CHARACTERIZATION OF ABDOMINAL ADIPOSITY IN PREGNANCY AND ITS RELATIONSHIP TO ADIPOKINES AND THE ADAPTIVE IMMUNE RESPONSE

By

Marlies K. Ozias

Submitted to the graduate degree program in Medical Nutrition Science and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Susan E. Carlson, Ph.D. (Advisor)

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Stephen H. Benedict, Ph.D.

___________________________________________________________

William M. Brooks, Ph.D.

___________________________________________________________

Holly R. Hull, Ph.D.

___________________________________________________________

Margaret G. Petroff, Ph.D.

Date Defended: September 24, 2013
The Dissertation Committee for Marlies K. Ozias

certifies that this is the approved version of the following dissertation:

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___________________________________________

Susan E. Carlson, Ph.D. (Advisor)

Date approved: September 24, 2013
ABSTRACT

A large body of evidence demonstrates that obese pregnant women have a greater risk of pregnancy and delivery complications and poorer health outcomes compared with normal weight pregnant women. Most research on the impact of obesity in pregnancy defines obesity by body mass index (BMI), a measurement that does not consider the location of body fat. Abdominal fat mass is associated with cardiometabolic diseases so it is reasonable to postulate that it plays a greater role in obesity-related pregnancy complications, yet the role of abdominal fat in pregnancy is not well defined. It is known that obese pregnant women have higher circulating interleukin 6, C-reactive protein, and higher macrophage expression in the placenta. It is also known that T cell populations increase in non-pregnant obese individuals. However, it is not known how T cells respond to the incidence of obesity in pregnancy.

The main purpose of this research is to examine the relationships between abdominal fat mass and circulating adipokines and peripheral CD4⁺ T cells in pregnancy. We recruited thirty-eight women from the University of Kansas Obstetrics and Gynecology Clinic in their third trimester for the Pregnancy Health Study. Recruitment was focused on women in healthy pregnancy and with respect to a full spectrum of pre-pregnancy BMI (18.5 – 40). Before delivery, blood was collected to measure circulating adipokines by ELISA and measure CD4⁺ T cell cytokine production by multiplex. CD4⁺ T cells were isolated from peripheral blood mononuclear cells by positive selection and stimulated ex vivo to examine cytokine production. Total body fat was measured by dual x-ray energy absorptiometry in the subjects within three-weeks postpartum. At the same visit, magnetic resonance imaging scans on the abdominal region
occurred in a subset of twenty subjects. ImageJ was used to quantify abdominal subcutaneous and visceral fat mass from the axial images.

Women categorized as overweight or obese by BMI had significantly greater amounts of abdominal subcutaneous and visceral fat mass compared to normal-weight BMI women, as well as greater total body fat. We found a strong relationship between abdominal visceral fat mass and resistin but no other adipokines measured (leptin, visfatin, and adiponectin) correlated with abdominal visceral fat mass. No relationships were observed between abdominal subcutaneous fat mass and any adipokine. Leptin was the only adipokine to correlate with total body fat and pre-pregnancy BMI. Additionally, there were no correlations between pre-pregnancy BMI, total body fat (with one exception), or abdominal subcutaneous fat with CD4⁺ T cell production of cytokines. We did observe a significant decrease in cytokine concentration in relationship to abdominal visceral adiposity. This dampening effect was observed across all categories of CD4⁺ T cell cytokines.

Together, these results suggest that visceral fat mass in pregnancy has the potential to play a role in obesity-related pregnancy complications whereas BMI is a poor indicator of the effect of fat on adaptive immunity. Even though women with an overweight or obese pre-pregnancy BMI have greater amounts of abdominal fat mass, BMI was unrelated to physiologically relevant adipokines (leptin as the exception) and CD4⁺ T cell cytokines. Visceral fat is an ectopic fat compartment and should be studied as a physiologically relevant factor in obesity-related pregnancy complications, regardless of a woman’s weight status.
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<tr>
<td>3T3-L1</td>
<td>adipocyte morphology cell line</td>
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<td>ADP</td>
<td>air displacement plethysmography</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BIA</td>
<td>bioelectrical impedance analysis</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>CCL</td>
<td>chemokine (C-C motif) ligand</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>CV</td>
<td>covariance</td>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
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<tr>
<td>DIO</td>
<td>diet-induced obesity</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<td>DM</td>
<td>diabetes mellitus type II</td>
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<td>DXA</td>
<td>dual-energy x-ray absorptiometry</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GDM</td>
<td>gestational diabetes mellitus</td>
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<td>GLUT</td>
<td>glucose transporter</td>
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<td>GWG</td>
<td>gestational weight gain</td>
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<td>HMW</td>
<td>high molecular weight</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IL</td>
<td>interleukin</td>
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<td>IUGR</td>
<td>intrauterine growth restriction</td>
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<td>Abbreviation</td>
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<td>L2</td>
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<td>LMW</td>
<td>low molecular weight</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MCP</td>
<td>monocyte chemotactic protein</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>MMW</td>
<td>medium molecular weight</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<td>Nampt</td>
<td>nicotinamide phosphoribosyltransferase</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NHANES</td>
<td>national health and nutrition examination survey</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>peripheral blood mononuclear cells</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>RBP</td>
<td>retinol binding protein</td>
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<td>S3</td>
<td>sacral 3</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SVF</td>
<td>stromal vascular fraction</td>
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<td>TBW</td>
<td>total body water</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>Th</td>
<td>T helper (cell)</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>Treg</td>
<td>regulatory T (cell)</td>
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<td>VEGF</td>
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CHAPTER 1

INTRODUCTION
I. Significance of Obesity in Pregnancy

Fifty percent of women of childbearing age in the United States are overweight or obese [1, 2], imposing a significant health risk to mother and offspring. Women with pregravid obesity, with or without excessive gestational weight gain (GWG), are at a greater risk for pregnancy-induced hypertension, preeclampsia, gestational diabetes mellitus (GDM), and cesarean delivery [3-5]. Additionally, after delivery there is a greater risk for postpartum infection, hemorrhage [5], and weight retention [6]. In addition, offspring of obese pregnant women are at increased risk of preterm birth [5], congenital abnormalities [7], and macrosomia [3-5], resulting in more admissions to neonatal intensive care units [8]. These health complications translate into higher medical costs, including more prenatal clinic visits, medications dispensed, more prenatal fetal tests and ultrasounds [9], increased prenatal hospital admissions, and prolonged hospitalization after delivery [10]. Long term, women with pregnancy complications such as preeclampsia or intrauterine growth restriction (IUGR) have a higher prevalence of metabolic syndrome seven years after delivery compared to women who had healthy pregnancies [11].

As pregnancy introduces dramatic change to the female body, the presence of pregravid obesity may increase the risk of complications in obese pregnant women. Altered placental environment and adverse neonatal delivery outcomes in obese pregnancies have led to the hypothesis that unfavorable in utero development may occur and have long-term implications [12]. Animal and epidemiological studies suggest a link between obese pregnant women and the risk of childhood obesity [13-15] and insulin dysregulation [16, 17]. Finally, offspring of obese mothers are at an increased risk of developing metabolic syndrome [18] and lower child
cognitive development [19]. A major goal is to elucidate the physiological role excess adiposity has on pregnancy.

Adipokines are hormones primarily expressed by adipose tissue. Leptin, adiponectin, resistin, and visfatin are adipokines that regulate appetite, glucose homeostasis [20-23], and immune function [24-27]. Coincidentally, the placenta either produces or has receptors to these adipokines. For example, adiponectin influences lipid and glucose metabolism [28] and leptin stimulates protein transport [29] to support the nutritional needs of the growing fetus. These adipokines are measured in maternal circulation during pregnancy as well as in the cord blood and in newborns to elucidate their roles in pregnancies with and without complications. Differences in circulating adipokine concentrations have been shown in pregnant women who are obese compared to normal weight [30-32] and it has been suggested that these adipokines play a role in pregnancy complications [33-35]. The roles of adipokines in obese-pregnancy complications and as well as long-term offspring health are not clearly defined.

A defining characteristic of obesity is low-grade inflammation. Elevated levels of circulating tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6) [36], C-reactive protein (CRP) [37] are typically found in obese individuals. These elevated levels in the presence of obesity increase the risk of atherosclerosis, with [38] or without diabetes mellitus type II (DM) [39]. In pregnancy, women with pregravid obesity have greater circulating IL-6 and CRP versus lean pregnant women in addition to placental inflammation [40-42]. These proteins are primarily associated with acute inflammation, macrophage-derived and indicative of innate immunity. The other arm of immunity is the adaptive immune response. T cells, a component of adaptive
immunity are also involved in obesity-induced inflammation. CD8+ T cells initiate the influx of monocytes into adipose tissue [43] and CD4+ T cell populations increase in adipose tissue [44] and in the circulation of obese subjects [45, 46]. The majority of research on the incidence of obesity in pregnancy focuses on the macrophage-mediated inflammation while the adaptive immune response to obesity is not as well characterized.

Excess adiposity in the abdominal region contributes a greater amount of inflammation compared to lower-body adiposity [47, 48] and increases the risk of cardiovascular disease (CVD), DM, dyslipidemia [49], and mortality [50]. Despite these risks, the effect of body fat mass in pregnancy is not well understood. The location of body fat may influence the degree of inflammation in pregnant women but no studies to date have addressed the relationship between body fat location and inflammation during pregnancy. This literature review will address these subjects with special consideration of the location of excess body fat.

II. Methods to Body Composition: Problems and Considerations in Pregnancy

The majority of obesity research utilizes body mass index (BMI) to categorize weight status. Although an easy calculation (weight divided by height squared, kg/m²; underweight < 18.5, normal weight 18.5 – 24.9, overweight 25.0 – 29.9, obese > 30), BMI has several limitations. First, BMI does not measure body composition or location of fat mass. Second, BMI does not take into consideration sex, age, or the amount of lean tissue. A person can have a normal-weight BMI and yet carry excessive body fat. An example of this is the increase in body fat relative to the loss of lean body mass that occurs in aging [51]. Furthermore, a healthy BMI is
not equivalent to a healthy metabolic profile. In a cross-section of the NHANES database, 24% of adults with normal-weight BMI had cardiometabolic abnormalities [52]. Therefore, BMI is not an ideal measure to categorize subjects in healthy or unhealthy groups for weight-related studies when the intent is to identify effects of excess adiposity [53]. Despite the limitations of BMI, most obesity research includes BMI as an indicator of body fatness because it is a quick and inexpensive measurement.

