MATERNAL CIRCULATING IMMUNE FACTORS AND FETAL GROWTH: C-REACTIVE PROTEIN, TUMOR NECROSIS FACTOR - α, INTERLEUKIN - 6, IN MID-PREGNANCY AND BIRTHWEIGHT FOR GESTATIONAL AGE

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ABSTRACT

MATERNAL CIRCULATING IMMUNE FACTORS AND FETAL GROWTH: C-REACTIVE PROTEIN, TUMOR NECROSIS FACTOR - α, INTERLEUKIN - 6, IN MID-PREGNANCY AND BIRTHWEIGHT FOR GESTATIONAL AGE

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Inflammation pathways may contribute to fetal growth restriction. Observations of immune molecules such as the acute phase reactant, C-reactive protein (CRP) and fetal growth have produced mixed results, therefore further investigation is warranted. We analyzed data from 1,308 sub-cohort women enrolled at 16 -27 weeks completed gestation in the Pregnancy Outcomes and Community Health (POUCH) Study from 52 prenatal clinics in 5 Michigan communities. Using a US population reference of birthweights for gestational age, POUCH infants were grouped as small for gestational age (SGA) (≤10th percentile), large for gestational age (LGA) (≥ 90th percentile), or appropriate for gestational age (AGA). Levels of inflammatory signaling molecules Tumor Necrosis Factor - α (TNF-α), Interleukin – 6 (IL-6), and CRP were measured in maternal blood collected at enrollment and compared across groups who delivered either SGA, LGA, or AGA infants. Maternal TNF- α and IL-6 levels did not differ across the three groups. Unadjusted Mean CRP level of the SGA, 3.76 µg/L, was significantly lower than that of the AGA (5.43 µg/L, p=0.002) or LGA (6.36 µg/L) in linear regression models. Following adjustment for maternal age, race/ethnicity, pre-pregnancy BMI, gestational age at enrollment, Mean CRP values were 3.89 µg/L for SGA 5.41 µg/L for LGA 5.1, and µg/L for AGA (p=0.008 for SGA vs AGA). In sensitivity analyses, differences remained after excluding women with a pre-pregnancy BMI <18.5, hypertensive disorders of pregnancy, extreme CRP values, or antibiotic prescription < 2 weeks before blood draw. Further analysis should assess characteristics of the maternal-fetal interface for clues to relationships between maternal inflammatory status at 16 – 27 weeks gestation and birthweight outcomes.

Dedicated to my beautiful, smart, and wonderfully weird children,
Xavian, Blaise, and Lillian Glazier.

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KEY TO ABBREVIATIONS

CRP	C-Reactive Protein
POUCH	Pregnancy Outcomes and Community Health
TNF - α	Tumor Necrosis Factor Alpha
IL - 6	Interleukin 6
SGA	Small for Gestational Age
LGA	Large for Gestational Age
AGA	Appropriate for Gestational Age
BMI	Body Mass Index
LBW	Low Birth Weight
IUGR	Intrauterine Growth Restriction
PE	Preeclampsia
kDa	kilodalton
PTD	Preterm Delivery
hs-CRP	high sensitivity C-Reactive Protein
IL - 1β	Interleukin 1 Beta
IL – 10	Interleukin 10
PTX3	Pentraxin 3
IL – 8	Interleukin 8
IP-10	interferon-γ-inducible Protein
suPAR	insoluble urokinase plasminogen activator receptor
DII	Dietary Inflammatory Index
MSAFP	Maternal Serum Alpha-Fetoprotein
MVM	Maternal Vascular Malperfusion

INTRODUCTION

World Health Organization estimates that 20 million infants per year are born low birth weight (LBW), i.e. preterm and term birthweight < 2500 g.[1-4] In 2012, six Global Nutrition Targets were collectively endorsed, the third goal being a 30% reduction in LBW by 2025.[1] According to systematic analysis of LBW described in Lancet Global Health by Blencowe et al., worldwide prevalence decreased from 17.5 per 100 live births in 2010 to 14.6 per 100 live births in 2015, when approximately 20.5 million cases of LBW occurred (reported uncertainty range, 17.4 to 24.0 million). [5] A complex factors impact fetal growth and reducing the prevalence of LBW requires global improvement in the collection and analysis of birthweight data.[5] (Figure A.1). A separate measure is used to describe fetal growth specifically, i.e. small for gestational age (SGA). SGA is most often defined as a birthweight of < 10th percentile at a given gestational age and is sex specific. Infants born SGA may be constitutionally small or small due to intrauterine growth restriction (IUGR), meaning a disruption from the expected rate of fetal growth.[2-3] IUGR is the second leading cause of perinatal morbidity and mortality, and occurring in 3 - 8% percent of pregnancies.[6,7] Furthermore, IUGR results in significantly higher risk of intrauterine death and stillbirth.[6,7] Maternal risk factors for IUGR include alcohol use, clotting disorders, illicit drug use, hypertension, heart disease, kidney disease, inadequate nutrition and smoking. [6,7] Established risk factors for disruption of fetal growth include high altitudes, multiple pregnancy, placental dysfunction, preeclampsia (PE) or eclampsia, chromosomal abnormalities, infections such as rubella, cytomegalovirus, toxoplasmosis, and syphilis.[6,7] Investigators hypothesize that higher levels of pro-inflammatory molecules, in maternal or fetal blood, will be associated with IUGR and underlying causes, e.g. placental ischemia, maternal infection, infection of the maternal-fetal interface, or maternal adiposity.[8,9,13-33] However, inconsistent results have emerged among studies of association between the commonly measured marker of acute inflammation, C-Reactive Protein (CRP), and outcomes such as SGA or IUGR.[13-33]

CHAPTER 1.

Inflammation is implicated in the pathogenesis of restricted fetal growth but heterogeneity of association limits interpretation.[13-33] Continued investigation of immune regulation during gestation is justified given inconsistencies of study design and observed effect.[13-33] Indeed, maternal immune signaling molecules do far more than simply facilitate response to infection. Longitudinal studies observe profiles of dynamic concentration for a variety of immune molecules throughout the course of uncomplicated gestation.[9-11] Integral to successful pregnancy, these molecules contribute to the immunologic mediation of embryogenesis and implantation, modulation of the maternal immune response, and inflammatory maintenance of placental structure and function.[8-11,29,34-43] Differences of inflammatory profiles may indicate disruption of normal immune mechanisms required for ongoing vascular remodeling of the maternal uterine blood vessels.[9,32-34] Pathophysiology at the maternal-fetal interface may alter the expected trajectory of uteroplacental blood flow over the course of pregnancy for restriction of fetal nutrition.[6-13,35] Comorbidities of IUGR, PE and maternal obesity, remain integral considerations for assessment of inflammation pathways and risk of restricted fetal nutrition.[12-38]

Select pleiotropic molecules, namely the cytokines Tumor Necrosis Factor α (TNF - α) and Interleukin – 6 (IL - 6), may act as endogenous pyrogens to regulate body temperature via the hypothalamus, facilitate metabolism of fat and protein for fever related energy expense, and activate the production and dissemination of acute phase proteins, such as CRP by the liver.[29,38-46] Concurrent measurement of CRP, TNF – α, and IL – 6 strengthens the assessment of maternal inflammatory status at a single timepoint and bolsters exploration of the relationship between inflammation and fetal growth.[19, 29, 38-46]

TNF – α is encoded at chromosome 6p21.3 and synthesized by tumor cells, macrophages, monocytes, natural killer cells, T helper cells, fibroblasts, adipocytes, muscle cells, and placental cells.[43-45] The 26 kDa (kilodalton) transmembrane protein may be found on the cell surface or processed and released into circulation in a 17 kDa, soluble form.[45] TNF – α

ligand binds to TNF Receptor -1 or TNF Receptor -2, each being present on almost all cell types, except erythrocytes.[43-45] Ligand binding facilitates apoptosis, targets gene activation to stimulate liver regeneration, promotes growth and differentiation of cells, and enables remodeling of extra cellular matrices. [43-45] Elevated TNF-α in the first trimester is associated with history of preterm birth.[10] TNF – α blockers may be prescribed for disorders of implantation and placentation associated with excess TNF – α including idiopathic recurrent pregnancy loss, recurrent failed implantation, preterm delivery (PTD), and more serious forms of PE with comorbid IUGR.[39]

IL – 6 is encoded at chromosome 7p21 and synthesized by immune mediated cells, mesenchymal cells, endothelial cells, fibroblasts, muscle cells, adipocytes, and placental cells.[40,41] Produced at the site of tissue damage as a 20 kDa core protein, glycosylation results in release of a 21 - 26 kDa protein into circulation.[41] Evidence suggests that IL - 6 participates in metabolic processes, the regulation of pain, and bone homeostasis.[40,41] IL-6 stimulation of hepatocytes, bone marrow, synovial fibroblasts, dermal fibroblasts, and B cells results in the production of various factors.[40] Hepatic stimulation with IL – 6 precedes decreased synthesis and release of fibronectin, albumin, transferrin with increased synthesis and release of fibrinogen, hepcidin, serum amyloid A, and CRP.[12,40] Among pregnant women, maternal adiposity with insulin resistance correlates with higher circulating levels of IL-6.[12,41] The multi-functional molecule is associated with subclinical chorioamnionitis among cases of preterm premature rupture of membranes.[46] Circulating TNF – α and IL – 6 are typically measured by enzyme linked immunosorbent assay with standardized values computed as the deviate score (observed value minus the mean) divided by the standard deviation for comparison across laboratories.

Serving as a key facilitator of immune defense, CRP is encoded at chromosome 1q23.2 and synthesized as a 24 kDa protein, primarily by hepatocytes in response to stimulation with IL-6. Although to a lesser extent, CRP is also synthesized and released by adipocytes and placental cells.[47,48] Following release into circulation, CRP binds various molecules including calcium iron, choline, complement component Cq1, identical protein, Low Density Lipoprotein (LDL) particle,

and LDL particle receptor.[47,48] CRP opsonizes and activates complement along the classical pathway and activates phagocytic cells for clearance of cellular debris.[32] Apoptotic processes at the maternal fetal interface may facilitate classical, lectin, or alternative pathways of complement activation, each being implicated in the pathogenesis of preeclampsia, a common comorbidity of growth restriction.[32]

Circulating CRP concentration is a biomarker of inflammatory status commonly measured in clinical settings by one of two immunoturbidimetric assay methods.[49] Traditional CRP captures the circulating levels, typically those > 5-10 mg/L, associated with the acute immune response to infection.[44] High-sensitivity CRP (hs - CRP) detects CRP < 10mg/L, i.e. the levels of inflammation associated with subclinical risk of cardiovascular disease or pregnancy complication.[13-33,47-49] CRP levels are not typically normally distributed for a given study population. Log transformation allows investigators to compute the arithmetic mean of the logarithms and simply take the antilogarithm of this arithmetic mean for return to the original scale of concentration.

Immune cells and non-immune cells (decidua and chorion) express immune signaling molecules in association with hormonal shifts throughout the reproductive cycle of non-pregnant women, independent from acute immune response to infection.[42] Given that the complex physiology of the maternal-fetal interface during gestation requires nuanced immune regulation, further differences are observed between non-pregnant and pregnant states.[9,10,42,43] Throughout the course of successful gestation, immune signaling molecules interact with maternal tissues to facilitate implantation of the blastocyst, mediate tissue remodeling associated with placental development, and simultaneously protect against infection. [8-11,33,42,43,47] Thus, a delicate balance between inhibition of the adaptive immune system and active protection by the innate immune system is necessary.[8] This balance is not constant, rather, inflammatory profiles exhibit notable changes throughout healthy pregnancy.[9-12] Therefore, the timing of measurement of maternal

inflammatory markers is relevant to the study of associations between the levels of specific markers and birthweight outcomes.

Longitudinal observation of maternal inflammatory molecules and fetal growth. Three longitudinal studies repeatedly measured maternal hs-CRP across gestation and looked for relationships with birthweight outcomes.[13-16] Costa de Oliveira et al. measured hs-CRP at 5 - 13 weeks, 20 - 26 weeks, and 30 - 36 weeks.[13] Ferguson et al. measured hs-CRP, IL-1β, lL-6, IL-10, and TNF-α along with markers of oxidative stress at 5 - 16 weeks, 15 - 22 weeks, 23 - 29 weeks, and 33 - 38 weeks.[14,15] Finklestein et al. measured hs-CRP along with haemoglobin (Hb), serum ferritin (SF), hepcidin, and alpha-1-acid glycoprotein (AGP) at 9 -13 weeks, 23 -25 weeks, and 32 – 34 weeks.[16] Ferguson reported that no association was observed for markers of oxidative stress, 8 - isoprostane and 8 - hydroxy-deoxyguanosine, and birthweight outcomes following adjustment.[14,15] Maternal inflammation was associated with preterm birth (n=35) due to preeclampsia or IUGR without PE in unadjusted models.[14,15] Results of unadjusted and adjusted linear mixed effects models reported by Costa de Olivera and Ferguson showed that hs-CRP levels were inversely associated with estimated fetal weight by ultrasound and birthweight Zscore. [13-15] Of the three identified longitudinal studies, only Finklestein observed no significant association between maternal inflammatory molecules over the course of pregnancy and outcomes indicative of restricted fetal growth.[13-16] Aside from the study described by Ferguson et al., we did not find other studies with longitudinal measures of maternal IL-6 or TNF - α and birthweight outcomes.

