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The Relationship of Mid-Pregnancy Levels of Cytokines, Stress, and Depression with Gestational Age at Delivery

Melissa Molinari Shelton

University of South Florida, mmolinar@health.usf.edu

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The Relationship of Mid-Pregnancy Levels of Cytokines, Stress, and Depression
with Gestational Age at Delivery

by

Melissa Molinari Shelton

A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Major Professor: Maureen Groer, Ph.D.
Jason W. Beckstead, Ph.D.
Cecilia Jevitt, CNM, Ph.D.
Cecile A. Lengacher, Ph.D., RN

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DEDICATION

To my husband, Stephen, who has been there to cheer me on through my successes and encourage and support me during times of challenge. You are my best friend and I could not have done this without you.

To my family and friends – Thank you for your patience and understanding when, “I can’t. I have to go to library,” became my standard response.

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LIST OF ACRONYMS

ACTH	adrenocorticotropin hormone
BMI	body mass index
CRH	corticotropin-releasing hormone (from hypothalamus)
dl	deciliters
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony-stimulating factor
HPA	Hypothalamic-Pituitary-Adrenal
IFN	Interferon
IL	Interleukin
IRB	Institutional Review Board
MCP	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
µg	micrograms
MIF	macrophage migration inhibitory factor
ml	milliliters
nm	nanometers
pCRH	corticotropin-releasing hormone (from placenta)
PI	Principal Investigator
pg	picograms

PLGF	placental growth factor
POMS	The Profile of Mood States
PPROM	preterm premature rupture of membranes
PSS	The Perceived Stress Scale
PTB	preterm birth
PTD	preterm delivery
PTL	preterm labor
rpm	revolutions per minute
SD	standard deviation
sPTD	spontaneous preterm delivery
TNF	Tumor Necrosis Factor
VEGF	vascular endothelial growth factor

ABSTRACT

Pregnancy is a time of alternating states of inflammation. The establishment of pregnancy is marked by controlled inflammation and transition toward an anti-inflammatory state for much of the gestational period before returning to an inflammatory state at the onset of labor. Stress and depression trigger the HPA Axis to produce cortisol and levels are maintained in a state of elevation during pregnancy and continue to rise before parturition.

The aim of this research was to explore the relationship of gestational age at delivery with mid-pregnancy levels of cytokines, stress and depression. Participant samples ($N = 122$) were collected between 16 and 26 weeks gestation and analyzed for 14 cytokines using a bead-based multiplex assay. Plasma cortisol was also measured along with demographic variables and measures of perceived stress and dysphoric mood.

Results of Pearson's correlations showed that gestational age at delivery was significantly inversely correlated with pro-inflammatory cytokine IFN- γ and anti-inflammatory IL-13. A significant positive correlation was noted with the number of pregnancies in the obstetric history and pregnancy length. Both cortisol and stress were not correlated with gestational age at delivery.

CHAPTER ONE

INTRODUCTION

Background of Problem

During pregnancy, activation of the immune system by inflammation and the Hypothalamic-Pituitary-Adrenal (HPA) axis by stress triggers the release of cytokines and hormones into the maternal-fetal circulation. Responses to such events vary by individual, but could mark the start of a cascade of events leading to a spontaneous birth or a medically indicated induced delivery. The length of time that a woman is pregnant is often directly related to the health and mortality of a newborn infant.

Compared with infants born at term, preterm infants have higher rates of disability and mortality (Behrman & Butler, 2007). Early delivery interferes with the fetus' development in utero, resulting in immature (and often impaired functioning) vital body systems. A child born prior to 37 weeks gestation may not have fully developed lungs that are able to function properly to provide adequate oxygenation to support life or the body's processes. Prolonged pregnancies also carry risks which are usually associated with the increased size of the infant at birth.

While there is no single cause, the incidence of delivery at an early gestational age has been attributed to a combination of idiopathic causes, inflammation, over-distention of the uterus, and psychosocial variables such as maternal stress and depression during pregnancy. Delivery prior to term development has long-lasting emotional and physical effects on the affected family, as well as an immense economic impact on the family and healthcare system. Similarly, the cause of post-term deliveries is not entirely understood but also poses harmful risks to the pregnant woman and fetus.

Just as there is no single cause for the early onset of labor and preterm delivery, one particular symptom does not precede its onset. Several conditions that may precede early labor and delivery are discussed in the literature. Vaginal infections have been noted in patients who have delivered prior to term (Hendler et al., 2007; Hitti et al., 2007) with the most common infection noted as bacterial vaginosis. Vaginal bleeding during pregnancy has also been associated with a higher incidence of early delivery (Kim et al., 2005; Weiss et al., 2004). Other conditions which contribute to a greater incidence of preterm delivery include stressful life conditions (Dasari & Kodenchery, 2007), a body mass index (BMI) greater than 30 (Kim, et al., 2005), and a history of induced abortion (Ancel, Lelong, Papiernik, Saurel-Cubizolles, & Kaminski, 2004; Moreau et al., 2005).

Maternal stress may also play a role in controlling the length of gestation. The inclusion of stress in the maternal environment and how women process stressful stimuli may alone, or in combination with other variables, contribute to onset of labor. During the body's normal response to stress, it releases

corticotropin-releasing hormone (CRH) into circulation. Studies have shown that levels of stress in conjunction with CRH levels may be a predictor of gestational age at delivery (Hobel, Dunkel-Schetter, Roesch, Castro, & Arora, 1999; Ruiz, Fullerton, Brown, & Dudley, 2002).

The delicate balance of cytokines of the immune system during pregnancy has also been investigated for its role in influencing the length of gestation. Immune mechanisms vary and adapt throughout pregnancy depending on an individual's genetic and environmental influences (Mor & Cardenas, 2010). An attack on the developing fetus by immune protectors resulting in a rise of inflammation could result in an early birth or miscarriage.

Purpose

The current investigation sought to examine the links between maternal physiological and psychological variables perceived stress, depressive symptoms, cytokines, and cortisol with gestational age at delivery. Biological markers of interest, such as maternal plasma levels of cytokines and cortisol, may aid in the early identification of women at risk for early delivery, or deliveries that extend beyond term. Figure 1 is a visual representation of the relationship of the variables of interest in the current study. The psychological variables, stress and depressive symptoms, may be related and the level of one may influence the level of the other. The presence of stress and depressive symptoms triggers the body's physiological response and release of cortisol by the HPA axis and cytokines by the immune system. During pregnancy, this state may influence the gestational age at which labor is initiated and birth occurs.

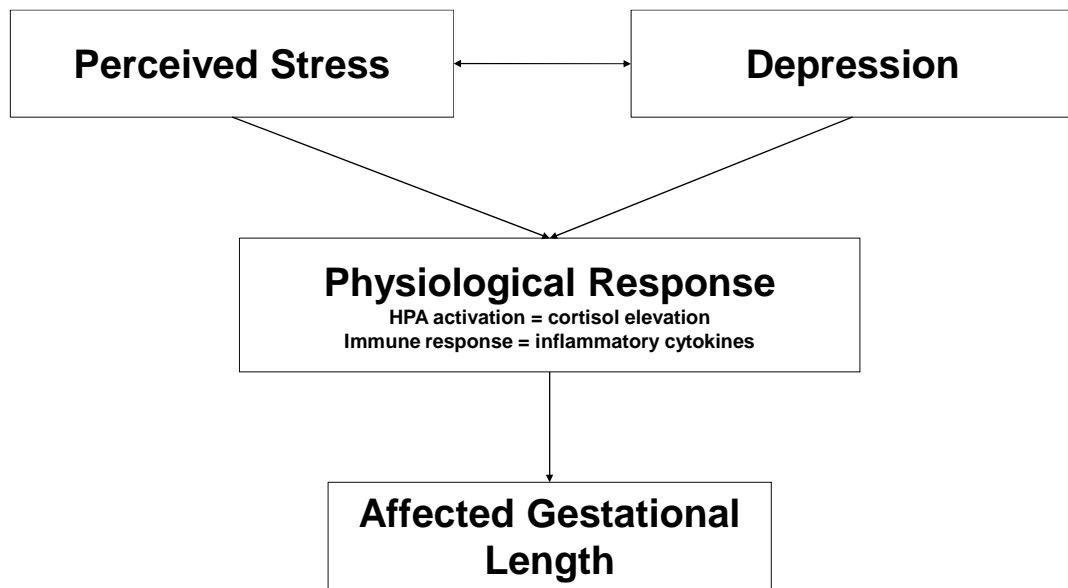


Figure 1. Conceptual Framework

Significance of Study

Investigation is necessary to understand how gestational length is affected and identify methods of predicting women who are at risk for a pre- or post-term delivery. The utility of such findings will translate into preventive interventions and treatments, thus saving thousands of lives and preventing future disability in children. This study builds on available findings, but is one of few investigations to explore mid-pregnancy plasma cytokine levels utilizing a multiplex, bead-based assay analyzed with Luminex technology.

Specific Aims and Research Questions

This study focused on the evaluation of physiological and psychological variables and gestational age at delivery. Specifically, this investigator proposed to:

Aim 1. Estimate the bivariate relationship between mid-pregnancy measures of physiological and psychological variables and gestational age at delivery.

Research Question 1: To what extent are mid-pregnancy maternal plasma cytokines correlated with gestational age at delivery?

Research Question 2: To what extent are mid-pregnancy maternal plasma cortisol levels correlated with gestational age at delivery?

Research Question 3: To what extent are mid-pregnancy perceived stress scores correlated with gestational age at delivery?

Research Question 4: To what extent are mid-pregnancy depressive symptom scores correlated with gestational age at delivery?

Aim 2. Examine the unique role played by physiological and psychological variables in predicting gestational age at delivery in a multivariate multiple regression model.

Definition of Terms

The following terms have been defined for the purposes of this study:

Apgar score is a summed rating assessment (0 to 2 per item) of a newborn infant based on five items: heart rate, respiratory effort, reflex irritability, muscle tone and color (Apgar, 1953). The assessment is completed at one minute after birth and repeated again at five minutes to assess for change in condition. The five minute Apgar score is a strong indicator of infant survival after birth (Cunningham et al., 2010).

Cytokines are “regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses” (Thomson & Lotze, 2003, p. 5).

Gestational length (or gestational age) refers to the length of time that a woman is pregnant. It is often calculated by using the date of a woman’s last menstrual period as the beginning of her pregnancy and may be confirmed by other means such as the use of ultrasound (Behrman & Butler, 2007).

Gravida refers to the number of times that a woman has been pregnant.

Medically indicated birth refers to the delivery of an infant prior to the spontaneous onset of labor due to risk for or presence of medical complications.

Nulliparity refers to a woman who has not previously given birth to a child.

Parturition is defined as the “bringing forth of young” (Cunningham, et al., 2010); the process of giving birth to offspring.

Postterm birth occurs at 42 weeks (294 days) gestation or greater (Brazelton, 1984).

Preterm birth may occur as early as 20 weeks (140 days) (Creasy & Iams, 1999) and prior to 37 weeks (259 days) gestation (Mathews & MacDorman, 2008).

Spontaneous birth is the natural onset of labor leading to delivery of the infant.

Term birth is defined as a pregnancy with delivery of the infant occurring between 37 and 41 weeks gestation (Mathews & MacDorman, 2008).

Chapter Summary

Chapter one provides an introduction to the problem and establishment of the significance of the study. Alterations in gestational length of pregnancy may have detrimental effects on families and the healthcare system, therefore establishment of effective means of identifying women at risk for shortened or prolonged pregnancy may provide an opportunity for intervention and prevention. The current study sought to examine the links between levels of maternal stress, depressive symptoms, cytokines, and cortisol with gestational age at delivery. Relationships among these variables, if established, may be utilized as markers of risk.

Chapter two will provide an overview of the physiological changes during pregnancy and explain the role of the immune system. Conditions which may contribute to alterations in gestational length will be examined. Relevant literature will be reviewed.

CHAPTER TWO

REVIEW OF LITERATURE

This chapter presents a review of the literature relevant to this study. Topics include an overview of the physiological mechanisms associated with pregnancy with an emphasis placed on identifying alterations among a pregnancy characterized as *normal* and that which has been complicated by various conditions affecting gestational length. An introduction to basic immunology has been included to establish a general understanding of cytokines and their function. To further support the hypothesized relationship between maternal cytokines, stress, and depressive symptoms with gestational age at delivery, the effect of cytokine, stress and depression levels on pregnancy outcome are also discussed.

Physiological Mechanisms of the Normal Pregnancy

Adaptations of the maternal body systems. The female body undergoes many changes as it prepares for conception, maintenance of pregnancy, parturition, and return to the non-pregnant state. Besides the obvious transformation in physical appearance, each body system of the pregnant female undergoes alterations throughout pregnancy to keep up with the demands of the developing fetus.

The most notable changes to the maternal reproductive system are the expansion in the size of the uterus and increased uterine blood flow. The uterus in the non-pregnant state weighs about 70 grams with a total cavity volume of 10 milliliters or less. At term, the pregnant uterus can reach up to 1100 grams in weight and total volume of five to 20 liters (Cunningham, et al., 2010).

Uterine blood flow is vitally important to the survival of the fetus. Blood flow to the uterus provides for adequate perfusion of the placenta which is the means of delivering oxygen, essential nutrients, and removal of waste from the fetus (Cunningham, et al., 2010; Torgersen & Curran, 2006). Factors that impact uterine blood flow including medical conditions such as hypertensive disorders, diabetes mellitus, and post-date gestation can have a detrimental effect on fetal development and survival (Torgersen & Curran, 2006).

In order to accommodate the increased blood flow to the uterus, placenta, and fetus and prepare for the loss of blood after delivery, the hematologic system must adapt by increasing the maternal volume of blood (Cunningham, et al., 2010; Torgersen & Curran, 2006). Although this state of hypervolemia varies per individual, blood volume increases (on average) 48 percent during pregnancy with a marked increase occurring during the second trimester. Pregnancies in women challenged with megaloblastic anemia or severe gestational hypertension may not appreciate the same expansion in blood volume as that in a normal pregnancy thus impairing the ability to tolerate blood loss at parturition (Pritchard, 1965).

Pregnancy affects metabolism of proteins, carbohydrates, and fats in response to the demands placed by the fetus on the mother. The maternal basal metabolic rate may increase 10 to 20 percent by the third trimester of pregnancy resulting in increased energy demands and caloric consumption (Cunningham, et al., 2010). Weight gain during pregnancy is the result of the expanding size of the uterus and its contents, increased blood and extracellular fluid volume, and additional stores of maternal fat and water in the body.

Studies have demonstrated that pre-pregnancy weight, as well as weight gained during pregnancy, are related to infant weight at birth (Cunningham, et al., 2010; Heude et al., 2011) and the risk for developing complications during pregnancy (Heude, et al., 2011). Low weight gain during pregnancy presents an increased risk for preterm delivery while the risk of gestational hypertension and diabetes is greater in women with a high pre-pregnancy body mass index (BMI) (Heude, et al., 2011).

The endocrine system functions to regulate the body by secreting hormones into the bloodstream. One of the earliest examples of this function during pregnancy is the pituitary's secretion of growth hormone. Maternal secretion of pituitary growth hormone into circulation is later replaced by placental growth hormone during pregnancy (Cunningham, et al., 2010). Findings by Mittal et al. (2007) support the relationship between placental growth hormone with fetal growth and the development of preeclampsia. In their study, women with preeclampsia had higher levels of placental growth hormone than women experiencing a normal pregnancy. Among women with preeclampsia,

lower levels of placental growth hormone were measured in women delivering infants who were small for gestational age than those delivering infants of average size.

The most important function of the maternal immune system to note is the suppression of a Th1 response and upregulation of Th2 cells. This balance is in contrast to the non-pregnant state, but vital to prevent rejection of the fetus (i.e. miscarriage) (Thellin & Heinen, 2003) or risk development of pregnancy related complications such as preeclampsia (Jonsson et al., 2006). An overview of the immune system and the immune system during pregnancy is described later.

Parturition. Four phases comprise parturition which begins at the point of conception and ends when fertility is restored and the woman is once again able to achieve pregnancy. In the normal pregnancy, Phase One (Quiescence) is characterized by a softening of the cervix while maintaining its structure (competence) and the absence of dilation. Ninety-five percent of the pregnancy is spent in this phase. Phase Two (Activation) is the period during which the body prepares for labor. This occurs during the last six to eight weeks of pregnancy and includes active changes in the uterus. The *ripening* of the cervix includes changes in connective tissue leading to further softening and thinning allowing for the passage of the fetus through the birth canal. Phase Three (Stimulation) includes all of the processes associated with labor; uterine contractions, cervical dilation, and delivery of the fetus and placenta. The final phase, Phase Four (Involution), is the recovery phase leading to a restored state of fertility. Phase Four begins immediately after the placenta is delivered and may last four to six

weeks (or longer if breastfeeding). During this time, the uterus and cervix return to the prepregnant state and fertility is restored once ovulation begins again (Cunningham, et al., 2010).

Alterations of these four phases may lead to difficult deliveries, preterm labor, or post-term delivery. An incompetent cervix, one that is dilating and thinning prematurely, may lead to miscarriage or preterm delivery. Owen and colleagues (2001) reported that a noted thinning, or shortening, in cervical length on ultrasound can be noted as early as 16 weeks gestation in women who will experience spontaneous preterm birth.

Complications in Pregnancy Affecting the Length of Gestation

Gestational length (or gestational age) refers to the time which has passed since the first day of the last menstrual period to the day of birth. For humans, the average length of a pregnancy is 40 weeks (280 days) (Cunningham, et al., 2010), however a term gestation includes births occurring between 37 and 41 completed weeks gestation (Fleischman, Oinuma, & Clark, 2010). During this time, the fetus undergoes a major transformation beginning with the union of the haploid gametes to form a diploid zygote, formation of functioning body systems, and growth from mere millimeters up to 14 to 20 inches at full term.

Shortened gestational length. Shortened gestational length interferes with the fetus' development of mature body systems vital for life outside of the womb. Immature neurological, immune, respiratory, urinary, and gastrointestinal systems leave the fetus at risk for numerous complications including infection

and death. A number of factors, a few of which are presented here, contribute to a shortened gestational length resulting from a birth prior to term.

Spontaneous abortion (miscarriage). Among known pregnancies, spontaneous abortion affects 15 to 20 percent of women, primarily in the first trimester of pregnancy. In addition to other variables, factors which may contribute to the occurrence of spontaneous abortion are advanced maternal age, previous history of miscarriage or ectopic pregnancy (outside of the uterus), maternal tobacco use, and chromosomal abnormalities (de la Rochebrochard & Thonneau, 2002). Failure to maintain a Th2 balanced state in pregnancy may also contribute to increased inflammation and subsequent attack on the developing *non-self* fetus leading to miscarriage.