The prevalence of obesity in pregnancy and the associated medical risks led the American College of Obstetricians and Gynecologists in January 2013 to publish recommendations for weight gain and nutritional consultation for both preconception and pregnancy of overweight and obese women [54]. Too much or too little weight gained during pregnancy increases the risk of complications during pregnancy and delivery and can adversely impact offspring health [55]. In 2009, the Institute of Medicine recommended GWG guidelines based on pre-pregnancy weight and defined the health risks of improper GWG. Nevertheless, the majority of obese women do not meet GWG guidelines and obstetric providers may not: a) follow clinical practice guidelines, b) be able to define overweight and obesity, or c) know recommended GWG for pre-pregnancy weight status [56].

Weight gain in pregnancy is composed of increases in adipose and mammary gland tissues, increased plasma volume, and the creation of the placenta, fetus, and amniotic fluid. These changes preclude the use of standard anthropometric measurements such as waist-to-hip ratio and skinfold thickness to determine excess adiposity in pregnancy [57]. The amount and location of adipose tissue gained is dependent on pre-pregnancy weight and parity and can start as early as gestational week six [58] while the greatest increases in adipose and maternal blood
volume occur between the second trimester and delivery [59]. Altogether, measuring body composition during pregnancy is difficult due to the dynamic changes the maternal body undergoes to support the fetus and elucidation of excess adiposity from pregravid obesity or excessive GWG can be difficult.

Given that 40% of all pregnancies are unintended [60], research that attempts to assess body composition before conception would require very significant effort. Methods to measure body composition during pregnancy are also difficult because most procedures have either not been validated in pregnancy or are contraindicated. For example, bioelectrical impedance analysis (BIA) measures electrical resistance in tissue and extracellular fluid and has been validated in estimating body fat percentage in non-pregnant adults [61, 62]. Although it has been used to compare increases in body water for pregnancy-induced hypertension [63], BIA measurement of adiposity has not been validated for pregnancy-related fat changes. Air-displacement plethysmography (ADP) measures volume displacement in an enclosed chamber and is safe to use during pregnancy, however, ADP estimates fat and fat-free mass based on assumptions of total body water (TBW) and bone density. Assumptions of TBW cannot be made because of the large variation in body water volume in pregnancy [64].

TBW is measured with the stable isotope deuterium (three- and four-compartment models) and can be measured along with fat and protein compartments during pregnancy. Bone density is measured postpartum by dual-energy x-ray absorptiometry (DXA) since bone density is assumed not to change during pregnancy [64]. Estimates of fat mass and its relationship to obesity-related pregnancy risks are difficult because of the physiological changes that occur throughout pregnancy. In a small study of nine pregnant women, GWG from fat mass was so
variable (measured with 4-compartment modeling) throughout pregnancy that researchers concluded adiposity accretion rates for each woman was under each subject’s unique hormonal influence [64].

III. Abdominal Adiposity: Measuring Its Impact

So far, only the measurement of whole-body fat mass has been discussed. The location of fat may play a role in pregnancy and delivery complications, however it is difficult to visualize abdominal fat mass during pregnancy because of the growing fetus. Nevertheless, studies of abdominal fat mass could lead to a clearer understanding of obesity-related pregnancy complications and long-term health risks.

Abdominal adiposity is divided into two compartments: subcutaneous and visceral (a sagittal view of abdominal adiposity is displayed in Figure 1.1). The subcutaneous fat layer is between the epidermis and the abdominal cavity whereas visceral is an ectopic fat storage in the intra-abdominal cavity and situated around the internal organs. Excess upper-body adiposity is a better predictor of metabolic complications compared to BMI [49] or lower-body adiposity [65].
and a greater waist circumference increases mortality risk even with a normal-weight BMI [50]. The metabolic dysfunction associated with ectopic visceral fat mass leads to systemic inflammation [48] while hypertrophic subcutaneous adipose stores correlate with metabolic syndrome [47, 66] and insulin resistance [67]. In pregnancy, there is a redistribution of lower-body adiposity toward more central, upper-body adiposity, hypothesized to increase fetal access to stored energy [68]. The newly deposited adipose tissue remains after pregnancy. Women giving birth during a 5-year period had a 26% increase in visceral fat compared to those who did not give birth despite the fact that their weight, BMI, and total abdominal fat did not change [69]. In addition, factors such as age, ethnicity, and genetics are linked to abdominal adiposity [70, 71] so general assumptions cannot be made for the amount of pregnant women’s abdominal fat mass. The ability to quantify this more metabolically active fat pool could aid in clarifying its role in obese pregnancy.

Computed tomography (CT) is one of the most accurate methods for measuring body composition and adiposity stores [72] but subjects are exposed to radiation. In order to reduce radiation exposure, prediction models are used to estimate total subcutaneous and visceral abdominal adiposity with a CT scan taken at lumbar 4-5 [73]. Ultrasound is another means of measuring of body composition [74]. The procedure is commonly used to measure fetal growth and movement throughout pregnancy. Ultrasound measurement of preperitoneal fat thickness (space between abdominal wall and visceral cavity) correlates well with visceral fat adiposity [75] and appears to be a convenient, safe way to estimate abdominal adiposity in pregnancy. Pregnant women have increased preperitoneal and subcutaneous adipose in the third trimester compared to the first two trimesters [76] and subcutaneous adipose thickness measured by
ultrasound between 18-22 weeks gestation is associated with an increased risk of GDM and cesarean delivery [77]. Two techniques used in whole body composition, BIA and DXA, have also been tested for their ability to measure abdominal adiposity. BIA correlates well with subcutaneous, but not visceral adiposity in non-pregnant adults [78]. DXA can quantify abdominal adiposity, but visceral adiposity is estimated from modeling equations [79]. Similar to the CT scan, DXA exposes the subject to radiation and is generally avoided during pregnancy. Finally, magnetic resonance imaging (MRI) is a valid measurement for whole-body adiposity [80, 81] as well as the abdominal adipose compartments, visceral and subcutaneous [82, 83] (Figure 1.2). The major limitations of MRI are instrument access and expense of the procedure. Additionally, the supine position needed for the MRI scan may cause unnecessary spinal column pressure. All the techniques mentioned above may be used to measure abdominal adiposity in the immediate postpartum period as abdominal fat mass does not change drastically after delivery and, in fact, can be present up to 5 years after pregnancy [69, 84].

The redistribution of lower-body adiposity to more centrally located abdominal adiposity in pregnancy is also hypothesized to aid in the physiologic insulin resistance needed to provide
more nutrients to the fetus [85]. These adipose mass changes were initially believed to be facilitated by pregnancy hormones (progesterone, placental lactogen, cortisol, prolactin) [86]. However, adipokines have been shown to have distinct roles. TNF-α and leptin are involved in the pathophysiology of pregnancy-induced insulin resistance [87] and after adjusting for fat mass, TNF-α was the most significant independent predictor of insulin sensitivity in 25 pregnant women (with or without GDM) [88]. The adipokines resistin [89] and visfatin [90] have also been implicated in insulin resistance. Adiponectin enhances insulin sensitivity in the muscle of women with GDM who also have lower circulating adiponectin in the first trimester [91]. The possible role of adipokines in the etiology of obesity complications during pregnancy is not well studied but of potential great importance.

**IV. Adipokines Role in Obese Pregnancy**

When energy consumption exceeds the body’s needs, adipocytes enlarge and proliferate to store energy for potential times of famine. Adipose tissue was previously believed to be merely a storage organ for excess energy. However, it is now understood to be a metabolically active organ producing hormones and inflammatory mediators. In response to excess energy, enlarged adipocytes secrete cytokines (termed adipokines) to engage the immune system to mediate the abnormal state. Adipocytes and the surrounding stromal vascular fraction (SVF) secrete adipokines. Leptin and adiponectin are secreted from adipocytes while most adipokines are secreted from the SVF in adipose [92], which is comprised of macrophages, T and B lymphocytes, mast cells [93], endothelial cells, preadipocytes, fibroblasts, and nerve fibers [94].
Adipokines work in paracrine and endocrine roles to regulate appetite, glucose homeostasis [20-23], and immune function [24-27]. They have also been linked to health complications in both non-pregnancy states [95-98] and in pregnancy [34, 99, 100]. Numerous adipokines have been analyzed for their role in pregnancy. This review focuses on leptin, adiponectin, resistin, and visfatin. Other adipokines that have been identified as having potential roles in obesity-related pregnancy complications include TNF-α, IL-6, retinol binding protein-4 (RBP-4), and apelin (Table 1.1). The role of each of these adipokines will be addressed in regards to their roles in obesity, pregnancy, and pregnancy complications.