The three studies described above each incorporated a different set of covariates in their adjusted models. Costa de Olivera and Ferguson considered maternal age, maternal education, smoking, alcohol, and maternal BMI.[13-15] Only Costa de Oliveira et al. measured gestational weight gain, fasting glucose; only Ferguson et al. measured sex, gestational age at ultrasound measurement, maternal race/ethnicity, insurance, parity, and assistive reproductive technologies.[13-15] Finklestein et al. most thoroughly considered maternal and infant

anthropometrics by observation of maternal mid-upper arm circumference and triceps, biceps, subscapular skinfold thickness, with not only birthweight, but infant supine length, mid-upper arm circumference, head and chest circumferences, and triceps and biceps skinfolds.[16]

Single early pregnancy observation of maternal inflammatory molecules and fetal growth. Three studies measured maternal hs-CRP at a single timepoint ≤ 14 weeks completed gestation with assessment for relationship to birthweight outcomes.[17-19] Tjoa et al. measured hs-CRP upon enrollment at 10 - 14 weeks for n = 107 grouped by status as healthy pregnant (92), PE (9), and IUGR without PE (9), defined as birthweight ≤ 10th percentile adjusted for sex and gestational age.[17] Mean CRP values were significantly higher for PE (6) and growth restricted (9) groups compared to all healthy index pregnancies (92), (P = 0.002 and P = 0.005, respectively).[17] The association persisted following matching of preeclampsia (n=12; 6 cases, 6 controls) and growth restricted (n=18; 9 cases, 9 controls) to healthy pregnant women, with matching based on gravidity, parity, maternal age at delivery and gestational age at delivery.[17] Cetin et al. measured hs-CRP and Pentraxin 3 (PTX3) upon enrollment at 11 - 14 weeks for n = 88 grouped by status as healthy pregnant (60), PE (16), and IUGR without PE (12).[18] Only severe cases of PE and IUGR without PE were included in this study, i.e. cases resulting in medically indicated preterm delivery < 37 weeks completed gestation.[18] Cetin et al. observed no significant difference in hs-CRP level between groups, significantly higher levels of PTX3 for PE and no difference for IUGR without PE, compared to healthy pregnant controls.[18] Wang et al. measured circulating serum Zinc, hs- CRP, TNF-α, IL8 in the first trimester, no value for completed gestational weeks at measurement was described.[19] The study included n = 150 grouped by status as healthy pregnant (100) and SGA (50), defined as birthweight ≤ 10th percentile adjusted for sex and gestational age.[19] Maternal serum zinc was significantly lower and hs-CRP, TNF-α, IL8 were significantly higher among SGA compared to healthy pregnant controls.[19] All three studies considered maternal age.[17-19] Gestational age, diastolic blood pressure, and placental weight were assessed by Tjoa et al.[17] Cetin et al. collected maternal race, parity, and BMI, although

without indication of whether they collected pre-pregnancy or pregnancy BMI.[18] Wang et al. included household income, pre-pregnancy BMI, parity, and gravidity, but did not exclude or differentiate based on gestational hypertensive status or PE diagnosis.[19]

Four studies measured maternal hs-CRP at single timepoint between 9 – 20 weeks completed gestation with assessment for relationship to birthweight outcomes.[20-23] Ernst et al. measured folate and hs-CRP levels for n = 5979 upon enrollment at 10 - 18 weeks and observed no association with fetal anthropometric measures collected by ultrasound in the 2nd and 3rd trimester.[20] However, hs-CRP ≥25 mg/L was significantly associated with SGA, defined in this study as birthweight for gestational age ≤ 5th percentile.[20] Gandevani et al. observed hs-CRP at 14 - 20 weeks for n = 778 grouped by status as healthy pregnant (715), mild PE (30), and severe PE (33).[21] Although investigators did not adjust birthweight for gestational age, there was a significant association between elevated hs-CRP and low birth weight.[21] Yang et al. observed hs-CRP once between 16-20 weeks for a cohort of 307 eligible pregnant women grouped by birthweight outcome as Low birthweight (< 2500g), Normal birthweight (2500-4000g), and High birthweight (> 4000g).[22] Dietary Inflammatory Index (DII) was calculated with 20 nutritional factors estimated by maternal self-report on a food frequency questionnaire using the population-based score well described through the work of Cavicchia et al. and published in 2009.[22] Yang et al. observed a statistically significant difference for both hs-CRP levels and DII scores measured at 16-20 weeks among Low birthweight (hs-CRP 4.37(mg/L); DII Score -1.44 ± 2.39) compared to Normal birthweight (hs-CRP 1.6 (mg/L); DII Score -3.47 ± 2.24), ($p < 0.05$).[22] Haedersal et al. performed an unmatched, nested case-control study of n = 209 grouped by status as uncomplicated pregnancy (127), spontaneous preterm birth < 34 weeks (n=9), PE +/- comorbid IUGR (29), and IUGR without PE (53), the latter defined as birthweight below two standard deviations of expected birthweight.[23] No association was observed between maternal circulating hs-CRP, interferon-γ-inducible Protein (IP-10), or insoluble urokinase plasminogen activator receptor (suPAR) at 16 - 20 weeks gestation and IUGR without PE.[23]

All four studies considered maternal age, smoking status, and parity.[20-23] Gandevani et al. considered maternal obesity, employment, and maternal supplement use with calcium, multivitamin, and folic acid.[20] Ernst et al. used data on maternal education, race/ethnicity, alcohol use, anthropometrics, and early pregnancy BMI.[21] While Ernst et al. did not exclude or differentiate based on gestational hypertensive status or PE, maternal blood pressure was collected for consideration.[21] Haedersal et al. considered maternal BMI, although it was not clear if maternal BMI was measured pre-pregnancy or during gestation.[23] For this gestational period, only Yang et al. considered maternal diet and the relationship between nutrition and inflammation.[22]

Single mid-pregnancy observation of maternal inflammatory molecules and fetal growth. Two studies measured maternal hs-CRP at a single timepoint between 22 - 33 weeks completed gestation and its relation to birthweight outcomes.[24,25] Sen et al. observed hs-CRP between 22 - 31 weeks for n = 853 enrolled in the Project Viva pregnancy cohort.[25] Dietary inflammatory index (DII) was calculated based on 28 dietary parameters collected by maternal self-report using a food frequency questionnaire.[24] Higher second trimester hs-CRP was associated with a higher DII score indicating a potentially proinflammatory diet., i.e. hs-CRP increased $\beta = 0.08$; 95 CI: 0.02, 0.14 mg/L for each DII unit increase.[24] Among obese mothers (BMI ≥ 30) a proinflammatory diet was associated with an increased likelihood of SGA (OR = 1.68; 95 CI: 1.09, 2.60).[24] Kuzawa et al. measured hs-CRP at 25-33 weeks for n = 429 in relation to neonatal anthropometrics and gestational age at birth.[25] Investigators dichotomized hs-CRP by median split, defined as CRP < 1.14 mg/L and CRP ≥ 1.14 mg/L, and observed no significant association with neonatal anthropometrics including birthweight, length, head circumference, or sum of skinfolds when bivariate regressions were clustered on the mother.[25] Following adjustment for maternal measures of adiposity and gestational age, continuous hs-CRP was significantly associated with measures of offspring size including sum of skin folds ($p < 0.0006$), birthweight and length ($p < 0.005$).[25] Both studies considered maternal age, pre-pregnancy BMI, household income, and

parity. Sen et al. included data on maternal education, race/ethnicity, smoking status, hypertensive status, white blood cell count, gestational weight gain, and glucose tolerance.[24,25] Kuzawa et al. did not determine maternal hypertensive status, however, adjusted models considered systolic blood pressure.[25] In this study, consideration of maternal adiposity includedmaternal triceps skinfold thickness in addition to adjustment for maternal pre-pregnancy BMI. [25]

Single mid/late-pregnancy observation of maternal inflammatory molecules and fetal growth. Eight studies evaluated single measures of maternal hs-CRP between 24 - 40 weeks completed gestation in relation to birthweight outcomes.[**26-33**] Ertas et al. measured hs-CRP at 24 - 40 weeks for n = 212 women grouped by status as healthy pregnancy (115), mild PE (63), and severe PE (34).[26] A significant association was observed between hs-CRP and birth of a growth restricted baby, when hs-CRP concentration was dichotomized into low CRP (< 9.66 mg/L) and high CRP (≥ 9.66 mg/L).[26] Ragsdale et al. measured hs-CRP, IL6, IL10, TNF-α for n=407 women at 26-36 weeks in relation to newborn anthropometric measures including weight, length, head circumference, and sum of five skinfold thicknesses (triceps, subscapular, suprailiac, bicep, and calf).[27] While no association between hs-CRP, IL6, IL10, TNF-α*,* as singular molecules, and growth was observed, the ratio of IL6:IL10 was significantly associated with birthweight. [27] Yeates et al. observed maternal circulating hs-CRP, IFN-γ, IL-1β, IL-2, TNF-α, IL-4, IL-5, IL-10, IL-6, MCP-1, TARC, sFlt-1 and VEGF-D at 28 weeks for n = 1411 women.[28] Cases of preterm birth were excluded and a significant association was observed between higher maternal hs-CRP and birthweight outcomes, LBW and SGA.[28] Guven et al. observed maternal circulating hs-CRP, IL-6, TNF-α, homocysteine, folic acid, and vitamin B12 at 28 - 40 weeks for n = 183 women grouped by status as healthy pregnant (62), mild PE (61), and severe PE (60).[29] A significant association was observed between elevated hs-CRP, IL-6, TNF-α and low birth weight among pregnancies complicated by severe preeclampsia.[29] Nazzari et al measured serum Interleukine-6 (IL-6), C-

Reactive Protein (CRP), salivary cortisol, alpha amylase (sAA), with collection of self-report symptoms of depression and anxiety among n = 97 (exclusion of PE) women at 34-36 weeks with follow up for assessment of birthweight, birth length, head circumference, newborn cortisol, and newborn behavior.[30] While investigators did not observe an association between maternal hs-CRP and measures of fetal growth, there was a significant association between maternal Il-6 in late pregnancy and smaller newborn head circumference.[30] A significant relationship was also observed between maternal diurnal sAA levels and birthweight.[30] Erkenekli et al. measured maternal circulating hs-CRP and serum neopterin at 36 - 39 weeks for n = 96 women grouped by status as healthy pregnant (62) and growth restricted without comorbid PE (34), the latter diagnosed by ultrasound, confirmed by birthweight for gestational age ≤ 10th percentile.[31] No association was observed between maternal hs-CRP and birthweight, however, there was a significant association between elevated maternal serum neopterin levels and fetal growth restriction.[31] Derzy et al. measured hs-CRP, C4d, Bb, C3a, SC5b9, C4, C3, C9, C1inhibitor, C4b-binding protein, factor H antigen at 36-39 weeks for n = 179 women grouped by status as non-pregnant (59), healthy pregnant (60), and PE (60).[32] No association was observed between hs-CRP levels and IUGR, however, there was a significant association between increased hs-CRP and PE.[32] Ali et al. assessed maternal hs-CRP levels upon third trimester enrollment for n = 120 women grouped by status as healthy pregnant (60) and PE (60).[33] Among PE women, hs-CRP negatively correlated significantly with birthweight.[33] Ali et al. observed higher levels of third trimester hs-CRP ($p<0.001$) for PE 8.8 (0.3-25.5) mg/L compared to normotensive 5.4 (0.24-9.8) mg/L pregnancies matched by maternal BMI.[33] All eight studies considered maternal age and gestational age at blood draw. Ertas et al, Guven et al., Erkenekli et al. assessed parity and gravidity, but Derzy et al. assessed only parity. Ragsdale et al. and Guven et al. considered maternal pre-pregnancy BMI; Ertas et al, Derzy et al., Yeates et al., Ali et al. considered maternal pregnancy BMI. Ertas et al., Guven et al., and Derzy et al. measured systolic blood pressure and diastolic blood pressure; only Ertas et al. also considered mean arterial pressure. Derzy et al.

and Yeates et al. assessed maternal smoking status, however, none of the other studies measuring maternal hs-CRP between 24 – 40 weeks considered maternal behavioral risk factors.

Overall, seventeen studies included single observation of hs-CRP in maternal blood, collected at a range of gestational ages, with assessments of their relations to birthweight outcomes.[17-33] Eleven studies observed an association between higher maternal serum hs-CRP and SGA or IUGR, however, description and analysis of hs-CRP levels varied considerably. Six studies found no association between maternal hs-CRP levels and birthweight outcomes. No study observed a significant relationship between lower levels of maternal hs-CRP and birthweight for gestational age. Fourteen studies considered maternal size, however, some elected to use pre-pregnancy BMI, others used maternal pregnancy BMI observed around the time of biomarker measurement, and very few considered additional anthropometric factors such as skin fold thickness or gestation weight gain. Yang et al. and Sen et al. elected to consider the participant diet and pro-inflammatory potential in the context of the relationship between maternal inflammatory status and birth outcomes. Kuzawa et al., Nazzari et al., Ragsdale et al. and Yeates et al. included measurements of adiposity that were considered by no other study identified in this review. Adjustment for maternal adiposity strengthened the effect observed between CRP and birth outcomes, however, further adjustment for gestational age resulted in a minor attenuation of the relationship.[25]

Nine studies considered diagnosis with PE, defined as increase in diastolic blood pressure of ≥15mmHg with end diastolic blood pressure ≥90 mmHg or as two measurements of systolic blood pressure ≥ 140/90 mMHg > 4 hr apart, with proteinuria ≥ 300 mg/L in 24 h. Gandaevani et al., Ertas et al., and Guven et al. further differentiated cases of PE as mild or severe. Mild preeclamptic cases were defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg first measured above these thresholds after 20 weeks' gestation with proteinuria > 300mg in two measures in samples collected > 6 hours apart. Severe preeclamptic

cases were defined as systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mmHg first measured above these thresholds after 20 weeks' gestation with proteinuria > 2g in a 24-hour period. Three studies excluded cases of PE, four studies made no mention of maternal hypertensive status, and one study measured only systolic blood pressure.