Preterm labor and birth. It is estimated that 12.3 percent of all live births in the United States occur preterm (Martin et al., 2010) contributing to total healthcare costs of over \$26 billion in the United States alone (Institute of Medicine, 2007). Prematurity is a leading cause of death in the first month of life and the percentage of preterm-related infant deaths continues to rise annually (Mathews & MacDorman, 2008). A major contributor to illness and disability among infants in this population, preterm birth can lead to developmental delays, chronic respiratory problems, and problems with vision and hearing. If the child survives beyond birth and infancy, affected families may be faced with the emotional, physical, and financial challenges of caring for a child with lifelong illness and disability.

There are four main reasons for a preterm delivery: (a) medically indicated induced vaginal or cesarean delivery for maternal or fetal indications (i.e. preeclampsia, placental abruption, fetal distress), (b) spontaneous preterm labor with intact membranes, (c) preterm premature rupture of the membranes (PPROM), and (d) multifetal gestation (Cunningham, et al., 2010; Goldenberg, Culhane, Iams, & Romero, 2008). Medically indicated deliveries account for 30 to 35 percent of preterm births, 40 to 45 percent result from spontaneous preterm labor, and PPRM accounts for 25 to 30 percent of preterm deliveries (Goldenberg, et al., 2008). Among pregnancies in the United States in 2008, a preterm delivery resulted from 10.6 percent of singleton pregnancies compared to 58.9 percent of twin pregnancies and 92.2 to 95.7 percent of triplet and higher order pregnancies (Martin, et al., 2010). Increased stretch of the uterus, greater production of corticotropin-releasing hormone (CRH), and elevated surfactant levels in the amniotic fluid resulting from a multifetal gestation may contribute to the disparate preterm rates compared to singleton pregnancies (Stock & Norman, 2010).

Research has focused on identifying causes, predictive biological markers, and interventions for preventing delivery prematurely. Despite a growing body of knowledge about the potential pathways (i.e. inflammation, Hypothalamic-Pituitary-Adrenal (HPA) axis activation, hemorrhage, and uterine distension), the exact cause of preterm delivery still remains unclear. These pathways often occur simultaneously, however the presence of inflammation and

activation of the HPA axis by stress account for the largest percentage (~70 percent) of preterm births (Lockwood & Kuczynski, 2001).

Gestational hypertension and preeclampsia. Gestational hypertension is a condition that occurs during pregnancy in the previously normotensive individual. It manifests after 20 weeks gestation and is no longer present after postpartum recovery. Gestational hypertension is characterized by measurement of a systolic blood pressure of 140mmHg or greater and diastolic blood pressure of 90mmHg or higher (American College of Obstetricians and Gynecologists, 2002).

Preeclampsia is a condition characterized by the changes in blood pressure as described above with the addition of 300mg of protein excretion in the urine in a 24-hour period. The condition affects five to nine percent of pregnancies. Preeclampsia may result in blood flow impairment to the placenta and lead to fetal intra-uterine growth restriction (IUGR), oligohydramnios (decreased amniotic fluid), placental abruption (placental lining separates from uterus before birth), and impaired fetal well-being. The maternal condition may be further complicated by the onset of seizures (eclampsia) and the development of HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets). The only treatment for this condition is delivery of the placenta which unfortunately may result in a preterm delivery (American College of Obstetricians and Gynecologists, 2002).

The onset of preeclampsia appears to be the result of multiple intersecting factors. Some of the causes include, but are not limited to, abnormal

trophoblastic invasion of the uterine arteries during implantation (Kharfi et al., 2003), an abnormal maternal immune response to paternal and fetal antigens, and genetic factors (Cunningham, et al., 2010).

Extended gestational length. A post-term pregnancy is one that extends beyond the length of a term gestation. According to the American College of Obstetricians and Gynecologists (2004), a pregnancy which has extended to or beyond 42 weeks of gestation (294 days) is considered to be post-term. In a review by Divon and Feldman-Leidner (2008), the authors indicate that an estimated four to 19 percent of pregnancies may reach or exceed 42 weeks gestation. In 2008, 5.7 percent of all live births in the United States reached or exceeded 42 weeks gestation (Martin, et al., 2010).

Although the factors that influence the occurrence of a post-term delivery are not entirely known, some known influences include a previous experience of a post-term pregnancy (Mogren, Stenlund, & Hogberg, 1999), pregnancy with a male fetus (Divon, Ferber, Nisell, & Westgren, 2002), and genetic factors (Olesen, Basso, & Olsen, 2003). Additionally, a high pre-pregnancy BMI and nulliparity (never given birth before) may also contribute (Caughey, Stotland, Washington, & Escobar, 2009; Olesen, Westergaard, & Olsen, 2006).

Pregnancy prolonged beyond term carries a significant risk to both mother and child. For the mother, a post-term delivery puts her at risk for labor dystocia (abnormal or difficult labor) and injury to the perineum resulting from the passage of a large baby. A post-term birth also carries an increased chance for a cesarean delivery which is associated with endometritis (inflamed or irritated

uterine lining), hemorrhage, and the development of blood clots which can block the flow of blood in arteries and veins (American College of Obstetricians and Gynecologists, 2004).

For the fetus, a prolonged pregnancy is associated with an increased risk of stillbirth, neonatal mortality, and post-neonatal mortality (Hilder, Costeloe, & Thilaganathan, 1998). Post-term infants risk passage of meconium (feces accumulated in the fetal intestines) in utero and then subsequent aspiration upon delivery leading to respiratory complications. They also risk development of neonatal acidemia (low umbilical artery blood pH) (Kitlinski, Kallen, Marsal, & Olofsson, 2003), oligohydramnios, distress/non-reassuring heart rate of the fetus during labor (Doherty & Norwitz, 2008), low Apgar scores, macrosomia (large for gestational age/increased birth weight), and injury during delivery (Doherty & Norwitz, 2008; Spellacy, Miller, Winegar, & Peterson, 1985). Post-term delivery has also been associated with a greater risk for developing cerebral palsy (Moster, Wilcox, Vollset, Markestad, & Lie, 2010) and lower cognitive development (Yang, Platt, & Kramer, 2010).

The Immune System in the Non-Pregnant State

The immune system is the body's defense against toxins, bacteria, cancer, and other infectious threats (Doan, Melvold, Viselli, & Waltenbaugh, 2008). Primary organs of the immune system include the bone marrow and the thymus while secondary organs are the lymph nodes, spleen, tonsils, adenoids, appendix, and Peyer's patches of the intestine (Moser & Leo, 2010). Three major defense mechanisms of the immune system include: (a) external barriers

(physical and chemical/biological), (b) the innate immune system, and (c) the adaptive immune system (Doan, et al., 2008; Moser & Leo, 2010).

An important function of the immune system is to recognize that which is *self* versus *non-self*. *Self* refers to cells, molecules, and tissues encountered during immune surveillance which contain recognition markers on their surfaces that are identified as belonging within the body. Under normal conditions, the body's immune system does not attack cells, molecules, and tissues identified as self. *Non-self* cells and molecules are viewed as foreign invaders of the body and will trigger the immune system to attack and eliminate the *non-self* from the body (Doan, et al., 2008).

The innate and adaptive immune responses. The first lines of defense against infection are the physical and chemical/biological barriers of the immune system. These barriers work to prevent or impair foreign cells and molecules from invading the body. Physical barriers include the skin, mucous membranes, respiratory tract, and urinary tract. The acidic pH of the stomach is an example of a chemical barrier preventing bacteria from invading. Another example of a chemical barrier is the presence of lysozyme in sweat and tears. Lysozyme contains an enzyme used to eliminate most bacteria by its ability to break down bacterial cell walls (Doan, et al., 2008). During pregnancy, the mucus plug serves as a physical and chemical barrier preventing entrance into the uterus via the vaginal canal (Hein, Petersen, Helmig, Uldbjerg, & Reinholdt, 2005).

If foreign cells move beyond the external barriers, then the innate immune response is next in line to protect the body. The innate immune system employs

an immediate (within a few hours), non-specific response to foreign invaders and uses pattern recognition receptors (PRR) to distinguish self from non-self. These PRRs recognize structures (pathogen-associated molecular patterns – PAMPs) on bacteria, viruses, parasites and other invaders that are not found on host cells or tissues. Binding of PRRs to the PAMPs triggers the launch of the innate immune response which involves the activation of the complement pathway, attack of phagocytic cells (cells that engulf and destroy) and activation of Natural Killer (NK) cells to destroy the body's invader. Activated phagocytes (such as macrophages, dendritic cells, and neutrophils) secrete cytokines (i.e. interleukin(IL)-1, IL-6, IL-8, IL12, and tumor necrosis factor(TNF)- α) and chemokines that produce inflammation, attract other immune cells to the site of the infection, and eventually activate the adaptive immune response (Dempsey, Vaidya, & Cheng, 2003; Doan, et al., 2008; Moser & Leo, 2010).

Unlike the innate immune response, the adaptive immune response is specific, develops with each exposure to foreign cells, and then creates a memory to that exposure. The specificity of its role results in a delayed response of up to 10 days. Memory results in specific immunity to the particular disease and a more rapid response to the disease if exposed again. The adaptive immune system relies on the recognition of foreign antigens on the surface of invading cells to identify *non-self*. A specific T cell or B cell with a matching antigen receptor is activated to respond and eliminate the threat (Elgert, 2009).

Cytokines. The relationship between cells, cytokines, and other biological agents is complex. First, cytokines are described as being *pleiotropic*, meaning a

single cytokine may have multiple target cells and multiple actions. Second, the actions of different cytokines are often redundant and therefore actions cannot be attributed to a single cytokine. Next, exposure of a cell to several cytokines may have a synergistic or antagonistic effect leading to quantitatively different responses. Also, a cytokine may increase (or decrease) the production of another cytokine. Finally, a cytokine may increase (or decrease) the expression of and signaling by receptors for other cytokines and growth factors (Thomson & Lotze, 2003).

Cytokines are often characterized by their secretion from different T helper (Th) lymphocytes. The Th1 cytokines (i.e. IFN- γ and IL-2) are related to cell-mediated immunity (primarily phagocyte dependent involving macrophages, NK cells, and cytotoxic T-lymphocytes; not antibodies) and inflammation. Cytokines of the Th2 group (e.g. IL-4, IL-5, IL-6, IL-10, IL-13) are associated with antibody-mediated humoral immunity. The antagonistic relationship between Th1 and Th2 cytokines results in the inhibition of Th2 cytokines by Th1 cytokines and vice versa. Th2 cells are inhibited by IFN- γ while IL-4 and IL10 inhibit Th1 cells (Fitzgerald, O'Neill, Gearing, & Callard, 2001)

The predominance of either a Th1 or Th2 cytokine subset has been helpful in explaining the underlying mechanisms of various human diseases. For example, a Th1 dominance is associated with many autoimmune diseases (Balkwill, 2000). In contrast, pregnancy has been long described as a Th2 phenomenon where successful pregnancies tend to be associated with greater Th2 cytokines and elevations in Th1 cytokines may have detrimental effects on

pregnancy outcome (Wegmann, Lin, Guilbert, & Mosmann, 1993). This shift toward a Th2 profile in pregnancy may also explain why women with cell-mediated autoimmune diseases such as rheumatoid arthritis demonstrate a remission of their symptoms during pregnancy.

Table 1 (at end of chapter) provides an overview of cytokines related to the current study. It identifies the source of cytokine secretion and the actions of the cytokine. This is not an all inclusive list of all cytokines, nor does it provide complete details on all sources and actions.

The Immune System During Pregnancy

The immune system during normal pregnancy. Even in the absence of complications, the body of a pregnant female undergoes much change in order to prevent rejection of the developing fetus. To prevent the body from viewing the fetus as a foreign invader (*non-self*), the maternal immune system must be modified. Alterations in both adaptive and innate immune mechanisms vary throughout pregnancy, depending on stage, exposures to certain microorganisms, preexisting diseases, and pathophysiological processes that might occur during the pregnancy (Mor & Cardenas, 2010).

Wegmann et al. (1993) first described pregnancy as a Th2-like phenomenon and possible adverse pregnancy outcomes, such as miscarriage, could result from a shift in cytokine balance toward a Th1 state. Recent research has partially supported Wegmann's theory in that a great majority of pregnancy is characterized by a Th2, or anti-inflammatory, state. In a recent review by Mor

and Cardenas (2010), they describe three distinct phases in pregnancy characterized by a shift in cytokine balance.

The first phase includes the period of implantation, placentation and the first and early second trimesters of pregnancy. They associate this period with an “open wound, ... a veritable battleground of invading cells, dying cells, and repairing cells.” While the woman’s body adjusts to the presence of the fetus, it remains in a pro-inflammatory state. This state of inflammation is evidenced by maternal symptoms such as nausea.

The second phase includes the second and third trimesters of pregnancy. During this time, cytokines have shifted toward an anti-inflammatory state. Maternal symptoms from inflammation have subsided and the mother and fetus live in harmony as the fetus is rapidly growing and developing. Delivery of the fetus (stage three) is characterized by the return of the pro-inflammatory state. Inflammation is achieved by activated immune cells in the myometrium and subsequent contraction of the uterus. Therefore, normal pregnancy is a state of shifting cytokine balance from a pro-inflammatory to an anti-inflammatory state, and return to a pro-inflammatory state at delivery.

Few studies have detailed the shifting levels of cytokines in maternal serum or plasma during a normal pregnancy. Recent completed studies vary greatly in sample size from less than 30 participants (Makhseed et al., 2000; Vassiliadis, Ranella, Papadimitriou, Makrygiannakis, & Athanassakis, 1998) to a larger study including over 1200 participants (Curry et al., 2008). Results are not congruent for all cytokines across studies.

Several studies have reported stable levels of TNF- α throughout pregnancy (Curry, et al., 2008; Makhseed, et al., 2000; Vassiliadis, et al., 1998). Increased levels of IFN- γ (Curry, et al., 2008; Vassiliadis, et al., 1998), IL-12 (Curry, et al., 2008), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Vassiliadis, et al., 1998) were recorded in some investigations while others found no significant differences during pregnancy for levels of IFN- γ (Makhseed, et al., 2000) and IL-12 (Vassiliadis, et al., 1998) and decreased levels of GM-CSF (Curry, et al., 2008). Levels of IL-6 were shown to increase, but at varying points during pregnancy; after the first trimester (Curry, et al., 2008; Vassiliadis, et al., 1998), after 24 weeks gestation (Curry, et al., 2008), and at the time of delivery (Makhseed, et al., 2000). During labor, IL-10 was detected at greater concentrations, but was otherwise stable throughout pregnancy (Makhseed, et al., 2000; Vassiliadis, et al., 1998).

Curry, et al. (2008) explored the relationship between cytokines and maternal variables during early- and mid-gestation of normal pregnancy. Their findings revealed that early levels of GM-CSF were lower in women with a Body Mass Index (BMI) below 18.5. Early levels of TNF- α , IL-2, IL-6, and IFN- γ were elevated in women age 35 years or older. Socioeconomic level, smoking status, and gravidity were not shown to be related to cytokine levels during their investigation. These results indicate that maternal factors influence cytokine levels in the normal, healthy pregnancy and may contribute to the shift in balance of the Th1 and Th2 state.

The immune system and complications affecting gestational length.

Table 2 (at end of chapter) summarizes recent investigations published on serum or plasma measures of cytokines and pregnancies complicated by preterm labor, threatened or spontaneous abortion (miscarriage), and preeclampsia.

Preterm birth. In a review of 50 human studies of cytokines and their association with preterm birth published prior to April 2008, Lyon, et al. (2010) concluded that the most consistent finding across all studies was an increase in levels of pro-inflammatory cytokines. In particular, this included increased levels of IL-6, IL-1 β , and TNF- α in preterm maternal serum samples verses term. In comparing recent studies on maternal serum or plasma cytokines and their relationship to preterm birth, inconsistencies in the timing of sample collection, methods used for cytokine analysis, and cytokine variables of interest make comparisons across studies challenging and conclusions difficult to formulate. For example, the timing of venipuncture may occur as early as six weeks gestation and up to 37 weeks gestation. Cytokine levels may be measured by Luminex technology (Ekelund et al., 2008; Kramer et al., 2010; Vogel et al., 2007), enzyme-linked immunosorbent assay (ELISA) (Hudic et al., 2009; Laudanski, Lemancewicz, Pierzynski, Akerlund, & Laudanski, 2006; Mercer et al., 2006; Pearce et al., 2008; Vitoratos, Papadias, Makrakis, et al., 2006; Whitcomb, Schisterman, Luo, & Chegini, 2009), or flow cytometry (Curry et al., 2009).

Pre-eclampsia. Saito, et al. (2007) summarized published findings supporting the relationship between preeclampsia and an altered immune state.

First, preeclampsia occurs more often in primigravid (first pregnancy) women or multigravid women with a new partner. Subsequent pregnancies with the same partner decrease the rate of preeclampsia indicating maternal recognition of paternal antigens and immune adaptation. Next, exposure to semen over an extended period of time appears to decrease the risk of preeclampsia. Effective exposure to paternal antigens in the seminal fluid may be by vaginal or oral contact. The risk of developing preeclampsia is very high (16 to 33%) when a woman receives a donated ovum or embryo as the donated products are foreign to the woman's body. Finally, the rates of preeclampsia are low in women with HIV who have T cell immune deficiency.

Preeclampsia is marked by the presence of elevated inflammatory proteins. In recent reported studies of women with preeclampsia, TNF- α , (Cackovic et al., 2008; Canakci et al., 2007; Laskowska, Leszczynska-Gorzela, Laskowska, & Oleszczuk, 2006), IL-1 β (Canakci, et al., 2007), IL-6 (Jonsson, et al., 2006), IL-8 (Jonsson, et al., 2006; Laskowska, Laskowska, Leszczynska-Gorzela, & Oleszczuk, 2007), IL-16 (Hu, Wang, Wang, Huang, & Dong, 2007), IL-18 (Huang, Huang, Dong, Yao, & Wang, 2005; Seol et al., 2009), and macrophage migration inhibitory factor (MIF) (Todros et al., 2005) were increased as compared to levels in normal, healthy controls. Interestingly, there is a decrease in the concentration of TNF- α in the urine of women with elevated serum levels of TNF- α (Cackovic, et al., 2008). The authors pose the question about whether serum values are affected by increased production of TNF- α in

circulation or decreased filtration and clearance by the kidneys. This is an area of further investigation.