A. Leptin

Leptin is primarily known for its role in energy homeostasis and a genetic leptin deficiency results in an obese phenotype [101]. Administration of leptin to ob/ob mice or humans with leptin-dependent obesity causes appetite suppression and subsequent weight loss [102]. Congenital leptin deficiency leads to decreased CD4+ T cell count, cytokine release, and impaired immune cell proliferation, all of which are reversed by leptin administration [103]. As an immunomodulator, leptin induces proliferation and survival of numerous immune cells such as monocytes [104], neutrophils [105], and T cells [106]. In leptin-independent obesity there is an increase in circulating leptin that leads to leptin resistance [107]. Obese women have increased leptin synthesis from subcutaneous and visceral adipose and higher circulating leptin compared to normal weight women [108], a phenomenon also observed in obese women with
### Table 1.1 Physiologic Effects of Adipokines

<table>
<thead>
<tr>
<th>Adipokine</th>
<th>Tissue Expression</th>
<th>Function</th>
<th>Response to Obesity</th>
<th>Proposed Roles in Pregnancy</th>
<th>Roles in Pregnancy Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leptin</strong></td>
<td>Adipocytes [92],syncytiotrophoblast, fetal tissue [151], stomach [152]</td>
<td>Satiety signal, promotes increased energy expenditure, stimulates immune system</td>
<td>↑ in obesity, resistance develops [107]</td>
<td>Trophoblast invasion [131], angiogenesis [132], nutrient transfer [29, 133], maternal fat deposition [85]</td>
<td>↑ macrosomia [109], ↓ IUGR [110], ↑ [111-113] &amp; ↓ [114] GDM, ↑ [34, 99, 115] &amp; ↓ [116] preeclampsia</td>
</tr>
<tr>
<td><strong>Visfatin</strong></td>
<td>Ubiquitous [156], fetal membrane [157], chorionic [31]</td>
<td>Possibly main mechanism as Nampt, glucose homeostasis [144]</td>
<td>↑ in obesity [141], insulin resistance [95]</td>
<td>Placental glucose transport [137], amnion permeability [138]</td>
<td>↓ &amp; ↑ GDM [35, 120], ↓ preeclampsia [121]</td>
</tr>
<tr>
<td><strong>Adiponectin</strong></td>
<td>Adipocytes [92], fetal tissue [158]</td>
<td>Improves energy homeostasis, insulin sensitivity [145], anti-inflammatory [146]</td>
<td>↓ in obesity and DM [97, 98]</td>
<td>Trophoblast insulin attenuation [139], regulate amino acid transport [139]</td>
<td>↓ macrosomia [111, 117], ↓ GDM [33], ↓ preeclampsia [34], ↔ pregnancy hypertension [122]</td>
</tr>
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</table>

#### Other Adipokines

- **TNF-α**: Adipose tissue macrophages [159]
  - Inflammation, reduces insulin sensitivity [147, 148]
  - ↑ in obesity, insulin resistance [36]
  - Maternal insulin resistance [88]
  - ↑ & ↔ macrosomia [111, 123], ↑ GDM [111], ↑ preeclampsia [124], ↑ IUGR [125]

- **IL-6**: Adipocytes [160], hepatocytes [161], macrophages [162]
  - Transports retinol from liver
  - ↑ in obesity, ↓ GLUT4 in adipocytes [142]
  - Maternal insulin resistance [126]
  - ↑ GDM [126], ↔ & ↓ preeclampsia [127, 128]

- **RBP-4**: Adipocytes [143], placental tissue [129]
  - Vascular development [149], cardiovascular function [150]
  - ↑ adipocyte differentiation & insulin resistance [143]
  - Angiogenesis [140]
  - ↑ & ↓ preeclampsia [129, 130]
Increased adipose mass and enlarged adipocyte cell size contribute to the increase in circulating leptin in humans [164].

In pregnancy, the placenta produces leptin [151] and 95% of placental leptin is released into circulation [165]. Highman et al. [166] reported increased maternal leptin levels at 12-14 weeks gestation, well before significant fat accumulation occurs. It has been hypothesized that placental production of leptin is needed early in pregnancy to increase fat mobilization stores to ensure energy availability to the fetus [85]. In the placenta, leptin promotes trophoblast invasion [131], potentially stimulates placental angiogenesis [132, 167], and nutrient transport [29, 133]. By gestational week 18, leptin is detected in fetal plasma [168] and the concentration is highest at term [169].

Hyperleptinemia is observed in obese pregnant women and women with pregnancy complications. Obese pregnant women compared to normal weight pregnant women have higher leptin levels [170] and leptin is associated with greater whole-body adiposity in pregnancy [32, 171]. In pregnancy complications, hyperleptinemia occurs in preeclamptic pregnant women independent of BMI status [34, 99, 115]. Conversely, in a study of pre-pregnancy BMI-matched women who were either normotensive or preeclamptic, preeclamptic women had lower leptin levels and umbilical cord leptin concentration was not different between the two groups [116]. Most studies have found increased circulating leptin in women with GDM when measured at 13 weeks gestation [113], at the time of the glucose tolerance test (~28 weeks) [112] and at delivery [111]. After adjusting for pre-pregnancy BMI, women with a leptin concentration $\geq 31$ ng/mL had a 4.7 fold increased risk of GDM compared to those with leptin concentrations of $\leq 14.3$
Conversely, hypoleptinemia in women with GDM has also been reported [114]. Miehle et al. [172] suggests an influence of ethnicity and severity of GDM may affect leptin’s role.

The difference in circulating leptin in women with GDM may come primarily from peripheral tissue rather than the placenta. Dysregulation of leptin metabolism from muscle and adipose tissue from pregnant women with GDM versus without GDM has been shown [173]. In newborns, umbilical cord leptin correlates with birth weight [168]. Macrosomic infants have increased circulating leptin at birth [109] and infants born to obese mothers have increased body fat and cord blood leptin in addition to increased insulin resistance [174]. Conversely, infants born with IUGR have low placental and umbilical cord leptin levels at delivery [110].

**B. Resistin**

Resistin is produced in adipose tissue by adipocytes [25], monocytes, and macrophages [153], and in other tissues such as pancreas and muscle [154]. Resistin-treated human adipocytes secrete higher amounts of IL-6 and TNF-α than untreated adipocytes [25]. Abdominal subcutaneous and visceral adipose depots express greater amounts of resistin mRNA and protein expression than other adipose sites [175]. A higher concentration of circulating resistin and adipose tissue mRNA expression have been observed in obese compared to normal-weight adults [176]. Greater total body adiposity is correlated with circulating resistin in some reports [177, 178], while another study found no relationship [179]. Resistin may be involved in insulin resistance. Administration of the anti-diabetic drug, rosiglitazone decreases circulating resistin in humans while administration of anti-resistin antibody improves blood sugar and insulin action in
the diet-induced obesity (DIO)-mouse model [89]. Obese diabetic women exhibit a 2-fold increase in resistin compared to normal-weight healthy women [96] however, obese, insulin-resistant adolescents do not have higher plasma resistin compared to age-matched, lean adolescents [179].

In pregnancy, first trimester resistin levels are higher than in non-pregnant women and reach peak concentration at delivery, and there is no difference between normal weight and overweight pregnant women [180]. Resistin is expressed in placental trophoblasts at a higher level in term placenta compared to first trimester chorionic tissue, however no change in resistin expression is observed in adipose tissue as pregnancy progresses [155]. Resistin can increase proliferation of endothelial cells [134] and induce expression of growth factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-1 and MMP-2 [135], suggesting a potential angiogenic role in pregnancy.

In women with pregnancy complications, resistin’s role and concentration remains inconclusive compared to healthy pregnancy. Women with GDM have been reported to have either increased [118] or decreased maternal resistin [119] at 24-31 weeks gestation compared to pregnant women with normal glucose tolerance. Tissue from pregnant women with and without GDM show no difference in resistin secretion by placental or subcutaneous adipose tissue [173]. If there is a change in resistin synthesis in women with GDM, it occurs in another tissue. Reports have shown no change [34] as well as a decrease [33] in circulating resistin in preeclamptic women versus healthy-pregnant women regardless of pre-pregnancy BMI. Finally, decreased maternal and cord blood resistin concentrations have been reported in macrosomia [117].
C. Visfatin

Visfatin is another inflammatory adipokine that is involved in glucose homeostasis. Originally called pre-B-cell colony-enhancing factor and identical to the protein nicotinamide phosphoribosyltransferase (Nampt), visfatin’s role is still controversial. Visfatin has been described as insulinomimetic, possibly through its connection to nicotinamide adenine dinucleotide (NAD) biosynthesis and stimulation of glucose uptake [144]. Insulin also induces visfatin protein expression in human subcutaneous adipocytes [181]. Circulating visfatin is not related to measures of insulin sensitivity in subjects having a wide spectrum of insulin sensitivity [141]; however, increased circulating visfatin has been observed in diabetic patients [95] and diabetic patients given rosiglitazone have reduced visfatin levels [181]. Abdominal subcutaneous and visceral adipose depots have similar visfatin mRNA expression and visfatin is correlated with BMI and body fat percentage [141]. Visfatin is an immunomodulator and has been shown in vitro to induce the secretion of IL-1β, TNF-α, and IL-6 in circulating CD14+ monocytes [26].

In pregnancy, visfatin may be involved in insulin resistance. Placental chorionic tissue expresses visfatin [31] and the exposure of pregnancy hormones estradiol, progesterone, and estriol to 3T3-L1 adipocytes increases visfatin gene expression [182]. Human amnion expresses visfatin and induces VEGF to increase amnion permeability [138], important in amniotic fluid control. Circulating visfatin increases throughout pregnancy and may play a role in parturition via the NF-κβ pathway [183]. Visfatin gene expression is greater in the visceral adipose of pregnant women compared to normal-weight non-pregnant women [31].
Placental visfatin mRNA expression does not change in GDM patients compared to those without GDM, but circulating visfatin was lower at delivery in women with GDM [35]. Conversely, visfatin was measured numerous times in women with GDM and it steadily increased from second to third trimester and stayed elevated two weeks after delivery compared to healthy pregnant women [120]. Additionally, lower circulating visfatin has been observed in preeclamptic women in the third trimester compared to healthy pregnant women [121]. In conclusion, visfatin may have a more paracrine or autocrine role rather than systemic function as an obesity and pregnancy hormone.

