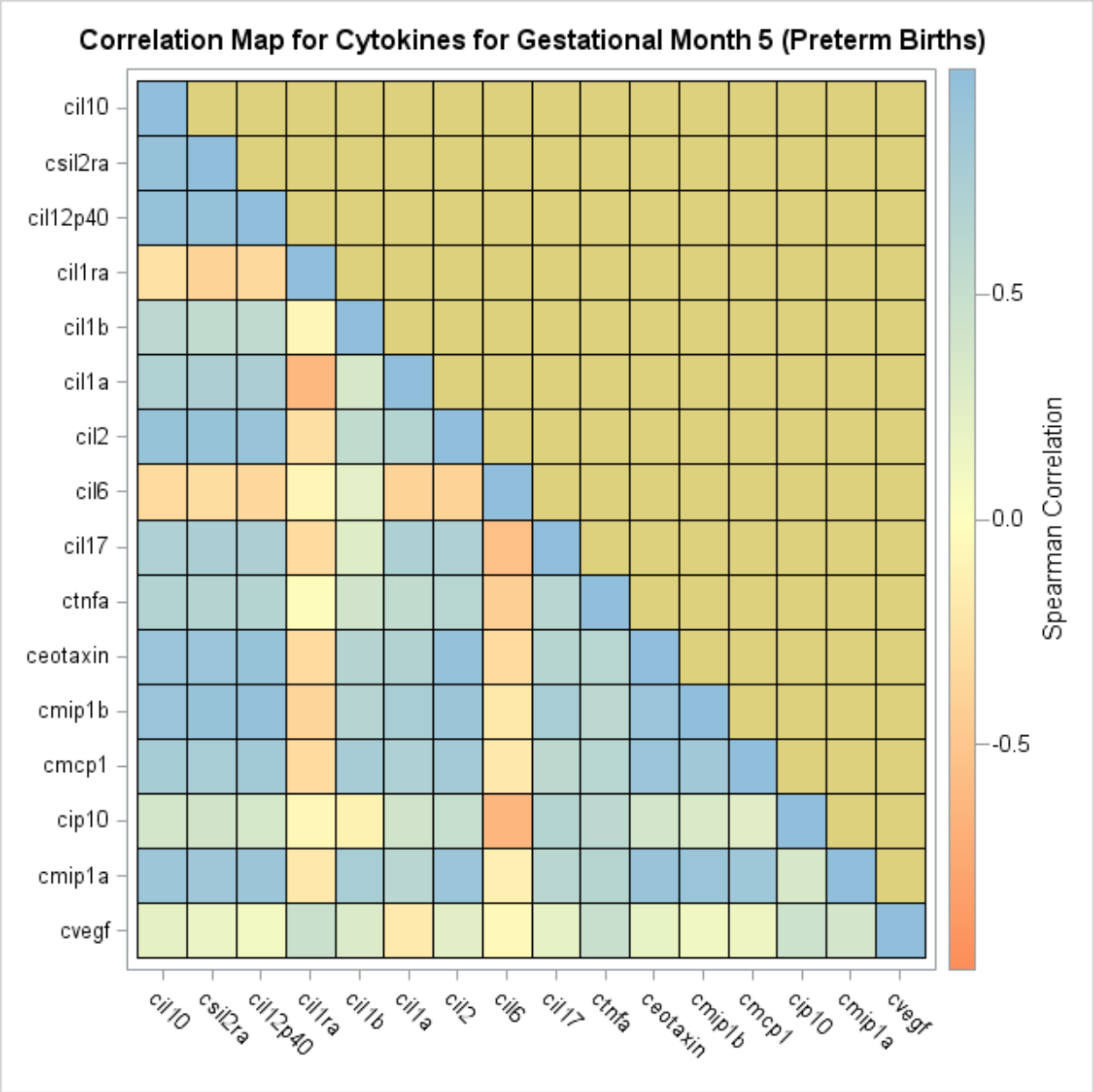

Figure 4.15: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered preterm using data from gestation month 5.

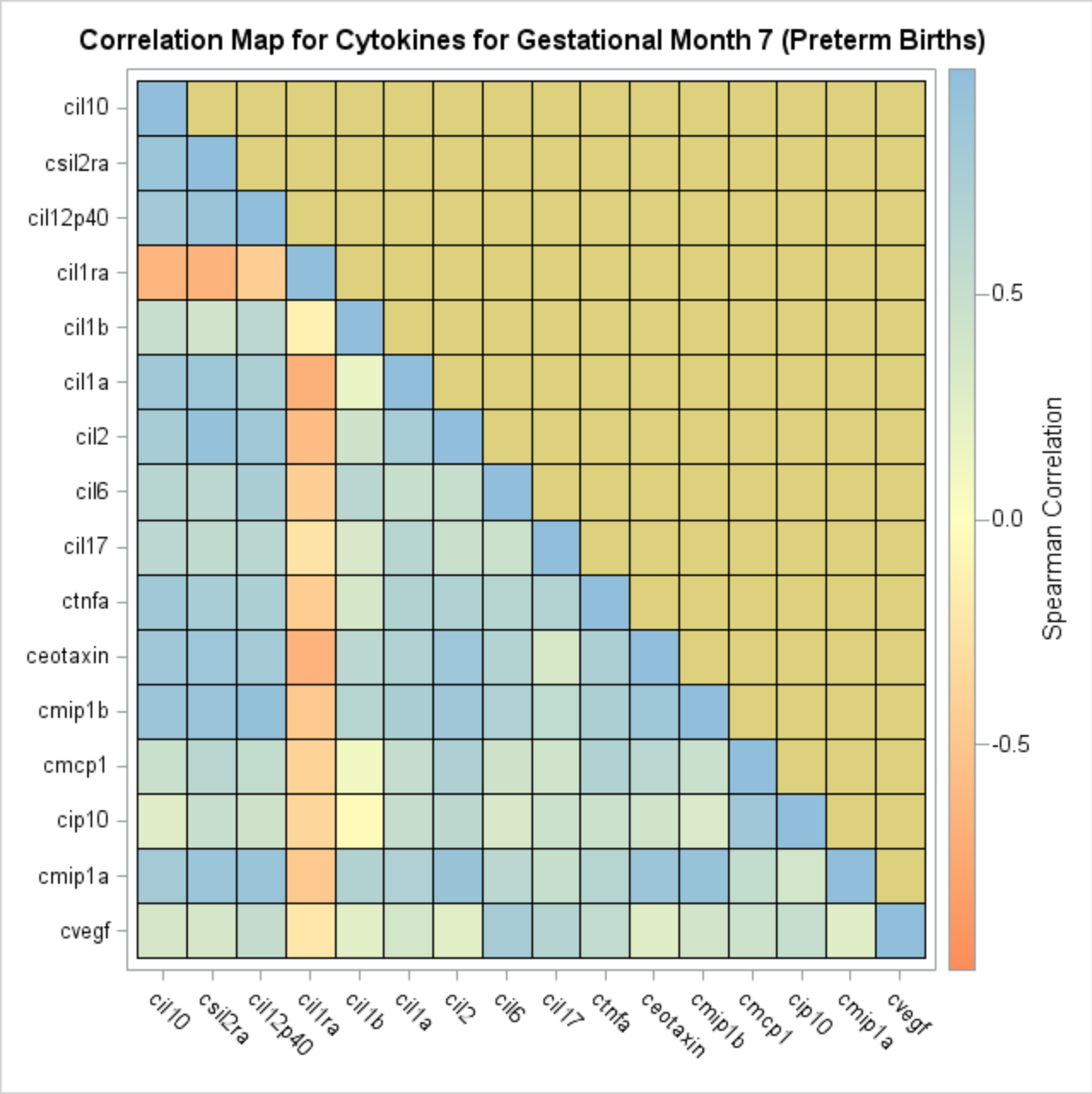

Figure 4.16: Correlation map based on Spearman coefficients among cervico-vaginal cytokines for women who delivered preterm using data from gestation month 7.