Various markers of maternal immune regulation are assessed in relation to low birthweight outcomes;[13-33] however, there is a paucity of observations including the cytokines, TNF-α or IL-6 and their relationship to birthweight for gestational age. Further, while we identified a small body of literature assessing CRP and birthweight for gestational age, divergence of effect is observed between the commonly measured marker and LBW outcomes.[13-33] Indeed, factors such as gestational age at measurement, maternal hypertensive status, maternal diet or maternal adiposity, may contribute to differences in the observed relationship between maternal immune signaling molecules and outcomes indicative of restricted fetal growth. Thus, there is evident need for further epidemiologic investigation of the relationship between the maternal immunologic milieu and birthweight for gestational age.

CHAPTER 2.

We measured maternal circulating CRP, TNF-α, and IL-6 levels in mid-pregnancy and assessed their relation to birthweight for gestational age, standardized to the national reference as described by Talge et al.[50] We hypothesized that *higher levels* of maternal mid-pregnancy CRP would be associated with delivery of a small for gestational age (SGA) infant and that immune molecules IL-6 and TNF-α would be significantly correlated with CRP and birthweight outcomes.

The Pregnancy Outcomes and Community Health (POUCH) Study enrolled 3038 women having a singleton pregnancy from among 52 clinics in 5 Michigan communities between 1998-2004.[51] Women who accessed prenatal care at one of the study clinics between 16 – 27 weeks completed gestation and for whom maternal serum α – fetoprotein (MSAFP) was measured, were eligible to enroll if they were able to communicate effectively in English, were ≥ 15 years, and had no identified congenital anomaly for the index pregnancy.[51] Women diagnosed with diabetes mellitus prior to the index pregnancy were excluded from the sampling frame. [51] Probability sampling was applied to the sampling frame for the POUCH cohort and sub-cohort.[51] Sampling weights were assigned to each sampling stratum so that the group of participants in that stratum was representative of the proportion of that stratum in the sampling frame.[51] After stratification by race/ethnicity, pregnant women with normal levels of MSAFP were randomly sampled from the sampling frame; all pregnancies with significantly high levels of MSAFP were presented with the opportunity to be in the study.[51] Following informed consent, participant data collected comprised demographic, anthropometric, behavioral, and clinical domains. [51]

The POUCH sub-cohort included selection of n = 1371 pregnancies for whom additional data was abstracted from the medical record of the index pregnancy and additional biospecimens retained. The sub-cohort included all women who delivered a preterm or SGA infant, all women with high MSAFP levels and a stratified random sample of women with term deliveries with

oversampling of African-American women in this latter category. Maternal blood was collected in an EDTA plasma tube at enrollment. After processing, plasma was aliquoted and stored frozen at -80 C. Women included in the sub-cohort had their placentas collected and examined following delivery. For a majority of these, placental pathology was completed.[52] Pregnancy complications and birth outcomes were abstracted from the maternal medical record and included maternal hypertensive status, gestational age at delivery, delivery type, and birthweight. Derivation of the analytical sample led to the assessment of n = 1308 mother child dyads for whom biomarkers were assessed in maternal circulation and birthweight for gestational age data were available (Figure A.2)

Three maternal immune-related biomarkers were included in this analysis, C-Reactive Protein (CRP), IL-6, and TNF-α. Deidentified aliquots of maternal plasma collected upon study enrollment were transported in dry ice to the laboratory.[51] The assay for CRP was performed in duplicate on the Luminex platform for which the Limit of Quantitation (LOQ) was 0.005 ug/mL. Method validation is well described with the intra-assay coefficient of variation (CV) of 5.3 % and the inter-assay CV of 15.6 %, both falling within the working range of CV < 20%.[53] CRP values below LOQ were assigned a level of 0.0025 ug/mL to prevent truncation.[53] In a separate laboratory analysis, L-6 and TNF-α levels in maternal circulation were determined by multiplex xMAP assay using the Luminex platform for which limit of detection (LOD) was 0.0000604 ug/mL and 0.000541 ug/mL, respectively. While intraassay CV of 9.3% for IL-6 and 13% for TNF-α indicated acceptable within batch variation, both pleiotropic cytokines had interassay CV outside the working range of < 20%.[53] Interassay CV of 23% for IL-6 and 25% for TNF-α illustrates the unacceptable batch to batch variation and justifies computation of the ZScore, a standardized value defined as deviate score (observed value minus the mean) divided by the standard deviation. [53] Standardization of IL-6 and TNF-α allowed for valid comparison of these biomarkers across batches.

Pre-pregnancy BMI was based on maternal self-report pre-pregnancy weight and maternal height (BMI; kg/m2) at enrollment. *Weeks at blood draw* was defined by gestational age at enrollment based on analysis of all available gestational age estimates. *Maternal Pre-pregnancy BMI* was measured as a continuous variable and grouped for analysis by CDC definition as Low (< 18.5), Normal (18.5 – 24.99), overweight (25.00 – 29.99), and Obese (≥ 30.00).[54] *Pregnancy weight gain* was assessed by maternal self-report of pre-pregnancy weight and weight upon admission for delivery abstracted from the maternal medical record for the index pregnancy. *Maternal smoking status* was assessed by self-report and categorized as follows: did not smoke during pregnancy, quit smoking before enrollment, smoked <1/2 pack per day at enrollment, or smoked at least 1/2 pack per day at enrollment. *Maternal hypertensive status* was dichotomized into any hypertensive disorder versus no hypertensive disorder. Any hypertensive disorder was defined as women diagnosed with pregnancy-induced hypertension (PIH), pre-eclampsia (PE), chronic hypertension (CHTN) or a diastolic blood pressure ≥90 mmHg and/or systolic blood pressure ≥140 mmHg on ≥2 days.[47] *Antibiotic exposure* was defined as antibiotic prescribed versus no antibiotic prescribed in the two weeks before maternal blood draw as abstracted for the medical record.

Gestational age at delivery was based on analysis of all available gestational age estimates, prioritizing consideration of last menstrual period over clinical estimation of gestational age by ultrasound. If both measures were available and there was an estimated gestational age difference of greater than two weeks between the two methods, then ultrasound was used to estimate gestational age. In this analysis, we considered gestational age at delivery as a continuous value and grouped as early preterm (< 34.00 weeks completed gestation), late preterm (34.01-36.99 weeks completed gestation), term (37.00-41.0 weeks completed gestation), and post-term (41.00-45.00 weeks completed gestation). *Preterm delivery circumstances* were grouped as: spontaneous preterm labor (PTL), defined by contractions and subsequent cervical changes of at least 2 cm dilation; premature rupture of membranes (PROM),

defined as the rupture of membranes before or with onset of contractions; and medically indicated (MI), defined as induction or C-section performed before onset of either PTL or PROM.

Infant birthweight (grams) was abstracted from hospital records with assessment as a continuous and categorical variable by CDC definition for low (<2500g), normal (2500g-4000g), and high (>4000g). *Birthweight for gestational age Z-Score* was a continuous value based upon means and standard deviations generated by Talge et al. (2014) who used USA vital records data, standardized for gestational age and sex; Z = (individual birth weight – M) / SD with estimates not provided for missing BWts (N = 2).[45] *Birthweight for gestational age categories* were assigned as appropriate for gestational age-AGA (10^{th} to < 90^{th} percentiles), small for gestational age - SGA (<10^{th} percentile), or large for gestational age - LGA (≥90^{th} percentile).

Due the probability sampling utilized for the POUCH study, weights were assigned to all analyses to ensure generalizability of results to the sampling frame and to ensure accurate standard errors and confidence intervals. Sampling weights were particularly assigned based on the sampling stratums designed to consider varied combination of three factors, i.e. maternal race (black vs. white/other), MSAFP level (normal vs. high), and gestational age at birth (preterm vs. term birth). Normality of continuous variables was assessed by Shapiro-Wilk *W test*. Log transformation of maternal circulating CRP levels approximated a normal distribution for analysis, interassay CV within the working range of 20% indicates acceptable batch to batch variation, thus, it was not necessary to utilize a standardized score for analysis of CRP values. Given that each interassay CV was above a working range of 20%, TNF-α and IL – 6 levels were necessarily standardized to the deviate score (observed value minus the mean) divided by the standard deviation for valid comparison from batch to batch. Computation of the Z-Score does not affect the nature of the distribution, which was parametric for TNF-α and IL – 6. Univariate weighted linear regression models assessed relations among maternal circulating immune molecules, CRP, IL-6, and TNF- α and characteristics of the analytic sample (PROC SURVEYREG in SAS 9.4, SAS Institute, Inc.). Pearson product moment correlation (PROC

CORR in SAS 9.4, SAS Institute, Inc.) was used to assessed the magnitude of any linear correlation between standardized values of birthweight for gestational age, logCRP, IL-6, TNF-α, pre-pregnancy BMI, maternal age, gestational weeks at blood draw, and gestational age at delivery. For unadjusted and adjusted models, linear contrast tested the null hypothesis that there is no difference in concentrations of maternal circulating inflammatory markers among the AGA, SGA, and LGA groups.

In the multi-variable regression models, we considered biological plausibility of biomarker relations to outcomes and covariates (e.g. linearity, threshold, u shape), interactions among covariates, model fit, and regression diagnostics to identify the most parsimonious models for associations with birthweight and birthweight for gestational age outcomes. Least square means of the logCRP were retransformed to the original scale for reporting as the Mean plasma CRP µg/L.

We conducted sensitivity analyses to test the robustness of our findings considering highly relevant covariate values as gleaned from the literature. The association of lower maternal CRP levels among the SGA group proved robust in models that removed women with a BMI <18.5, or women any hypertensive disorder, or women with an antibiotic prescription within two weeks prior to enrollment, given that effective antibiotic treatment is known to reduce circulating levels of CRP. Sensitivity analysis also tested the exclusion of CRP values less than 1% of the distribution observed in the analytical sample, or CRP values less than 5% of the of the distribution observed in the analytical sample, considering the LOD of 0.005 ug/mL and the assigned value of 0.0025 ug/mL that were substituted for measurements falling below LOD. (Table A.6, A.7, Figure A.6).

This analysis includes 1,308 mother-infant dyads for whom there was maternal plasma for measurement of biomarkers and completeness of data for calculation of birthweight for gestational age (Figure A.1). Differences of least square means, tested by linear contrast, showed significantly higher CRP levels for maternal age 20-29 years (6.0 µg/mL), compared to

< 20 years (4.2 µg/mL) and ≥ 30 years (4.8 µg/mL). As expected, higher CRP was associated with higher pre-pregnancy BMI, i.e. Gmean CRP values were significantly different for underweight (2.0 µg/mL), overweight (6.8 µg/mL), and obese (9.3 for µg/mL) compared to normal weight (3.8 µg/ mL), ($p=0.00005$, $p<0.0001$, and $p<0.0001$, respectively). When maternal hypertensive status was dichotomized, a statistically significant difference was observed between CRP levels among women with no hypertensive disorder (5.0 µg/mL) compared to women with any hypertensive disorder (5.3 µg/mL), ($p=0.022$). No significant difference was observed for CRP among categories of self-reported maternal education, maternal race, Medicaid status, maternal smoking status, or weeks completed gestation at blood sample collection. (Table A.1)

Maternal levels of TNF – α (Z-score ± SD) were not associated with maternal age, education, race, Medicaid status, or gestational age at blood draw (modeled as tertiles), but did differ by BMI; the underweight group was significantly lower (-0.32 ± 4.38) than the normal weight (-0.03 ± 1.82), ($p=0.03$) (Table A.2).TNF – α levels varied by smoking status, < ½ pack per day at enrollment (0.13 ± 3.55) or ≥ ½ pack per day at enrollment (-0.11 ± 3.95) compared to no smoking during pregnancy (-0.05 ± 1.39), though comparisons were not quite statistically significant ($p=0.08$ and $p=0.06$, respectively). Maternal IL-6 (Zscore ± SD) was not related to maternal age, education, race, pre-pregnancy BMI, hypertensive status, smoking status, or gestational weeks at blood draw. However, maternal IL-6 levels were higher among women insured by Medicaid (0.01 ± 1.82) vs those not insured by Medicaid (-0.17 ± 1.55), ($p=0.01$).