D. Adiponectin

Adiponectin is known as an anti-inflammatory adipokine; lower plasma concentration is associated with insulin resistance, DM, obesity, and CVD [97, 98]. Adiponectin is synthesized in adipocytes [92] and two forms have been identified, full-length [184] and globular [185]. Though both forms stimulate glucose uptake in myocytes [185], globular adiponectin is found in very low concentration in circulation [145]. Full-length adiponectin is composed of a basic trimer unit and multiple trimers bind together via disulfide bonds to form low, medium, and high molecular weight (LMW, MMW, and HMW) adiponectin [186]. HMW adiponectin is the most active form with respect to insulin sensitivity. DM is associated with a lower concentration of circulating HMW adiponectin and levels increase with treatment of the anti-diabetic thiazolidinediones [186]. Adiponectin-deficient mice have impaired insulin sensitivity compared to wild type mice [145], while overexpression of adiponectin in mice enhances energy expenditure and reduces adipocyte differentiation [187]. The two adiponectin receptors,
AdipoR1 and AdipoR2 are proposed to aid in adiponectin-modulated glucose and lipid metabolism [188]. AdipoR1 is expressed ubiquitously whereas AdipoR2 is expressed primarily in heart and liver [189]. Adipose tissue has both receptors but AdipoR1 expression is 10-fold higher than AdipoR2 [190]. BMI is inversely related to AdipoR1 and weight loss increases AdipoR1 expression [190].

As an immunomodulator, adiponectin induces IL-10 and IL-1RA secretion in monocytes and dendritic cells while inhibiting interferon gamma (IFN)-γ in macrophages [146]. Adiponectin inhibits mRNA expression of T-lymphocyte chemokine (C-X-C motif) ligand (CXCL) 10, CXCL9, and CXCL11 in lipopolysaccharide (LPS)-stimulated macrophages [27]. Additionally, inflammation results in lower adiponectin in adipocytes. TNF-α [191], IL-6 [192], and CRP [193] inhibit adiponectin synthesis in murine and human adipocytes in vitro while adiponectin increased IL-1β and IL-8 expression in term placental trophoblasts [194]. These site-specific actions of adiponectin suggest a complex role in both obesity and pregnancy.

Adiponectin is proposed to regulate fetal growth. It has been reported that the placenta produces adiponectin [158, 173, 195], however other studies have not been able to confirm this [196, 197]. Like adipose tissue, the term placenta has both types of adiponectin receptor [196]. The two forms of adiponectin perform different actions in the placenta. Full-length adiponectin prevents insulin-stimulated amino acid transport while globular adiponectin stimulates insulin-independent trophoblast amino acid transport [139]. Fetal adiponectin expression starts at 14 weeks gestation and is found in numerous tissues such as adipocytes, and skeletal and smooth muscle [197]. By delivery, the concentration of cord blood adiponectin is 3-fold greater than that
of maternal adiponectin [197]. Catalano et al. [30] followed ten women with a normal-weight BMI throughout their pregnancy and reported a 25% increase in body fat by the third trimester which was assumed to cause the observed 2-fold decrease in adipocyte mRNA expression and subsequent decrease in circulating adiponectin and insulin sensitivity.

Pregnant women with greater adiposity do not have significantly different circulating adiponectin concentrations but do have lower umbilical cord blood concentration compared to pregnant women with lower adiposity [32]. There are conflicting reports on maternal adiponectin in preeclampsia. While obese women with preeclampsia have lower circulating adiponectin than normal-weight women with preeclampsia [34], another report indicates that circulating adiponectin is unchanged in women with pregnancy-induced hypertension regardless of body fat percentage [122]. Women with GDM have lower circulating adiponectin [33] as do macrosomic neonates born to mothers with GDM compared to women and neonates from pregnancies without GDM [111]. Additionally, lower adiponectin is observed in both the umbilical cord and maternal circulation in cases of macrosomia without other pregnancy complications [198].

**E. Conclusion**

Adipokines aid in metabolism by promoting satiety and regulating glucose homeostasis. Abnormal levels in obese humans have been reported which may contribute to metabolic dysfunction. The four adipokines discussed here are believed to play a role in pregnancy and pregnancy complications. Hyperleptinemia occurs in women with preeclampsia and GDM independent of BMI status and women with GDM have leptin metabolism dysregulation in peripheral tissue. Thus, factors other than adipose’s production of leptin may play a larger role
in leptin-related pregnancy complications. Visfatin and resistin are implicated in obesity-induced insulin resistance and are potentially needed for pregnancy-induced insulin resistance. Because the relationships of visfatin and resistin in maternal circulation and pregnancy complications are inconclusive, it remains to be determined what their roles are in pregnancy and pregnancy complications. Adiponectin is involved in insulin regulation and decreases in the state of excess adiposity. While a maternal decrease may be needed to promote pregnancy-induced insulin resistance, greater reductions are seen in women with preeclampsia and GDM. Lastly, most studies use pre-pregnancy BMI to calculate excess adiposity. Very few studies have assessed the relationship of abdominal adiposity to these adipokines so what relationship these adipokines have to the more metabolically active abdominal fat is an answer needed in pregnancy research. These four adipokines are also immunomodulators and it is unknown how they affect the immune system in pregnancy. Their relationship to pregnancy and immunity may provide insight to understand the impact of excessive adiposity and inflammation in pregnant women.

V. Immune Response to Obesity and Pregnancy

A. Immune Response to Obesity

A defining characteristic of obesity is low-grade, chronic inflammation. It is implicated in metabolic syndrome, which is a cluster of cardiometabolic risk markers, insulin resistance, and obesity [47]. People with obesity-induced insulin resistance have elevated circulating TNF-α and IL-6 [36], which are predictors for macrovascular complications in individuals with DM [38]. Obese individuals also have greater levels of circulating CRP [37] which is associated with
atherosclerosis [39]. This inflammation is created and maintained primarily in the expanding adipose. As adipocytes enlarge, there is increased IL-6 [199], TNF-α [147], and chemokine (C-C motif) ligand 2 [(CCL2); also known as monocyte chemotactic protein (MCP)-1] [200] in adipose tissue. This is the systemic result from the accumulation of adipose tissue macrophages [159] (Figure 1.3).

Two types of adipose tissue macrophages have been discovered: M1 macrophages respond to enlarged adipocytes and secrete inflammatory cytokines such as IL-6, TNF-α, and CCL2; and M2 which is involved in tissue remodeling and clearance of necrotic adipocytes [201]. One of the primary roles of macrophages in adipose tissue is to clear the necrotic adipocytes that occur at a greater rate in expanding adiposity [202]. Additionally, obese subjects have elevated circulating CCL2 and IL-8 [203]. CCL2 also aids in tissue remodeling and clearance of necrotic adipocytes [201] and in circulation, CCL2 and IL-8 trigger adhesion of monocytes to endothelium [204], a citing factor in atherosclerosis. Adipose tissue macrophages are thought to be primarily responsible for the low-grade inflammation. However, T cells are also involved in obesity-induced inflammation.

Obesity results in an increase of CD8+ and CD4+ T cells in adipose and in circulation. It was originally thought that macrophages initiated the influx of other immune cells into adipose but Nishimura et al. [43] showed a significant increase in CD8+ T cells occurs prior to macrophage infiltration and the induction of insulin resistance in DIO mice. CD4+ T helper (Th) and regulatory T (Treg) cells also contribute to the obesity-induced inflammatory response. Th1 cells are proinflammatory and strengthen macrophage secretion of inflammatory cytokines while
Figure 1.3 Modulation of adipose tissue in obesity. Three phenotypes of adipose tissue: lean with normal metabolic function, obese with mild metabolic dysfunction, and obese with full metabolic dysfunction. Lean phenotype has relatively healthy metabolic function, exhibit low levels of immune cells, and adipocytes secrete the anti-inflammatory adipokine adiponectin. In the state of excess energy, enlarged adipocytes stimulate transition towards metabolic dysfunction. Macrophages and CD8+ T cells begin to accumulate and the CD4+ T cell subpopulations change. Secretion of proinflammatory adipokines increase and metabolic control is lost. Metabolically dysfunctional adipose tissue is characterized with higher levels of adipocyte necrosis that manifests as crown-like structures and decreased vascular function. Abbreviations: CCL2, chemokine (C-C motif) ligand 2; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha. Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology, [205], copyright 2011.
Th2 and Treg cells, in contrast, induce the anti-inflammatory M2 macrophage phenotype. It was first shown in DIO mouse models that expanding fat compartments had increased adipose tissue T cell populations compared to control groups [206, 207]. Additionally, the induction of insulin resistance in DIO is associated with an increase in visceral adipose Th1 cell mRNA expression, before the increase in macrophage F4/80+ mRNA expression (while Th2 and Treg cells decrease) [208].

In humans, a larger waist circumference correlates with a greater adipose CD4+ T cell population in individuals with DM [209]. The CD4+ T cell subpopulations in obese subjects with DM are inflammatory compared to insulin-sensitive lean and obese subjects [210]. In the DIO mouse model, insulin resistance results from high fat diet study regimen; in humans, insulin resistance does not always occur with obesity. Therefore, it appears that there may be a difference in adipose inflammation in those with and without insulin resistance.

In obese humans with normoglycemic status, CD4+ T cell populations increase in adipose tissue [44] and in the circulation of obese subjects [45, 46]. Indeed, BMI positively correlates with peripheral CD4+ and CD8+ T cell populations [46]. However, when the CD4+ T cell subpopulations are measured, it appears there may be a dampening effect in the T cell response to obesity. van der Weerd et al. [45] showed that circulating Th2 and Treg cell populations are responsible for the elevated CD4+ T cell population in normoglycemic, morbidly obese adults while Th1 and Th17 populations are not different comparable to lean subjects. In adipose tissue, elevated CD3+ T cell populations are seen in both abdominal visceral and subcutaneous fat depots of obese subjects compared to lean controls, but the proportion of CD8+ T cells and Th1...
are unchanged while Th2 and Treg cells increase [44]. These studies indicate that in obesity not accompanied by insulin resistance, T cells respond to expanding adiposity with increased anti-inflammatory Th2 and Treg cell populations.