Table 4.2a. Categories based on pro-inflammatory and anti-inflammatory ratio scores by outcome among 90 pregnant women in Mexico City, 2009-2014		
Inflammatory State	**Term N(%)**	**Preterm (N%)**
	Trimester 1*	
High anti-/low pro-inflammatory	12(15.4)	-
High pro-/low anti-inflammatory	7(9.0)	5(41.7)
Balanced	59(75.6)	7(58.3)
	Trimester 2*	
High anti-/low pro-inflammatory	9(11.5)	-
High pro-/low anti-inflammatory	6(7.7)	4(33.3)
Balanced	63(80.8)	8(66.7)
	Trimester 3*	
High anti-/low pro-inflammatory	8(10.3)	1(8.3)
High pro-/low anti-inflammatory	5(6.4)	4(33.3)
Balanced	65(83.3)	7(58.3)

*Fisher's Exact test p-value < 0.05

Table 4.2b. Categories based on pro-inflammatory and anti-inflammatory ratio scores by outcome among pregnant women in Mexico City, 2009-2014 using data with varying degrees of follow up across trimesters.		
Inflammatory State	**Term N(%)**	**Preterm N(%)**
	Trimester 1* (N=98)	
High anti-/low pro-inflammatory	12(14.5)	-
High pro-/low anti-inflammatory	7(8.4)	6(40.0)
Balanced	64(77.1)	9(60.0)
	Trimester 2* (N=208)	
High anti-/low pro-inflammatory	21(12.8)	1(2.3)
High pro-/low anti-inflammatory	8(4.9)	13(29.6)
Balanced	135(82.3)	30(68.2)
	Trimester 3* (N=188)	
High anti-/low pro-inflammatory	14(9.3)	1(2.6)
High pro-/low anti-inflammatory	7(4.7)	7(18.4)
Balanced	129(86.0)	30(79.0)

*Fisher's Exact test p-value < 0.05

Table 4.3. Adjusted* odds ratios(OR) (95% confidence intervals) for preterm birth per log unit increase in cervico-vaginal cytokine concentration(pg/mL) from models† including one trimester alone (individual), and overall average (average), from a cohort in Mexico City, 2009-14.

		Individual Model Trimester Estimates OR (95% CI)			Average Estimate OR (95% CI)
Cytokine	**N**	**1st**	**2nd**	**3rd**	**Average**
Eotaxin	90	**1.40 (1.05, 1.87)**	1.30 (0.99, 1.71)	1.18 (0.88, 1.57)	1.30 (0.97, 1.74)
IL-10	90	**1.41 (1.07, 1.84)**	**1.34 (1.02, 1.74)**	1.21 (0.91, 1.62)	**1.37 (1.03, 1.83)**
IL-12p40	90	**1.40 (1.06, 1.84)**	**1.35 (1.03, 1.76)**	1.25 (0.96, 1.62)	**1.35 (1.02, 1.78)**
IL-17	90	**1.39 (1.04, 1.86)**	1.30 (0.98, 1.74)	1.35 (0.99, 1.85)	1.38 (1.01, 1.89)
IL-1α	90	0.79 (0.61, 1.02)	**0.72 (0.54, 0.97)**	**0.72 (0.54, 0.95)**	**0.69 (0.51, 0.94)**
IL-1β	90	**1.44 (1.07, 1.92)**	**1.35 (1.03, 1.76)**	1.26 (0.99, 1.60)	**1.40 (1.05, 1.87)**
IL-1RA	90	1.02 (0.79, 1.32)	1.10 (0.81, 1.49)	1.01 (0.79, 1.29)	1.05 (0.78, 1.41)
IL-2	90	**1.59 (1.14, 2.21)**	**1.55 (1.13, 2.13)**	1.40 (1.01, 1.94)	**1.61 (1.14, 2.29)**
IL-6	90	**1.76 (1.28, 2.42)**	1.32 (0.97, 1.78)	**1.43 (1.11, 1.83)**	**1.53 (1.13, 2.07)**
IP-10	90	**1.68 (1.19, 2.38)**	**1.62 (1.12, 2.34)**	1.35 (0.96, 1.88)	**1.66 (1.13, 2.43)**
MCP-1	90	**1.62 (1.17, 2.24)**	1.31 (0.94, 1.82)	1.26 (0.94, 1.70)	**1.45 (1.02, 2.06)**
MIP-1α	90	**1.63 (1.23, 2.16)**	**1.41 (1.09, 1.83)**	1.27 (1.01, 1.61)	**1.47 (1.12, 1.92)**
MIP-1β	90	**1.43 (1.05, 1.96)**	1.28 (0.96, 1.70)	1.20 (0.90, 1.60)	1.32 (0.98, 1.78)
sIL-2Rα	90	**1.39 (1.05, 1.85)**	1.32 (0.97, 1.79)	1.22 (0.89, 1.66)	1.34 (0.97, 1.84)
TNFα	90	**1.34 (1.04, 1.73)**	1.32 (1.01, 1.72)	1.28 (0.97, 1.68)	**1.38 (1.04, 1.84)**
VEGF	90	**2.11 (1.43, 3.10)**	**1.83 (1.29, 2.59)**	**1.49 (1.07, 2.09)**	**1.95 (1.34, 2.84)**

*Models adjusted for age

† Cytokine measures up to eight months were included in the models

Green font $p \leq 0.05$ after False Discovery Rate (FDR) adjustment for multiple testing

Blue font ≤ 0.1 after FDR adjustment for multiple testing

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CHAPTER 5

Dissertation Conclusion

5.1 Research Overview and Implications

Despite decades of research, preterm birth remains a public health problem of global significance with far reaching implications. These implications, which include permanent consequences such as long term morbidity and death, highlight that prevention rather than treatment of those with threatened labor is crucial in limiting the burden of preterm birth. Prevention is especially crucial in resource limited settings that lack the capacity (both in terms of medical personnel and facilities) to handle complications of pregnancy. However, even in resource rich settings, prevention is still important because treatment options for threatened labor [1] or for those at high risk have limitations, and subsequent preterm birth is associated with enormous medical and societal costs.[2]

This dissertation evaluated systemic and cervico-vaginal inflammation during pregnancy by characterizing cytokines over the course of gestation and explored how such information may be used to identify persons at risk for preterm birth. The findings that gestational inflammation is essential for the successful progression of pregnancy, that mean cytokine concentrations were stable over time, and that first trimester cytokines were most frequently predictive of preterm birth are especially important for preterm birth epidemiology. Together, these three findings imply that there is potential utility in screening asymptomatic women to

evaluate for risk of preterm birth early in pregnancy. Additionally, we found that inflammation from systemic and lower reproductive tract sources were not correlated, and were differently associated with air pollution exposure. This suggests that systemic cytokines may represent processes that are less specific than those occurring in the immediate surroundings of the fetus, and the protection provided to the developing fetus by gestational organs might make systemic inflammatory markers too imprecise for use in spontaneous preterm birth studies, or for studies linking oxidative stress from environmental exposures to adverse birth outcomes.

5.2 Strengths and Limitations

The use of monthly data on 20 inflammatory markers obtained from cervico-vaginal exudates and serum is a major strength of this study. The combination of cytokines from both systemic circulation and the lower reproductive tract, and frequency of measurement during gestation, is rare in inflammation research, and this provided a unique opportunity to simultaneously evaluate the effects of cytokines from different points during pregnancy in the same women. Additionally, analyses included methods that appropriately accounted for characteristics of the data, rather than dropping observations and losing information as is sometimes done.

However, a number of limitations must be noted. First trimester data were limited due to an enrollment criterion that allowed participants to be enrolled up to eighteen weeks of gestation. Most participants enrolled during the third month of gestation; therefore the first trimester was represented by data from the third month of gestation. Also, although the inflammatory markers we measured were lower in the systemic circulation compared to the corresponding cervico-vaginal cytokines, other sources of systemic inflammation were not

accounted for in the evaluation of the association between air pollution and systemic inflammation. In addition, use of anti-inflammatory medication was not accounted for and this might have impacted cytokine levels in this population. Finally, the low power resulting from the small number of preterm births precluded the evaluation of preterm birth by etiology, and the ability to control for known confounders of the association between inflammation and preterm birth.

5.3 Future Research /Directions

The observation that the direction of the association between air pollution and cervico-vaginal inflammation was inconsistent with the expectation of increased inflammation levels requires further study. For now, these findings suggest that future studies should consider quantifying inflammation with a particular focus on the type of pollutant evaluated, as gaseous pollutants might operate through pathways that are different from particle-based pollutants. Specifically, a larger sample size will be required to evaluate these associations with appropriate statistical power, and to include the evaluation of both pro- and anti-inflammatory cytokines.