Miscarriage. Similarly, increased inflammation is also seen in cases of spontaneous abortion. Pro-inflammatory IL-1 β and TNF- α are elevated in women with threatened abortion and subsequent miscarriage as compared to women who carried their pregnancy to completion (Vitoratos, Papadias, Makrakis, et al., 2006). This may indicate a shift toward a Th1-type response and inflammation incompatible with successful pregnancy.

Identification of novel cytokine markers in maternal serum/plasma would provide a feasible means of identifying women at risk for inflammation associated complications in pregnancy. Among recent studies of preterm birth, spontaneous abortion, and preeclampsia, TNF- α was shown to be significantly elevated in cases versus controls across all conditions. A significant rise in IL-6 was commonly shared in cases of preterm birth and preeclampsia; and was elevated in spontaneous abortion and preeclampsia. Further investigation with human subjects is needed to completely understand the Th1/Th2 dynamic in pregnancy and parturition and more specifically in conditions leading to adverse pregnancy outcomes.

These three cytokines commonly found to be elevated, IL-1 β , IL6, and TNF- α , are all proinflammatory cytokines indicating a possible association between these conditions and an increased state of inflammation. As previously mentioned, two of the four potential pathways to early delivery include

inflammation and HPA Axis activation which account for approximately 70 percent of preterm deliveries (Lockwood & Kuczynski, 2001).

Psychological Stress and Depression During Pregnancy

The term *stress* carries meaning that is different among most individuals. As a noun, stress may take on a negative meaning such as “ a physical, chemical, or emotional factor that causes bodily or mental tension and may be a factor in disease” (Merriam-Webster, 2011). Stress may also take action as a verb as in, to place emphasis on something. Selye (1976, p. 15) defined stress as “the non-specific response of the body to any demand for change.” In his description, stress may have a desirable (eustress) effect or undesirable (distress) influence on an individual.

The body’s response to stress is complex and involves the interaction between the nervous, immune, endocrine, cardiovascular, and metabolic systems in positive and negative feedback loops. Whenever the body encounters stress, good or bad, it will initially respond in the same manner. The stressful stimuli will stimulate the HPA Axis beginning with the release of corticotropin-releasing hormone (CRH) from the hypothalamus. The anterior pituitary is triggered by CRH to release adrenocorticosteroid hormone (ACTH) which then acts on the adrenal glands to secrete cortisol into circulation. The feedback loops help the body to terminate the stress response when the stressful situation is no longer present. The rise in cortisol negatively influences the hypothalamus to cease from secreting CRH. Figure 2 provides a basic

illustration of the HPA Axis in response to stress in a non-pregnant individual. Positive and negative feedback loops have been indicated.

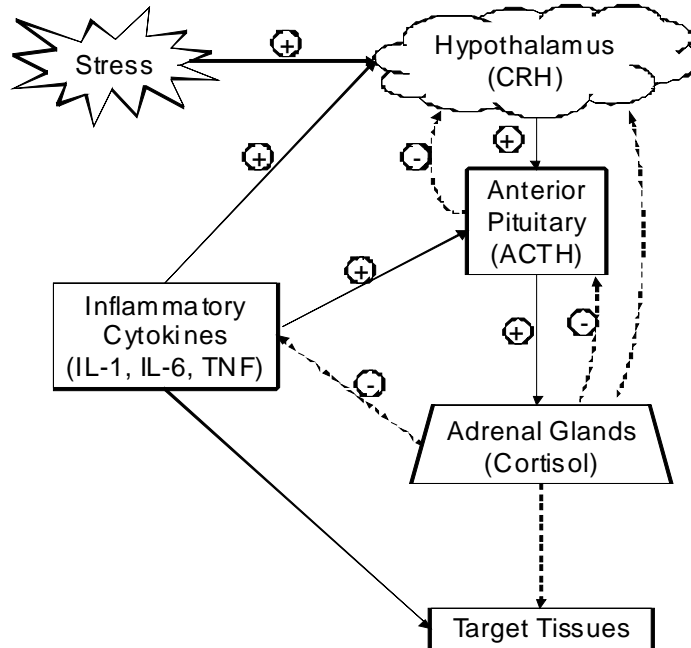


Figure 2. Hypothalamic-Pituitary-Adrenal (HPA) Axis in response to stress. Stress triggers the release of CRH which acts on the pituitary to release ACTH and subsequently the adrenal glands to release cortisol into circulation. Solid lines represent positive feedback and dashed lines are negative feedback relationships.

During pregnancy, however, the placenta also produces CRH (pCRH) (see Figure 3). The relationship of cortisol to pCRH is in contrast to that which is produced in the hypothalamus. A rise in cortisol during pregnancy increases the production of pCRH (Sandman, Davis, Buss, & Glynn, 2011). There is a steady rise in pCRH and managed state of hypercortisolemia during pregnancy. Sufficient levels of CRH binding protein (CRH-BP) manage the circulating levels of pCRH. In the final weeks of pregnancy, CRH-BP levels decrease as pCRH levels continue to rise in excess influencing the onset of parturition (McLean et al., 1995; Wadhwa, Porto, Garite, Chicz-DeMet, & Sandman, 1998).

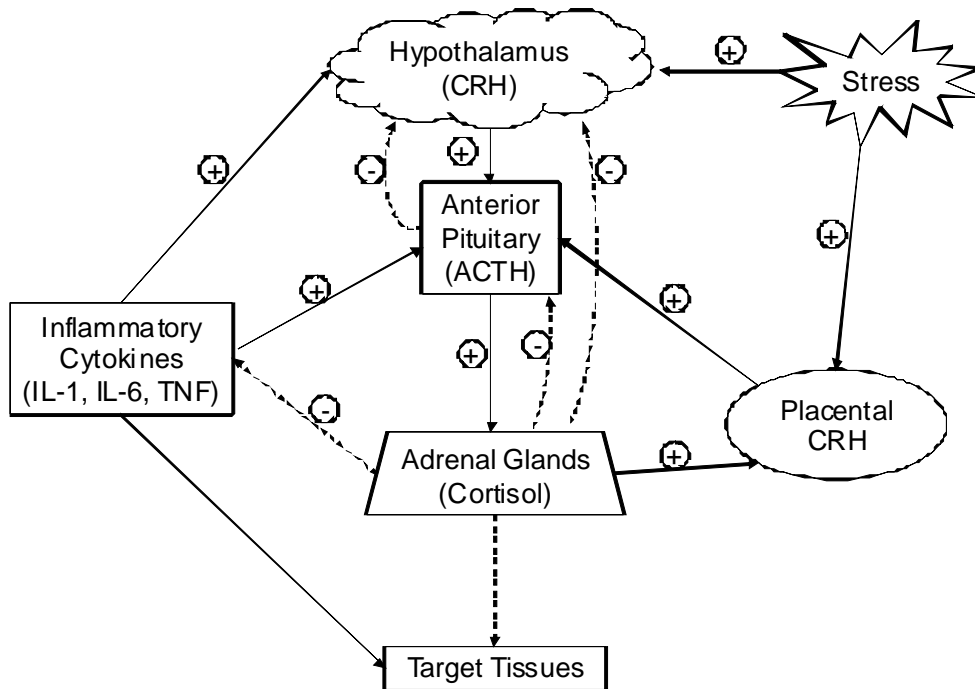


Figure 3. Effect of stress on the HPA Axis and immune mediators during pregnancy. In contrast to CRH from the hypothalamus, placental CRH stimulates the release of ACTH and cortisol into circulation. Solid lines indicate positive feedback and dashed lines indicate negative feedback relationships.

Numerous studies have investigated the relationship between CRH levels and the timing of parturition. In a study of 485 women recruited in their first trimester of pregnancy, McLean et al. (1995) concluded that pCRH is a marker of gestational length and measurement of maternal plasma CRH between 16 and 20 weeks gestation may help identify women who will deliver at term, preterm, and postterm. Significant elevations of CRH early in pregnancy (~20 weeks gestation) have been shown to be present in women who experience preterm labor (Hobel, Dunkel-Schetter, et al., 1999) and gestational hypertension (Hobel, Arora, & Korst, 1999). Sandman and colleagues (2006) also support the relationship in the rise of pCRH and the onset of labor. In their study, however, only CRH levels at 31 weeks gestation were predictive of preterm birth.

Additionally, they reported that elevated cortisol levels at 15 weeks gestation predicted the excessive rise in CRH leading to shortened gestation.

As discussed, physiological measures (e.g. hormones of the HPA Axis) have been investigated to describe the role that stress plays in predicting gestational length. Psychological measures may also serve as a useful tool. The challenge with psychological measures is the varying definitions of stress. Previous studies conducted during pregnancy have utilized various measures for stress including levels of anxiety (Hobel, Dunkel-Schetter, et al., 1999), work stress (Lee et al., 2011), and perceived stress (Krabbendam et al., 2005)

Perceived stress measures an individual's appraisal of their life's stressors (Cohen, Kamarck, & Mermelstein, 1983). In pregnancy, a reduction of perceived stress over the course of the pregnancy has been inversely related to gestational length. Measures of perceived stress in conjunction with CRH levels have been shown to be predictive of gestational age at delivery (Hobel, Dunkel-Schetter, et al., 1999; Ruiz, et al., 2002).

Prenatal depression is a significant problem as depressed women are more likely to experience preterm labor, preeclampsia, diabetes, Cesarean section, anemia, and infections during labor. Infants born to depressed women are also at greater risk for fetal growth restriction, abnormalities, distress, and death (Bansil et al., 2010). Depression has been found to be positively related to preterm delivery (Fransson, Ortenstrand, & Hjelmstedt, 2011; Phillips, Wise, Rich-Edwards, Stampfer, & Rosenberg, 2010; Smith, Shao, Howell, Lin, &

Yonkers, 2011) and cortisol elevation has been noted in women with prenatal depression (Field & Diego, 2008; O'Keane et al., 2011).

Chapter Summary

Chapter two provided a review of literature related to the topic of study and provided an overview of the physiological changes which occur during pregnancy. Pregnancy may be impacted by conditions such as preterm labor, spontaneous abortion, and gestational hypertension which may interfere with the successful pregnancy ending at term. Research has demonstrated that maternal levels of perceived stress and depression can negatively impact the outcome of pregnancy.

Stress triggers the stress response which involves the HPA Axis. Levels of CRH, which are normally secreted from the hypothalamus, are exponentially increased during the course of gestation due to the concurrent production of CRH in the placenta. Additionally, while cortisol has a negative influence on CRH secretion from the hypothalamus in the non-pregnant individual, placental CRH is further secreted in the presence of cortisol. This is important to note as cortisol levels rise in response to HPA activation by stress. Thus, stress experienced during pregnancy may increase levels of circulating CRH which has been referred to as the “placental clock”, a trigger for the onset of labor.

While there are numerous variables to consider that may affect the gestational length of pregnancy (the outcome variable), the current variables under study were chosen based on a review of the literature and data obtained to complete a secondary data analysis. Figure 4 summarizes the independent

variables (inputs) and the dependent variable (output). First, we will consider the influence that certain maternal characteristics (i.e. age, race, ethnicity, and gravida) have on the length of gestation. Second, the influence of maternal depressive symptoms and perceived stress (psychological variables) will be investigated. Finally, physiological measures of maternal plasma cytokine and cortisol levels will also be considered.

A detailed description of the study method executed in the current study has been described in Chapter three. Further information about the source of the data for the secondary analysis has been discussed.

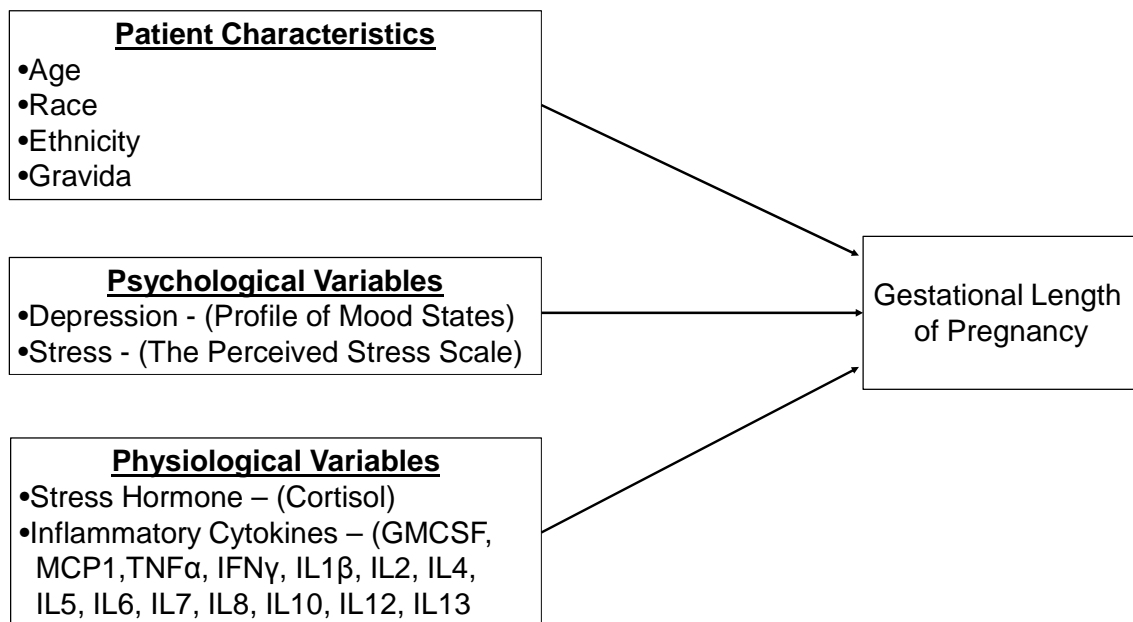


Figure 4. Input and Output Variables under Study

TABLE 1. Overview of cytokine source and function

Cytokine	Sources	Action
GM-CSF	T cells, macrophages, fibroblasts, and endothelial cells	Growth factor for haematopoietic progenitor cells; activating factor for granulocytic and monocytic cells; growth factor for several cells including T cells
IFN- γ	CD8+ and CD4+ T cells, NK cells	Involved in nearly all phases of immune and inflammatory responses; activation, growth, and differentiation of T cells, B cells, macrophages, NK cells, and others. Hallmark of Th1 differentiation
IL-1 β	A variety of cells including monocytes, macrophages, dendritic cells, T lymphocytes, B lymphocytes, and NK cells	Induces fever, hypotension, weight loss, neutrophilia, and acute phase response
IL-2	T cells	Stimulates growth and differentiation of T cells, B cells, NK cells, LAK cells, monocytes, macrophages, and oligodendrocytes
IL-4	T cells, mast cells, eosinophils, and basophils	Effects on B cells, T cells, nonlymphoid cells including monocytes, endothelial cells and fibroblasts; induces secretion of IgG4 and IgE
IL-5	Mast cells, T cells, eosinophils	Stimulates eosinophils colony formation and is an eosinophil differentiation factor
IL-6	T cells, B cells, and nonlymphoid cells such as macrophages, bone marrow stromal cells, and endothelial cells	Regulates B and T cell function, hematopoiesis and acute phase reactions
IL-7	Bone marrow, thymic stromal cells, and spleen cells	Growth factor for progenitor B cells and T cells; also stimulates proliferation and differentiation of mature T cells
IL-8	Multiple cells including monocytes, lymphocytes, granulocytes, and endothelial cells	Inflammatory chemokine; functions as neutrophil chemoattractant and activating factor. Also attracts basophils, and some lymphocytes.
IL-10	Th0 and Th2 subsets of CD4+ T lymphocytes; T-reg cells	Blocks activation of cytokine synthesis by Th1 T cells, activated monocytes, and NK cells; stimulates proliferation of B cells, thymocytes, and mast cells
IL-12	B lymphoblastoid cells, dendritic cells (most potent producers), and B cells	Induces IFN- γ production by T cells and NK cells, enhances NK activity, and induces differentiation of the Th1 subset of T lymphocytes
IL-13	Activated Th2 cells, mast cells, and NK cells	Important in Th2 response; upregulation of IgE; suppression of inflammatory response; inhibits production of inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-8)
MCP-1	Produced by wide range of tissues; upregulated by proinflammatory stimuli such as IFN- γ	Monocyte chemoattractant and activating factor for monocytes, basophils, T cells, NK cells, and immature dendritic cells
TNF- α	Activated monocytes and macrophages and other cells including B cells, T cells, and fibroblasts	Mediator of inflammatory and immune functions; regulates growth and differentiation of a variety of cell types

Note. CD = cluster of differentiation; NK = Natural Killer; LAK = lymphokine activated killer; IgG = Immunoglobulin G; IgE = Immunoglobulin E

TABLE 2. Recent cytokine research related to preterm birth, miscarriage, and preeclampsia

Year	Author(s)	Sample	Cytokines Studied	Outcome
Preterm Labor and Preterm Birth				
2010	(Lyon, et al., 2010)	**State of the Science – Integrated Review Human studies for association of cytokines in blood with preterm birth (PTB)	Pro-inflammatory – IL1β , IL6 , IL8 , IL11 , IL12 , IL17 , IL18 , TNFα , IFNγ Anti-inflammatory – IL4 , IL10 , IL13 , IL16 , IFNα , TGFβ Colony Stimulating Factors – G-CSF , GM-CSF , M-CSF	Most consistent finding = increased level of proinflammatory cytokines, particularly IL6 , IL1β , TNFα associated with PTB *Relatively few studies and results not consistent
2010	(Kramer, et al., 2010)	Spontaneous preterm delivery (sPTD) (n=207) and term controls (n=444) frozen plasma samples collected prospectively at 24-26 weeks gestation prior to onset of preterm labor (PTL)	IL1β , IL4 , IL5 , IL6 , IL8 , IL10 , IL12 , IL17 , IL18 , sIL6ra , IFNγ , TNFα , TNFβ , sTNFrl , MCP1 , TGFβ , MIP1a , MIP1B , MIF , MMP9 , TREM1 , RANTES , BDNF , NT3 , NT4 , GM-CSF , CRP cytometric sandwich assay with Luminex	High MMP-9 concentrations associated with PTB
2009	(Curry, et al., 2008)	sPTD – early 24-29 weeks (n=107), moderate 30-33 weeks (n=353), and late 34-36 weeks (n=422); term (n=1372). Plasma collected prospectively during 1 st trimester (\leq 16 weeks - - median 8 weeks)	IL2 , IL6 , TNFα , INFγ , GM-CSF Measured with flow cytometry	Elevated TNFα and GM-CSF associated with increased risk of delivery at 34-36 weeks. sPTD <34 weeks associated with elevated IL6 and TNFα in underweight women. Elevated IL2 and TNFα reduced risk of delivery <34 weeks in obese women. **Cytokines not related to delivery <34 weeks in normal and overweight women. 1 st trimester cytokine levels and risk of PTD may depend on pre-pregnancy BMI.