B. Physiology of Obese Pregnancy

The biological mechanisms responsible for pregnancy complications in obese women are not well characterized although there is a strong link to the increased inflammation that occurs in obesity [211]. The potential effect of obesity-derived inflammation in pregnancy interfaces with the dynamic immune system response to pregnancy, which goes through three phases during healthy pregnancy (reviewed by Mor et al. [212]). During the first and early in the second trimester period, pregnancy resembles an open wound that requires a proinflammatory response. Implantation and the creation of the placenta cause damage to the endometrial tissue and an inflammatory response is required to repair the uterine epithelium and remove cellular debris. A high level of proinflammatory CD4\(^+\)Th1 cells, uterine-specific natural killer cells, macrophages, and dendritic cells regulate trophoblast invasion and help promote angiogenesis for placenta development [212].

The second immunological period of pregnancy is an anti-inflammatory state and a time of rapid growth and development where the mother, placenta, and fetus are in symbiosis. Described as the “Th2 shift”, Th2 cells suppress Th1 cells to promote pregnancy [213]. The final phase of pregnancy, a build-up to parturition, is achieved with renewed inflammation. The hormone relaxin causes an influx of macrophages and neutrophils into the myometrium initiating labor, while MMPs and prostaglandins rupture the membrane and facilitate myometrial
contractility for delivery. Adaptive immunity also plays a part in parturition. Memory T cells expressing CXCL8, CXCL10, IL-1β, TNF-α, and MMP-9 are present in the choriodecidua in spontaneous labor versus planned cesarean delivery [214].

Changes to the placental environment have been shown in obese pregnant women compared to lean, pregnant counterparts. Obese women have: 1) increased placental IL-1β, IL-8, CCL2, and CXCR2 mRNA expression, 2) increased neutrophils in the interstitial space, and 3) greater muscularity of the placental vessel walls compared to healthy-weight pregnant women [41]. In addition, there is a greater number of CD14+/CD68+ macrophages and increased expression of IL-1β, TNF-α, and IL-6 in the placenta of obese compared to normal weight pregnant women [40]. Oxidative and nitrative stress has been found in placentas of obese women without pregnancy complications [215], an environment that is typically found in women with preeclampsia [216, 217]. Preeclampsia is characterized by endothelial dysfunction, and obese pregnant women have decreased endothelium-dependent and independent vasodilation compared to non-obese counterparts [218]. An altered placental environment and adverse delivery outcomes in obese pregnant women have led to the hypothesis that fetal exposure in utero to altered maternal metabolism and inflammation could lead to a long-term risk of obesity [14, 16], insulin dysregulation [16, 17], and metabolic syndrome [18], reduced childhood cognition [19], and DNA hypomethylation of inflammation-related genes [15].

In light of the placental changes that have been seen in pregnant obese women and obese pregnancy models, the changes to the immune system in pregnancy add an additional nuance to understanding the effect of obesity in pregnancy. Circulating immunity has been measured to
understand immunologic adaptation to pregnancy and potential disease and infection susceptibility. The peripheral CD4\(^+\) T cell population decreases during the first and second trimester and returns to pre-pregnancy levels by the third trimester [219]. Also, third trimester peripheral blood mononuclear (PBMC) cytokine expression exhibits a dampened inflammatory response, characterized by decreased IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\), and IL-6 and increased IL-10 compared to the previous two trimesters [220]. These pregnancy-induced immunologic changes add a facet not seen in measuring obesity-related effects in non-pregnancy populations. Considering this provides insight as to when and how researchers should assess adiposity-induced inflammation in pregnancy.

Given the immune changes that occur during pregnancy, differences in circulating inflammation have been reported between obese and lean pregnant women. In accordance with the immune changes that occur during pregnancy, differences in circulating CRP, IL-6, and CCL2 observed in the first and second trimesters in pregnant women categorized by their pre-pregnancy BMI disappear by the third trimester [42]. However, increased circulating IL-6 and CRP at delivery have been reported in numerous studies in obese compared to lean pregnant women [40, 218, 221]. In addition, peripheral monocyte differentiation and maturation markers CD14\(^+\) and C68\(^+\) are greater in obese pregnant women than lean pregnant women [40]. In the adipose SVF, inflammation characterized by accumulation of CD68\(^+\) macrophages and increased gene expression of IL-6, TNF-\(\alpha\), IL-8, and CCL2 are 2-fold higher in obese pregnant women compared to lean pregnant women [221].
In pregnancy complications, relationships to obesity-induced inflammation are not as apparent. Higher levels of IL-6 have been reported in women with GDM compared to healthy pregnancies, independent of pre-pregnancy BMI status [118, 222]. In preeclampsia, pre-pregnancy BMI status also does not appear to influence circulating cytokines. Preeclamptic women have higher levels of circulating IL-6, IL-8, and CCL2 compared to healthy pregnant women, independent of pre-pregnancy BMI status [115]. Amongst obese and lean preeclamptic women with higher plasma TNF-α than healthy pregnant women at delivery, pre-pregnancy BMI did not correlate with TNF-α [223]. Finally, a panel of plasma cytokines measured at 30-33 weeks gestation did not reveal any difference between women with and without preeclampsia when they had a similar BMI [224]. Similar to adipokines’ relationship to pregnancy complications, simply assessing pre-pregnancy BMI and circulating cytokines cannot predict a woman’s risk for pregnancy complications. Other factors must be taken into account such as age, genetic predisposition and potentially, the presence of the metabolically-active abdominal adiposity.

VI. Conclusions

Half of women of childbearing age in the United States are categorized as overweight or obese and their risk of pregnancy and delivery complications are greater than normal-weight pregnant women. These complications affect the offspring as well as an increased risk of long-term maternal health complications. Obesity in most research is defined by BMI, a measurement that does not directly measure body fat nor consider the location of body fat. Abdominal
adiposity is associated with obesity-induced metabolic disease so it is reasonable to suggest that abdominal adiposity is influential in obese pregnancy, yet abdominal obesity in pregnancy is not well defined. Measurement of abdominal obesity, particularly distinguishing between subcutaneous and visceral fat mass could lead to determining if abdominal adiposity promotes complications in obese pregnancy and help identify women and infants most likely to benefit from interventions before and during pregnancy.

Pregnancy-induced changes to the immune system create a dynamic situation with the low-grade inflammation present from pregravid obesity. Adipokines and the immune system work together in healthy pregnancy to promote insulin resistance needed for fetal development. In obese pregnancy, an abnormal increase in proinflammatory adipokines may be part of the etiology of pregnancy complications while it is unknown what the nature of circulating CD4$^+$ T cells in relationship to body fat compartments. Excess adiposity in pregnancy is associated with a suboptimal uterine environment that is hypothesized to influence fetal development and play a role in the development of childhood obesity and subsequent metabolic syndrome in the mother. Knowledge of the role of adipose tissue-generated inflammation and its link to metabolic complications will provide insight into the inflammation cascade and opportunities for therapeutic potential.

VII. Significance of Present Work

A large body of evidence demonstrates that obese pregnant women have a greater risk of obesity-related pregnancy and delivery complications and poorer health outcomes compared with
normal weight pregnant women. Because 40% of US pregnancies are unplanned and half the childbearing-women in the United States are categorized as overweight or obese, proposing weight reduction strategies before conception may have little impact. In contrast, a better understanding of the inflammatory process during obese pregnancy may allow implementation of a pharmaceutical or dietary intervention strategy to ameliorate inflammation and obesity-related pregnancy complications. As will be presented, understanding the etiology of obesity-induced inflammation during pregnancy could help these women and their medical providers to take appropriate measures to prevent or mitigate inflammation-induced complications and potentially minimize long-term health consequences to the child. Furthermore, pregnant women with abdominal obesity, but without medical complications should be aware of the impact this fat and its inflammatory components have on both them and their offspring’s health.

The outcomes of this current study may highlight the need for more research in pregnancy targeting the role of fat mass in adverse pregnancy outcomes. Ultimately, this research could change the way obstetricians and pregnant women see the impact of excess adiposity in pregnancy compared to reliance on BMI categories. For example, the adipokines leptin, adiponectin, visfatin, and resistin are implicated in obesity-induced inflammation. Because the placenta utilizes these adipokines, assessing their relationship in obese pregnancy may provide insight into pregnancy and delivery complications associated with obesity. Understanding the adipokines’ relationship to abdominal adiposity in regards to adverse pregnancy outcomes may provide obstetricians with greater understanding of their obese patients’ health and their potential pregnancy risk factors.

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The results of this study may also influence the manner in which other researchers perform their studies on the impact of adiposity in pregnancy. For example, the majority of research focuses on innate immunity. Addressing the role adaptive CD4\(^+\) T cells have on systemic inflammation in pregnant women may help answer some of the larger issues of obesity-induced inflammation. Determining CD4\(^+\) T cells’ role in relationship to abdominal adiposity will contribute to the growing body of literature on the short- and long-term effects of obesity in pregnancy.

VIII. Specific Aims and Statement of Hypotheses

The main purpose of this work is to examine the role of abdominal adiposity on circulating adipokines and the CD4\(^+\) T cell cytokines in obese pregnancy. The majority of obese pregnancy research relies on pre-pregnancy BMI classification and growing evidence indicates excess abdominal adiposity may have a greater physiologic impact. Assessing the relationship between abdominal adiposity, adipokine concentrations, and adaptive immune response may help provide significant insight into obesity-related pregnancy complications. Additionally, assessment of inflammation’s impact in obese pregnancy has occurred primarily in the macrophages. It is established that obese pregnant women have higher circulation of IL-6 and CRP. Understanding how CD4\(^+\) T cells respond to excess adiposity in healthy pregnant women will further our understanding of short- and long-term effects to both mother and offspring.

**Specific Aim 1: To quantify abdominal visceral and subcutaneous fat mass in pregnancy.**

Because the majority of obese pregnancy research uses pre-pregnancy BMI to identify
obesity, we hypothesized that there would be a greater impact on inflammatory status from abdominal fat mass independent of a woman’s BMI. Our working hypothesis is that BMI is a poor indicator of abdominal adiposity in overweight/obese pregnant women. To achieve this aim, we quantified the amount of subcutaneous and visceral adipose tissue in the abdomen by MRI from 20 women (9 normal-weight pre-pregnancy BMI, 11 overweight and obese pre-pregnancy BMI) within 3-weeks postpartum. Surprisingly, we found women considered overweight or obese by BMI had significantly greater amounts of abdominal subcutaneous and visceral fat mass compared to normal-weight BMI women, which correlated to their significantly larger amount of total body fat measured by DXA. However, there was a large amount of variation within the two groups’ visceral adiposity indicating genetics and environment play a large role in accumulation of ectopic visceral fat mass.