Currently, cytokines are not routinely evaluated in prenatal care. The majority of preterm birth cases occur spontaneously among women who are asymptomatic (preterm labor resulting in preterm birth and preterm premature rupture of membranes (PPROM) account for up to an average of 75% of preterm birth cases in some populations in the United States [3]); it is in this group that efforts to reduce preterm birth rates should be aimed as it will most likely effect preterm birth rates.

Current strategies used to treat women who present with threatened labor usually prolong gestation briefly but not long enough to change delivery status from preterm to term, and thus have not had a major impact in reducing preterm rates.[1] Therefore, it is time for the focus to shift to screening of asymptomatic women before they become symptomatic. With earlier detection of risk, treatment and prevention strategies might be more effective in reducing preterm birth rates, and also minimize the adverse impacts of preterm birth. Even in instances where persons with identified risks are refractory to treatment, the identification of a health facility with specialized services (both in terms of clinical personnel and advanced treatment options) will potentially prevent some of the sequelae of preterm birth such as mortality for mother and child.

Recommendations to screen asymptomatic women during the first trimester of pregnancy must consider the complexity of cytokines, and the costs associated with quantifying cytokines. Quantification of cytokines is expensive [4] and may play a role in the lack of interest in considering screening of asymptomatic women at least once during pregnancy. However, when compared to the 26 billion in 2005 dollars in estimated cost associated with preterm birth in the United States alone,[2] and the unquantifiable burden associated with morbidity leading to a reduced quality of life, and loss of a mother and/or a child, the costs seems minimal.

Additionally, since cytokines are pleiotropic and redundant in action, and because quantification of cytokines is expensive, future studies should aim to evaluate cytokines that represent a summary of inflammatory processes – that is, select cytokines that can be used to assess deviations from a balanced system. In this dissertation, we found differences in the

expected expression of IL-1 family cytokines anti-inflammatory IL-1RA and pro-inflammatory IL-1α among term and preterm birth as early as the first trimester. These cytokines may represent a cost efficient opportunity to use only a minimal number of cytokines to screen for preterm birth risk in the first trimester.

5.4 References

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APPENDICES

A. Table S2.1 Cytokine, the minimum detectable concentration in pg/ml, intra-assay coefficient of variation (CV) %, and inter-assay CV % respectively for each cytokine.

Table S2.1. Minimal detectable concentrations, intra- and inter-assay coefficients of variation for 20 human cytokines/chemokines using a MILLIPLEX® MAP kit). [30]

Analyte	Lower LOD	Intra-/ inter-assay CV	Analyte	Lower LOD	Intra-assay CV
Eotaxin	4.0 pg/ml	(7.20% , 10.8%)	IL-2	1.0 pg/ml	(2.10% , 6.3%)
IFNγ	0.8 pg/ml	(1.60% , 12.0%)	IL-4	4.5 pg/ml	(2.90% , 14.2%)
IL-10	1.1 pg/ml	(1.60% , 16.8%)	Il-6	0.9 pg/ml	(2.00% , 18.3%)
IL-12p40	7.4 pg/ml	(2.80% , 12.4%)	IL-8	0.4 pg/ml	(1.90% , 3.5%)
IL-12p70	0.6 pg/ml	(2.20% , 16.7%)	IP-10	8.6 pg/ml	(2.60% , 15.3%)
IL-17	0.7 pg/ml	(0.70% , 1.2%)	MCP-1	1.9 pg/ml	(1.50% , 7.9%)
IL-1RA	8.3 pg/ml	(2.10% , 10.7%)	MIP-1α	2.9 pg/ml	(1.90% , 14.5%)
sIL-2rα	11.2 pg/ml	(2.40% , 8.0%)	MIP-1β	3.0 pg/ml	(2.40% , 8.8%)
IL-1α	9.4 pg/ml	(3.30% , 12.8%)	TNFα	0.7 pg/ml	(2.60% , 13.0%)
IL-1β	0.8 pg/ml	(2.30% , 6.7%)	VEGF	6.3pg/ml	(3.70% , 10.4%)

B. Table S2.2. Spearman correlation coefficients, corresponding number of observations, and p-values adjusted for false discovery rate* for pairs of cervico-vaginal cytokines for months 3, 5 and 7 of gestation for term births in Mexico City

Table S2.2. Spearman correlation coefficients, corresponding number of observations, and p-values adjusted for false discovery rate* for pairs of cervico-vaginal cytokines for months 3, 5 and 7 of gestation for term births in Mexico City.

		Month 3			Month 5			Month 7		
Cytokine 1	**Cytokine 2**	**Rho**	**N**	**P-value**	**Rho**	**N**	**P-value**	**Rho**	**N**	**P-value**
cil4	cil10	0.52	31	0.00	0.48	73	0.00	0.55	53	0.00
cil4	csil2ra	0.71	31	0.00	0.51	73	0.00	0.65	53	0.00
cil10	csil2ra	0.66	86	0.00	0.67	157	0.00	0.79	122	0.00
cil4	cil12p40	0.71	31	0.00	0.64	73	0.00	0.66	53	0.00
cil10	cil12p40	0.65	86	0.00	0.65	157	0.00	0.66	122	0.00
csil2ra	cil12p40	0.79	86	0.00	0.67	157	0.00	0.66	122	0.00
cil4	cil1ra	0.25	31	0.20	0.09	73	0.49	0.14	53	0.36
cil10	cil1ra	0.08	86	0.53	0.06	157	0.52	0.00	122	0.97
csil2ra	cil1ra	0.05	86	0.67	0.15	157	0.07	0.09	122	0.34
cil12p40	cil1ra	0.12	86	0.29	0.01	157	0.88	0.09	122	0.33
cil4	cil1b	0.64	31	0.00	0.47	73	0.00	0.55	53	0.00
cil10	cil1b	0.60	86	0.00	0.67	157	0.00	0.61	122	0.00
csil2ra	cil1b	0.62	86	0.00	0.48	157	0.00	0.55	122	0.00
cil12p40	cil1b	0.77	86	0.00	0.63	157	0.00	0.64	122	0.00
cil1ra	cil1b	0.33	86	0.00	0.37	157	0.00	0.42	122	0.00
cil4	cil1a	0.71	31	0.00	0.52	73	0.00	0.63	53	0.00
cil10	cil1a	0.44	86	0.00	0.51	157	0.00	0.44	122	0.00
csil2ra	cil1a	0.41	86	0.00	0.38	157	0.00	0.41	122	0.00
cil12p40	cil1a	0.64	86	0.00	0.52	157	0.00	0.54	122	0.00
cil1ra	cil1a	0.43	86	0.00	0.51	157	0.00	0.52	122	0.00
cil1b	cil1a	0.80	86	0.00	0.81	157	0.00	0.81	122	0.00
cil4	cil2	0.66	31	0.00	0.69	73	0.00	0.63	53	0.00
cil10	cil2	0.64	86	0.00	0.62	157	0.00	0.68	122	0.00
csil2ra	cil2	0.74	86	0.00	0.67	157	0.00	0.73	122	0.00
cil12p40	cil2	0.84	86	0.00	0.71	157	0.00	0.84	122	0.00
cil1ra	cil2	0.03	86	0.77	0.04	157	0.61	0.11	122	0.27
cil1b	cil2	0.66	86	0.00	0.64	157	0.00	0.64	122	0.00
cil1a	cil2	0.52	86	0.00	0.45	157	0.00	0.54	122	0.00
cil4	cil6	0.25	31	0.21	0.41	73	0.00	0.58	53	0.00
cil10	cil6	0.53	86	0.00	0.50	157	0.00	0.67	122	0.00