Year	Author(s)	Sample	Cytokines Studied	Outcome
2009	(Whitcomb, et al., 2009)	Normal pregnancy (n=394) and PTB (n=31) Serum collected prospectively in 1 st and 2 nd trimester between 6-18 weeks (during years 1959-1966)	G-CSF Measured by sandwich ELISA-based approach	Higher serum levels of G-CSF 6-18 weeks (greater risk if elevated 11-18 weeks) significantly associated with gestational age at delivery and PTB.
2009	(Hudic, et al., 2009)	Threatened PTD (n=30) and healthy pregnant women (n=20) serum collected prospectively at 24-37 weeks gestation after hospitalization for threatened PTD (case) and control collected 24-37 weeks with no risk factors for PTD	Progesterone Induced Blocking Factor (PIBF) – a protein released by lymphocytes during pregnancy in presence of progesterone. Progesterone thought to establish adequate immune environment, IL10, IL6, TNFα, IFNγ Measured by ELISA	PIBF lower in preterm subjects; lower IL10 in preterm, higher IL6 and IFNγ in preterm
2008	(Ekelund, et al., 2008)	Threatened PTD (n=93) admitted for PTL, PPRM, or cervical ripening Serum collected on admission to hospital with threatened PTD between 24 weeks and <34 weeks (during years 1997-1999)	IL12 and L18 reported here, but also IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL17, sIL6ra, TNFα, TNFβ, sTNFR1, IFNγ, GM-CSF, MMP9 Measured by 15-plex sandwich assay with Luminex	Correlations between low levels of IL18 and PTD; women delivering before 34 weeks had lower IL18 than women >34 weeks; Women with IL18 level <95pg/ml had 3-fold increased risk of delivering before 34 weeks No difference in IL12 levels in those delivering <34 or >34 weeks Possible interaction – patients with low IL18 and high IL12 had increased risk of delivery <34 wks

Year	Author(s)	Sample	Cytokines Studied	Outcome
2008	(Pearce, et al., 2008)	Preterm (n=60) and term (n=122) Serum collected prospectively between 9-23 weeks	Macrophage migration inhibitory factor (MIF) – soluble mediator that helps govern interaction between cytokines and stress hormones (i.e. cortisol) Measured by sandwich ELISA	MIF elevated in PTB cases High MIF associated with maternal risk-taking behavior
2007	(Vogel, et al., 2007)	Asymptomatic pregnant women 12-25 weeks gestation with at least 1 prior sPTD between 16-30 weeks	IL1β, IL2, IL5, IL6, IL8, IL12 , IL18, TNFα , TGF β , sTNFR1, GM-CSF , TREM1 Measured by xMAP technology with Luminex	Serum IL1β, IL2, IL5, IL6, IL8, IL12 , IL18, TNFα , TGF β , sTNFR1, GM-CSF , and TREM1 individually associated with sPTB. Stepwise linear model = high serum TNFα , high cervicovaginal sIL6Ra, and short cervical length contributed to prediction of PTB <35 weeks (false positive 5% able to predict 69%)
2006	(Mercer, et al., 2006)	Included were women with 2 or 3 completed pregnancies (including current) delivering at 20 or more weeks divided into 3 groups: Recurrent sPTD (2 or 3 consecutive sPTD with no term births (TB)) (n=47), isolated sPTD (1 sPTD and 1 or 2 TB) (n=241), recurrent term birth (2 or 3 consecutive TB and no sPTD) (n=969) Plasma collected prospectively at 22-24 weeks	Inflammatory Cytokines – IL1β, IL6, IL10 Stress marker – CRH and cortisol Measured with ELISA	Cortisol and CRH higher in isolated PTB and recurrent PTB than in recurrent TB. Cytokines not increased in either isolated sPTD or recurrent sPTD.

Year	Author(s)	Sample	Cytokines Studied	Outcome
2006	(Laudanski, et al., 2006)	Preterm labor (PTL) who delivered (n=17), preterm pregnancy not in labor (n=13), and labor at term (n=8) Serum collected 26-36 weeks for those in labor and delivered preterm and those not in labor and delivered term; collected >37 weeks for those in labor at term	Chemokines – MDC/CCL22, TARC/CCL17, ITAC/CXCL11, I-309/CCL1, IP-10/CCL10, MIP-1a/CCL3, -1B/CCL4, -3a/CCL20, -3B/CCL19 Measured with multiplexed, mini-array, sandwich-type ELISA	MIP-3B/CCL19 significantly lower in PTD group than other 2 groups.
2006	(Vitoratos, Papadias, Makrakis, et al., 2006)	PTL (n=30) and normal control (n=19) Serum collected from PTL group upon presentation to hospital with symptoms of PTL >24 and ≤34 weeks; normal group collected at clinic appointment >24 and =34 weeks.	Corticotropin-releasing hormone (CRH) and TNFα CRH and TNFα measured with ELISA	PTL group had higher CRH levels and TNFα than normal group. Positive correlation between CRH and TNFα in both groups but correlation stronger in PTL group.
Threatened Abortion/Miscarriage				
2009	(Barrientos et al., 2009)	Subsequent spontaneous abortion (SA) (n=42) and normal pregnancy (n=42) Serum collected prospectively between 4 and 12 weeks	Asymmetric IgG antibodies (AAb) Indoleamine 2,3-dioxygenase (IDO) Cytokines – IL4, IL12, TNFα, and IFNγ IgG antibodies measured with ELISA Cytokines measured by sandwich ELISA	Levels of IL10, IL12 , and IDO activity were lower in SA cases but not significantly lower

Year	Author(s)	Sample	Cytokines Studied	Outcome
2008	(Whitcomb et al., 2008)	Miscarriages (n=439) and controls (n=373) Serum collected prior to report of miscarriage (loss < 140 days) at entry into study and bimonthly but not less than 10 days prior to miscarriage Samples collected between years 1959 and 1974	IL1ra, IL1β , IL4 , IL6 , IFNγ , TNFα , thrombopoietin (TPO), G-CSF	Increased risk of miscarriage associated with elevated levels of TPO
2007	(Whitcomb et al., 2007)	Miscarriages (n=439) and controls (n=373) Serum collected prior to report of miscarriage (loss < 140 days) at entry into study and bimonthly but not less than 10 days prior to miscarriage	Chemokines (CC type) – MIP1a, MIP1B, MCP1, RANTES Chemokines (CXC type) – IL8, ENA-78 Chemokines measured with Fleurokine MAP Multiplex Human Cytokine Panel with Luminex	ENA-78 levels associated with increased risk of miscarriage as collection-outcome interval increased in those samples collected >35 days prior to pregnancy outcome
2006	(Vitoratos, Papadias, Economou, et al., 2006)	Three groups: (1)Threatened Aborters (TA) discharged after resolution with viable fetus (n=31), (2)TAs with subsequent loss (n=22), (3)asymptomatic controls (n=22) Serum samples 7-10 weeks gestation collected once on admission (groups 2 and 3) or twice (group a on admission and discharge)	IL1β , TNFα , IL6 Measured by ELISA	IL1β and TNFα significantly higher in TAs with subsequent loss. No difference in levels of IL6

Year	Author(s)	Sample	Cytokines Studied	Outcome
2006	(Kalinka & Radwan, 2006)	Threatened Aborters (TA) (n=27) and control (n=16) 2 Serum samples collected between 6 and 12 weeks gestation	TNFα, IL12, IL10 Measured by ELISA	Cytokines did not differ in TA group and normal group at first and second sampling
2009	(Seol, et al., 2009)	Preeclampsia (n=24) and healthy pregnant controls (n=13) Serum collected from case group in 3 rd trimester after admission to hospital for preeclampsia and before delivery. Serum from control group collected before elective csection or induction at term without labor	Pre-eclampsia Pro-inflammatory cytokine IL18 – may promote atherosclerotic plaque formation Chitinase-like glycoprotein YKL-40 – inflammatory marker r/t extracellular remodeling and angiogenesis, elevated in acute and chronic inflammation. Both measured with ELISA	YKL-40 levels significantly elevated in preeclampsia Median IL18 higher in preeclampsia group. YKL-40 higher in patients with more severe proteinuria may be related to severity of disease
2008	(Montagnana et al., 2008)	Preeclamptic women (n=14), 1 st trimester normotensive women (n=20), 2 nd trimester normotensive women (n=20), 3 rd trimester normotensive women (n=17), non-pregnant women (n=21) Serum collection	TNFα, IL1, IL6, IFNγ Measured by multiplexed sandwich ELISA	Concentrations of TNFα lower than sensitivity limit of detection. IL1 and IL6 increased significantly in 2 nd and 3 rd trimester normotensive pregnancy Results of this study do not support screening for TNFα, IL6, IL1, or IFNγ for preeclampsia

Year	Author(s)	Sample	Cytokines Studied	Outcome
2008	(Cackovic, et al., 2008)	Severe Preeclampsia (n=45) and healthy controls (n=45) Serum and urine samples collected at same time as clinical assessment or admission to hospital	TNFα Measured with ELISA	Serum TNFα elevated in preeclampsia group Women with severe preeclampsia had significantly reduced fractional excretion of TNFα . Urinary levels of TNFα were decreased in preeclampsia group. *Fractional excretion helps to understand if elevated serum levels are caused by increased production or decreased excretion. **therefore – impaired urinary clearance may contribute to elevation of serum levels of TNFα
2007	(Borekci, Aksoy, Al, Demircan, & Kadanali, 2007)	Mild preeclampsia (n=38), severe preeclampsia (n=20), eclampsia (n=11), normotensive controls (n=20) Serum collected prior to planned c-section, induction of labor, or active labor. Collected within 24 hours of birth in 72% of patients and 48 hours of birth in 28%. Samples collected within 12 hours of convulsions in patients with eclampsia	IL2, IL6, IL10 Measured with ELISA	Mean concentrations of IL2 and IL6 were not different between groups. Median concentrations of IL10 in mild and severe preeclampsia were similar and significantly lower than controls and patients with eclampsia Median concentrations of IL10 significantly lower in eclampsia than all other groups. *Pre-eclampsia associated with deficient IL10 . High serum IL10 correlated with presence of eclampsia

Year	Author(s)	Sample	Cytokines Studied	Outcome
2007	(Canakci, et al., 2007)	Mild preeclampsia (n=20), severe preeclampsia (n=18), healthy pregnant (n=21) Gingival cervicular fluid (GCF) and serum collected 48 hours prior to delivery	IL1β, TNFα Prostaglandins (PGE2) Serum IL1β, TNFα , and PGE2 measured with ELISA	IL1β, TNFα , and PGE2 levels in serum and GCF were significantly higher in pre-eclamptic women than normal controls Serum TNFα influenced by severity of preeclampsia
2007	(Hu, et al., 2007)	Severe Preeclampsia (n=22), Mild preeclampsia (n=15) and normal controls (n=36). Serum samples collected after onset of preeclamptic symptoms	IL15 and IL16 IL15 measured by chemiluminescent immunoassay IL16 measure by ELISA	Levels of IL15 and IL16 significantly higher in preeclampsia than controls IL15 and IL16 significantly higher in severe preeclampsia than mild preeclampsia indicating IL15 and IL16 associated with severity.
2007	(Laskowska, et al., 2007)	Preeclampsia with normal fetal weight (n=15), preeclampsia with intrauterine growth restriction (IUGR) (n=12), normal controls (n=10) Serum from mothers collected before active labor phase. Umbilical cord serum collected immediately after delivery.	IL8 – strong neutrophil chemoattractant and activator Measured by sandwich ELISA	Increased IL8 found in both preeclamptic patient groups compared to control group Umbilical cord concentrations tended to be higher than maternal blood concentrations
2006	(Laskowska, et al., 2006)	Preeclampsia with normal fetal weight (n=18), preeclampsia with IUGR (n=8), normal controls (n=18) Serum from mothers collected before active labor phase. Umbilical cord serum collected immediately after delivery.	TNFα Measured with sandwich ELISA	Higher maternal and umbilical serum TNFα levels in severe preeclampsia

Year	Author(s)	Sample	Cytokines Studied	Outcome
2006	(Bachmayer, Rafik Hamad, Liszka, Bremme, & Sverremark-Ekstrom, 2006)	<p>Preeclamptic women (n=22) and healthy controls (n=24)</p> <p>Serum collected when mothers arrived to delivery unit</p> <p>Also looked at deciduas natural killer (NK) cells and placental expression of cytokines</p>	<p>NK cell activating cytokines – IL12, IL15, IL18</p> <p>Anti-inflammatory cytokines – IL10</p> <p>Serum cytokines measured by sandwich ELISA</p>	<p>Due to insufficient amount of material – all cytokines could not be measured in all people</p> <p>IL12 significantly elevated in severe and mild cases versus controls.</p> <p>IL15 significantly elevated in severe cases versus controls.</p> <p>IL18 did not differ per group No significant differences in IL10</p>
2006	(Jonsson, et al., 2006)	<p>Moderate or Severe Preeclampsia (n=15) and normal controls (n=15) **due to insufficient sample volume, not all measures were complete on all cytokines/chemokines</p> <p>Serum samples collected after diagnoses and admission to the hospital for preeclamptic group. Control group samples collected in antenatal clinic at pregnancy week matching case group.</p>	<p>Cytokines and chemokines - IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12 p40, IL13, IL17, IFNα, IFNγ, GM-CSF, MIP1a, MIP1B, MCP1, eotaxin, RANTES</p> <p>Measured with Luminex</p> <p>Soluble CD14 and soluble IL4 receptor measured with ELISA</p>	<p>IL13 not detectable in any samples</p> <p>Higher serum concentrations of pro-inflammatory cytokine IL6 in preeclamptic group.</p> <p>Elevated levels of pro-inflammatory chemokines IL8 in preeclamptic group.</p> <p>**Elevated IL6 and IL8 support hypothesis of increased inflammatory response in preeclampsia</p> <p>Higher serum soluble IL4 receptor in preeclamptic patients</p>

Year	Author(s)	Sample	Cytokines Studied	Outcome
2005	(Bersinger & Odegard, 2005)	Mild Preeclampsia (PE) without fetal growth restriction (n=23), Fetal growth restricted (FGR) without Preeclampsia (n=9), matched controls at 17, 25, and 33 weeks gestations (n=40) Serum collected prospectively at 17 and 33 weeks. 25 week sample collected on 12 cases in PE group, 6 cases in FGR group, and all 40 controls.	M-CSF, VEGF, and placental growth factor (PLGF) All measured with ELISA	VEGF did not differ between PE group and controls at any time point tested, but FGR group was significantly lower throughout. No significant differences in M-CSF levels between groups at any gestational age
2005	(Huang, et al., 2005)	Mild preeclampsia (n=13), severe preeclampsia (n=14), and normal controls (n=28) Serum and placental samples from women in 3 rd trimester with elective c-section prior to labor	Pro-inflammatory cytokine IL18 Measured by ELISA	Serum and placental IL18 significantly higher in preeclampsia than controls Preeclamptic women who delivered before or after 36 weeks had comparable serum and placental IL18 levels. Preeclamptic women who delivered after 36 weeks had significantly higher serum and placental IL18 levels than controls IL18 levels not significantly different between mild or severe preeclampsia

Year	Author(s)	Sample	Cytokines Studied	Outcome
2005	(Kaleli et al., 2005)	Preeclamptic women (n=14) and normal controls (n=14) Serum collected around 34 weeks gestation	Neopterin and IL2R Neopterin measured with ELISA IL2R measured with Immulite analyzer	Neopterin and IL2R levels significantly higher in women with preeclampsia than normal controls. Neopterin and IL2R levels significantly higher in women with severe preeclampsia. Results indicate a Th1-type immune mechanism in pathogenesis of preeclampsia
2005	(Todros, et al., 2005)	Severe preeclampsia delivering <34 weeks, Severe preeclampsia delivering ≥34 weeks, 41 term controls, 41 non-pregnant women Serum collected at time of vaginal delivery or before c-section	Proinflammatory cytokine – Migration Inhibitory Factor (MIF) Measured by colorimetric sandwich ELISA	MIF significantly higher in preeclamptic patients than control MIF significantly higher in preeclamptic patients who delivered <34 weeks than those who deliver ≥34 weeks. *High MIF supports role of inflammation in pathogenesis on disease MIF similar in normal pregnancy and non-pregnant controls

Note. Cytokines and chemokines highlighted in bold text indicate they were included in the current study

CHAPTER THREE

METHOD

Sample

This study was a secondary analysis of data collected as part of a larger longitudinal investigation of stress, immunity and thyroid disease in pregnant and postpartum women (herein after referred to as the Source Study). Participants were recruited from two prenatal clinics affiliated with a large university practice in the southeastern United States. Institutional Review Board approval was obtained at both the university and clinical sites and informed consent was received prior to the collection of data.

During the four year Source Study, 631 women were enrolled. Although the postpartum period was the actual focus of the Source Study, a single collection of demographic information, two psychological surveys, and blood were collected during the prenatal time of enrollment between 16 and 26 weeks gestation. This prenatal data is the focus of the current study.

Initially, blood collected during the prenatal stage was analyzed for a panel of 14 cytokines. Due to budget restrictions, the Source Study later eliminated the prenatal analysis of cytokines from their study. Therefore, prenatal cytokine data on only 159 participants were available for inclusion in this study.

Sampling Method

Sample size estimate. The estimated sample size for the secondary analysis was based on the use of multiple regression analyses as stated in aim two. As this aim is largely exploratory, a standard rule-of-thumb, $N \geq 104 + m$ (where m is the number of predictors), was used initially and suggested that 125 participants was sufficient (Tabachnick & Fidell, 2007). A more precise sample size estimate was calculated assuming a modest value of $R^2 = .06$, $\alpha = .05$ and $\beta = .20$. This second estimate indicated that a sample of 122 participants would be sufficient to assess the study aims.

Inclusion criteria. Healthy pregnant women were enrolled in the Source Study at their prenatal appointment between 16 and 26 weeks gestation. English and Spanish speaking participants were considered eligible for inclusion if they were less than 26 weeks pregnant and did not have any of the criteria for exclusion.