In unadjusted weighted linear models, maternal levels of CRP, IL-6, and TNF- α were not significantly associated with timing or delivery circumstances of the birth, i.e. PTL, PROM, MI, or with birthweight groups, i.e. low birth weight (<2500g), normal birthweight (2500g-4000g), or high birthweight (>4000g) (Table A.3). However, unweighted correlation analyses suggested maternal CRP was positively correlated with birth weight ($r = 0.10518$, $p = 0.0001$), maternal pre-pregnancy BMI ($r = 0.4256$, $p <0.0001$), and maternal age ($r = 0.0601$, 0.0298), and negatively

correlated with GA at measurement (r = -0.0688, p = 0.0128) (Table A.4.) IL-6 and TNF- α levels were not correlated with maternal CRP levels, birthweight for gestational age, maternal age, or gestational age at birth. However, IL-6 was positively correlated with TNF- α (r = 0.4893, p <.0001) and maternal pre-pregnancy BMI (r = 0.06271, p = 0.0233).

Unadjusted weighted Gmean CRP (µg/ml) for SGA (3.8 µg/mL) and LGA (6.4 µg/mL) were compared to AGA (5.4 µg/mL) by linear contrast. Maternal mid-pregnancy levels of CRP were significantly lower among SGA, (p=0.002), and borderline significantly higher among LGA (p=0.082) (Table A.5.). When women were stratified by maternal pre-pregnancy BMI, and the relation between maternal CRP levels and infant group (SGA, AGA, LGA) was examined within each stratum, the pattern of significantly lower CRP levels in the SGA group remained, though it was less pronounced among women with low pre-pregnancy BMI (Figure A.3).

Final adjusted weighted models resulted from purposeful selection considering linearity, interactions among covariates, model fit, and regression diagnostics. Significantly lower maternal CRP levels in SGA group persisted after adjustment for continuous BMI only (Model 1. Table A.5) as well as continuous BMI, categorical maternal age, maternal race/ethnicity, and gestational age at sample collection (Model 2. Table A.5). Effect estimates of the adjusted models had narrower confidence intervals indicating that inclusion of select variables provides a more precise estimate (Figure A.4). No significant associations were observed for unadjusted and adjusted IL-6 Z-Score and TNF-a Z-Score models when SGA, AGA, LGA are compared (Figure A.5).

Among those women included in the POUCH sub-cohort, 1070 mother-infant dyads had data available for investigating antibiotic prescription in relation to maternal immune system biomarkers, birth weight, gestational age, and fetal sex (Figure A.6). In unadjusted weighted linear models, CRP was significantly higher among women with antibiotic prescription (5.8 µg/mL) versus no antibiotic (5.0 µg/mL), (p=0.036) (Table A.7); this relation was most evident within the AGA and SGA groups (Table A. 8) There was no clear evidence of an association

between antibiotic prescription within two weeks of enrollment and the infant growth groups, AGA, SGA, LGA (OR 1.2, 95 % CI 0.75,1.96). (Table A.9).

Our prospective design included a diverse sample of women and collection of maternal biospecimens prior to the outcome of interest. Contrary to our expectations, we observed that *lower* maternal plasma levels of CRP, a systemic inflammatory mediator, were associated with SGA. Studies with multiple measures of CRP across pregnancy note that normal pregnancies experience a rise in maternal blood CRP in mid-pregnancy, therefore a departure from this trajectory, i.e. lower CRP, may reflect underlying pathology related to fetal growth. However, unlike our study, most, but not all studies have reported an inverse relation between maternal CRP and birthweight for gestational age.

Gestational age and select immune biomarkers. Our measurement of CRP, IL-6, and TNF- α at a single time point results in a limited interpretation of the observed association to birthweight for gestational age. Longitudinal studies observed an inverse association or no association between maternal CRP levels and birthweight Z-Score in unadjusted and adjusted models.[13-16] These studies considered maternal age, maternal education, smoking, alcohol, and maternal BMI, while inclusion of other factors in study design varied.[13-16] No additional literature included longitudinal measurement of maternal CRP, IL-6 or TNF - α with subsequent birthweight outcome.[13-16] Repeat measures of select maternal immune molecules during uncomplicated gestation indicate gestational age associated trends, independent from pregnancy complications.[10-12] Curry et al. assessed n = 1274 uncomplicated pregnancies and observed trends of *increased* IL-6, IFN-λ, IL-12, along with *decreased* GM-CSF, and *no change* in TNF-α, IL – 2 between repeat measures at 7 - 10 weeks and 24 - 26 weeks.[10] Among n = 45 uncomplicated pregnancies, observed by Denney et al., generalized linear models indicate *decreased* IL-6, IFN- γ, IL-4, TNF-a, IL-1b, and IL-10 across repeat measures at 8 - 14 weeks, 18 - 22 weeks, and 28 - 32 weeks completed gestation.[11] Observation of n = 57 uncomplicated pregnancies by Costa de Olivera et al. found *decreased* CRP levels, *increased* TNF-α and IL- 6,

and *u-shaped* curves for IL-8 and IL-1 β measured at 9 – 13 weeks, 21 – 25 weeks, 30 – 33 weeks, and 2 - 6 weeks postpartum.[12] Although statistically significant trends were observed, the direction of effect was divergent. Longitudinal studies of uncomplicated pregnancies exhibited inconsistent consideration of confounding covariates and differences in gestational weeks at measurement of immune markers.

Maternal hypertensive status and select immune biomarkers. Similar assumptions of pathogenesis in preeclampsia and IUGR may explain the simultaneous occurrence of these complications. Evidence indicates that dysregulation of innate and adaptive maternal immune response contributes to the pathology of preeclampsia. We did not have sufficient power to test for an association between maternal immunologic milieu and SGA accompanied by PE. When we adjusted for maternal hypertensive status in our multi-covariate models, our results of lower CRP levels among the SGA group remained.

Investigators study markers of inflammation, endothelial activation or injury, oxidative stress, and trophoblast debris in maternal circulation and their relation to PE and birthweight outcomes. Stenczer et al. observed significantly higher hs-CRP, von Willebrand factor antigen, fibronectin, malondialdehyde, and cell-free fetal DNA among PE (n = 44) compared to healthy pregnant (n = 44) women.[55] Among PE cases, 25% included comorbid IUGR, however, investigators did not distinguish this subset for analysis.[55] Enrollment and biomarker measurement occurred at 36-37 weeks completed gestation with consideration of maternal age, BMI at blood draw, smoking status, primiparas, systolic blood pressure, diastolic blood pressure, and gestational age at blood draw.[55] Investigators followed enrolled women and assessed gestational age at delivery, birthweight, and fetal growth restriction.[55]

Mihu et al. measured hs-CRP, leukocytes, neutrophils, IL-6, TNF-α, and markers of oxidative stress for n = 230 women including non-pregnant controls (n = 72), healthy pregnant (n = 78), and PE (n = 80).[56] All factors were significantly different when non-pregnant controls were compared to healthy and preeclamptic pregnancies, however, only hs-CRP, IL-6, and TNF-

α levels were significantly higher at 28 - 40 weeks completed gestation when PE diagnosis was compared to uncomplicated pregnancy.[56] Investigators considered gestational age at birth, birthweight, Apgar score, and mode of delivery, but did not assess preterm/term outcomes.[56]

Maternal adiposity and select immune biomarkers. Cytokines produced by adipose tissue such as leptin, CRP, TNF-α and circulating adiponectin may be related to fetal weight, either directly or indirectly by confounding. [13] Adipokines (adipocyte derived signaling molecules) are implicated in disorders of fetal growth restriction.[13,26] Maternal body mass index (BMI) does not entirely account for the physiologic complexity of factors that influence the distribution of maternal adipose tissue, however, maternal BMI is associated with differences of observed cytokines during pregnancy and with birthweight outcomes.[13,26,37] Direct correlations exist between adiposity with insulin resistance and circulating levels of cytokines IL-6 and TNF-α. Paradoxically, restricted fetal growth alters the development of fetal adipose tissue which has multiple purposes including fat storage, endocrine regulation of body metabolism and energy homeostasis.[13,26] Due to the unique physiology of pregnancy, factors related to maternal BMI measurement or failure to observe any measure of maternal size may result in bias between immune profiles and birthweight.[12,26] Further, there may be effect modification by maternal hypertensive status.[26]

In our sample, the unadjusted association between maternal CRP in mid-pregnancy and birthweight for gestational age was persistent across strata of maternal BMI. As expected, increasing maternal BMI resulted in higher GMean CRP across birthweight for gestational age groups. Consideration did not change direction or strength of effects among linear models adjusted for continuous pre-pregnancy BMI only and continuous pre-pregnancy BMI along with other variables.

Christian and Porter observed significantly higher IL-6, TNF-α, and hs-CRP across repeat measurements among women with pre-pregnancy BMI ≥ 30 (obese) compared to women with pre-pregnancy BMI 18.5 – 24.9 (normal weight).[12] Observation of maternal (n = 830) hs-CRP, homocysteine, folate, and fetal fibronectin by Han et al. found higher hs-CRP and lower

birthweight among obese pregnant women.[57] Ertas et al. found that elevated maternal hs-CRP was associated with BMI ≥ 25 kg/m^2 among severe preeclampsia versus mild preeclampsia and uncomplicated controls.[26] Sen et al. assessed the role of maternal diet in relation to maternal inflammation, indication of fetal growth restriction, and likelihood of breastfeeding success ≥ 1 month. [24] Maternal self-report to food frequency questionnaire, validated for use in pregnancy, provided data for estimation of nutrition status for 28 parameters by reference to the Harvard nutrient composition database.[24] Investigators then calculated dietary inflammatory index (DII) scores, validated among non-pregnant adults by measurement of circulating immune molecules CRP, IL-6, TNF-α.[24] Among those enrolled in the Project Viva cohort, higher DII was associated with lower birthweight for sex-adjusted gestational age and breastfeeding outcomes.[24] The analysis of dietary inflammatory potential could be explored in other cohorts with requisite data available and has been more recently attempted by Yang et al. Further investigation of the complex relationships between nutrition, inflammation, and fetal growth patterns may lend greater insight to understanding the role of maternal systemic inflammation as it pertains to offspring birthweight for gestational age.[22]

Maternal antibiotic prescription and select immune biomarkers. Antibiotics used during gestation are typically classified as FDA category B medications, meaning that animal studies have failed to demonstrate a risk to the fetus, but there are not adequate, well controlled studies in pregnant women.[58] Woman might be prescribed antibiotics during gestation to treat infections or during labor as prophylaxis. Maternal infections such as chlamydia, gonorrhea, gram-positive upper respiratory infections, subclinical bacteriuria, and syphilis are associated with fetal growth and are likely to be treated with antibiotic regimen at various stages of gestation.[58] Crucial areas of concern for maternal antibiotic use during the perinatal period include allergy, teratogenic potential, placental transfer, altered organogenesis, disrupted fetal development, and interaction with environmental or genetic factors. Administration of antibiotics during labor provides primary

prevention of neonatal infection and is ordered most commonly due to a positive Group B Streptococcus screen between 36 – 38 weeks.

We expected that rates of antibiotic usage would be higher among women with SGA vs AGA infants. However, the data from our cohort did not allow for definitive determination of indications for antibiotic prescription or assessment of maternal compliance. Further, we expect that antibiotic exposure at different gestational weeks, i.e. earlier or later during pregnancy, may result in different effects on birthweight for gestational age, but that these timepoints of exposure may not be associated with maternal circulating immune molecules measured at 16 – 27 weeks' gestation. Our analysis suggested that the association we observed between CRP levels and size for gestational age was not confounded by antibiotics prescribed within 2 weeks prior to enrollment (and therefore not by the indication for those antibiotics).

There is divergence in the literature regarding the potential relationship between maternal antibiotic usage and LBW; only one study has attempted observation of this relationship as possibly mediated by epigenetic impact.[58] Vidal et al. found that Maternal self-reported antibiotic prescription during gestation is associated with altered methylation at 1 of 9 differentially methylated regions (specifically the *PLAGL1* DMR) and *lower* birthweight with the strongest association for non-penicillin antibiotic use and *lower* birthweight.[58] Bahat Dinur et al. reported *no association* between maternal macrolide prescription in the 1st or 3rd trimesters and incidence of LBW.[60] Jepsen et al. observed a *higher* mean birthweight among amoxicillin (penicillin) exposed pregnant women compared to unexposed pregnant women.[62] Analysis described by Ciezal et al. indicates *lower* mean birthweight for women treated with aminoglycosides and cefalexin, and *higher* mean birthweight for women reporting any antibiotic treatment compared to no antibiotic treatment.[63] Differences in effect may arise based on the type of antibiotic prescribed, such that penicillins might be associated with higher birthweight for gestational age and non-penicillins associated with lower birthweight for gestational age.