csil2ra	cil6	0.41	86	0.00	0.48	157	0.00	0.64	122	0.00
cil12p40	cil6	0.50	86	0.00	0.36	157	0.00	0.47	122	0.00
cil1ra	cil6	0.06	86	0.65	0.05	157	0.55	0.03	122	0.78
cil1b	cil6	0.51	86	0.00	0.46	157	0.00	0.56	122	0.00
cil1a	cil6	0.39	86	0.00	0.30	157	0.00	0.41	122	0.00
cil2	cil6	0.50	86	0.00	0.45	157	0.00	0.55	122	0.00
cil4	cil17	0.75	31	0.00	0.51	72	0.00	0.60	51	0.00
cil10	cil17	0.73	86	0.00	0.74	156	0.00	0.80	120	0.00
csil2ra	cil17	0.75	86	0.00	0.66	156	0.00	0.72	120	0.00
cil12p40	cil17	0.73	86	0.00	0.73	156	0.00	0.78	120	0.00
cil1ra	cil17	0.05	86	0.66	0.07	156	0.42	0.11	120	0.26
cil1b	cil17	0.62	86	0.00	0.65	156	0.00	0.70	120	0.00
cil1a	cil17	0.45	86	0.00	0.49	156	0.00	0.57	120	0.00
cil2	cil17	0.73	86	0.00	0.76	156	0.00	0.82	120	0.00
cil6	cil17	0.40	86	0.00	0.42	156	0.00	0.60	120	0.00
cil4	cil12p70	0.67	31	0.00	0.60	73	0.00	0.78	53	0.00
cil10	cil12p70	0.52	31	0.00	0.63	73	0.00	0.66	53	0.00
csil2ra	cil12p70	0.73	31	0.00	0.37	73	0.00	0.57	53	0.00
cil12p40	cil12p70	0.83	31	0.00	0.62	73	0.00	0.70	53	0.00
cil1ra	cil12p70	0.15	31	0.47	0.01	73	0.91	0.12	53	0.42
cil1b	cil12p70	0.66	31	0.00	0.57	73	0.00	0.72	53	0.00
cil1a	cil12p70	0.75	31	0.00	0.60	73	0.00	0.72	53	0.00
cil2	cil12p70	0.60	31	0.00	0.47	73	0.00	0.67	53	0.00
cil6	cil12p70	0.32	31	0.10	0.34	73	0.00	0.51	53	0.00
cil17	cil12p70	0.78	31	0.00	0.56	72	0.00	0.70	51	0.00
cil4	ctnfa	0.09	31	0.66	0.26	73	0.03	0.44	53	0.00
cil10	ctnfa	0.60	86	0.00	0.36	157	0.00	0.54	122	0.00
csil2ra	ctnfa	0.43	86	0.00	0.48	157	0.00	0.53	122	0.00
cil12p40	ctnfa	0.36	86	0.00	0.28	157	0.00	0.34	122	0.00
cil1ra	ctnfa	0.43	86	0.00	0.51	157	0.00	0.42	122	0.00
cil1b	ctnfa	0.24	86	0.04	0.02	157	0.84	0.07	122	0.44
cil1a	ctnfa	0.09	86	0.45	0.11	157	0.17	0.03	122	0.78
cil2	ctnfa	0.47	86	0.00	0.31	157	0.00	0.42	122	0.00
cil6	ctnfa	0.53	86	0.00	0.41	157	0.00	0.49	122	0.00
cil17	ctnfa	0.45	86	0.00	0.29	156	0.00	0.41	120	0.00
cil12p70	ctnfa	0.20	31	0.31	0.17	73	0.17	0.25	53	0.08
cil4	cil8	0.34	31	0.08	0.27	73	0.03	0.32	53	0.02
cil10	cil8	0.70	31	0.00	0.51	73	0.00	0.69	53	0.00
csil2ra	cil8	0.49	31	0.01	0.33	73	0.01	0.59	53	0.00
cil12p40	cil8	0.44	31	0.02	0.31	73	0.01	0.52	53	0.00
cil1ra	cil8	0.13	31	0.53	0.11	73	0.38	0.22	53	0.13
cil1b	cil8	0.68	31	0.00	0.64	73	0.00	0.69	53	0.00
cil1a	cil8	0.46	31	0.01	0.36	73	0.00	0.64	53	0.00

cil2	cil8	0.41	31	0.03	0.55	73	0.00	0.65	53	0.00
cil6	cil8	0.72	31	0.00	0.55	73	0.00	0.72	53	0.00
cil17	cil8	0.55	31	0.00	0.53	72	0.00	0.70	51	0.00
cil12p70	cil8	0.38	31	0.04	0.24	73	0.05	0.44	53	0.00
ctnfa	cil8	0.56	31	0.00	0.42	73	0.00	0.44	53	0.00
cil4	ceotaxin	0.59	31	0.00	0.60	73	0.00	0.81	53	0.00
cil10	ceotaxin	0.42	86	0.00	0.36	157	0.00	0.42	122	0.00
csil2ra	ceotaxin	0.67	86	0.00	0.55	157	0.00	0.55	122	0.00
cil12p40	ceotaxin	0.61	86	0.00	0.58	157	0.00	0.59	122	0.00
cil1ra	ceotaxin	0.14	86	0.23	0.36	157	0.00	0.26	122	0.00
cil1b	ceotaxin	0.45	86	0.00	0.26	157	0.00	0.30	122	0.00
cil1a	ceotaxin	0.32	86	0.00	0.16	157	0.06	0.19	122	0.04
cil2	ceotaxin	0.67	86	0.00	0.47	157	0.00	0.60	122	0.00
cil6	ceotaxin	0.31	86	0.01	0.36	157	0.00	0.37	122	0.00
cil17	ceotaxin	0.62	86	0.00	0.49	156	0.00	0.60	120	0.00
cil12p70	ceotaxin	0.74	31	0.00	0.52	73	0.00	0.63	53	0.00
ctnfa	ceotaxin	0.46	86	0.00	0.50	157	0.00	0.56	122	0.00
cil8	ceotaxin	0.24	31	0.24	0.04	73	0.77	0.24	53	0.10
cil4	cmip1b	0.11	31	0.59	0.35	73	0.00	0.52	53	0.00
cil10	cmip1b	0.48	86	0.00	0.38	157	0.00	0.53	122	0.00
csil2ra	cmip1b	0.45	86	0.00	0.57	157	0.00	0.60	122	0.00
cil12p40	cmip1b	0.45	86	0.00	0.43	157	0.00	0.42	122	0.00
cil1ra	cmip1b	0.16	86	0.19	0.52	157	0.00	0.39	122	0.00
cil1b	cmip1b	0.44	86	0.00	0.17	157	0.05	0.22	122	0.02
cil1a	cmip1b	0.28	86	0.01	0.03	157	0.76	0.11	122	0.26
cil2	cmip1b	0.45	86	0.00	0.38	157	0.00	0.49	122	0.00
cil6	cmip1b	0.67	86	0.00	0.54	157	0.00	0.57	122	0.00
cil17	cmip1b	0.33	86	0.00	0.36	156	0.00	0.41	120	0.00
cil12p70	cmip1b	0.33	31	0.09	0.30	73	0.01	0.34	53	0.02
ctnfa	cmip1b	0.69	86	0.00	0.76	157	0.00	0.79	122	0.00
cil8	cmip1b	0.61	31	0.00	0.47	73	0.00	0.54	53	0.00
ceotaxin	cmip1b	0.29	86	0.01	0.52	157	0.00	0.54	122	0.00
cil4	cmcp1	0.03	31	0.88	0.22	73	0.07	0.50	53	0.00
cil10	cmcp1	0.26	86	0.02	0.33	157	0.00	0.49	122	0.00
csil2ra	cmcp1	0.27	86	0.02	0.34	157	0.00	0.53	122	0.00
cil12p40	cmcp1	0.15	86	0.20	0.20	157	0.02	0.31	122	0.00
cil1ra	cmcp1	0.11	86	0.33	0.34	157	0.00	0.28	122	0.00
cil1b	cmcp1	0.15	86	0.21	0.09	157	0.31	0.19	122	0.04
cil1a	cmcp1	0.02	86	0.88	0.07	157	0.40	0.09	122	0.37
cil2	cmcp1	0.24	86	0.04	0.28	157	0.00	0.40	122	0.00
cil6	cmcp1	0.58	86	0.00	0.46	157	0.00	0.60	122	0.00
cil17	cmcp1	0.17	86	0.16	0.33	156	0.00	0.39	120	0.00
cil12p70	cmcp1	0.06	31	0.78	0.24	73	0.05	0.37	53	0.01