Exclusion criteria. Criteria for exclusion included: age less than 18 and greater than 45 years; a body mass index (BMI) less than 20 at time of venipuncture; problems with drugs or alcohol or the use of drugs at any time during the current pregnancy; current use of immunosuppressant, hormonal or anticoagulant medications; personal history of thyroid or any autoimmune disease; serious mental illness; chronic disease; use of in-vitro fertilization for the current pregnancy; multiple gestation in the current pregnancy; hyperemesis; anemia; and known genetic abnormalities in the developing fetus. Additionally,

women who indicated that they could not participate in the Source Study's six month postpartum follow-up were not eligible for enrollment.

Measures

Demographic form. A brief demographic survey was administered to each participant at the prenatal time of enrollment to capture information about age, race, ethnicity, education, income, marital status, employment status, and pregnancy history. Smoking status prior to and during pregnancy was also noted. Recorded measures of height, pre-pregnancy weight, and weight at the time of blood collection were used to calculate body mass index (BMI). The BMI values were calculated by the Source Study and included in the data file received for the secondary analysis.

Following delivery of the baby, the Research Coordinator of the Source Study updated the Demographic Form. Information about the labor and delivery including the presence of any complications was noted. See Appendix A for a copy of the demographic form.

The Perceived Stress Scale. Self-reported levels of maternal stress were measured using The Perceived Stress Scale (PSS) (Cohen, et al., 1983). The 14-item version of the scale measures how often the individual felt or thought a certain way with Likert-type responses ranging from 0 (never) to 4 (very often). It is a measure of the degree to which an individual perceives their life as stressful.

Developed for use with individuals with at least a junior high school education, the scale items are of a general focus and applicable to all sub-populations of use. Internal consistency reliability testing in a population of

college-age students was found to have a coefficient alpha of .84 to .86.

Congruent and criterion validity for the scale has been shown to be excellent, although predictive validity falls with time (Cohen, et al., 1983).

The Perceived Stress Scale is not a diagnostic instrument and therefore there are no established cut-off values for stress measurement. The scale does not require permission for utilization when used for academic research purposes. See Appendix B for a copy of the Perceived Stress Scale.

Profile of Mood States. The Profile of Mood States (POMS) (McNair, Lorr, & Droppleman, 1992) was also completed by each participant at the time of prenatal enrollment. The tool provides for a multi-dimensional assessment of mood. A 65-item adjective rating scale measuring how often a feeling was experienced in the past week including the day of measurement, the POMS has a total mood disturbance score and six subscales: tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia, and confusion-bewilderment. Responses range from 0 (not at all) to 4 (extremely). The validity of the scale (face validity, factorial validity, predictive validity, and construct validity) is reported to be excellent (McNair, et al., 1992).

Only the depression-dejection scale (POMS-D), the sum of 15 related items on the total scale, was used in this analysis. The POMS-D has an internal consistency reliability of .84 and .95 in an antepartum population (Heaman, 1992). The POMS-D score may range between zero and 60 however a cut-off for depression has not been established. See Appendix C for a copy of the POMS.

In the Source Study, the researchers established a POMS-D score of 20 as their cut-off value for screening for severe depressive symptoms. The primary obstetrician or midwife providing care for women with a value of 21 or greater received written and verbal communication that their patient scored a 21 or greater on the POMS-D subscale. Further care and/or referral was at the discretion of the healthcare provider.

Cortisol and cytokine measures. Plasma cortisol levels ($\mu\text{g}/\text{dl}$) were evaluated as a measure of HPA activation and stress. Cortisol was measured with ELISA assay kits from ALPCO Immunoassays (Salem, NH) and Calbiotech (Spring Valley, CA) per kit instructions. A total of four ELISA plates were completed in total. The first three plates of samples were completed with ALPCO kits from the same lot. A fourth plate was originally completed with an ALPCO kit from a different lot, but results were found not to be congruent (greatly increased) with the earlier samples. The fourth plate was completed again with the Calbiotech kit which yielded results similar to the first three plates thus a decision was made to include the Calbiotech results.

A multiplex panel of cytokines was used in this study as a measure of immune system activation. The cytokines chosen for this multiplex by the Source Study are signature pro-inflammatory and anti-inflammatory cytokines, as well as chemokines of importance to immune responses. Cytokines (pg/ml) measured were granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)- α , Interferon (IFN)- γ , Interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, and IL-13. A

bead-based assay was completed with Milliplex Map kits (Billerica, MA), and analyzed on a Luminex-200. Standards and controls were completed with the samples for each plate used to measure cytokines and cortisol. All analyses were done in duplicate on each plate. Intraassay coefficients of variation for all laboratory measures were assessed with an acceptable variation goal of less than 10 percent.

Procedures

Institutional Review Board Approval. Information for the current study was submitted for review to the Institutional Review Board (IRB). Since a de-identified data set would be obtained for a secondary analysis, it was determined by the IRB that “the described research activities do not meet the definition of USF HRPP Policy for Human Subjects Research” and there was no need for “IRB review for this Not Human Subjects Research Activities.” After review from the IRB, a request for Demographic, Psychological, and Physiological data on the 159 participants with cytokine data was made to the Principal Investigator (PI) of the Source Study and the data were provided in Excel format.

Informed Consent and Collection of Psychological Data. Institutional Review Board (IRB) approval was obtained at both the university and clinical sites for the Source Study and consent was received from each participant prior to the collection of data in the prenatal clinic. Additional consent was not required to complete the secondary analysis.

Eligible participants interested in participating in the Source Study were provided with the consent document to read and review. After reading the

document, discussing the information, and allowing time for questions with the study recruiter, patients were provided with an opportunity to sign the consent document or take the document home for further review (and signature at a future appointment). If consent was provided, and the woman was at least 16 weeks pregnant, then blood, psychological, and demographic data were concurrently collected in the clinic setting at the patient's regularly scheduled prenatal appointment. If the woman was not yet 16 weeks at the time of consent, then the participant was met at a future clinic appointment after 16 weeks gestation for data collection.

Collection of Physiological Data. Blood was collected in three 5 milliliter heparinized tubes (green top) and transported to the lab in a chilled cooler. Upon receipt in the lab, plasma was collected by first placing tubes in the centrifuge at 3800rpm at 0°C for 25 minutes. The plasma was aliquoted into Eppendorf tubes and stored in a -80°C freezer until the batched completion of cytokine and cortisol analyses.

Cortisol Analysis. Calibrators (standards) and controls were run for every plate and all analyses were completed in duplicate. On the day the assay for the first three plates was completed, all kit (ALPCO) reagents and samples were allowed to reach room temperature and placed on a vortex to mix tube contents prior to initiation of the procedures. Cortisol-horseradish peroxidase (HRP) conjugate (120 microliters) was mixed with 12 milliliters of assay buffer to prepare a conjugate working solution. A wash buffer dilution was also prepared

by adding 50 milliliters of wash buffer concentrate to 450 milliliters of deionized water.

A pipette was used to transfer 20 microliters of each calibrator (seven in total), control, and sample into duplicate wells. A multichannel pipette was then used to transfer 100 microliters of conjugate working solution into each well. The plate was sealed and incubated on a plate shaker at 200 rpm for 45 minutes at room temperature.

Following incubation, a plate washer was used to wash the wells three times with diluted wash buffer. After the final wash, the plate was gently tapped against absorbent paper to ensure dryness. The lights in the laboratory were dimmed and a multichannel pipette was used to add 150 microliters of TMB solution to each well. The plate was sealed, covered with aluminum foil and incubated on a plate shaker. After a 15 minute incubation at room temperature, the foil was removed and a multichannel pipette was used to add 50 microliters of stop solution to each well. The plate was immediately read on a microwell plate reader at 450nm. Duplicate data were entered into GraphPad Prism 3.03 (San Diego, CA) and analyzed for final results.

For the final plate, all kit (Calbiotech) reagents and samples were allowed to reach room temperature and placed on a vortex to mix tube contents prior to initiation of the procedures. Cortisol-enzyme conjugate (1 milliliter) was mixed with 20 milliliters of assay diluents buffer to prepare a conjugate solution. A wash buffer dilution was prepared by adding 25 milliliters of wash buffer to 475

milliliters of deionized water. The laboratory lights were dimmed during the entire assay process.

A pipette was used to transfer 25 microliters of each cortisol standard (seven in total), high and low controls, and patient samples into duplicate wells. A multichannel pipette was then used to transfer 200 microliters of cortisol-enzyme conjugate solution into each well. The plate was sealed and mixed thoroughly for 10 seconds and incubated in a drawer at room temperature for 60 minutes.

Following incubation, a plate washer was used to remove all liquid from the wells and then wash three times with diluted wash buffer. After the final wash, the plate was gently tapped against absorbent paper to ensure dryness. A multichannel pipette was used to add 100 microliters of TMB substrate to each well. The plate was sealed and placed in a drawer at room temperature to incubate for 15 minutes. A multichannel pipette was used to add 50 microliters of stop solution to all wells. The plate was immediately read on a microwell plate reader at 450nm. Duplicate data were entered into GraphPad Prism 3.03 (San Diego, CA) and analyzed for final results.

Cytokine Analysis. On the day the immunoassay was completed, all kit reagents and samples were allowed to reach room temperature prior to initiation of the protocol. The 10x wash buffer (30 milliliters) was diluted with 270 milliliters of deionized water. Quality controls were reconstituted with 250 milliliters of deionized water, inverted several times and placed on a vortex to mix. Controls were allowed to sit for 10 minutes and then were placed in labeled

microcentrifuge tubes. A serum matrix reconstitution was prepared by mixing one milliliter of deionized water with lyophilized serum matrix. Finally, human cytokine standards were prepared by reconstituting the human cytokine standard with 250 milliliters deionized water to prepare a 10,000 pg/ml concentrate and serial dilutions completed to result in standard concentrations of 2,000 pg/ml, 400 pg/ml, 80 pg/ml, 16 pg/ml, and 3.2 pg/ml. During some of the analyses, an additional dilution was completed to achieve a concentration of 1.6 pg/ml.

Assay buffer (200 microliters) was added to each well of the filter plate. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature. A vacuum was used to remove the assay buffer and excess buffer was blotted from the bottom of the plate on absorbent paper. Twenty-five microliters of each standard and control was added to appropriate wells and 25 microliters of assay buffer was added to the sample wells. Twenty-five microliters of the prepared serum matrix was added to the standards and control wells and 25 microliters of each sample were added to the appropriate wells.

The kit bead bottle was placed on a vortex to mix and then 25 microliters of the beads were added to each well. The plate was sealed, covered with a plate cover, wrapped with a rubber band and incubated overnight at 4°C on a plate shaker. Following the overnight incubation, the fluid was removed with a vacuum and the plate was washed two times with a plate washer and excess fluid blotted on an absorbent towel.

Detection antibodies (25 microliters) were added to each well and the plate was sealed, covered with the plate cover, and incubated on a plate shaker

at room temperature for one hour. Streptavidin-Phycoerythrin (25 microliters) was added to each well and the plate was sealed, covered with a plate cover and incubated for an additional 30 minutes on a plate shaker at room temperature. All well contents were then removed with a vacuum and the plate was washed two times with a plate washer. Sheath fluid (150 microliters) was added to all wells and a plate shaker was used to resuspend the beads prior to running the plate on the Luminex-200. Median fluorescence intensity was converted into picograms per milliliters.

Storage of study materials. Study forms were stored at the university where the Source Study was conducted. Forms are locked in a filing cabinet draw accessible only to the PI and study personnel. Files will be maintained for three years following completion of the final report of the Source Study. Original data forms were not accessed for the purposes of the secondary analysis. Electronic data files from the current study were password protected when possible (i.e. Excel files) and do not contain personal identifiers of the participants.

Data Analysis

Only participants with complete data on the key variables of interest were included in the analysis. Patient characteristics of interest included age, ethnicity, race, and gravidity (number of times pregnant). Perceived stress and depressive symptoms were key psychological variables. Physiological variables under study were cortisol (a stress hormone and marker of HPA activation) and inflammatory cytokines (markers of immune status).

Since the collection of data occurred between 16 and 26 weeks, it was important to note the exact gestational age at the time of data collection to determine if the variation among collection times had any effect on the outcomes. This was especially important in relation to the physiological variables whose levels are known to fluctuate throughout pregnancy. Finally, since gestational age at the time of delivery is the main outcome variable, information about the estimated date of delivery and the actual date of delivery were required so gestational age could be calculated.

Preliminary Analyses. Prior to analysis, data were reviewed for accuracy of data entry, missing values, and outliers. Statistical analyses were conducted using SPSS statistical software (IBM SPSS Statistics 19).

Gestational age at delivery was calculated by first subtracting the actual date of delivery from the estimated date of delivery identified in the patient's prenatal record and recorded in the Source Study data file. In relation to the estimated date of delivery, positive values identified the number of days the pregnancy was shortened and negative values were the result in longer pregnancies. These positive and negative values were then subtracted from 280 days (i.e. the number of days equal to a full term pregnancy of 40 weeks) to determine the gestational age at delivery.

The gestational age at the time that the data were collected was calculated in the same manner. The date that the blood was collected was subtracted from the estimated date of delivery. That value was then subtracted from 280.

As previously mentioned, serial dilutions were completed resulting in the lowest standard concentration of 3.2 pg/ml or 1.6 pg/ml. These values were considered to be the lowest limit of detection (LOD) for each laboratory plate. Using conventional methods, cytokine values indicated by Luminex output to be lower than the LOD were replaced with a value that was one-half the lowest limit of detection. Therefore, one-half of the LOD was either 1.6 pg/ml (i.e. one-half of 3.2) or .8 pg/ml (i.e. one-half of 1.6) depending upon which dilution method was utilized.

After reviewing the cytokine data, it was noted that some samples had been assayed on two separate occasions. Data from both assays were available in the Excel data file obtained from the Source Study. If two measurements were available, then they were averaged to obtain a single value which was utilized for analysis.

Cytokine and cortisol data were first evaluated for extreme outliers that were more than three standard deviations from the mean. Cases greater or less than three standard deviations on any of the variables were excluded from the analysis. Next, the cytokine and cortisol data were evaluated for skewness by calculating the skewness measure and visualizing a histogram using SPSS. Log₁₀ transformation of physiological variables was used to reduce skewness and normalize the distribution (Tabachnick & Fidell, 2007). Following transformation, a ratio of IL2 to IL4 was calculated to determine the Th1/Th2 state of each individual at the prenatal time of data collection.

Individual item responses on the Perceived Stress Scale were made available in the Excel data file received from the Source Study. Item responses were used to calculate the total score on the Perceived Stress Scale per author instructions. Since the data received were on a scale of 1 to 5 instead of 0 to 4, the responses were first recoded to agree with the original scale measurement. The scores on the seven positive items were reversed (0=4, 1=3, 2=2, 3=1, and 4=0) and then all 14 items were summed to calculate a total score (Cohen, et al., 1983). The Cronbach's alpha for the current sample was .77 for the 14-item scale.

The POMS-D subscale score is the sum of the 15 related items from the total POMS scale responses (McNair, et al., 1992). Individual scores on the scale's 65 items were not available in the data set received from the Source Study. The subscale scores were calculated by the Source Study personnel and only the six subscale scores and the total mood disturbance score were identified in the received data set.

Review of the POMS-D data revealed that the data were positively skewed. A square root transformation method was utilized to achieve a normal distribution. Reliability estimates for the current sample were not able to be calculated since only subscale scores were provided and individual item scores were not available.

Descriptive statistics were calculated to summarize the participant characteristic information, total PSS score, and POMS-D subscale. Mean scores and standard deviations (*SD*) were calculated for the PSS, POMS-D, cortisol and

individual cytokines. Untransformed raw data were used to calculate and report the descriptive results. All comparative analyses were conducted using transformed data.

Analyses to Address the Aims. To address the aims, the following data analysis methods were executed:

Aim 1. Estimate the bivariate relationship between mid-pregnancy measures of physiological and psychological variables and gestational age at delivery.

Bivariate methods were utilized to explore the association between the variables of interest. The Pearson correlation coefficient (r) was calculated to estimate the relationship between the variables of interest and gestational age at delivery. Correlations measure the size and direction of the linear relationship between two variables. Also, the squared correlation yields the measure of strength of association between the variables (Tabachnick & Fidell, 2007).

Values for the correlation coefficient always lie between +1 and -1. If all values lie on a straight line with a negative slope, then r would equal -1 and a straight line with a positive slope equals +1. A value of zero is interpreted as the absence of a linear relationship between the variables (Dunn & Clark, 2009). If r is less than zero, then the dependent variable decreases as the independent variable increases and vice versa. If r is greater than zero, then as the independent variable increases (or decreases), then so does the dependent variable (Wilcox, 2009).

Scatter plots were created to graphically depict the linear relationship between the variables. Gestational age at delivery represented the dependent (Y) variable and the psychological and physiological variables were the predictor (X) variables. Significant correlations were identified as calculated using SPSS statistical methods.

It was considered to be important to remove the influence of medically indicated deliveries on gestational age at delivery. Therefore, if necessary, we planned to calculate semipartial correlation coefficients to estimate the relationship between each physiological and psychological variable and gestational age at delivery after controlling for the medically indicated deliveries. However, since a linear relationship between medically indicated deliveries and gestational age at delivery was not found to exist (using Pearson's r), this method was not implemented.

Aim 2. Examine the unique role played by physiological and psychological variables in predicting gestational age at delivery in a multivariate model.

Multiple linear regression techniques were utilized to examine the unique role of physiological and psychological variables with gestational age at delivery. A forward regression analysis was used with a liberal probability level of entry set at .20. The squared semi-partial correlations estimate the amount of variance that each independent variable adds to R^2 at the point that it enters the regression equation (Tabachnick & Fidell, 2007). The contribution of an independent variable to the prediction of the dependent variable depends upon the point of entry into the equation that is automatically selected by SPSS.

Starting with only the constant, each potential predictor variable is entered until the best model is achieved. Irrelevant variables are rejected from the model. Order was based on the partial correlations using the FORWARD method in SPSS. Predictors were added to the model one-at-a-time until there was no improvement in fit.

To enhance the predictive relationship of the independent variables on the dependent variable, several assumptions must be met. First, a linear relationship must exist between the predictors and the outcome. Linearity was assessed by review of scatterplots described in Aim 1 above. Second, a normal distribution of variables must exist. Assessment of normality was also conducted by review of scatterplots and consideration of skewness and outliers. Linearity and distribution of the physiological variables was improved by employing Log10 transformation methods. Homogeneity of variance is also assumed whereby the variance of scores of one variable is approximately that of the other variables. The final assumption is the absence of multicollinearity. Multicollinearity exists when independent (predictor) variables are too highly correlated (Logan, 2010). Multicollinearity was evaluated using SPSS collinearity diagnostics. Criteria included a conditioning index greater than 30 for a given dimension coupled with variance proportions greater than .50 for at least two different variables (Tabachnick & Fidell, 2007). Among the current set of variables, condition indices were not found to be greater than 12 therefore multicollinearity was not found to be present.