**Specific Aim 2: To determine the relationship between abdominal subcutaneous and visceral adipose tissues and circulating adipokines in pregnancy.**

Because the majority of obesity research uses BMI classification to identify obesity yet abdominal fat mass is proposed to have a greater impact on health, we hypothesized that measuring circulating adipokines would provide better insight into abdominal adiposity’s contribution. Our working hypothesis is that increased abdominal fat mass has a strong relationship with circulating adipokines in pregnant women. To achieve this aim, we measured plasma levels of leptin, resistin, visfatin, and adiponectin in the third trimester and compared the results to abdominal adiposity measured in Specific Aim #1. Contrary to this hypothesis, we found no relationship between subcutaneous abdominal fat mass and adipokine concentrations. Similarly, we observed no correlation between visceral abdominal fat mass and leptin, visfatin,
and adiponectin. However, we did find a strong relationship between visceral abdominal adiposity and resistin. Leptin also correlated with total body fat. As expected, there were no differences between the BMI categories in regards to the adipokines except for a 2-fold increase of leptin in overweight/obese pregnant women compared to normal-weight pregnant women.

**Specific Aim 3: To determine the peripheral CD4\(^+\) T cell cytokines to abdominal adiposity in pregnancy.**

The majority of obese pregnancy research focuses on macrophage-derived inflammation. We hypothesized that the CD4\(^+\) T cell population plays a role in obesity-induced inflammation in pregnancy. Our working hypothesis is that excess fat mass increased the inflammatory CD4\(^+\) T cell cytokine profile. To achieve this aim, we measured peripheral CD4\(^+\) T cell cytokines from third trimester PBMCs. We observed no difference between the BMI categories and in relationship to abdominal subcutaneous fat mass or total body fat mass (with one exception). Surprisingly, we observed a significant decrease in CD4\(^+\) T cell cytokine profile in relationship to abdominal visceral fat mass. This dampening effect was observed throughout all CD4\(^+\) T cell cytokine categories.

Two manuscripts based on the work presented in this dissertation will be submitted for publication. The first manuscript was based on Specific Aims 1 and 2, looking at the relationships between body composition and adipokines (Chapter 2; was submitted to the *Metabolism*). The second manuscript focused on the relationship between body composition and circulating CD4\(^+\) T cell-expressed cytokines of Specific Aim 3 (Chapter 3; to be submitted to *Metabolism*).
CHAPTER 2

LEPTIN AND RESISTIN ARE RELIABLE CIRCULATING BIOMARKERS FOR ADIPOSITY IN PREGNANCY
I. Abstract

Objective: Circulating adipokines are associated with physiological and pathophysiological processes in both obesity and pregnancy. Obesity in pregnancy increases the risk of pregnancy complications for both mother and infant but the majority of research uses BMI to assess fatness. Specific fat compartments are associated with obesity-induced health risks yet it is not known how abdominal fat mass in pregnancy is related to circulating adipokines. The purpose of this study was to determine the relationship between total and abdominal body fat and adipokines in normal weight compared to overweight or obese pregnant woman.

Materials/Methods: Plasma leptin, resistin, visfatin, and adiponectin were measured by ELISA in healthy pregnant women of normal weight (BMI 18.5 – 24.9; n = 17) and overweight/obese (BMI 25.0 – 40, n = 21) in the third trimester. Total body and abdominal subcutaneous and visceral fat mass were measured at 1-3 weeks postpartum by DXA and MRI, respectively.

Results: Women with a pre-pregnancy overweight/obese BMI compared to normal-weight BMI had greater total body fat mass (39.0 ± 8.5 vs. 22.8 ± 4.6 kg, P < 0.001), abdominal subcutaneous fat mass (1502.8 ± 507.6 vs. 685.3 ± 179.9 g, P < 0.001) and abdominal visceral fat mass (214.2 ± 159.9 vs. 94.2 ± 33.8 g, P = 0.024). They also had higher plasma leptin (66.3 ± 34.2 vs. 35.7 ± 19.3 ng/mL, P < 0.001). Leptin was associated with total body fat mass (r = 0.782, P < 0.001). After controlling for total body fat mass, resistin was associated with abdominal visceral fat mass (r = 0.569, P = 0.009). No significant correlations were observed between adiponectin or visfatin and any measure of body composition.

Conclusions: In pregnant women, leptin predicts total body fat mass while resistin is a biomarker for visceral fat mass, an ectopic fat compartment.
II. Introduction

Women of childbearing age in the United States have an obesity rate of 29% [1]. Pregravid obesity increases the risk of maternal preeclampsia, GDM, cesarean delivery [3-5], postpartum infection, hemorrhage during delivery [5], and postpartum weight retention [6]. Additionally, the offspring are at greater risk of preterm birth [5], congenital abnormalities [7], macrosomia [3-5], and admission to neonatal intensive care units [8]. A low-grade inflammation occurs in obesity and the combination of obesity and pregnancy may be a factor in these medical risks. Excess body fat is associated with altered levels of adipokines such as leptin, adiponectin, resistin, and visfatin, which function as important regulators of appetite, glucose homeostasis [20-23], and immune function [24-27]. Differences in circulating adipokine concentrations have been shown in pregnant women who are obese compared to normal weight [30-32] and it has been suggested that these adipokines play a role in pregnancy complications [33-35].

Obesity is generally determined by BMI, [body weight (kg) divided by height (m^2)]. However, it is known that individuals with the same BMI can have large differences in body fat – both in amount and anatomical location. The amount and anatomical location of fat mass may be the essential factor related to health risks of obesity because abdominal fat is highly inflammatory [49, 50]. Abdominal fat includes both subcutaneous and visceral fat and can be quantified individually by MRI. Additionally, there is a redistribution of lower-body adiposity toward more central, upper-body adiposity during pregnancy, proposed to increase fetal access to stored energy [68], potentially increasing a woman’s abdominal adipose stores. The aim of the current study was to explore the relationship among circulating adipokines to total fat mass and
abdominal fat mass in pregnant woman entering pregnancy with a normal weight or overweight/obese pre-pregnancy BMI.

III. Materials and Methods

Subjects

Women from the Kansas City metropolitan area were enrolled between February 2012 and March 2013 as part of the Pregnancy Health Study. Women were eligible if they were English-speaking, 18-44 years old, with a singleton pregnancy, > 28 gestational weeks, planning to delivery at the University of Kansas Hospital, and had a pre-pregnancy BMI between 18.5 – 40 kg/m² based on self-reported weight and height. Exclusion criteria included diabetes, hypertension, smoking or illegal drug use during pregnancy, chronic medical condition known to influence inflammation status, or fear of enclosed spaces. Gestational weight gain was determined by subtracting self-reported pre-pregnancy weight from the highest weight recorded before delivery in the subjects’ medical record. This study was approved by the University of Kansas Medical Center Human Subjects Committee (#12793), and the research protocol and informed consent process conducted according to the Declaration of Helsinki as amended in October 1996.

Adipokines

Blood samples were obtained from women between 35 and 39 weeks gestation (n = 36) or the morning following birth (n = 2) between 10 a.m. and 4 p.m. to avoid the circadian influence of circulating leptin [225]. Blood was collected in an EDTA-coated tube and plasma
separated from red blood cells and buffy coat (3000 x g, 10 min, 4°C). Women were asked to consume a light meal before their visit to prevent dietary influence on adipokines [226]. Plasma concentrations of leptin, resistin, and total and HMW adiponectin were determined by ELISA (AlpcO Immunoassays, Salem, NH). The lower limits of detection were 0.50, 0.012 ng/mL, and 0.019 µg/mL for leptin, resistin, and adiponectin, respectively, and the inter-assay coefficient of variance (CV) was 1.9%, 2.4%, and 1.7%, respectively. Visfatin was measured by ELISA (Phoenix Pharmaceuticals Inc, Burlingame, CA) with a lower limit of detection of 1.85 ng/mL and an inter-assay CV of 8.7%. All samples were assayed in duplicate.

**Body Fat Mass**

Total body fat mass was measured between 1-3 weeks postpartum by dual-energy x-ray absorptiometry (iDXA, GE Healthcare, Madison, WI) with encore software (Version 13.50). Subjects removed all metal artifacts from their body and were scanned according to standard imaging and positioning protocols. Half of the women were selected for measurement of visceral and subcutaneous abdominal fat mass determined by MRI at 3 Tesla (Skyra, Siemens Medical Solutions, Erlangen, Germany) [227] at the same visit. To measure abdominal fat mass, we obtained T2-weighted spin echo localizer axial, coronal, and sagittal images while subjects lay on the table in a supine position with arms placed beside their head. Sagittal images were used to identify lumbar vertebrae 2 (L2) and sacral vertebrae 3 (S3) as the landmarks for abdominal fat mass. Blocks of high-resolution axial HASTE TSE T2-weighted images were acquired during two 16-second breath-holds to minimize breathing motion artifacts. Each acquisition block was comprised of ~20 axial image slices (5 mm thick, 5 mm gaps). Anatomical locations of the two blocks were noted in order to find the consecutive images for analysis and prevent duplication.
Segmentation and quantification of the axial images were performed with ImageJ [228, 229]. Briefly, a mask was applied to enhance edge definition and adipose and non-adipose tissues were segmented by k-means clustering using custom-designed ImageJ scripts. Subcutaneous and visceral adipose tissues were delineated within each slice and each segmented image was reviewed to correct misclassified pixels caused by blurring of tissue space. Subcutaneous and visceral adipose tissue areas for each image were calculated by summing the highlighted pixels and multiplying by the pixel surface area. The volumes (cm$^3$) were calculated by multiplying the tissue area (cm$^2$) by the slice thickness (5 mm). The adipose tissue volume in the 5.0 mm gap was estimated by averaging the results from the adjacent slices. Finally, the volumes were multiplied by 0.916 g/cm$^3$, the density of adipose tissue, to obtain total mass (g) of the visceral and subcutaneous adipose tissue. All scans were read by a single observer. The intra-rater reliability coefficients of variation for subcutaneous and visceral adipose tissue mass measurements were 0.3% and 5.0%, respectively, based on two repeated, blinded, analyses of five randomly-selected sets of MRI images by a single observer.