Few studies have assessed the relationship between antibiotic use during gestation and fetal growth measures, none have assessed birthweight for gestational age. Among studies that attempt observation of this relationship, confounding bias introduced by indication or by compliance are of notable concern.[58-63]

Placental pathology and select immune biomarkers. Wang et al. observed higher maternal inflammation and Zinc deficiency among cases of SGA compared to healthy pregnancy controls.[34] In this same study, immunohistochemistry of placental tissue indicated increased NF-κB p65 positive cells and nuclear translocation of placental NF-κB p65 in the trophoblasts of SGA placentas compared to control placentas.[34] In the present analysis we observed lower levels of maternal CRP among SGA versus health pregnancy controls, but did not assess the maternal fetal interface for evidence of upregulation of the inflammatory regulator, NF-κB. Immunohistochemistry of placental tissues for NF-κB p65 positive cells could shed light on the possible mediation of maternal inflammatory status and birthweight outcomes. In a subset of POUCH pregnancies, histologic chorioamnionitis, determined by blinded placental pathology, was significantly associated with higher maternal circulating cytokine levels and higher likelihood of preterm delivery having comorbid infection of the fetal membranes.[64] It is not clear if this type of perinatal complication is associated with the outcome of SGA among mother-infant dyads in the POUCH cohort. Future investigation should consider inflammation at the maternal-fetal interface at the time of birth as it relates to indices of maternal inflammation in mid-pregnancy and birthweight for gestational age.

Maternal vascular malperfusion (MVM) of the placental bed is the recently adopted term (previously called maternal vascular underperfusion) for particular patterns of pathology at the maternal-fetal interface related to poor maternal blood flow and risk of restricted fetal nutrition.[65] MVM is grossly defined by - *placental hypoplasia*, i.e. placental weight < 10^{th} percentile for gestational age, with or without presence of umbilical cord width < 10^{th} percentile for gestational age or < 8mm at term; *placental infarction,* i.e. loss of blood flow due to congestion and

hemorrhage of villi; *retroplacental hemorrhage/hematoma*, i.e. blood clot adjacent to the basal plate with size/duration variable parenchymal compression.[65] MVM is histologically defined by – *villous lesions,* i.e. stunted chorionic villi with more syncytial knots than expected based on gestational age; *decidual vascular lesions*, i.e. whole/partially persistent muscularized basal plate arterioles or endovascular thrombectomy within the central maternal surface.[65]

Tjoa et al. observed significantly lower birthweight and placental weight for the IUGR without PE group, but not for the PE group, compared to the control group.[17] Some evidence indicates that the human placenta produces and releases CRP into the maternal circulation [47] and placental size/function among SGA fetuses may differ, thereby affecting maternal CRP level. Few studies of CRP and outcomes indicative of fetal growth restriction have assessed meditation of the relationship between systemic inflammation and fetal nutrition by placental size, structure and function. Observation and assessment of MVM among cases of SGA and health pregnancy controls could lend insight into the structural and functional differences at the maternal fetal interface impacting fetal nutrition. See Figure A.11.

Data available in this cohort may allow us to test further hypotheses of the dynamic factors associated with nourishment of the developing fetus. Future investigation could include evaluation of placental size, placental pathology and other markers of placental function in association with maternal inflammatory markers and SGA. Determination and grading of placental pathology in relation to definition of maternal vascular maIperfusion may lend to a greatly increased comprehension of the dynamic interplay between maternal systemic inflammation in mid-pregnancy, the structure and function of the maternal fetal interface, the adequacy of fetal nutrition given maternal blood flow, and fetal growth outcomes. While the current analysis did not assess these variables by quantile regression of continuous variables, this method could be applied, if appropriate, for further investigations of maternal immune status and birthweight outcomes. Maternal immune milieu affects the placental and fetal milieu in such a way as to influence fetal growth. Design of a future pregnancy cohort could include serial,

concurrent collection of maternal blood (whole, serum, plasma, lysates), maternal urine, fetal anthropometric estimates by ultrasound with birth collection of the cord blood. Nutritional factors, inflammatory factors, and environmental factors could be measured in maternal biospecimens. Fetal growth trajectory tracked by ultrasound in utero and could be assessed with maternal and fetal biomarkers for association to sudden percentile rank change.

Conflict of Interest

The author(s) declare no conflict of interest.

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APPENDIX

Figure A.1: Considerations with association to maternal circulating immune factors and fetal growth for Hypothesized Growth Restriction Pathways

Maternal Factors	**Chronic Disease**	**Gestational Factors**	**Acute Infection**	**Fetal Factors**
Alcohol use	Hypertension	High altitudes	HCA	
Smoking	Diabetes Mellitus	Preeclampsia	Rubella	Chromosomal Abnormality
Drug use	Obesity	Eclampsia	Cytomegalovirus	Fetal adiposity
	Clotting disorder	Multiple pregnancy	Toxoplasmosis	
	Kidney Disease	Nutrition Status	Syphilis	
		Weight Gain		

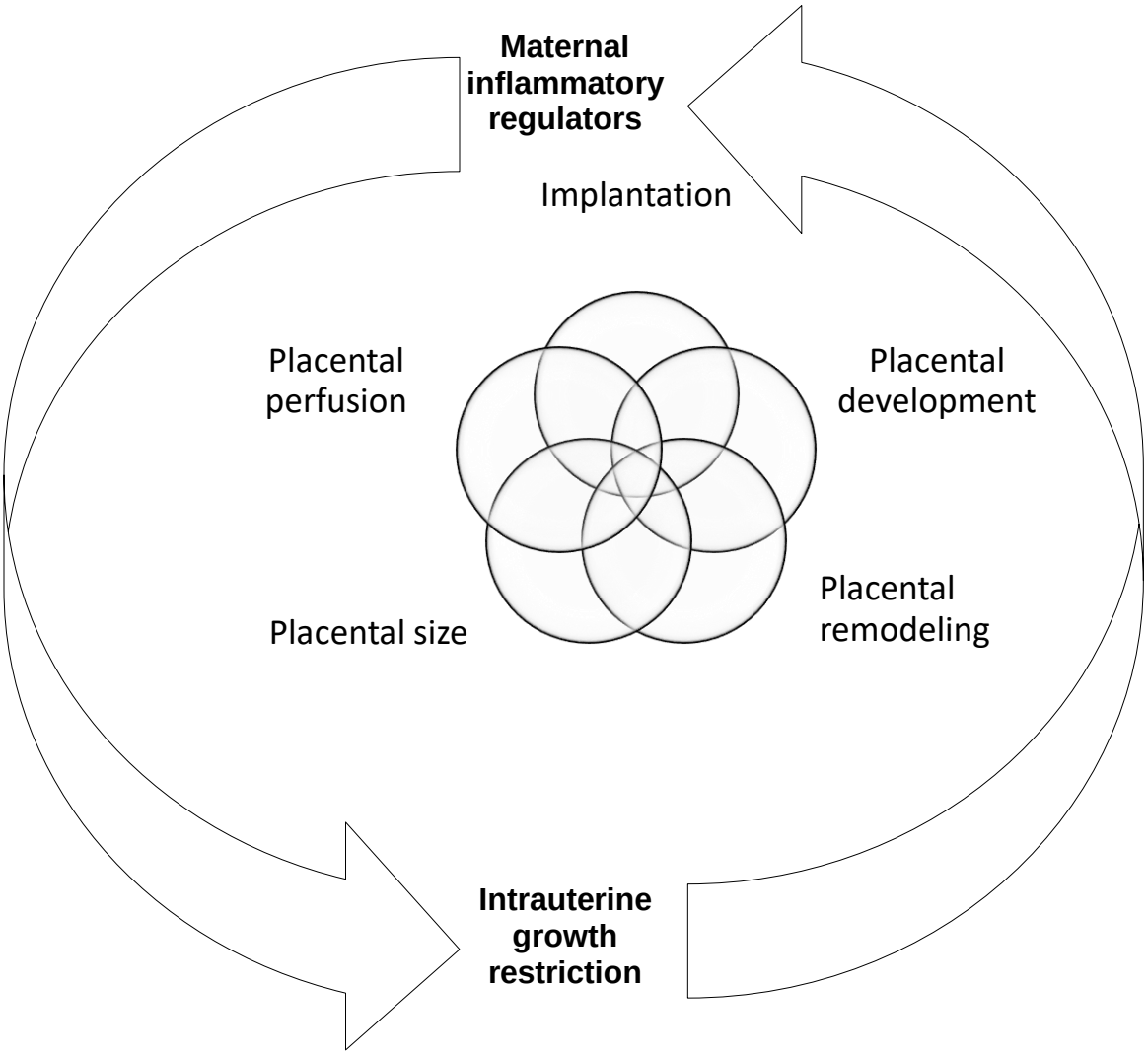

Figure A.2. Derivation of the analytical sample from the Pregnancy Outcomes and Community Health (POUCH) Study, n=1308 participants having maternal plasma and birthweight data

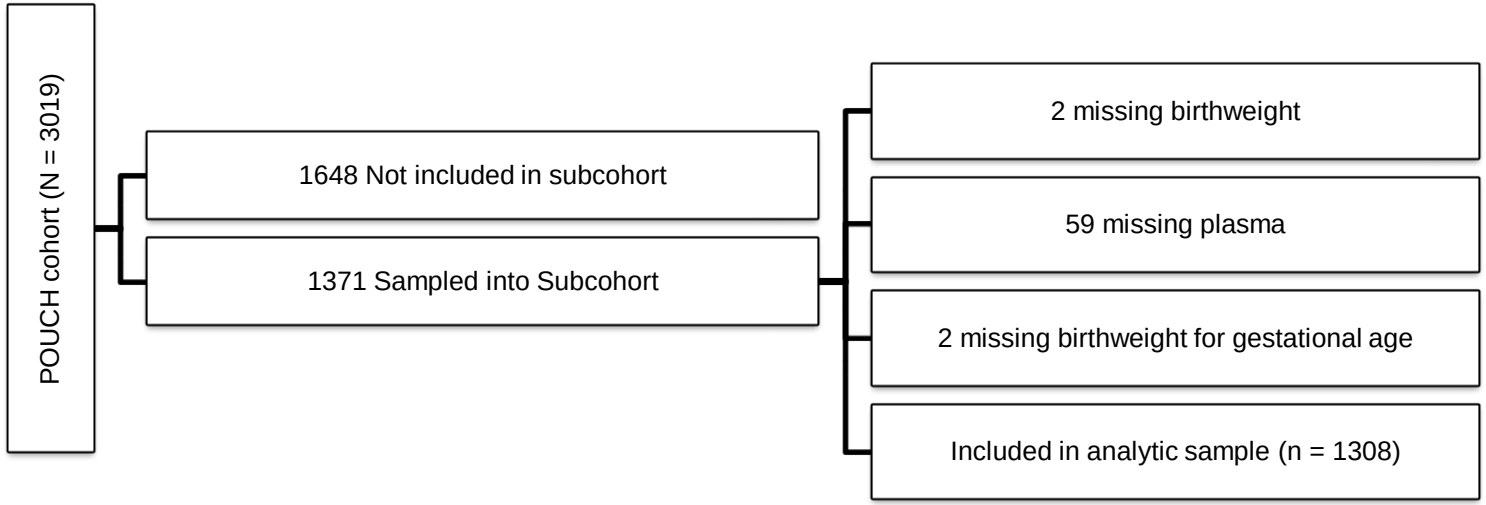

Figure A.3 Unadjusted Mean CRP Hs-CRP (µg/mL) with 95% CI for AGA, SGA, LGA by maternal Pre-pregnancy BMI for n=1308 dyads enrolled in the Pregnancy Outcomes and Community Health (POUCH) Study

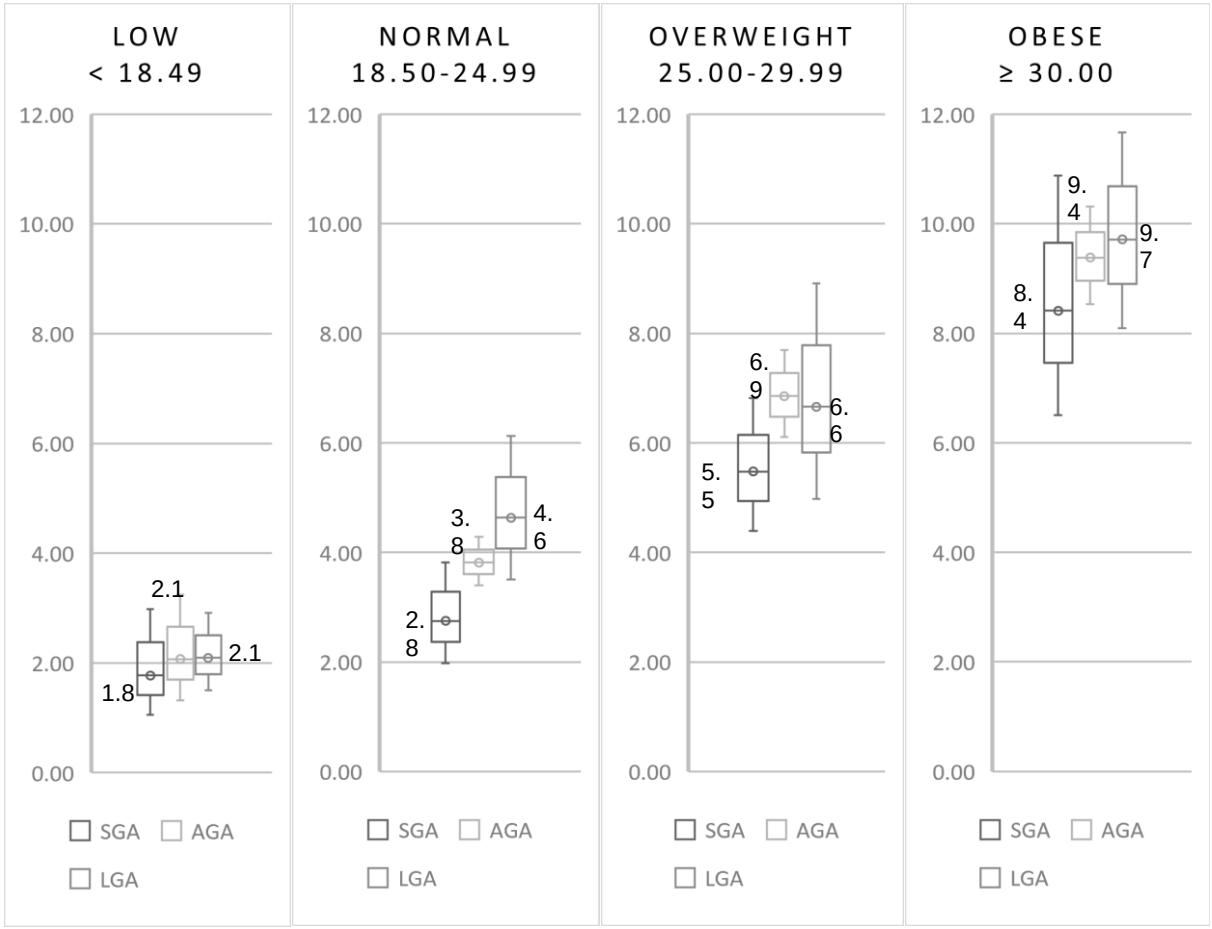

Figure A.4: Unadjusted and Adjusted Mean CRP Hs-CRP (µg/mL) with 95% CI for AGA, SGA, LGA for n=1308 dyads enrolled in the Pregnancy Outcomes and Community Health (POUCH) Study