Chapter Summary

This chapter summarized the methods implemented in the current research. This study was a secondary analysis of data obtained during pregnancy from women participating in a larger study of stress, immunity, and thyroid disease in the postpartum period. The relationship of participant characteristics, psychological variables (perceived stress and depressive symptoms), and physiological variables (cortisol and cytokines) to gestational age at the time of delivery was investigated.

Chapter four contains the results of Pearson's Correlations and Forward Regression. Additionally, sample characteristics have also been summarized in the following chapter.

CHAPTER FOUR

RESULTS

Preliminary Analyses

Missing data, normality and outliers. A total of 25 cases were not included in the analysis due to incomplete data on key variables or failing to meet inclusion criteria. Twelve cases were missing some or all of the psychological data. One case was missing cortisol results and another was missing values for seven of the 14 cytokines. Other cases excluded from the analyses included 10 individuals for which delivery information was not available and one person who delivered twins instead of a singleton birth. An additional 12 cases were lost after excluding individuals with extreme outliers. The final sample was 122 participants who were included in all of the descriptive and comparative analyses.

Valid data on only 12 of the 14 cytokines were available for all participants. Eighteen (14.8%) and two (1.6%) participants were missing data for GM-CSF and MCP-1, respectively. Instead of further eliminating cases and risk affecting statistical power and increase chances of making a Type I or Type II error, these two cytokines were not included in the final analyses.

The remaining 12 cytokines and cortisol results were assessed for skewness. All were found to be positively skewed (cases were piled on the left of the distribution with a long right tail) with skewness values all greater than zero. The Log10 transformation was the method utilized to correct the skewness and achieve a normal distribution.

Participant age, gestational age at the time of enrollment, and gestational age at delivery were also assessed for normal distribution. Gestational age at the time of delivery was found to be negatively skewed and was corrected using Log10 transformation with reflection. The remaining two variables did not require correction.

Description of the sample. Participants ranged in age from 18 to 43 years ($M = 27.39$; $SD = 5.44$). The most frequent race reported was Caucasian ($n = 84$; 68.9%), while 23.0% identified themselves as African-American/Black ($n = 28$), 4.9% as 'Other' ($n = 6$), and 3.3% as Asian/Pacific Islander ($n = 4$). The ethnicity of the sample was primarily Non-Hispanic ($n = 90$; 73.8%).

Previous pregnancy history (including the current pregnancy) reported by women ranged in number from one ($n = 34$; 27.9%) to nine ($n = 1$; <1.0%). Most women ($n = 49$; 40.2%) had no living children at home, 43 women (35.2%) currently had one child in the home, and 21 women (17.2%) had two children. For one woman (1.0%), the current pregnancy would add to her seventh child to her home.

Of the babies born to this sample, 59 were male (48.4%) and 60 were female (49.2%). The gender of three babies (2.5%) was not identified in the data

file from the Source Study. The majority of babies were delivered vaginally ($n = 86$; 70.5%) and most women entered into labor spontaneously ($n = 84$; 68.9%). The pregnancy history was reviewed for diagnosis of preterm labor, gestational hypertension, or gestational diabetes. Twenty-seven women (22.1%) experienced one or more of those conditions during the current pregnancy.

The study protocol defined the eligibility period for prenatal enrollment and data collection from 16 weeks to 26 weeks gestation. The actual gestational age at the time of enrollment ranged from 97 days (13 weeks and 6 days) to 182 days (26 weeks) with a mean gestational age of 140 days (20 weeks). Gestational age at the time of delivery ranged from 154 days (22 weeks) to 291 days (41 weeks and 4 days). The mean gestational age at the time of delivery was 269.95 days (38 weeks and 3 days).

Psychological and physiological variables. The mean score on The Perceived Stress Scale was 23.40 ($SD = 6.59$) with a range in scores from 6 to 45. Scores on the Profile of Mood States Depression subscale ranged from 0 to 42 ($M = 6.65$; $SD = 8.87$). Eleven women had depressive symptom scores of 21 or greater. These scores were reported to their physician or midwife for further treatment or referral.

Table 3 summarizes the physiological variables in the study. The untransformed raw data were used to calculate this descriptive information. As mentioned previously, data were not available for all participants for GM-CSF and MCP-1 and were excluded from comparative analyses. Of the remaining 12 cytokines and cortisol, four were undetectable in more than half of the plasma

samples. These undetectable cases were assigned a value prior to Log10 transformation that was one-half the limit of detection (either .80 or 1.6) as described in Chapter 3.

TABLE 3. Summary of physiological results

Cytokine	Mean (SD)	Min/Max	# Below LOD*
TNF-alpha	3.55 pg/ml (3.06)	1.20/ 24.20 pg/ml	13 (10.66%)
IFN-gamma	1.76 pg/ml (2.29)	.22/ 21.36 pg/ml	90 (73.77%)
IL-1beta	2.04 pg/ml (1.99)	.40/ 12.84 pg/ml	75 (61.48%)
IL-2	4.02 pg/ml (6.27)	.40/ 55.22 pg/ml	53 (43.44%)
IL-4	23.45 pg/ml (55.11)	.99/ 398.62 pg/ml	45 (36.89%)
IL-5	1.49 pg/ml (.77)	.40/ 5.79 pg/ml	96 (78.69%)
IL-6	4.12 pg/ml (6.67)	.93/ 49.91 pg/ml	45 (36.89%)
IL-7	15.03 pg/ml (31.10)	1.06/ 166.21 pg/ml	56 (45.90%)
IL-8	11.85 pg/ml (19.78)	1.20/ 166.87 pg/ml	5 (4.10%)
IL-10	5.42 pg/ml (7.92)	.80/ 37.45 pg/ml	42 (34.43%)
IL-12	6.88 pg/ml (15.55)	.23/ 98.27 pg/ml	60 (49.18%)
IL-13	2.93 pg/ml (4.75)	1.18/ 37.95 pg/ml	78 (63.93%)
Cortisol	15.66 µg/dl (6.97)	6.12/ 43.54 µg/dl	0 (0 %)

*LOD = Limit of Detection

Analyses Addressing the Study Aims

Aim 1. Estimate the bivariate relationship between mid-pregnancy measures of physiological and psychological variables and gestational age at delivery.

Research Question 1. To what extent are mid-pregnancy maternal plasma cytokines correlated with gestational age at delivery? Two of the 12 cytokines included in the correlation analysis were significantly correlated with gestational age at delivery. Both were negatively correlated indicating that as

gestational age increased, cytokine levels decreased. Interferon gamma had the strongest correlation ($r = -.22$) with gestational age at delivery.

Research Question 2. To what extent are mid-pregnancy maternal plasma cortisol levels correlated with gestational age at delivery? Plasma cortisol was not significantly correlated with gestational age at delivery ($r = .06, p = .53$). As discussed in the review of the literature, cortisol levels increase as gestational age increases and until delivery.

Research Question 3. To what extent are mid-pregnancy perceived stress scores correlated with gestational age at delivery? Self-reported levels of perceived stress were not significantly correlated with gestational age at delivery ($r = -.07, p = .44$).

Research Question 4. To what extent are mid-pregnancy depressive symptoms scores correlated with gestational age at delivery? Self-reported levels of depressive symptoms were also not significantly correlated with gestational age ($r = .07, p = .44$).

Table 4 identifies the Pearson's r values between all variables in the current study. Of the independent variables of interest, only 2 cytokines (IL13 and IFN γ) and gravida ($r = .28, p = .002$) resulted in significant correlations with the dependent variable.

Aim 2. Examine the unique role played by physiological and psychological variables in predicting gestational age at delivery in a multivariate multiple regression model.

TABLE 4. Correlations of key variables

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 GA (days)[^]	1.00	0.14	0.13	0.12	-0.08	0.28*	0.15	-0.07	0.06	-0.14	-0.12	-0.10	0.03	-0.11	-0.13	0.04	-0.08	-0.11	-0.18*	-0.22*	-0.11	0.10
2 Enroll (days)[#]		1.00	-0.02	-0.05	-0.05	0.02	-0.09	-0.06	0.37†	-0.05	-0.03	0.09	-0.09	-0.03	-0.05	-0.07	0.05	0.00	-0.02	-0.01	-0.08	-0.07
3 Age			1.00	-0.02	0.01	0.36†	0.06	-0.09	-0.09	0.00	0.00	0.12	0.06	0.08	0.13	0.11	0.18*	0.03	-0.06	-0.11	-0.05	0.09
4 Race				1.00	-0.10	-0.06	0.02	0.00	0.11	-0.08	-0.08	0.02	-0.08	0.11	0.03	-0.06	0.08	0.03	-0.05	-0.01	-0.04	0.00
5 Ethnicity					1.00	0.07	0.17	0.00	0.08	-0.13	-0.19*	-0.10	-0.02	-0.04	-0.09	0.08	-0.14	-0.02	-0.02	-0.07	-0.15	-0.01
6 Gravida						1.00	0.36†	0.21*	0.04	-0.04	0.05	-0.07	-0.02	0.00	-0.04	0.10	-0.03	0.07	0.00	0.00	-0.10	0.17
7 POMS-D							1.00	0.64†	0.05	-0.08	-0.14	-0.10	-0.05	-0.13	-0.16	-0.01	-0.18*	-0.13	-0.11	-0.08	-0.26*	0.03
8 PSS								1.00	0.05	-0.03	-0.04	-0.10	-0.12	-0.01	-0.11	-0.05	-0.09	-0.16	0.05	-0.07	-0.15	0.06
9 Cortisol									1.00	-0.14	0.06	-0.25*	-0.20*	-0.01	-0.10	-0.07	-0.10	-0.11	-0.10	0.00	-0.06	0.18*
10 IL1β										1.00	0.49†	0.38†	0.60†	0.43†	0.41†	0.09	0.38†	0.29†	0.49†	0.43†	0.00	-0.06
11 IL2											1.00	0.06	0.22*	0.52†	0.23*	0.00	0.16	0.29†	0.46†	0.35†	0.05	0.53†
12 IL4												1.00	0.30†	0.29†	0.73†	0.41†	0.73†	0.37†	0.40†	0.15	0.13	-0.39†
13 IL5													1.00	0.13	0.37†	0.08	0.31†	0.46†	0.35†	0.39†	-0.04	-0.16
14 IL6														1.00	0.37†	0.28*	0.47†	0.40†	0.63†	0.32†	0.16	0.16
15 IL7															1.00	0.36†	0.68†	0.29†	0.40†	0.18*	0.22*	-0.21*
16 IL8																1.00	0.36†	0.25*	0.26*	0.09	0.36†	-0.16
17 IL10																	1.00	0.24*	0.51†	0.16	0.22*	-0.24*
18 IL12																		1.00	0.40†	0.55†	0.01	-0.06
19 IL13																			1.00	0.43†	0.24*	-0.11
20 IFNγ																				1.00	0.07	-0.13
21 TNFα																					1.00	-0.14
22 Th1/Th2																						1.00

[^]Gestational age at delivery, [#]Gestational age at enrollment, *p \leq .05 †p \leq .001

The data were reviewed to assess if the assumptions of regression had been met. The linear relationship between the physiological predictors and outcome was confirmed by review of the scatterplots in Figures 5 through 17 (at end of chapter). Log10 transformation was used to correct skewness of the variables to achieve a normal distribution. Although some variables were correlated with one another, multicollinearity was not found to be present.

Forward (Stepwise) regression was then employed to examine the unique role of the cytokines with gestational age at delivery. Three models were identified with multiple correlations coefficients (R) ranging from .22 to .29. The third model included the variables IFN γ ($\beta = -.26, p = .01$), IL5 ($\beta = .19, p = .07$), and IL7 ($\beta = -.16, p = .10$). The combination of these three variables significantly explained a proportion of the variance of gestational age at delivery ($R^2 = .06, F = 3.61, p = .02$). Six percent of the variability of the dependent variable (gestational age at delivery) can be explained by the variability of the combination of these three cytokines in the model.

Additional Testing

The variables of interest were further described based on the point at which the prenatal samples were collected. The sample was first divided into two groups since levels of cytokines and cortisol fluctuate and often increase during pregnancy and there was a wide variation in the gestational age at which the data were collected (between 16 and 26 weeks). Group one ($n = 58$) included all of the data that were collected between 16 and 20 weeks gestation. Group two

($n = 64$) was made up of women who enrolled between 20 and 26 weeks gestation.

Results of ANOVA showed that gestational age at enrollment had a significant effect on cortisol ($F(1, 121) = 12.47, \eta^2 = .09, p = .001$) and IL7 levels ($F(1, 121) = 3.962, \eta^2 = .03, p = .05$). Mean cortisol levels of women in group one were $13.42\mu\text{g/dl}$ ($SD = 5.65$) and group two was $17.69\mu\text{g/dl}$ ($SD = 7.46$). For women enrolled between 16 and 20 weeks, the average IL7 levels were 20.85pg/ml ($SD = 41.40$). Mean IL7 levels were 9.76pg/ml ($SD = 15.75$) for those enrolled between 20 to 26 weeks gestation. The gestational age at enrollment did not significantly effect on any of the remaining psychological or physiological variables in the study.

The Th1/Th2 ratio was also calculated. This ratio is an indicator of whether the individual's immune system favors a Th1 or Th2 state at the time that blood was collected. As indicated in Chapter 3, it was calculated using one key cytokine from each Th state. Interleukin-2 was selected as the key indicator of Th1 and IL-4 for Th2. A value greater than 1.0 results when Th1 (IL-2) is greater and less than 1.0 when Th2 (IL-4) is greater.

The ratio ranged from .01 to 11.02 ($M = 1.47; SD = 2.28$) in the sample of women. Thirty-eight women favored a Th1 state with a ratio greater than 1.0 and 64 favored a Th2 state. Twenty women actually had a ratio equal to 1.0 indicating a balanced Th1/Th2 state.

Chapter Summary

Chapter four included the results of the descriptive analyses, testing for the two study aims and additional descriptive analyses. Pearson's correlation methods were carried out to examine the relationships investigated in the first aim. Forward linear regression was used to explore the second aim and identify the best fit model to explain the relationship between the predictor variables and the outcome variable (gestational age at delivery).

Chapter five will discuss the findings presented in the current chapter. The chapter will interpret how these findings relate to previous research and how they present the foundation for future research.