**Statistical Analyses**

Descriptive statistics are presented as mean values ± standard deviation. Linear relationships between body composition and circulating markers were described with Pearson correlation coefficients. Differences between the normal-weight and overweight/obese BMI categories were analyzed with one-way ANOVA. Each abdominal fat compartment (subcutaneous and visceral) was normalized to total body fat mass. Normalizing the abdominal fat compartments to total fat mass reduced variability caused by overall fatness and allowed for assessment of each abdominal adipose tissue compartment to each adipokine. The Shapiro-Wilk
test was used to test normality. Outcome variables that were not normally distributed (leptin, resistin, adiponectin, visceral adipose, subcutaneous adipose) were log-transformed prior to analyses. All data were analyzed with IBM SPSS Statistics 17 software (SPSS, Chicago, IL). P-values presented are two-tailed and statistical significance was set at 0.05.

IV. Results

The women in each group were of similar age, parity status, gestational time and weight gain (Table 2.1). Overweight/obese subjects had a 1.7-fold higher total body fat mass compared to normal-weight subjects \((F = 38.566, P < 0.001)\) and 2-fold greater abdominal subcutaneous and visceral fat mass \((F = 28.555, P < 0.001 \text{ and } F = 6.073, P = 0.024, \text{ respectively})\). Although abdominal visceral fat mass was different between the two BMI categories, there was a large amount of variability and the median visceral fat mass of the overweight/obese and normal weight groups were similar \((134 \text{ vs. } 87 \text{ g})\). The overweight/obese group had a 1.8-fold greater concentration of plasma leptin compared to normal-weight subjects \((F = 13.22, P = 0.001)\) and leptin was the only measured adipokine that differed significantly between groups.

Bivariate correlation analysis showed that leptin correlated positively with BMI and total body fat mass \((P = 0.001 \text{ and } P < 0.001, \text{ respectively})\) (Table 2.2). Additionally, leptin correlated positively with both abdominal subcutaneous and visceral fat mass \((P < 0.001 \text{ and } P = 0.006, \text{ respectively})\). No other adipokines correlated with total body fat mass of measured compartments.

However, when subcutaneous and visceral fat were normalized to total body fat mass to assess the relationship between each fat compartment and the adipokines, resistin correlated
positively with visceral fat mass \((P = 0.009)\) (Table 2.3). Additionally, the correlations between leptin and abdominal subcutaneous and visceral fat mass disappeared \((P = 0.128\) and \(P = 0.249,\) respectively). Finally, neither adiponectin nor visfatin correlated with any measure of fat mass.

V. Discussion

In this observational study, we found normal weight and overweight/obese pregnant women had significantly different abdominal fat mass. The most interesting, and potentially important finding in the study from a metabolic perspective was that resistin predicted abdominal visceral adiposity.

Resistin is primarily synthesized and secreted by macrophages in the stromal vascular fraction of adipose tissue [92] and, during pregnancy, resistin is produced by the placenta [155]. Resistin is generally thought to play a role in insulin resistance [89] and during pregnancy, resistin is hypothesized to contribute to the pregnancy-induced insulin resistance needed for fetal development [180]. Fat biopsies have shown greater resistin mRNA expression in both abdominal adipose compartments compared to thigh and breast adipose in adults. Previous studies found a strong association between resistin and total body fat mass [177, 178, 230] and abdominal subcutaneous fat mass [178] in healthy non-pregnant adults. However, because these studies were performed on non-pregnant women, comparisons between studies are difficult. To our knowledge, our study is the first study to measure both plasma resistin and fat mass in pregnancy.

Three studies have found a relationship between resistin and total body fat mass in non-pregnant populations. Yannakoulia et al. [177] and Vozarova de Courten et al. [230] measured
total body fat mass in adult men and women with bioelectrical impedance analysis (BIA) and DXA, respectively. In addition to a correlation between resistin and total body fat mass, Won et al. [178] found a relationship between resistin and abdominal subcutaneous fat mass but not visceral fat mass measured by computed tomography (CT) in adult men and women. The increase in resistin that occurs in pregnancy may affect its relationship to body composition compared to studies performed in a non-pregnant population. Additionally, our study quantified MRI images from the entire abdominal area (L2 to S3) whereas single-image protocols localized to L4-L5 are employed in CT prediction of total abdominal adiposity and are comparable only to MRI single image scan prediction models [73].

Our finding of correlation between circulating leptin and total body fat measured by DXA confirms previous findings that used estimates of total body fat. The function of leptin is to regulate appetite and body weight [101]. Leptin is secreted primarily from adipocytes [92] and abdominal subcutaneous adipocytes exhibit greater leptin mRNA expression than visceral adipocytes [108]. Leptin is also synthesized in the placenta [151] and in vitro perfusion of term placenta releases 98% of synthesized leptin into maternal circulation [165]. Maternal circulation of leptin increases by the end of the first trimester, before the increase in weight gain from adipose [166]. It is hypothesized that maternal circulation of leptin is important for mobilization of fat deposits to ensure energy availability for the fetus [85]. Because measurement of plasma leptin in this study occurred within a narrow gestational timeframe (35-39 weeks), we do not believe differences in placental leptin production were responsible for the differences in leptin concentration seen between normal weight and overweight/obese pregnant women.
We used DXA, a validated procedure for measuring total fat mass. Others have reported an association between leptin in the first and third trimester and whole-body fat mass in pregnancy by BIA [32, 171]. While it has been validated as a reliable estimation for body fat percentage outside of pregnancy [61], BIA has not been validated to measure body fat mass during pregnancy.

The strengths of this study include the detailed collection of subject information, the variety of circulating adipokines measured and the variety of body adiposity measurements. The wealth of data allowed us to investigate numerous relationships between subject characteristics, adipokines, and body composition. A limitation was that all of our subjects had generally healthy pregnancies. Future studies should include assessing the relationship of abdominal adiposity and adipokines in women with pregnancy complications.

In conclusion, the current study provides significant evidence for leptin as a reliable biomarker for whole body fat mass. Resistin was an indicator of abdominal visceral fat mass after controlling for the effect of total body fat mass. At least during pregnancy, resistin appears to be a useful clinical marker of visceral fat mass. In contrast to leptin and resistin, visfatin and adiponectin were not associated with adipose tissue mass although they may be important in the pathogenesis of obesity-related pregnancy complications.

Acknowledgments

The authors wish to acknowledge the nursing staffs at our participating clinics and hospital whose support was essential to recruitment and availability of blood samples; Hoglund Brain Imaging Center for the creation of the MRI adipose protocol and financial support; Kansas
Intellectual and Developmental Disabilities Research Center for adipokine analysis; and our subjects for their time.
<table>
<thead>
<tr>
<th></th>
<th>Normal(^a)</th>
<th>Range</th>
<th>Overweight/obese</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>28 ± 6(^b)</td>
<td>19 - 42</td>
<td>29 ± 5</td>
<td>21 - 37</td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>39 ± 1</td>
<td>37 - 41</td>
<td>39 ± 1</td>
<td>37 - 41</td>
</tr>
<tr>
<td>Nulliparity (n)</td>
<td>8 (47%)</td>
<td></td>
<td>8 (38%)</td>
<td></td>
</tr>
<tr>
<td><strong>Body composition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m(^2))</td>
<td>21.5 ± 1.4</td>
<td>18.9 – 23.6</td>
<td>31.7 ± 3.8</td>
<td>25.1 – 38.6</td>
</tr>
<tr>
<td>Total body fat (kg)</td>
<td>22.8 ± 4.6(^c)</td>
<td>14.0 – 32.8</td>
<td>39.0 ± 8.5(^\dagger)</td>
<td>27.5 – 53.5</td>
</tr>
<tr>
<td><strong>Abdominal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>subcutaneous (g)</td>
<td>685.3 ± 179.9(^d)</td>
<td>410.9 – 935.5</td>
<td>1502.8 ± 507.7(^\dagger)</td>
<td>891.6 – 2466.4</td>
</tr>
<tr>
<td>visceral fat (g)</td>
<td>94.2 ± 33.8</td>
<td>54.4 – 167.2</td>
<td>214.2 ± 159.9(^*)</td>
<td>58.3 – 554.0</td>
</tr>
<tr>
<td>GWG (kg)</td>
<td>15.5 ± 1.3</td>
<td>6.8 – 22.3</td>
<td>14.6 ± 7.5</td>
<td>1.8 – 29.1</td>
</tr>
<tr>
<td><strong>Adipokines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>35.7 ± 19.3</td>
<td>7.4 – 76.8</td>
<td>66.3 ± 34.2(^\dagger)</td>
<td>22.1 – 139.6</td>
</tr>
<tr>
<td>Resistin (ng/mL)</td>
<td>7.6 ± 2.9</td>
<td>3.7 – 15.8</td>
<td>7.6 ± 4.3</td>
<td>1.8 – 19.4</td>
</tr>
<tr>
<td>Visfatin (ng/mL)</td>
<td>16.9 ± 8.8</td>
<td>2.2 – 33.1</td>
<td>19.0 ± 9.3</td>
<td>2.1 – 36.4</td>
</tr>
<tr>
<td>Total adiponectin (µg/mL)</td>
<td>4.9 ± 1.9</td>
<td>2.4 – 9.1</td>
<td>4.7 ± 2.2</td>
<td>1.4 – 9.1</td>
</tr>
<tr>
<td>HMW adiponectin (µg/mL)</td>
<td>2.8 ± 1.7</td>
<td>0.6 – 6.8</td>
<td>2.3 ± 1.5</td>
<td>0.2 – 4.6</td>
</tr>
</tbody>
</table>

\(^a\) n = 17 normal, n = 21 overweight/obese; \(^b\) mean ± SD, one-way ANOVA was performed between groups; \(^c\) n = 13 normal, n = 18 overweight/obese; \(^d\) n = 9 normal weight, n = 11 overweight/obese.