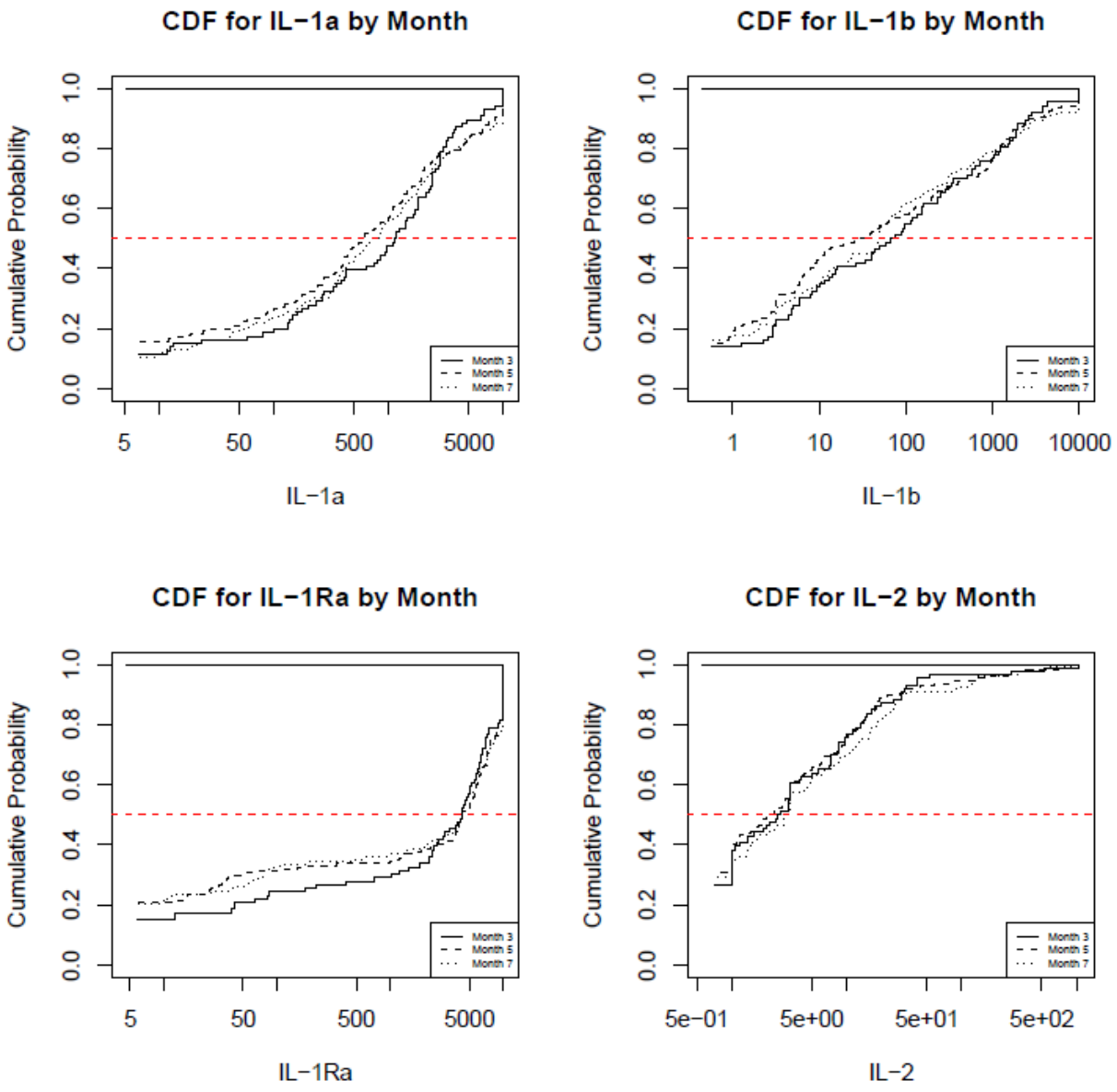
ctnfa	cmcp1	0.51	86	0.00	0.57	157	0.00	0.70	122	0.00
cil8	cmcp1	0.70	31	0.00	0.55	73	0.00	0.56	53	0.00
ceotaxin	cmcp1	0.26	86	0.02	0.47	157	0.00	0.54	122	0.00
cmip1b	cmcp1	0.63	86	0.00	0.65	157	0.00	0.72	122	0.00
cil4	cip10	0.59	31	0.00	0.46	73	0.00	0.64	53	0.00
cil10	cip10	0.05	86	0.67	0.04	157	0.62	0.03	122	0.75
csil2ra	cip10	0.08	86	0.52	0.11	157	0.18	0.01	122	0.89
cil12p40	cip10	0.13	86	0.27	0.12	157	0.15	0.08	122	0.40
cil1ra	cip10	0.17	86	0.14	0.05	157	0.57	0.07	122	0.47
cil1b	cip10	0.05	86	0.70	0.03	157	0.74	0.04	122	0.65
cil1a	cip10	0.17	86	0.16	0.14	157	0.10	0.22	122	0.02
cil2	cip10	0.06	86	0.65	0.09	157	0.31	0.10	122	0.30
cil6	cip10	0.27	86	0.02	0.26	157	0.00	0.15	122	0.11
cil17	cip10	0.04	86	0.73	0.09	156	0.28	0.04	120	0.65
cil12p70	cip10	0.61	31	0.00	0.55	73	0.00	0.59	53	0.00
Ctnfa	cip10	0.04	86	0.74	0.21	157	0.01	0.22	122	0.02
cil8	cip10	0.42	31	0.03	0.20	73	0.11	0.38	53	0.01
ceotaxin	cip10	0.33	86	0.00	0.44	157	0.00	0.35	122	0.00
cmip1b	cip10	0.17	86	0.16	0.23	157	0.00	0.17	122	0.07
cmcp1	cip10	0.35	86	0.00	0.41	157	0.00	0.33	122	0.00
cil4	cinfg	0.54	31	0.00	0.49	73	0.00	0.75	53	0.00
cil10	cinfg	0.56	31	0.00	0.47	73	0.00	0.62	53	0.00
csil2ra	cinfg	0.67	31	0.00	0.61	73	0.00	0.71	53	0.00
cil12p40	cinfg	0.82	31	0.00	0.68	73	0.00	0.71	53	0.00
cil1ra	cinfg	0.05	31	0.78	0.19	73	0.11	0.03	53	0.84
cil1b	cinfg	0.67	31	0.00	0.40	73	0.00	0.70	53	0.00
cil1a	cinfg	0.60	31	0.00	0.42	73	0.00	0.65	53	0.00
cil2	cinfg	0.79	31	0.00	0.51	73	0.00	0.72	53	0.00
cil6	cinfg	0.55	31	0.00	0.56	73	0.00	0.71	53	0.00
cil17	cinfg	0.61	31	0.00	0.32	72	0.01	0.72	51	0.00
cil12p70	cinfg	0.76	31	0.00	0.45	73	0.00	0.64	53	0.00
ctnfa	cinfg	0.38	31	0.05	0.54	73	0.00	0.47	53	0.00
cil8	cinfg	0.40	31	0.03	0.31	73	0.01	0.49	53	0.00
ceotaxin	cinfg	0.83	31	0.00	0.65	73	0.00	0.79	53	0.00
cmip1b	cinfg	0.54	31	0.00	0.62	73	0.00	0.57	53	0.00
cmcp1	cinfg	0.25	31	0.21	0.49	73	0.00	0.60	53	0.00
cip10	cinfg	0.64	31	0.00	0.57	73	0.00	0.54	53	0.00
cil4	cmip1a	0.05	31	0.78	0.24	73	0.05	0.46	53	0.00
cil10	cmip1a	0.41	86	0.00	0.29	157	0.00	0.46	122	0.00
csil2ra	cmip1a	0.31	86	0.01	0.46	157	0.00	0.50	122	0.00
cil12p40	cmip1a	0.36	86	0.00	0.26	157	0.00	0.34	122	0.00
cil1ra	cmip1a	0.09	86	0.46	0.29	157	0.00	0.20	122	0.04
cil1b	cmip1a	0.38	86	0.00	0.18	157	0.03	0.27	122	0.00