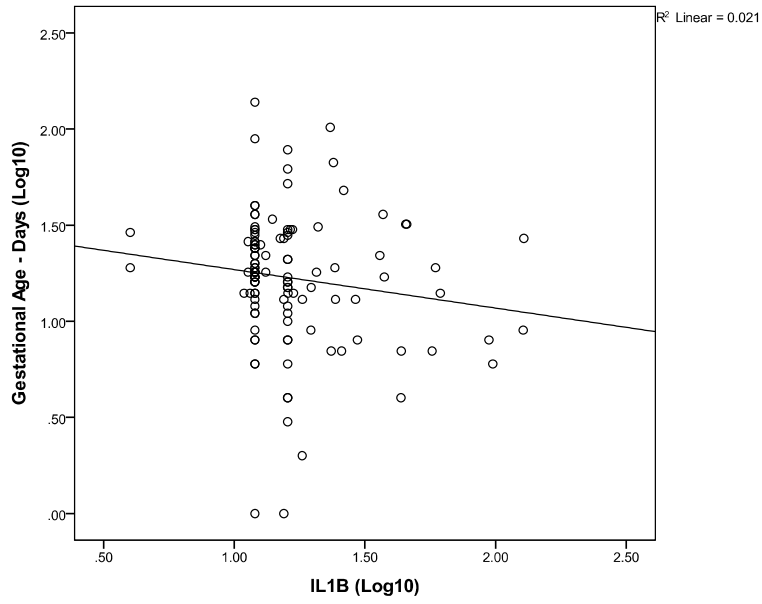


Figure 5. Scatterplot for Gestational Age and IL1 β

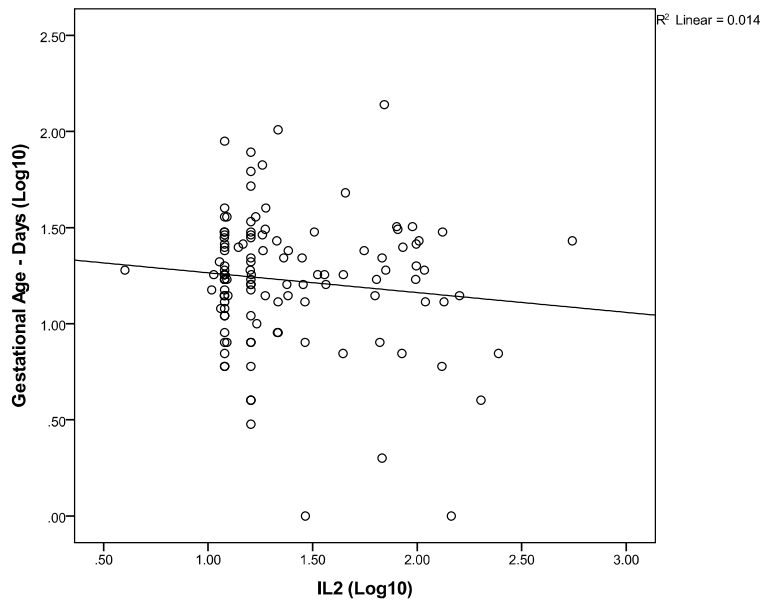


Figure 6. Scatterplot for Gestational Age and IL2

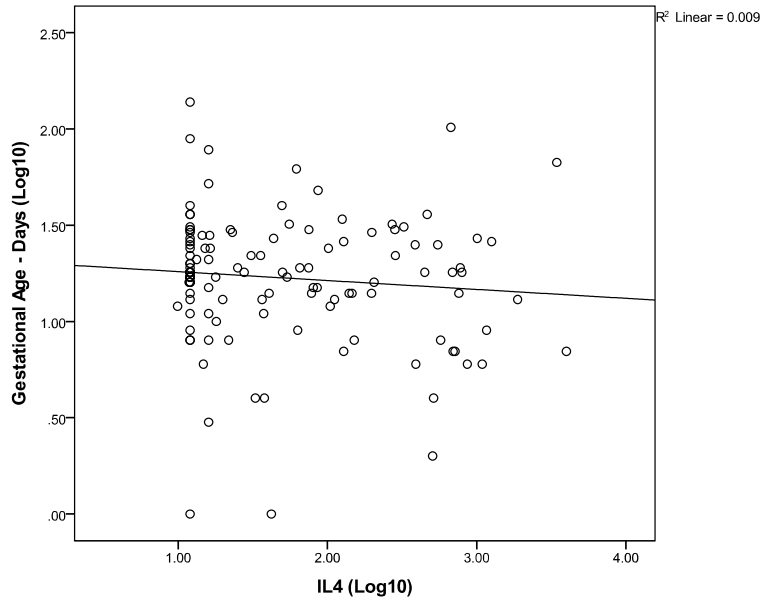


Figure 7. Scatterplot for Gestational Age and IL4

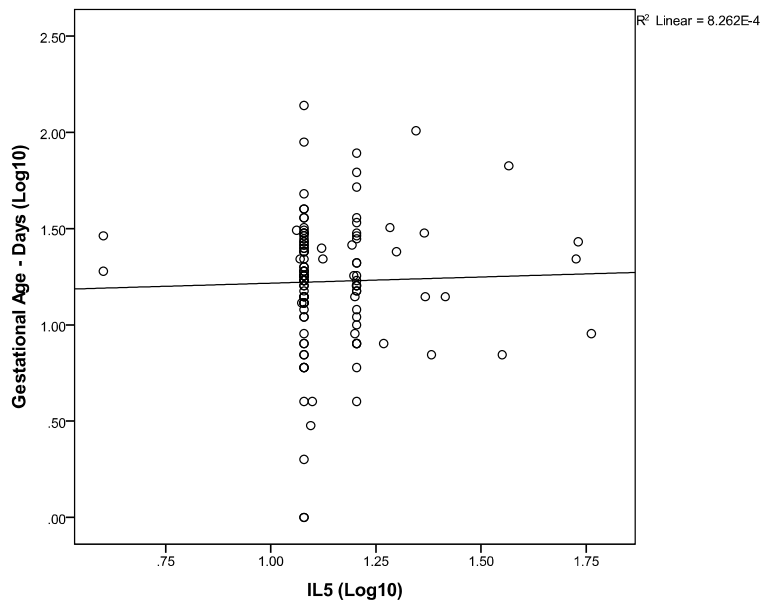


Figure 8. Scatterplot for Gestational Age and IL5

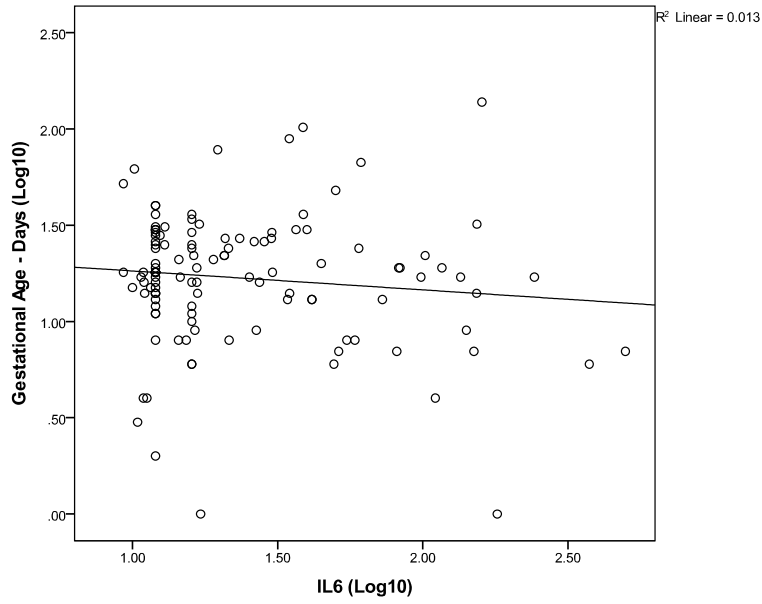


Figure 9. Scatterplot for Gestational Age and IL6

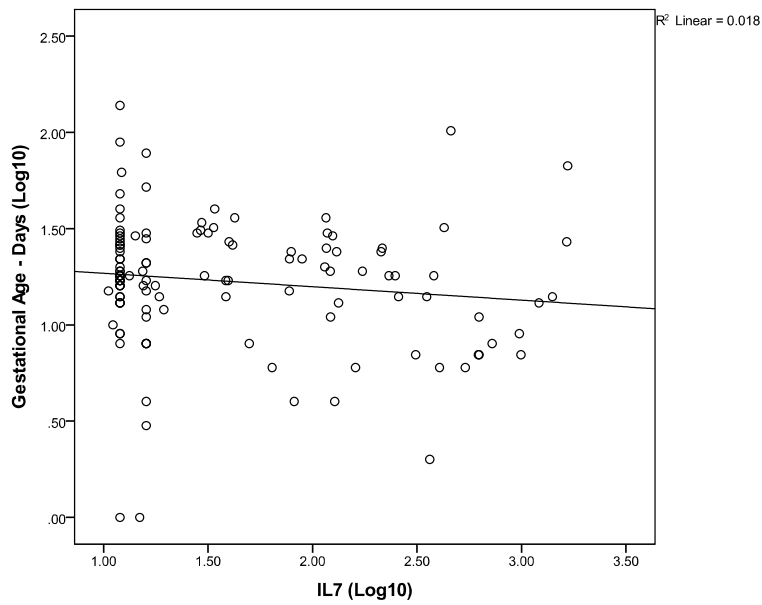


Figure 10. Scatterplot for Gestational Age and IL7

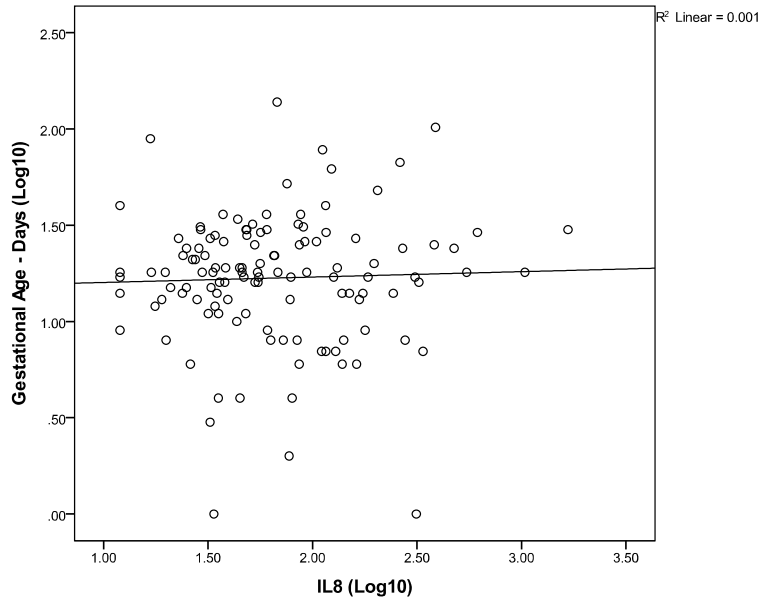


Figure 11. Scatterplot for Gestational Age and IL8

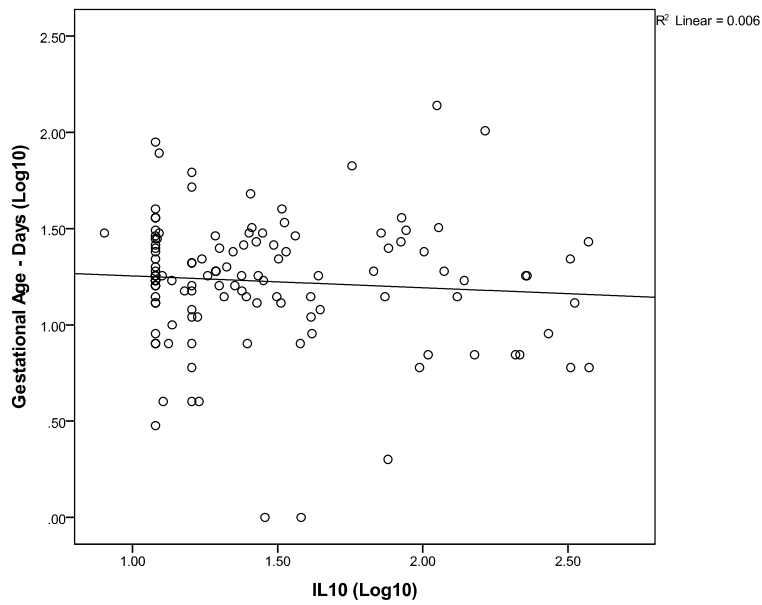


Figure 12. Scatterplot for Gestational Age and IL10

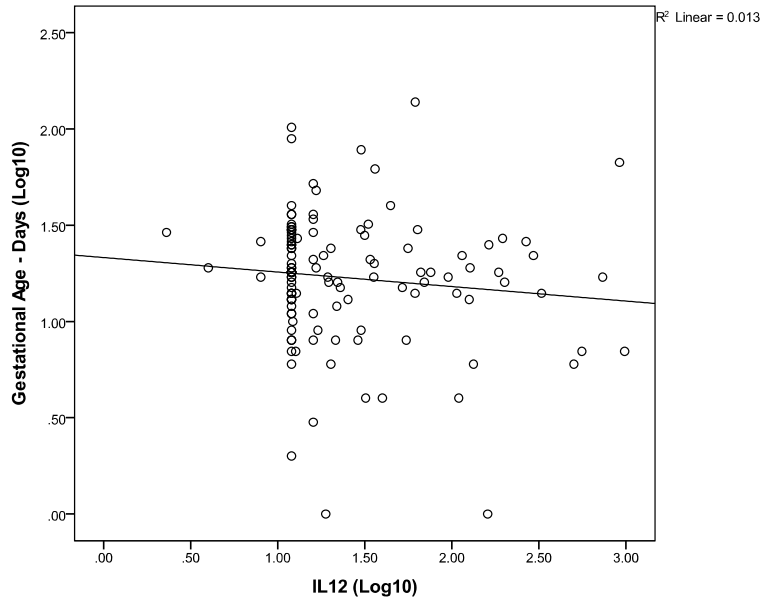


Figure 13. Scatterplot for Gestational Age and IL12

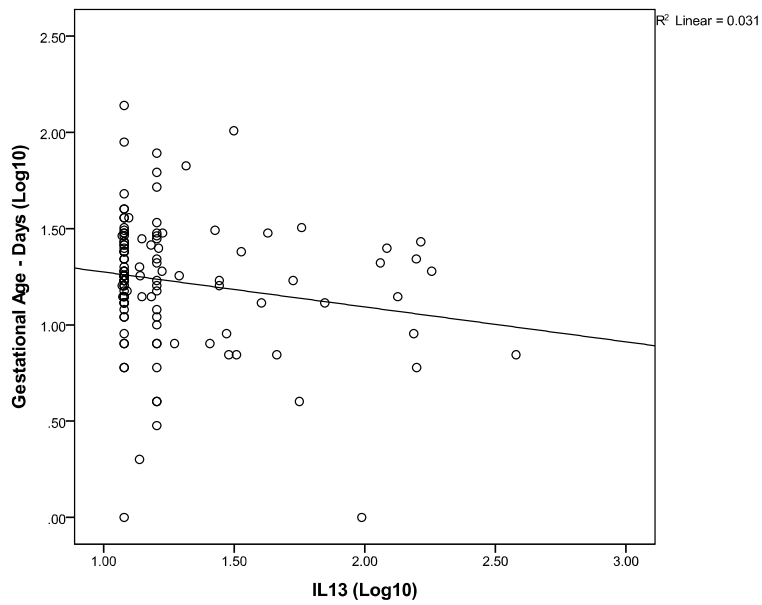


Figure 14. Scatterplot for Gestational Age and IL13

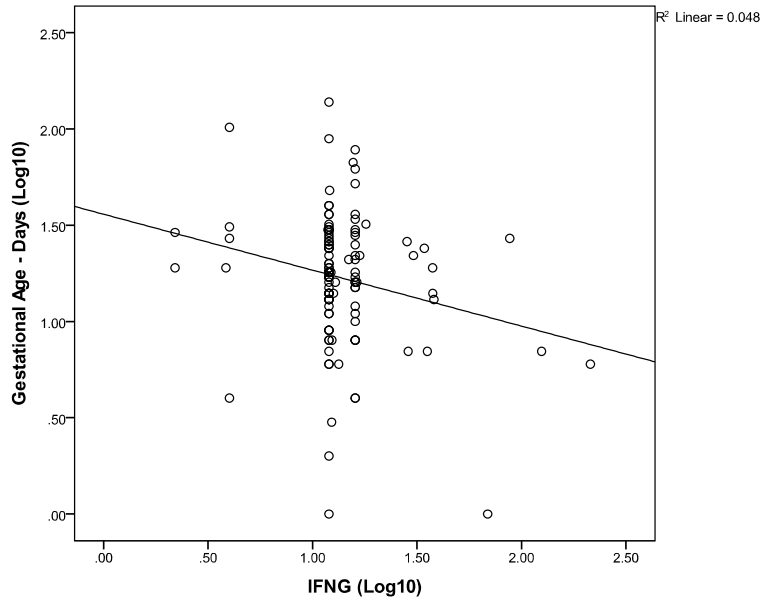


Figure 15. Scatterplot for Gestational Age and IFN γ

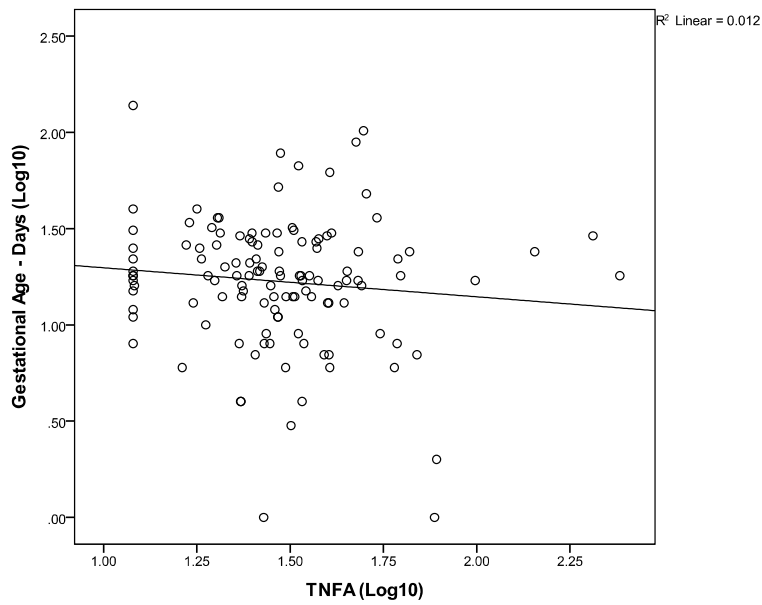


Figure 16. Scatterplot for Gestational Age and TNF α

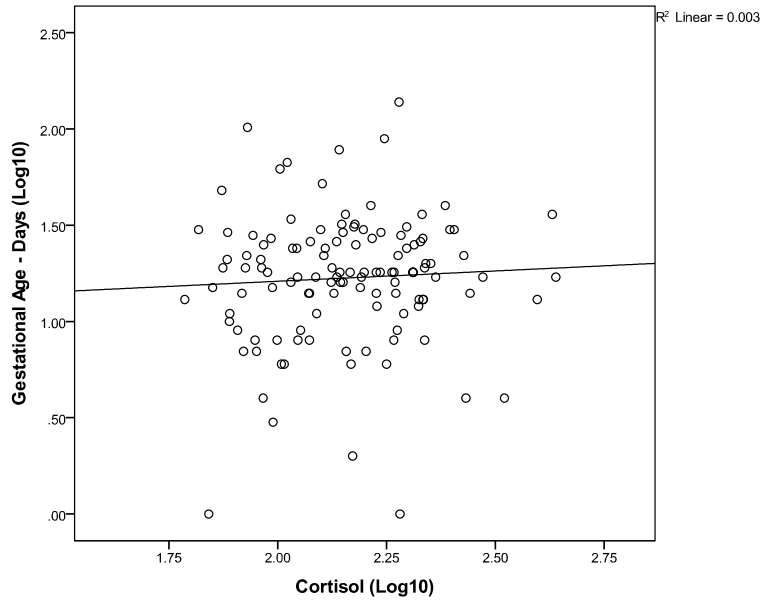


Figure 17. Scatterplot for Gestational Age and Cortisol

CHAPTER FIVE

DISCUSSION

Discussion of Findings

Measures were carried out to examine the relationship between psychological and physiological variables and the outcome variable, gestational age at delivery. Pearson correlations showed a significant negative linear relationship between IFN- γ and IL-13 with gestational age. This relationship indicates that women with higher levels of these cytokines have shorter pregnancies than do women with lower levels.

Interferon gamma is a key pro-inflammatory cytokine produced by Th1 cells while IL-13 is an anti-inflammatory cytokine usually associated with a Th2-like state. Natural Killer (NK) cells are a predominant producer of IFN- γ , and IL-13 to a lesser extent (Fitzgerald, et al., 2001). These cytokines generally work in opposition so as levels of one cytokine increases, then the level of the other decreases. Concurrent elevation of IFN- γ and IL-13 was an unexpected result, however similar findings have been reported.

In a study by Gargano et al. (2008), Th1 (IL-1, IL-2, IL12, IL-18, IFN- γ , TNF- α , and TNF- β), Th2 (IL-4, IL-6, and IL-10), and Th17 (IL-17, TGF- β , and GM-CSF) cytokines were investigated at mid-pregnancy to examine their

relationship to spontaneous preterm labor. Maternal plasma samples were collected between 15 to 27 weeks gestation (prior to the onset of preterm labor). Their findings showed concurrent elevations in levels of Th1 and Th2 cytokines in women who delivered prior to term (i.e. shortened gestation), although their findings were only in the presence of histologic chorioamnionitis.

In other studies of samples collected at mid-gestation, levels of IFN- γ have been shown to increase from the first to second trimester in normal, uncomplicated pregnancies carried to term (Curry, et al., 2008). Elevated levels of IFN- γ and IL-6 in plasma samples collected at 25 weeks gestation were shown to be weakly associated with an increased risk for delivering between 34 to 36 weeks gestation, but not to a degree that would be useful in the prediction of preterm delivery (Curry et al., 2007).