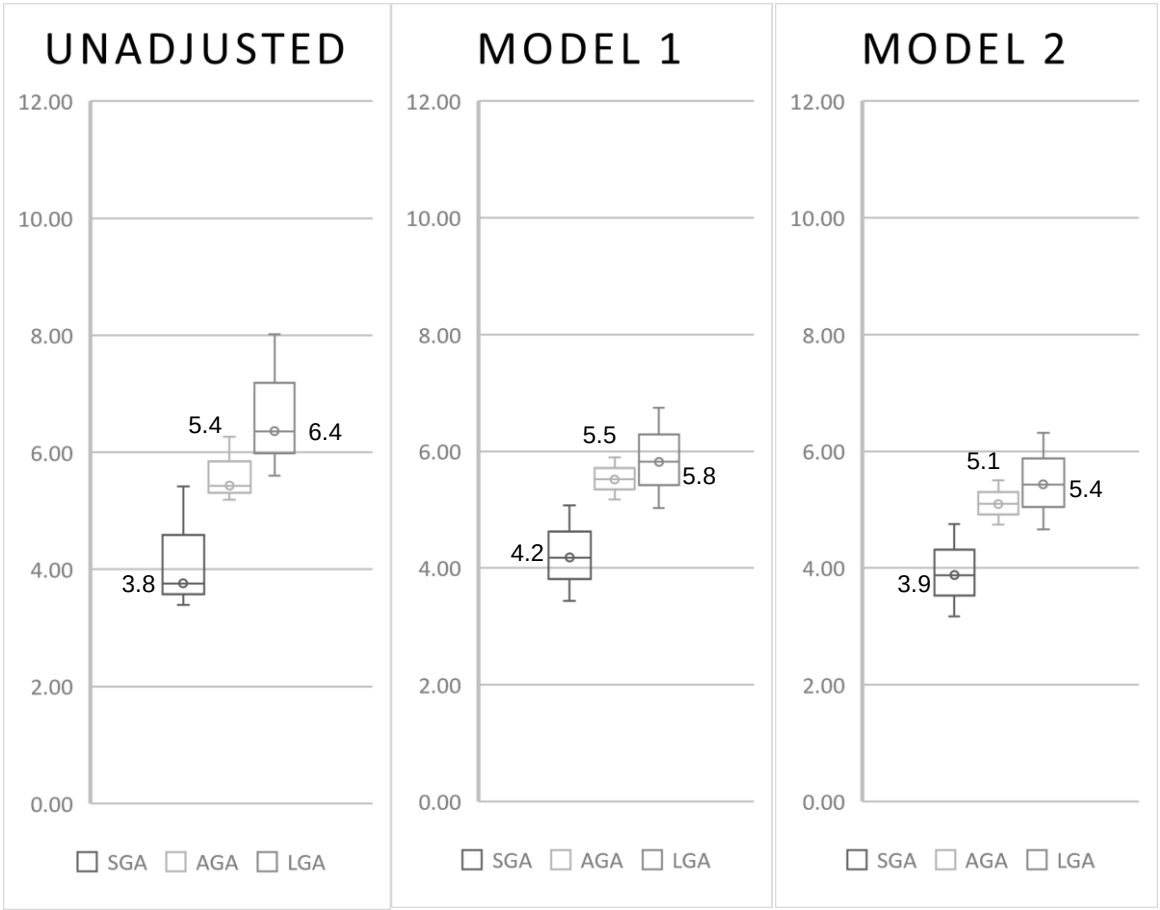

Figure A.5: Unadjusted IL-6 ZScore, TNF-a ZScore with 95% CI for AGA (n = 1038), SGA (n = 141), LGA (n = 129) and All Samples (n=1308) dyads enrolled in the Pregnancy Outcomes and Community Health (POUCH) Study

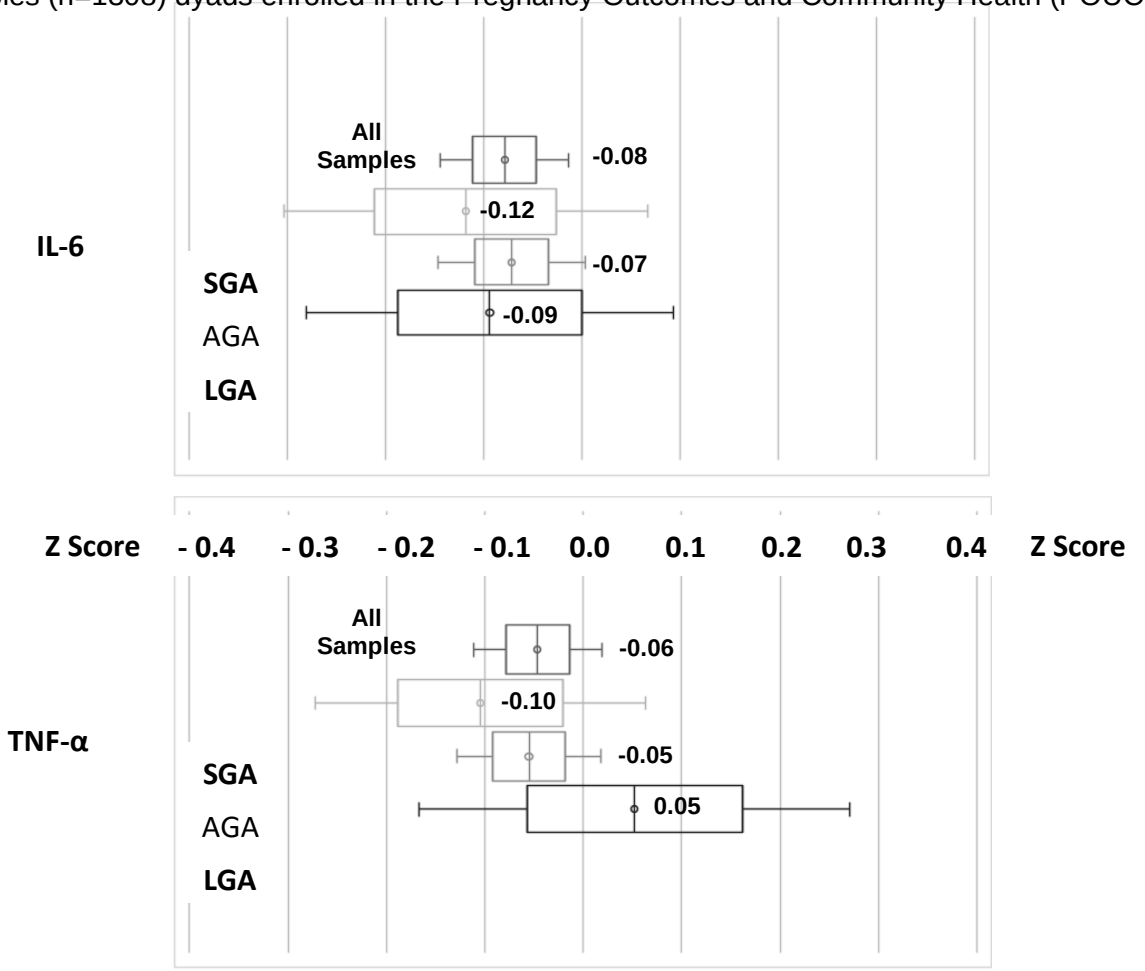

Figure A.6: Sensitivity Analysis - Unadjusted Mean CRP Hs-CRP (µg/mL) with 95% CI for AGA, SGA, LGA for dyads enrolled in the Pregnancy Outcomes and Community Health (POUCH) Study

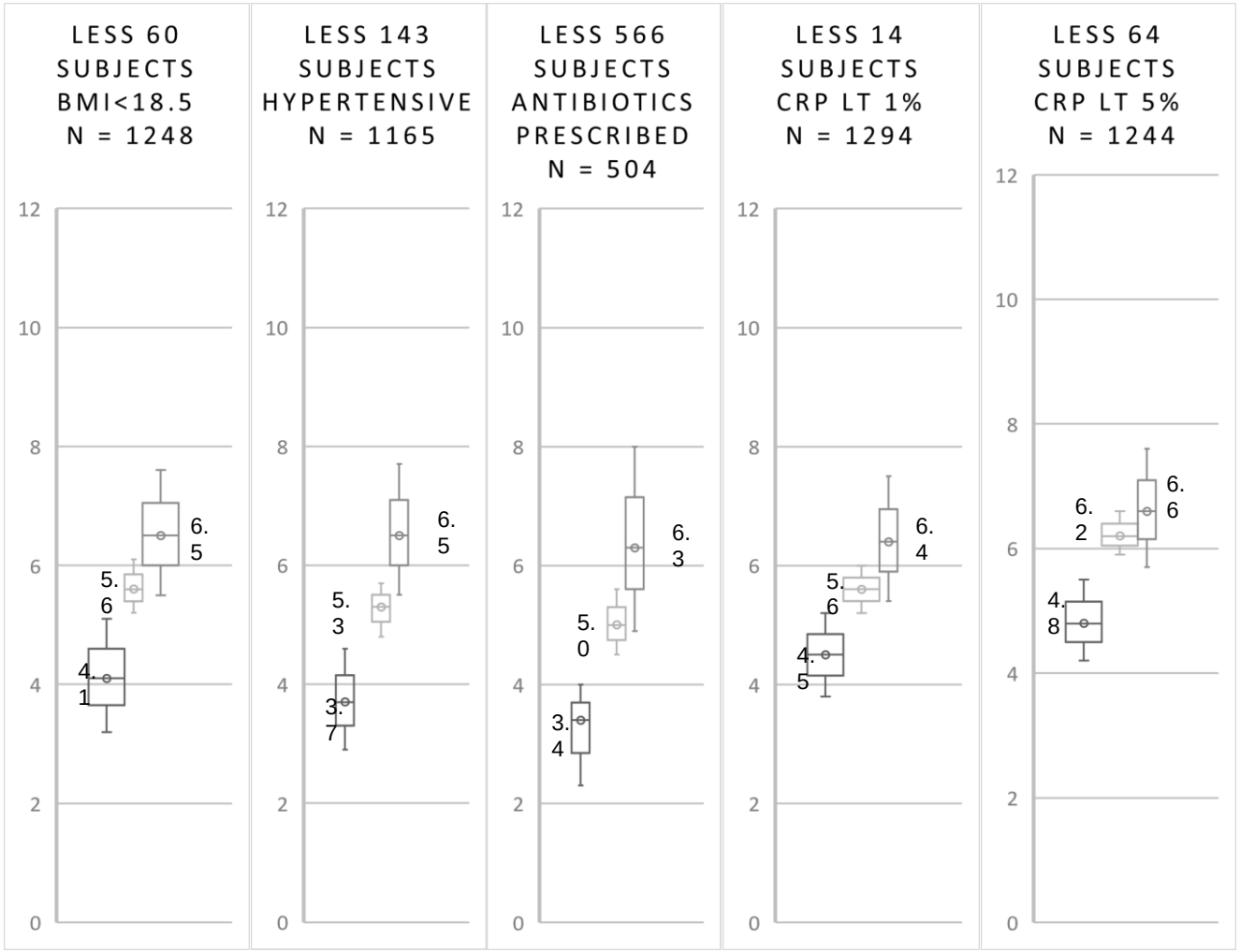

Figure A.7: Derivation of the analytical samples from the Pregnancy Outcomes and Community Health (POUCH) Study, n=1070 participants having maternal antibiotic prescription data, maternal plasma, and birthweight data