cil1a	cmip1a	0.29	86	0.01	0.12	157	0.15	0.21	122	0.03
cil2	cmip1a	0.40	86	0.00	0.32	157	0.00	0.45	122	0.00
cil6	cmip1a	0.73	86	0.00	0.58	157	0.00	0.60	122	0.00
cil17	cmip1a	0.26	86	0.02	0.27	156	0.00	0.33	120	0.00
cil12p70	cmip1a	0.25	31	0.21	0.25	73	0.04	0.26	53	0.07
ctnfa	cmip1a	0.55	86	0.00	0.61	157	0.00	0.66	122	0.00
cil8	cmip1a	0.59	31	0.00	0.44	73	0.00	0.49	53	0.00
ceotaxin	cmip1a	0.20	86	0.08	0.26	157	0.00	0.34	122	0.00
cmip1b	cmip1a	0.83	86	0.00	0.79	157	0.00	0.78	122	0.00
cmcp1	cmip1a	0.56	86	0.00	0.50	157	0.00	0.58	122	0.00
cip10	cmip1a	0.13	86	0.28	0.21	157	0.01	0.22	122	0.02
cinfg	cmip1a	0.37	31	0.05	0.52	73	0.00	0.48	53	0.00
cil4	cvegf	0.66	31	0.00	0.39	73	0.00	0.55	53	0.00
cil10	cvegf	0.43	86	0.00	0.51	157	0.00	0.51	122	0.00
csil2ra	cvegf	0.56	86	0.00	0.43	157	0.00	0.39	122	0.00
cil12p40	cvegf	0.63	86	0.00	0.52	157	0.00	0.58	122	0.00
cil1ra	cvegf	0.10	86	0.42	0.06	157	0.46	0.07	122	0.44
cil1b	cvegf	0.49	86	0.00	0.56	157	0.00	0.53	122	0.00
cil1a	cvegf	0.39	86	0.00	0.40	157	0.00	0.41	122	0.00
cil2	cvegf	0.59	86	0.00	0.58	157	0.00	0.51	122	0.00
cil6	cvegf	0.39	86	0.00	0.39	157	0.00	0.46	122	0.00
cil17	cvegf	0.52	86	0.00	0.63	156	0.00	0.60	120	0.00
cil12p70	cvegf	0.67	31	0.00	0.60	73	0.00	0.69	53	0.00
ctnfa	cvegf	0.21	86	0.07	0.10	157	0.22	0.14	122	0.13
cil8	cvegf	0.52	31	0.00	0.50	73	0.00	0.68	53	0.00
ceotaxin	cvegf	0.50	86	0.00	0.42	157	0.00	0.42	122	0.00
cmip1b	cvegf	0.32	86	0.00	0.26	157	0.00	0.20	122	0.04
cmcp1	cvegf	0.32	86	0.00	0.33	157	0.00	0.26	122	0.00
cip10	cvegf	0.36	86	0.00	0.26	157	0.00	0.21	122	0.02
cinfg	cvegf	0.84	31	0.00	0.39	73	0.00	0.64	53	0.00
cmip1a	cvegf	0.19	86	0.09	0.14	157	0.10	0.14	122	0.15

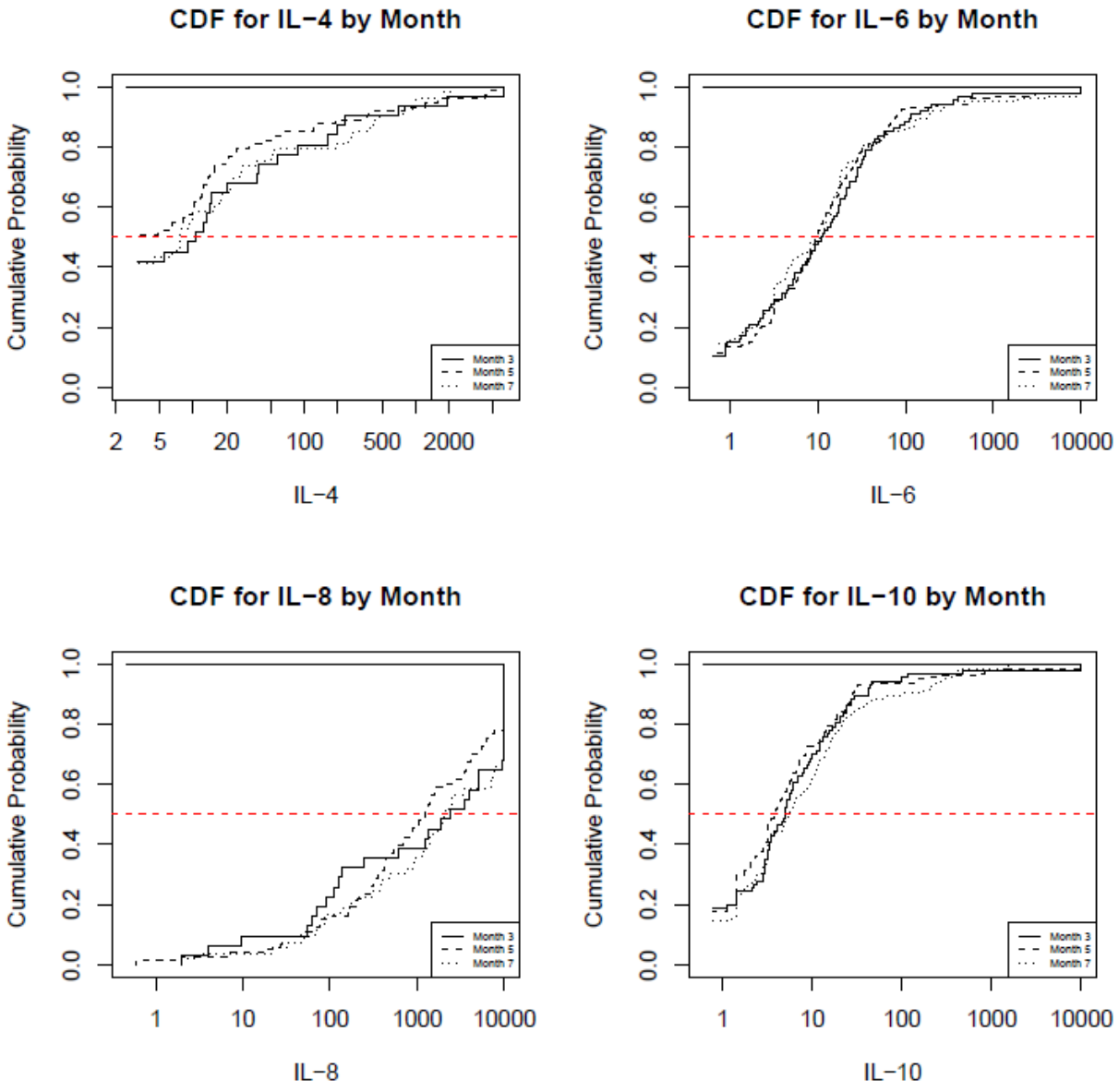
*False discovery rate based on Benjamini-Hochberg corrections for multiple testing