Since pregnancy is characterized by a shifting state of pro-inflammation during the first trimester, anti-inflammation during trimesters two and three, and then pro-inflammation at parturition (Mor & Cardenas, 2010), comparison of current findings to studies conducted at a time other than mid-pregnancy is a challenge. The analysis of only one time-point during pregnancy limits the ability to assess the shifting balance of cytokines, but the current findings may be representative of the time of transition from the pro-inflammatory state (indicated by the IFN- γ levels) to the anti-inflammatory state (IL-13 levels). Additionally, inter-study comparison is made more difficult by the varied methods of sampling sources (amniotic fluid, circulation, cervical fluid, placental tissue, etc) and method of analyses (flow cytometry, ELISA, or Luminex).

Strong correlations among cytokines (both pro-inflammatory and anti-inflammatory) were also shown in the current study. This relationship may be due to the regulation of cytokines by their interaction with one another. Methods carried out during this study were not able to decipher the source of secretion of the circulating cytokines which may have originated from the maternal-fetal unit or another source of inflammation in the body.

A weak positive correlation was shown to be present between gravidity and pregnancy length indicating that the length of the pregnancy increased as the number of pregnancies increased. However, when gestational age at delivery was categorized into three groups (those who delivered less than 38 weeks, 38 to 40 weeks, and 40 weeks or greater) and analyzed with ANOVA, the mean gravidity was highest in those women who delivered at less than 38 weeks and lowest in the over 40 week group. In a previous study, women delivering prior to term were more likely to be multi-gravid and nulliparous (i.e. more than one pregnancy without a subsequent delivery) (Curry et al., 2009). Further analysis of the current study sample showed no differences between the gestational age at delivery and women who were primigravid, multi-gravid women who were nulliparous, and multi-gravid women who were multiparous. Gravidity has not been shown to have an association with maternal cytokine levels during pregnancy (Curry, et al., 2009; Curry, et al., 2007; Curry, et al., 2008) although, in the current sample, IL-6 was significantly elevated in multi-gravid/nulliparous women.

The second aim was to examine the unique role played by physiological and psychological variables in predicting gestational age at delivery. The best model to predict gestational age at delivery (y) given (x), was from the combined variables, IFN- γ , IL-5, and IL-7. Six percent of the variability of the dependent variable (gestational age at delivery) can be explained by the variability of the combination of these three cytokines in the model.

Perceived stress and depression were key psychological variables of interest in the current study. Neither demonstrated a positive correlation with gestational age at delivery, nor were they identified as a good predictor in the regression model. This is an unexpected finding since perceived stress has been previously shown to be inversely related to gestational length (Hobel, Dunkel-Schetter, et al., 1999; Ruiz, et al., 2002) and prenatal depression has been found to be positively related to preterm delivery (Fransson, et al., 2011; Phillips, et al., 2010; Smith, et al., 2011). The absence of a correlation may be due to the small number of women reporting depressive symptoms in the current population. There may have not been enough variation in the stress and depression scores to identify a relationship with gestational age. Interesting to note, both depression and perceived stress had significant positive correlations with gravidity which was correlated with gestational age at delivery.

Not unexpected was the finding that the gestational age at which the blood sample was collected had a significant effect on the level of cortisol measured. Women whose blood was collected between 20 to 26 weeks had higher cortisol levels than those taken between 16 to 20 weeks. Pregnancy is characterized by

a managed state of cortisolemia and a rise in cortisol during pregnancy increases the production of pCRH (Sandman, et al., 2011). These levels continue to rise until the onset of parturition (McLean, et al., 1995; Wadhwa, et al., 1998).

Limitations

This study was conceived after data collection had taken place during the Source Study on postpartum thyroiditis. As mentioned, the current analyses were limited to a single blood sample obtained during the second trimester of pregnancy. It was not possible to describe the individuals' baseline status as a comparison for change, nor could a trend in levels of cortisol and cytokines during pregnancy and until delivery be estimated.

Also, since the time of day that blood samples were collected was not recorded for all patients, it was not possible to control for the diurnal variation in the physiological variables. Blood samples were collected at participants' regularly scheduled prenatal appointments which occurred between 8:30am and 6:00pm in those with times recorded. Differences in biological outcomes or relationships may be impacted based on sampling time.

Assessment of cytokines in this study was by analyses of systemic levels found in plasma. Many cytokine measurements were below the limit of detection and an estimated value had to be used for the analysis. Improvements in detection methods need to be identified for future studies. Also, it was not possible to identify the source of cytokine secretion. Plasma cytokine levels may be indicative of actions occurring elsewhere in the body other than at the maternal-fetal interface. Although peripheral collection of venous blood can be

done with ease, perhaps concurrent assessment of cervical fluid throughout the pregnancy would provide a better assessment of the cytokine profile related to pregnancy.

Gestational age in the current study was calculated by subtracting the exact date of delivery from the estimated date of delivery as noted in the participant's chart. The estimated date of delivery in the chart was calculated based on the woman's last menstrual period and confirmed with ultrasound. Even with the use of technology, the estimated date of delivery is just that - - an estimate. Women usually give birth +/- seven to 10 days from their estimated date of delivery. This known variability should be acknowledged since women who appeared to have delivered pre- or post-term may have actually been closer to term than estimated.

The Profile of Mood States Depression-Dejection subscale is a measure of dysphoric mood and is a screening tool for clinical depression. This study should be repeated with a depression instrument validated in a pregnant sample. An instrument such as the Edinburgh Postnatal Depression Scale (Cox, Holden, & Sagovsky, 1987) may be a more appropriate alternative.

Conclusions

Pregnancy is a dynamic state characterized by obvious changes in maternal characteristics, as well as alterations within the body that go unnoticed. A successful pregnancy to term gestation relies on the delicate balance of pro- and anti-inflammatory cytokines of the immune system. Alterations in this balance may lead to complications such as preterm labor, preeclampsia, and

gestational diabetes. Despite advances in medical research, the rate of early delivery in the United States continues to rise. Continued research is needed to identify early indicators of an altered gestational length so preventive interventions may be developed.

Implications for Future Research

A longitudinal study should be completed to accurately describe the shifting levels of cytokines and cortisol throughout pregnancy and postpartum recovery. Women should be enrolled and measurements completed beginning at their first prenatal appointment and monthly until delivery and six months postpartum. Non-pregnant controls would provide a comparison of pregnant to non-pregnant cytokine levels and findings may be used as a benchmark for future studies.

The current variables under study should be included in future research to further investigate their relationship to preterm delivery versus term delivery. The study would require a larger sample size and one with fairly equal cases of term and preterm pregnancies. Key Th1 and Th2 cytokines should be chosen for analysis based on the success of detection in pilot testing, therefore only those analytes with successful detection in ~75% of cases tested should be considered. Perhaps diurnal variation of cytokine levels should be considered and blood collected at a time of day when concentrations are greatest and detection would be best.

Acknowledgement

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APPENDICES

Appendix A
Demographic Form

Last Name: _____		First Name: _____		Initial: _____	
DOB: ____/____/____		Address:		Phone Number:	
Due Date: ____/____/____		Street: _____		Home: _____	
Pre-Pregnancy Wt: _____		City: _____		Cell: _____	
		State: _____		Emergency Contact	
		Zip Code: _____		Name: _____	
		Ht: _____ BMI: _____		Phone	
				Number: _____	
				Email: _____	
Race/Ethnicity:		Education:		Household Income (Yearly):	
<input type="radio"/> Caucasian <input type="radio"/> African-American <input type="radio"/> Asian/Pacific Islander <input type="radio"/> Native American <input type="radio"/> Hispanic Origin <input type="radio"/> White <input type="radio"/> Black <input type="radio"/> Other		<input type="radio"/> Grammar School <input type="radio"/> Middle School <input type="radio"/> High School Graduate <input type="radio"/> College Graduate <input type="radio"/> Post Graduate <input type="radio"/> Type of work _____		<input type="radio"/> Under \$4,999 <input type="radio"/> \$5,000-14,999 <input type="radio"/> \$15,000-24,999 <input type="radio"/> \$25,000-39,999 <input type="radio"/> \$40,000-69,999 <input type="radio"/> \$70,000+	
Marital Status:		Current Working Status:		Number of Pregnancies:	
<input type="radio"/> Single <input type="radio"/> Married <input type="radio"/> Divorced <input type="radio"/> Widowed		<input type="radio"/> Part time <input type="radio"/> Full time <input type="radio"/> Not at all Hours/week: _____		_____ Total pregnancies _____ Full Term _____ Premature _____ AB, induced _____ AB, Spontaneous _____ Ectopics _____ Multiple Births _____ Living _____	
				How many children do you have?	

				Number living in household:	

<p>Do you Smoke?</p> <ul style="list-style-type: none"> <input type="radio"/> Yes <input type="radio"/> No <p>If Yes, how many cigarettes/day:</p> <p>_____</p>	<p>Did you smoke during your pregnancy?</p> <ul style="list-style-type: none"> <input type="radio"/> Yes <input type="radio"/> No <p>If Yes, how many cigarettes/day:</p> <p>_____</p>	<p>Are you currently exercising?</p> <ul style="list-style-type: none"> <input type="radio"/> None <input type="radio"/> 0-30 min. per week <input type="radio"/> 30-60 min. per week <input type="radio"/> 60-90 min. per week <input type="radio"/> 90-120 min. per week <input type="radio"/> 120+ min per week 	<p>Type of Exercise:</p> <ul style="list-style-type: none"> <input type="radio"/> Very Strenuous (Jogging) <input type="radio"/> Pretty Strenuous (Walking up a hill) <input type="radio"/> Mildly Strenuous (Walking your pet) <input type="radio"/> Not Strenuous (Light housework)
<p>Are you currently receiving any medical treatment for any health problems? If so please list.</p> <ul style="list-style-type: none"> <input type="radio"/> No <input type="radio"/> Yes, please list <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>1.</p> <p>2.</p> <p>3.</p> <p>4.</p> </div> <p>Do any of your relatives have thyroid disease? Please describe.</p> <p>Allergy History; Have you ever been told by a health professional (doctor or nurse) that you have nasal allergy, allergic rhinitis , or hay fever? Yes No Have you ever been told b a health professional (doctor or nurse) that you have asthma? Yes No Have you been symptomatic with asthma during the past year? (12 months from today) Yes No Are you currently symptomatic? Yes No Are you currently using asthma medicine/ Yes No Have you had an asthma flare or worsening of symptoms requiring asthma medication during the past year? (12 months from today).</p>		<p>Are you taking any prescription medications?</p> <ul style="list-style-type: none"> <input type="radio"/> No <input type="radio"/> Yes, please list <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <p>1.</p> <p>2.</p> <p>3.</p> <p>4.</p> </div>	

Delivery Information:				
Date of baby's birth?	Sex of baby? <input type="radio"/> Male <input type="radio"/> Female	Type of delivery: <input type="radio"/> Vaginal <input type="radio"/> Caesarean	Any difficulties or complications with delivery <input type="radio"/> No <input type="radio"/> Yes	If yes, please circle: Bleeding, Infections, Abscesses, Severe Hemorrhoids, other <hr/>
Length of labor: <hr/>	Are you currently breastfeeding? <input type="radio"/> Never breastfed <input type="radio"/> Partial (ounces per day: _____) <input type="radio"/> Totally (never used formula) <input type="radio"/> Started then stopped Date stopped: _____		If Breastfeeding: Time of last feeding: _____ am _____ pm	

Appendix B
The Perceived Stress Scale

Stress Since We Last Visited with You					
Study ID #: _____	Date: _____				
<p>The questions in this scale ask you about your feelings and thoughts since we last visited with you. In each case, you will be asked to indicate how often you felt or thought a certain way. Although some of the questions are similar, there are differences between them and you should treat each one as a separate question. The best approach is to answer each question fairly quickly. That is, don't try to count up the number of times you felt a particular way, but rather make a reasonable estimate.</p>					
<p>Please check the correct answer: 1 = Never 2 = Almost never 3 = Sometimes 4 = Fairly often 5 = Very often</p>					
	1	2	3	4	5
How often have you been upset because of something that happened unexpectedly?					
How often have you felt that you were unable to control the important things in your life?					
How often have you felt nervous and "stressed"?					
How often have you dealt successfully with irritating life hassles?					
How often have you felt that you were effectively coping with important changes that were occurring in your life?					
How often have you felt confident about your ability to handle your personal problems?					
How often have you felt that things were going your way?					
How often have you found that you could not cope with all the things that you had to do?					
How often have you been able to control irritations in your life?					
How often have you felt that you were on top of things?					
How often have you been angered because of things that happened that were outside of your control?					
How often have you found yourself thinking about things that you have to accomplish?					
How often have you been able to control the way you spend your time?					
How often have you felt difficulties were piling up so high that you could not overcome them?					

Appendix C
The Profile of Mood States

POMS™ Standard Form


BY DOUGLAS M. McNAIR, Ph.D., MAURICE LORR, Ph.D., JW P. HEUCHERT, Ph.D., & LEO F. DROPPLEMAN, Ph.D.

Name: _____ Age: _____ Gender: Male Female
(Circle one)

Birth Date: ____/____/____ Today's Date: ____/____/____
Month Day Year Month Day Year

To the Administrator:

Place a checkmark ✓
in one box to specify the
time period of interest.

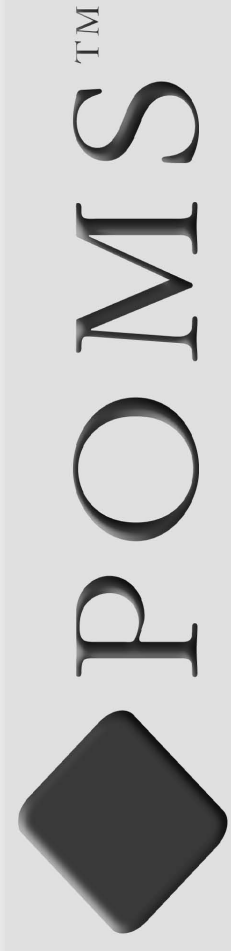


To the Respondent:

Below is a list of words that describe feelings that people have. Please read each word carefully. Then circle the number that best describes

how you have been feeling during the PAST WEEK, INCLUDING TODAY.
 how you feel RIGHT NOW.
 other: _____

If no box is marked, please follow the instructions for the first box.

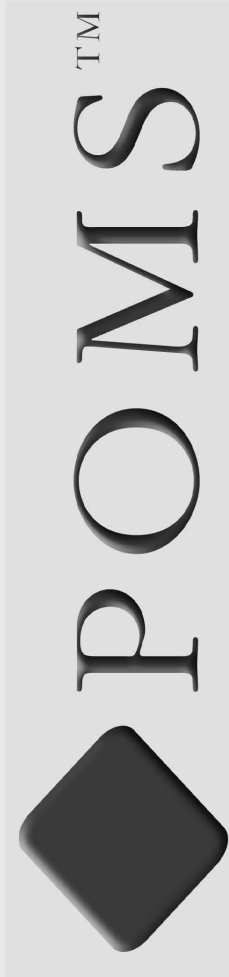


	Not at all	A little	Moderately	Quite a bit	Extremely
1. Friendly	0	1	2	3	4
2. Tense	0	1	2	3	4
3. Angry	0	1	2	3	4
4. Worn out	0	1	2	3	4
5. Unhappy	0	1	2	3	4
6. Clear-headed	0	1	2	3	4
7. Lively	0	1	2	3	4
8. Confused	0	1	2	3	4
9. Sorry for things done	0	1	2	3	4
10. Shaky	0	1	2	3	4
11. Listless	0	1	2	3	4
12. Peeved	0	1	2	3	4
13. Considerate	0	1	2	3	4
14. Sad	0	1	2	3	4
15. Active	0	1	2	3	4
16. On edge	0	1	2	3	4
17. Grouchy	0	1	2	3	4
18. Blue	0	1	2	3	4
19. Energetic	0	1	2	3	4
20. Panicky	0	1	2	3	4
21. Hopeless	0	1	2	3	4
22. Relaxed	0	1	2	3	4
23. Unworthy	0	1	2	3	4
24. Spiteful	0	1	2	3	4
25. Sympathetic	0	1	2	3	4
26. Uneasy	0	1	2	3	4
27. Restless	0	1	2	3	4
28. Unable to concentrate	0	1	2	3	4
29. Fatigued	0	1	2	3	4
30. Helpful	0	1	2	3	4

*Please flip over.
Items continue on the back page...*

POMS™ Standard Form

BY DOUGLAS M. McNAIR, Ph.D., MAURICE LORR, Ph.D., JW P. HEUCHERT, Ph.D., & LEO F. DROPPLEMAN, Ph.D.



	Not at all	A little	Moderately	Quite a bit	Extremely
31. Annoyed	0	1	2	3	4
32. Discouraged	0	1	2	3	4
33. Resentful	0	1	2	3	4
34. Nervous	0	1	2	3	4
35. Lonely	0	1	2	3	4
36. Miserable	0	1	2	3	4
37. Muddled	0	1	2	3	4
38. Cheerful	0	1	2	3	4
39. Bitter	0	1	2	3	4
40. Exhausted	0	1	2	3	4
41. Anxious	0	1	2	3	4
42. Ready to fight	0	1	2	3	4
43. Good natured	0	1	2	3	4
44. Gloomy	0	1	2	3	4
45. Desperate	0	1	2	3	4
46. Sluggish	0	1	2	3	4
47. Rebellious	0	1	2	3	4
48. Helpless	0	1	2	3	4
49. Weary	0	1	2	3	4
50. Bewildered	0	1	2	3	4
51. Alert	0	1	2	3	4
52. Deceived	0	1	2	3	4
53. Furious	0	1	2	3	4
54. Efficient	0	1	2	3	4
55. Trusting	0	1	2	3	4
56. Full of pep	0	1	2	3	4
57. Bad-tempered	0	1	2	3	4
58. Worthless	0	1	2	3	4
59. Forgetful	0	1	2	3	4
60. Carefree	0	1	2	3	4
61. Terrified	0	1	2	3	4
62. Guilty	0	1	2	3	4
63. Vigorous	0	1	2	3	4
64. Uncertain about things	0	1	2	3	4
65. Bushed	0	1	2	3	4

*Please ensure you have answered every item.
Thank you for completing this questionnaire.*



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ABOUT THE AUTHOR

Melissa Molinari Shelton was born in Islip, New York and grew up in Port Charlotte, Florida. She earned a B.S. in Elementary Education and B.S. in Nursing from the University of South Florida, Tampa. She also earned a M.S. in Nursing Education and Ph.D in Nursing Science from the University of South Florida. She has been a research assistant and project director on several studies including those funded by the National Institutes of Health. In 2007, she was selected as a fellow of the National Institute of Nursing Research and attended their prestigious Summer Genetics Institute. She has presented at numerous local and national conferences and has over 10 published abstracts and manuscripts.