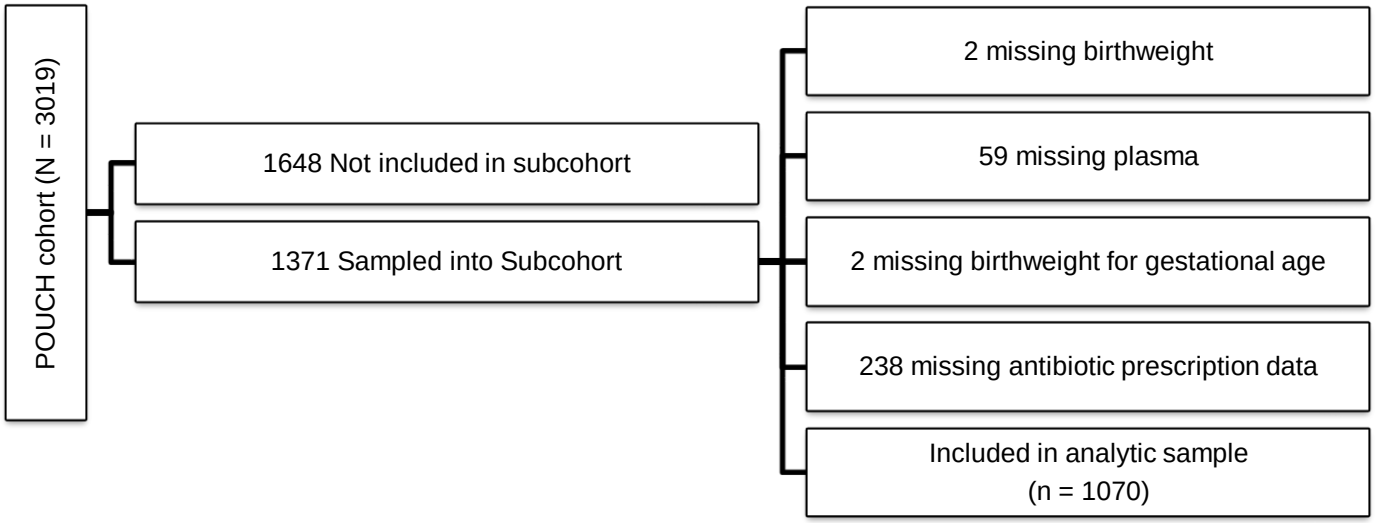

Figure A.8: Unadjusted Mean CRP (µg/mL) with 95% CI for AGA, SGA, LGA by antibiotic prescription within 2 weeks of blood draw for n=1070 maternal child dyads enrolled in the Pregnancy Outcomes and Community Health (POUCH) Study

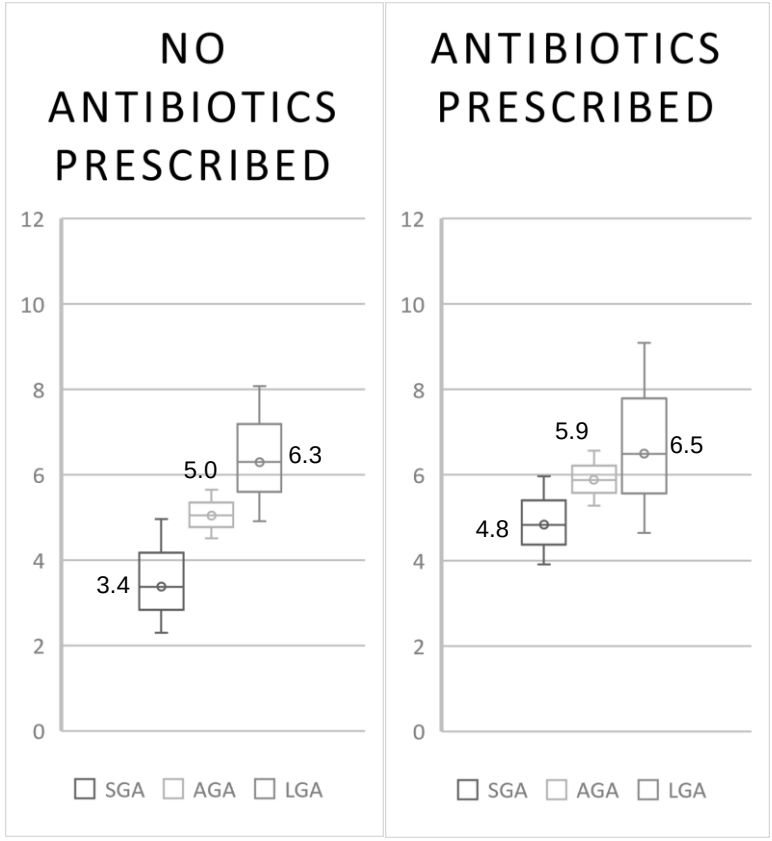

Figure A.9: Considerations for future work: potential antecedents or mediators of the relationship between the maternal immunologic milieu and birthweight for gestational age

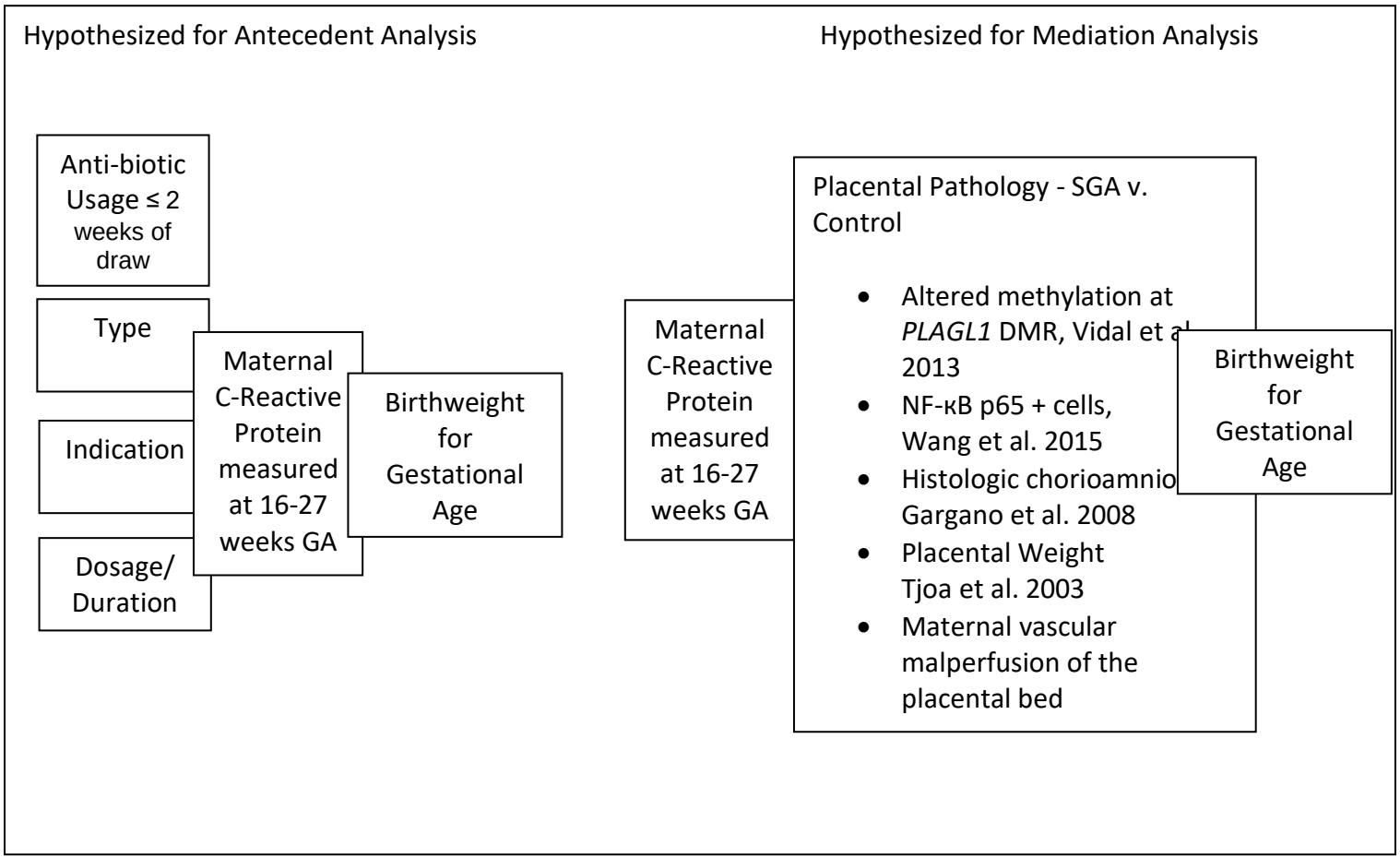

Figure A.10: Future Work - Derivation of the analytical samples from the Pregnancy Outcomes and Community Health (POUCH) Study cohort, n=1172? participants having maternal plasma, birthweight data, and placental pathology completed

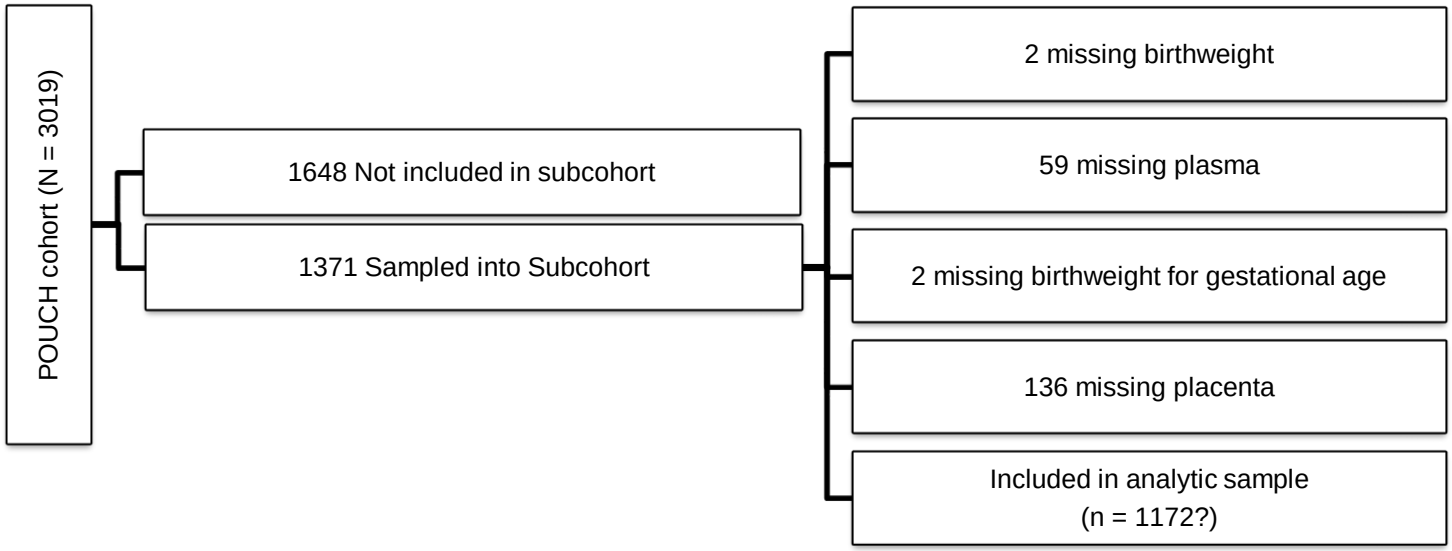

Figure A.11: Proposed data abstraction for investigation of hypothesized placental mediation of fetal growth restriction

Maternal vascular malperfusion (MVM)	**Patterns**	Global/partial	pathological villous maturation, distal hypoplasia with partial occlusion of vessels	
		Segmental/complete	Villous infarct with complete spiral artery occlusion	
	Gross	Placental hypoplasia, defined as placental weight < 10th percentile for gestational age and/or Thin umbilical cord, defined as width below the 10th percentile for gestational age or<8 mm at term		
		Placental Weight (g) _____ _____		Umbilical Cord Width (mm)
	Villous changes	Villous infarct(s), acute/subacute/remote, _____ cm in greatest dimension, occupying _____% of parenchyma.		Retroplacental hematoma, acute/subacute/remote, _____ cm in greatest dimension, occupying _____% of maternal surface.
		Accelerated villous maturation. YES NO		Distal villous hypoplasia. YES NO
	Vascular lesions	Acute atherosis/fibrinoid necrosis, basal and/or parietal.		Basal decidual vessel thrombosis.
		Persistent muscularization of basal plate arterioles.		Mural hypertrophy of membrane arterioles.
MVM Severity		**Placental weight**	**Parenchymal Involvement**	**Infarcts**
	Grade 1	Normal	< 30%	No more than 1 marginal infarct
	Grade 2	< 10th percentile for gestational age	≥ 30%	> 1 non-marginal infarct