c prefix indicates cervico-vaginal cytokine

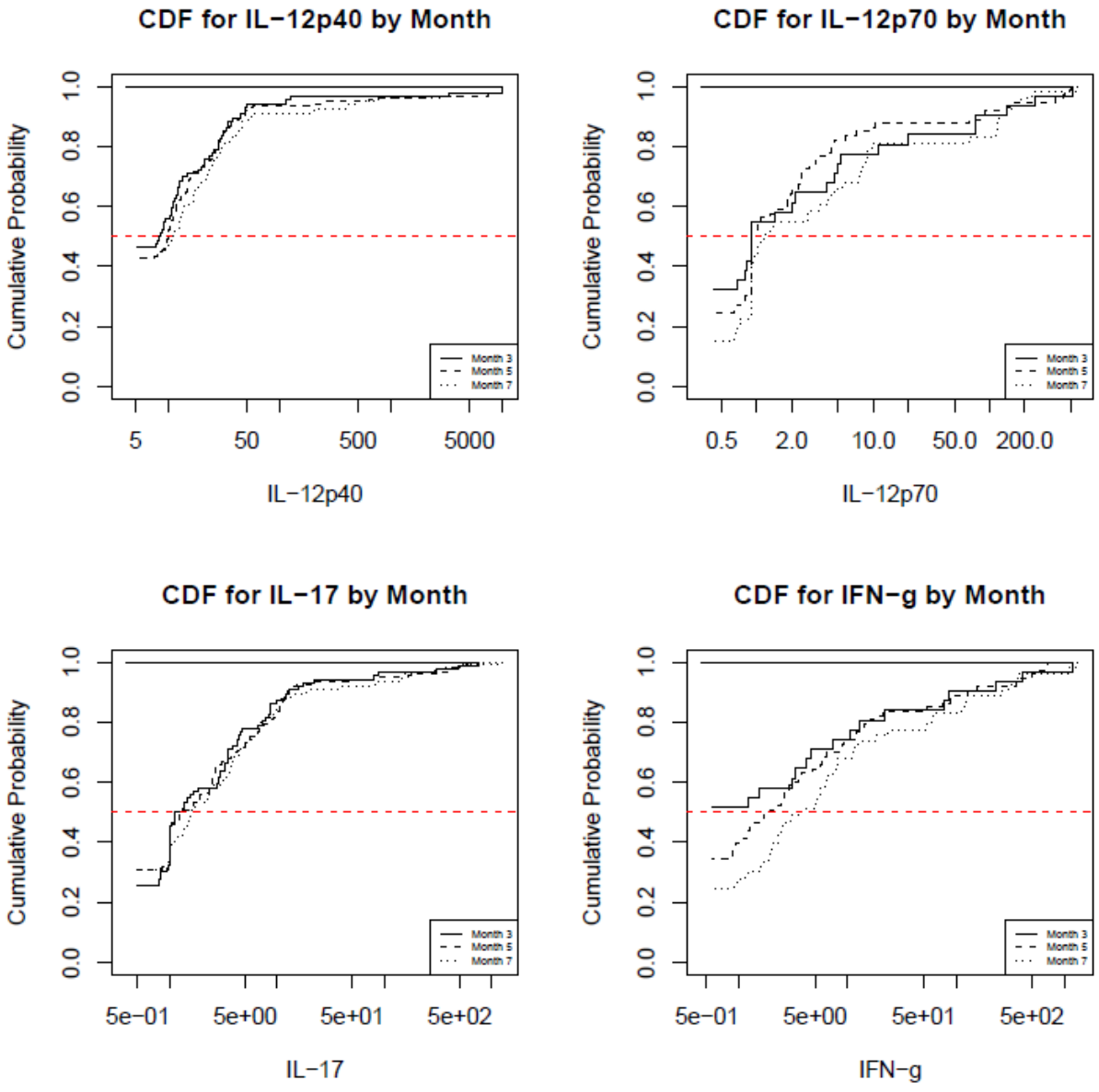
C. Figures S2.1. Cumulative distribution functions of cytokines (IL-1α, IL-1β, IL-1RA, and IL-2) evaluated among term births at three time points during pregnancy among 181 Mexico City-based pregnant women, 2009-2014.



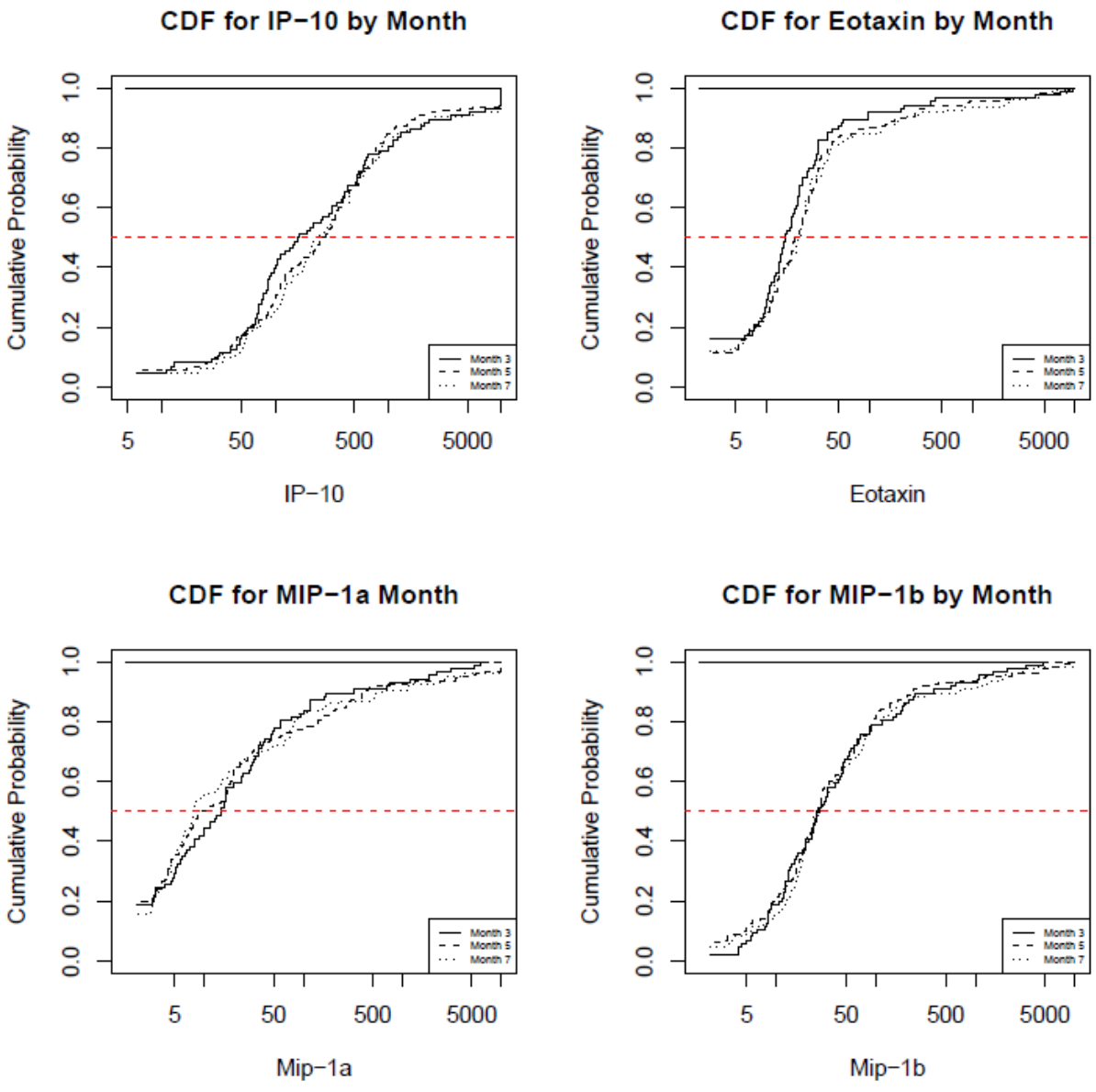
D. Figures S2.2. Cumulative distribution functions of cytokines (IL-4, IL-6, IL-8, and IL-10) evaluated among term births at three time points during pregnancy among 181 Mexico City-based pregnant women, 2009-2014.



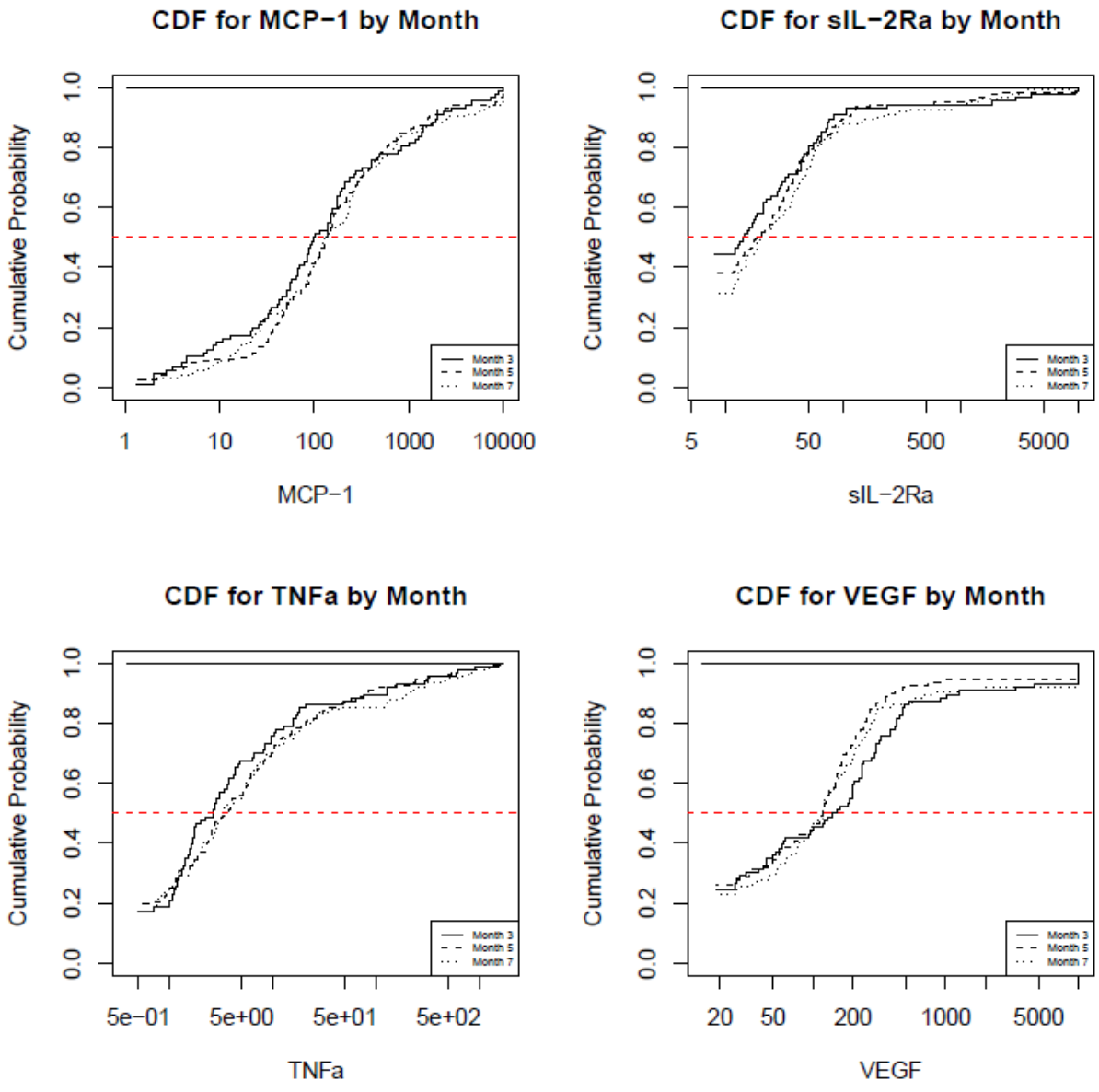
E. Figures S2.3. Cumulative distribution functions of cytokines (IL-12p40, IL-12p70, IL-17, and INF-γ) evaluated among term births at three time points during pregnancy among 181 Mexico City-based pregnant women, 2009-2014.



F. Figures S2.4. Cumulative distribution functions of cytokines (IP-10, Eotaxin, MIP-1α, and MIP-1β) evaluated among term births at three time points during pregnancy among 181 Mexico City-based pregnant women, 2009-2014.



G. Figures S2.5. Cumulative distribution functions of cytokines (MCP-1, sIL-2Rα, TNFα, and VEGF) evaluated among term births at three time points during pregnancy among 181 Mexico City-based pregnant women, 2009-2014.



H. Table S4.1. Table 4.3. Adjusted* odds ratios (OR) and 95% confidence intervals for preterm birth per log unit increase in cervico-vaginal cytokine concentration (pg/mL) from models† including one trimester alone (individual), all three trimesters entered (combined), and overall average (average), from a cohort in Mexico City, 2009-2014.

Table S4.1. Adjusted* odds ratios (OR) and 95% confidence intervals for preterm birth per log unit increase in cervico-vaginal cytokine concentration (pg/mL) from models† including one trimester alone (individual), all three trimesters entered (combined), and overall average (average), from a cohort in Mexico City, 2009-14.

		Individual Model Trimester Estimates OR (95% CI)			Combined Model Estimates OR (95% CI)			Average Estimate OR (95% CI)
Cytokine	**N**	**1st**	**2nd**	**3rd**	**1st**	**2nd**	**3rd**	**Average**
Eotaxin	90	**1.40 (1.05, 1.87)**	1.30 (0.99, 1.71)	1.18 (0.88, 1.57)	1.68 (0.65, 4.34)	1.82 (0.51, 6.56)	0.44 (0.14, 1.37)	1.30 (0.97, 1.74)
IL-10	90	**1.41 (1.07, 1.84)**	**1.34 (1.02, 1.74)**	1.21 (0.91, 1.62)	1.35 (0.86, 2.14)	1.31 (0.67, 2.55)	0.77 (0.42, 1.40)	**1.37 (1.03, 1.83)**
IL-12p40	90	**1.40 (1.06, 1.84)**	**1.35 (1.03, 1.76)**	1.25 (0.96, 1.62)	1.32 (0.73, 2.39)	1.42 (0.58, 3.47)	0.75 (0.36, 1.53)	**1.35 (1.02, 1.78)**
IL-17	90	**1.39 (1.04, 1.86)**	1.30 (0.98, 1.74)	1.35 (0.99, 1.85)	1.34 (0.78, 2.31)	0.92 (0.47, 1.83)	1.15 (0.62, 2.14)	1.38 (1.01, 1.89)
IL-1α	90	0.79 (0.61, 1.02)	**0.72 (0.54, 0.97)**	**0.72 (0.54, 0.95)**	0.98 (0.64, 1.48)	0.92 (0.51, 1.64)	0.78 (0.47, 1.30)	**0.69 (0.51, 0.94)**
IL-1β	90	**1.44 (1.07, 1.92)**	**1.35 (1.03, 1.76)**	1.26 (0.99, 1.60)	1.34 (0.90, 2.00)	1.07 (0.68, 1.69)	1.03 (0.71, 1.48)	**1.40 (1.05, 1.87)**
IL-1RA	90	1.02 (0.79, 1.32)	1.10 (0.81, 1.49)	1.01 (0.79, 1.29)	0.87 (0.56, 1.34)	1.54 (0.73, 3.24)	0.80 (0.49, 1.32)	1.05 (0.78, 1.41)
IL-2	90	**1.59 (1.14, 2.21)**	**1.55 (1.13, 2.13)**	1.40 (1.01, 1.94)	1.35 (0.84, 2.17)	1.51 (0.78, 2.91)	0.81 (0.45, 1.46)	**1.61 (1.14, 2.29)**
IL-6	90	**1.76 (1.28, 2.42)**	1.32 (0.97, 1.78)	**1.43 (1.11, 1.83)**	**1.74 (1.15, 2.63)**	0.69 (0.34, 1.41)	1.31 (0.71, 2.41)	**1.53 (1.13, 2.07)**
IP-10	90	**1.68 (1.19, 2.38)**	**1.62 (1.12, 2.34)**	1.35 (0.96, 1.88)	1.50 (0.97, 2.33)	1.28 (0.64, 2.55)	0.94 (0.56, 1.56)	**1.66 (1.13, 2.43)**
MCP-1	90	**1.62 (1.17, 2.24)**	1.31 (0.94, 1.82)	1.26 (0.94, 1.70)	**1.71 (1.15, 2.54)**	0.82 (0.49, 1.36)	1.19 (0.82, 1.72)	**1.45 (1.02, 2.06)**
MIP-1α	90	**1.63 (1.23, 2.16)**	**1.41 (1.09, 1.83)**	1.27 (1.01, 1.61)	**1.66 (1.13, 2.45)**	1.20 (0.67, 2.15)	0.81 (0.49, 1.36)	**1.47 (1.12, 1.92)**
MIP-1β	90	**1.43 (1.05, 1.96)**	1.28 (0.96, 1.70)	1.20 (0.90, 1.60)	1.46 (0.93, 2.30)	1.16 (0.57, 2.34)	0.83 (0.43, 1.60)	1.32 (0.98, 1.78)
sIL-2Rα	90	**1.39 (1.05, 1.85)**	1.32 (0.97, 1.79)	1.22 (0.89, 1.66)	1.55 (0.84, 2.85)	1.27 (0.52, 3.15)	0.68 (0.31, 1.49)	1.34 (0.97, 1.84)
TNFα	90	**1.34 (1.04, 1.73)**	1.32 (1.01, 1.72)	1.28 (0.97, 1.68)	1.26 (0.86, 1.85)	1.06 (0.62, 1.81)	1.04 (0.66, 1.65)	**1.38 (1.04, 1.84)**
VEGF	90	**2.11 (1.43, 3.10)**	**1.83 (1.29, 2.59)**	**1.49 (1.07, 2.09)**	**2.07 (1.16, 3.72)**	1.38 (0.68, 2.82)	0.72 (0.40, 1.28)	**1.95 (1.34, 2.84)**

*Models adjusted for age

† Cytokine measures up to eight months were included in the models

Green font p ≤ 0.05 after False Discovery Rate (FDR) adjustment for multiple testing

Blue font ≤ 0.1 after FDR adjustment for multiple testing