Table A.1 Unadjusted Mean CRP by maternal characteristic

	N	CRP (µg/mL)	95% CI	P>|t|
All Women	1308			
Maternal Age				
< 20	231	4.2	3.6, 4.9	<0.0001
20 - 29	741	6.0	5.5, 6.5	REF
≥ 30	336	4.8	4.2, 5.6	0.01
Maternal Education				
< 12	302	5.3	4.6, 6.1	0.98
12	368	5.3	4.6, 6.2	0.85
≥ 12	638	5.4	5.0, 5.9	REF
Maternal Race				
White	666	5.2	4.7, 5.7	REF
Non-white	642	5.4	5.0, 5.9	0.6289
Maternal Pre-pregnancy BMI				
Underweight <18.5	60	2.0	1.4, 2.8	0.0005
Normal 18.5-24.99	581	3.8	3.4, 4.2	REF
Overweight 25.00-29.99	293	6.8	6.1, 7.5	<0.0001
Obese ≥ 30.00	374	9.3	8.6, 10.1	<0.0001
Medicaid Status				
Never Medicaid	563	5.0	4.6, 5.5	REF
Ever Medicaid	743	5.7	5.2, 6.3	0.06
Hypertensive Status				
No Hypertensive Disorder	1165	5.0	4.5, 5.5	REF
Any Hypertensive Disorder	143	5.3	4.0, 7.1	0.0022
Smoking				
Did not smoke during pregnancy	937	5.3	5.0, 5.7	REF
Stopped before enrollment	125	5.8	4.8, 7.0	0.40
Smoked < ½ pack per day at enrollment	169	4.7	3.6, 6.2	0.37
Smoked ≥ ½ pack per day at enrollment	77	6.3	5.0, 8.1	0.18
Gestational weeks at blood draw (Range 15.0-27.3 weeks)				
Tertile 1: <21.2	435	5.5	5.0, 6.1	0.96
Tertile 2: 21.2 to < 24.3	578	5.5	5.0, 6.1	REF
Tertile 3: ≥ 24.3	295	4.9	4.1, 5.7	0.19

Table A.2 Unadjusted TNF - α and IL – 6 by maternal characteristic							
	N	TNF - α	± SD	P>|t|	IL – 6	± SD	P>|t|
All Women	1308	-0.05	± 1.20		-0.07	± 1.04	
Maternal Age							
<20	231	0.04	± 2.72	0.35	-0.06	± 3.14	0.72
20-29	741	-0.05	± 1.59	REF	-0.09	± 2.18	REF
≥30	336	-0.09	± 2.39	0.59	-0.06	± 1.62	0.70
Maternal Education							
<12	302	0.03	± 2.19	0.19	-0.08	± 2.33	0.20
12	368	-0.04	± 2.35	0.68	-0.07	± 2.44	0.62
>12	638	-0.07	± 1.72	REF	-0.11	± 1.68	REF
Maternal Race							
White	666	-0.05	± 1.61	REF	-0.07	± 1.64	REF
Non-white	642	-0.04	± 1.65	0.92	-0.10	± 1.60	0.69
Maternal Pre-pregnancy BMI							
Underweight <18.5	60	-0.32	± 4.38	0.03	-0.26	± 2.36	0.33
Normal 18.5-24.99	581	-0.03	± 1.82	REF	-0.13	± 2.58	REF
Overweight 25.00-29.99	293	0.01	± 2.71	0.65	-0.02	± 4.25	0.21
Obese ≥ 30.00	374	-0.09	± 2.02	0.37	0.00	± 1.76	0.11
Medicaid Status							
Never Medicaid	563	0.01	± 1.69	0.10	-0.17	± 1.55	0.01
Ever Medicaid	743	-0.10	± 1.70	REF	0.01	± 1.82	REF
Hypertensive Status							
Any Hypertensive Disorder	143	-0.05	± 2.93	0.09	-0.01	± 3.26	0.41
No Hypertensive Disorder	1165	-0.03	± 1.29	REF	-0.87	± 1.29	REF
Smoking							
Did not smoke during pregnancy	937	-0.05	± 1.39	REF	-0.10	± 3.68	REF
Stopped before enrollment	125	-0.19	± 4.52	0.29	-0.10	± 2.80	0.95
Smoked < ½ pack per day at enrollment	169	0.13	± 3.55	0.08	0.04	± 4.03	0.10
Smoked ≥ ½ pack per day at enrollment	77	-0.11	± 3.95	0.06	0.01	± 1.47	0.31
Gestational weeks at blood draw (Range 15.0-27.3 weeks)							
Tertile 1: <21.2	435	-0.10	± 1.91	0.14	-0.06	± 2.63	0.44
Tertile 2: 21.2 to < 24.3	578	0.01	± 1.87	REF	-0.12	± 1.91	REF
Tertile 3: ≥ 24.3	295	-0.08	± 2.62	0.30	-0.02	± 1.88	0.27

Table A.3 Unadjusted CRP, TNF – α, and IL – 6 by birth outcome

	n	CRP (µg/mL)	95% CI	Pr > |t|	TNF - α Z Score	± SD	Pr > |t|	IL – 6 Z Score	± SD	Pr > |t|
All Women	1308									
Delivery Circumstance										
Spontaneous Preterm Labor	131	5.5	4.7, 6.4	0.6880	0.04	± 3.54	0.3956	-0.17	± 3.53	0.3349
Premature Rupture of Membranes	88	6.0	5.0, 7.2	0.2106	-0.02	± 3.83	0.7808	0.03	± 4.81	0.4356
Medically Indicated Preterm Delivery	98	5.9	5.0, 7.1	0.2187	-0.07	± 4.44	0.8563	-0.23	± 4.69	0.236
Term	991	5.3	4.9, 5.7	REF	-0.05	± 1.32	REF	0.07	± 1.32	REF
Birthweight (CDC Category)										
Low Birth Weight (<2500g)	161	5.1	4.2, 6.1	0.6489	-0.03	± 3.81	0.791	-0.08	± 3.84	0.9492
Normal Birthweight (2500g-4000g)	1053	5.3	5.0, 5.7	REF	-0.06	± 1.30	REF	-0.09	± 1.38	REF
High Birthweight (>4000g)	94	5.8	4.7, 7.1	0.4462	0.06	± 4.26	0.3454	-0.002	± 3.62	0.4162

Table A.4 Pearson correlation coefficients for standardized continuous variables

n = 1308	BW	CRP	IL-6	TNF- α	PrePreg BMI	Maternal Age	Weeks at draw
BW	1.0000						
CRP	0.1052 0.0001	1.0000					
IL-6	-0.0379 0.1713	0.0517 0.0618	1.0000				
TNF- α	-0.0237 0.3918	-0.029 0.2951	0.4893 <.0001	1.0000			
PrePreg BMI	0.0958 0.0005	0.4256 <.0001	0.0627 0.0233	-0.01824 0.5099	1.0000		
Maternal Age	0.2089 <.0001	0.0601 0.0298	0.0059 0.8320	-0.0206 0.4559	0.0705 0.0108	1.0000	
Weeks at draw	-0.0895 0.0012	-0.0688 0.0128	0.0231 0.4045	0.0070 0.8002	-0.0774 0.0051	-0.0193 0.4849	1.0000

Table A.5 Unadjusted and Adjusted Mean CRP by AGA, SGA, LGA

	Size for Gestational Age**	n	CRP (*μ*g/l)	95% CI	Pr > |t|
All Women		1308			
Unadjusted	SGA	141	3.8	3.4, 5.4	0.002
	AGA	1038	5.4	5.2, 6.3	REF
	LGA	129	6.4	5.6, 8.0	0.082
Model 1	SGA	141	4.2	3.4, 5.1	0.0080
	AGA	1038	5.5	5.2, 5.9	REF
	LGA	129	5.8	5.0, 6.8	0.5156
Model 2	SGA	141	3.9	3.2, 4.8	0.0124
	AGA	1038	5.1	4.7, 5.5	REF
	LGA	129	5.4	4.7, 6.3	0.4583

** Size for Gestational Age: SGA - Small for Gestational Age (<10th percentile), AGA - Appropriate for Gestational Age, LGA - Large for Gestational Age (≥90th percentile)
Model 1: Adjusted for maternal pre-pregnancy BMI
Model 2: Adjusted for maternal race, pre-pregnancy BMI, maternal age at enrollment, gestational weeks at blood draw

Table A.6 Unadjusted and Adjusted Mean CRP by AGA, SGA, LGA with sensitivity analyses for maternal BMI or hypertensive disorder

	Size for Gestational Age**	n	GMean CRP (*μ*g/l)	95% CI	Pr > |t|
less 60 women with BMI<18.5 for n = 1248					
Unadjusted	SGA	127	4.1	3.2, 5.1	0.0090
	AGA	996	5.6	5.2, 6.1	REF
	LGA	125	6.5	5.5, 7.6	0.1237
Model 1	SGA	127	4.4	3.6, 5.4	0.017
	AGA	996	5.8	5.4, 6.2	REF
	LGA	125	6.0	5.2, 7.0	0.5541
Model 2	SGA	127	4.1	3.3, 5.1	0.0279
	AGA	996	5.3	5.0, 5.8	REF
	LGA	125	5.6	4.8, 6.6	0.5033
less 143 women with any hypertensive disorder for n = 1165					
Unadjusted	SGA	123	3.7	2.9, 4.6	0.0047
	AGA	920	5.3	4.8, 5.7	REF
	LGA	122	6.5	5.5, 7.7	0.0219
Model 1	SGA	123	4.0	3.3, 5.0	0.0126
	AGA	920	5.4	5.0, 5.8	REF
	LGA	122	5.8	5.0, 6.7	0.3642
Model 2	SGA	123	3.7	3.0, 4.6	0.0181
	AGA	920	5.0	4.5, 5.3	REF
	LGA	122	5.4	4.6, 6.3	0.257

** Size for Gestational Age: SGA - Small for Gestational Age (<10th percentile), AGA - Appropriate for Gestational Age, LGA - Large for Gestational Age (≥90th percentile)
Model 1: Adjusted for maternal pre-pregnancy BMI
Model 2: Adjusted for maternal race, pre-pregnancy BMI, maternal age at enrollment, gestational weeks at blood draw

Table A.7 Unadjusted and Adjusted Mean CRP by AGA, SGA, LGA with sensitivity analyses for CRP outliers					
	Size for Gestational Age**	n	CRP (*μ*g/l)	95% CI	Pr > |t|
less 14 women with CRP LT 1% for n = 1294					
Unadjusted	SGA	135	4.5	3.8, 5.2	0.0062
	AGA	1030	5.6	5.2, 6.0	REF
	LGA	129	6.4	5.4, 7.5	0.1513
Model 1	SGA	135	4.9	4.3, 5.0	0.0254
	AGA	1030	5.7	5.4, 6.1	REF
	LGA	129	5.9	5.1, 6.8	0.7065
Model 2	SGA	135	4.6	4.1, 5.2	0.0377
	AGA	1030	5.3	5.0, 5.7	REF
	LGA	129	5.6	4.8, 6.5	0.6005
less 64 women with CRP LT 5% for n = 1244					
Unadjusted	SGA	130	4.8	4.2, 5.5	0.0006
	AGA	986	6.2	5.9, 6.6	REF
	LGA	128	6.6	5.7, 7.6	0.4866
Model 1	SGA	130	5.2	4.7, 5.8	0.0017
	AGA	986	6.3	6.0, 6.6	REF
	LGA	128	6.2	5.4, 7.1	0.7896
Model 2	SGA	130	5.0	4.4, 5.6	0.0026
	AGA	986	6.0	5.7, 6.3	REF
	LGA	128	6.0	5.2, 6.8	0.9249

** Size for Gestational Age: SGA - Small for Gestational Age (<10th percentile), AGA - Appropriate for Gestational Age, LGA - Large for Gestational Age (≥90th percentile)
Model 1: Adjusted for maternal pre-pregnancy BMI
Model 2: Adjusted for maternal race, pre-pregnancy BMI, maternal age at enrollment, gestational weeks at blood draw

Table A.8 Unadjusted Mean CRP and antibiotic prescription					
		n	CRP (µg/l)	95% CI	Pr > |t|
	All Women	1070			
Prescribed*	No	504	5.0	4.6, 5.6	REF
	Yes	566	5.8	5.3, 6.4	0.036

* Antibiotics prescribed within 2 weeks prior to enrollment

Table A.9 Antibiotic prescription* by AGA, SGA, LGA						
		n	Prescribed*		Odds Ratio	95% CI
			No	Yes		
	All Women	1070	504	566		
Size for Gestational Age**	SGA	117	55	62	1.213	0.75, 1.96
	AGA	854	394	460	1.0	(Reference)
	LGA	99	55	44	0.702	0.43, 1.16

* Antibiotics prescribed within 2 weeks prior to enrollment
** Size for Gestational Age: SGA - Small for Gestational Age (<10th percentile), AGA - Appropriate for Gestational Age, LGA - Large for Gestational Age (≥90th percentile)

Table A.10 Unadjusted Mean CRP and antibiotic prescription by AGA, SGA, LGA				
Size for Gestational Age**	n	CRP (µg/l)	95% CI	Pr > |t|
SGA	117			0.109
No Antibiotics Prescribed	55	3.4	2.3, 5.0	
Antibiotics Prescribed	62	4.8	3.9, 6.0	
AGA	854			0.054
No Antibiotics Prescribed	394	5.0	4.5, 5.6	
Antibiotics Prescribed	460	5.8	5.3, 6.6	
LGA	99			0.884
No Antibiotics Prescribed	55	6.3	5.0, 8.1	
Antibiotics Prescribed	44	6.5	4.6, 9.1	

* Antibiotics prescribed within 2 weeks prior to enrollment
** Size for Gestational Age: SGA - Small for Gestational Age (<10th percentile), AGA - Appropriate for Gestational Age, LGA - Large for Gestational Age (≥90th percentile)

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