\(^*\) \(P \leq 0.05\); \(^\dagger\) \(P \leq 0.001\).
Table 2.2 Pearson’s correlation coefficients between adipokines and fat mass compartments

<table>
<thead>
<tr>
<th></th>
<th>Total BMI body fat mass (kg)</th>
<th>Abdominal Adiposity</th>
<th>Subcutaneous fat mass (g)</th>
<th>Visceral fat mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>0.512†</td>
<td>0.782†</td>
<td>0.765†</td>
<td>0.594*</td>
</tr>
<tr>
<td>Resistin</td>
<td>-0.049</td>
<td>-0.065</td>
<td>0.037</td>
<td>0.369</td>
</tr>
<tr>
<td>Total adiponectin</td>
<td>-0.209</td>
<td>-0.053</td>
<td>0.144</td>
<td>-0.015</td>
</tr>
<tr>
<td>HMW adiponectin</td>
<td>-0.296</td>
<td>-0.062</td>
<td>0.056</td>
<td>0.078</td>
</tr>
<tr>
<td>Visfatin</td>
<td>0.085</td>
<td>0.271</td>
<td>0.127</td>
<td>0.275</td>
</tr>
</tbody>
</table>

*P ≤ 0.01; † P ≤ 0.001.

Table 2.3 Pearson’s correlation coefficients between adipokines and abdominal fat compartments relative to total body fat mass

<table>
<thead>
<tr>
<th></th>
<th>Subcutaneous (g) / total body fat mass (kg)</th>
<th>Visceral (g) / total body fat mass (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>0.352</td>
<td>0.270</td>
</tr>
<tr>
<td>Resistin</td>
<td>0.324</td>
<td>0.569*</td>
</tr>
<tr>
<td>Total adiponectin</td>
<td>0.403</td>
<td>-0.024</td>
</tr>
<tr>
<td>HMW adiponectin</td>
<td>0.245</td>
<td>0.142</td>
</tr>
<tr>
<td>Visfatin</td>
<td>-0.113</td>
<td>0.181</td>
</tr>
</tbody>
</table>

* P = 0.009.
CHAPTER 3

ABDOMINAL VISCERAL ADIPOSITY INFLUENCES CD4$^+$ T CELL CYTOKINE PRODUCTION IN PREGNANCY
I. Abstract

Objective: Women with pre-gravid obesity are at risk for pregnancy complications. While the macrophage response in obese pregnant women categorized by BMI has been documented, the relationship between the peripheral CD4+ T cell cytokine profile and body fat compartments during pregnancy is unknown.

Materials/Methods: Third trimester peripheral CD4+ T cell cytokine profiles were measured in healthy pregnant women (n = 37; pre-pregnancy BMI: 18.5 – 40). CD4+ T cells were isolated from PBMC and stimulated to examine their capacity to generate cytokines. Between 1-3 weeks postpartum, total body fat was determined by DXA and abdominal subcutaneous and visceral fat masses were determined by MRI. Pearson’s correlation and simple regression analyses were performed to assess relationships between cytokines and fat mass.

Results: Greater abdominal visceral fat mass was associated with a decrease in stimulated CD4+ T cell cytokine expression. P values ranged from 0.001 to 0.046. T helper (Th) 1-related (IFN-γ, TNF-α, TNF-β, IL-12p40, and IL-12p70), Th2-related (IL-4, IL-10, and IL-13), and Th17-related (IL-17A) cytokines were inversely related to visceral fat mass. Chemokines (CCL3, IL-8, and CXCL10) and growth factors (FGF-2, G-CSF, GM-CSF, FLT-3L) were also inversely correlated. Additionally, total body fat mass was inversely correlated with FGF-2 while abdominal subcutaneous fat mass and BMI were unrelated to any CD4+ T cell cytokine.

Conclusion: The general lower responsiveness of CD4+ T cell cytokines associated with abdominal visceral fat mass is a novel finding in pregnant women, potentially revealing visceral adiposity as immunosuppressive, at least in pregnancy.
II. Introduction

Women with pregravid obesity are at a greater risk for pregnancy-induced hypertension, preeclampsia, and gestational diabetes mellitus [4, 5]. The biological mechanisms responsible for these clinical outcomes are not well characterized, although there is a strong link to the increased inflammation that occurs in obesity [211]. Obese pregnant women categorized by their pre-pregnancy BMI have greater circulating CRP and IL-6 accompanied by both adipose and placental innate inflammation at delivery compared to healthy weight pregnant women [40, 41, 221]. Another investigation found differences in circulating CRP, IL-6, and CCL2 associated with excess adiposity in the first and second trimesters which disappeared by the third trimester [42]. While that study did measure adiposity by skinfold thickness, the majority of research on the impact of obesity in pregnancy uses pre-pregnancy BMI to categorize obesity. Abdominal adiposity is more physiologically relevant to obesity-induced health risks. Abdominal subcutaneous [47] and visceral [48] adiposity contribute to the systemic inflammation seen in obesity-induced health risks and have the potential to play a greater role than whole-body adiposity in obesity-related pregnancy complications. For example, greater abdominal subcutaneous fat thickness measured at 18-22 gestational weeks by ultrasound increases the risk of GDM, cesarean delivery, and fetal macrosomia [77].

Chronic, low-grade inflammation occurs in obesity. In addition to higher circulating IL-6, TNF-α [36], and CRP [37], PBMC in obese individuals have elevated production of TNF-α and IFN-γ and decreased IL-10 compared to lean individuals [231]. Additionally, peripheral CD4+ [45, 46] and adipose CD3+ [44] T cell populations increase in individuals with an obese BMI. However, the CD4+ T cell subpopulations change depending on the severity of the obesity-
induced complications. In DIO mice, CD4+ Th1 cells initiate obesity-induced insulin resistance [208] and obese individuals with diabetes mellitus have a larger adipose CD4+ Th1 cell population than normoglycemic lean and obese subjects [210]. In contrast, the increase in CD4+ T cell population in metabolically healthy obese individuals comes from circulating Th2 and Treg cell populations while the Th1 and Th17 cell counts remain similar to those in lean subjects [45].

T cells are routinely characterized by their secretion of cytokines, chemokines, and growth factors (collectively referred to as cytokines herein). Cytokines are a diverse family of soluble proteins expressed by various cell and tissue types and function as important signaling messengers within the immune system. The majority of research on the impact of obesity in pregnancy focuses on macrophage-related inflammation. In addition, obesity categorized by BMI is characterized by increased circulating inflammation and increased CD4+ T cell population. It is not known how the CD4+ T cell profile changes in pregnant women based on their weight status (BMI) or adipose tissue mass. The intent of this study was to compare weight status and several measures of adipose tissue mass, including abdominal visceral and subcutaneous fat mass with the stimulated CD4+ T cell cytokine profile in women during the third trimester of pregnancy.
III. Materials and Methods

Participants

Women from the Kansas City metropolitan area were enrolled between February 2012 and March 2013 the Pregnancy Health Study. Women were eligible if they were English-speaking, 18-44 years old with a singleton pregnancy, planning to delivery at the University of Kansas Hospital, and had a pre-pregnancy BMI between \(18.5 \text{ – 40 kg/m}^2\) based on self-reported height and weight. Exclusion criteria included diabetes, hypertension, smoking or drug use during pregnancy, chronic medical conditions known to influence inflammation status, or fear of enclosed spaces. This study was approved by the University of Kansas Medical Center Human Subjects Committee (#12793). The research protocol and informed consent process adhered to the Declaration of Helsinki and were approved by the Institutional Review Boards/Human Subjects Committees at KUMC.

Isolation and Stimulation of CD4\(^+\) T cells

Blood samples were obtained from subjects in the third trimester (n = 35; between 35 to 39 weeks gestation) or the morning after delivery (n = 2). PBMC were isolated from blood collected in heparin-coated tubes (BD Vacutainer, Franklin Lakes, NJ). Blood was layered onto Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged for 30 minutes at 400 \(x\) g. Mononuclear cell fraction was collected and washed with complete RPMI before cell viability and count were assessed. Cells were resuspended in 10% DMSO for long-term storage. CD4\(^+\) T cells were isolated from PBMC by positive selection (Miltenyi Biotec Inc., Auburn, CA) within a year of collection. CD4\(^+\) purity was 98 ± 1\% and 96 ± 1\% in fresh and frozen samples, respectively. In duplicate, cells were stimulated with PMA (50 ng/mL) plus A23187 (250
ng/mL) for 72 hours. Supernates were collected by centrifugation at 18,400 \(x\) g for 10 minutes at 4\(^\circ\) C and stored at -20\(^\circ\) C until analysis.

**Cytokine Multiplex Analysis**

CD4\(^+\) T cell cytokine expression was determined by Milliplex® xMAP technology (Invitrogen Corp, Camarillo, CA) according to manufacturer’s instructions. Samples were processed on a Luminex 200 (Austin, TX). All samples were analyzed on one plate to eliminate intervariability.

**Body Fat Measurement**

Total body fat mass was measured between 1-3 weeks postpartum by dual-energy x-ray absorptiometry (iDXA, GE Healthcare, Madison, WI) with encore software (Version 13.50). Subjects removed all metal artifacts from their body and were scanned according to standard imaging and positioning protocols. Half of the women were selected for measurement of visceral and subcutaneous abdominal fat mass determined by MRI at 3 Tesla (Skyra, Siemens Medical Solutions, Erlangen, Germany) [227] at the same visit. To measure abdominal fat mass, we obtained \(T_2\)-weighted spin echo localizer axial, coronal, and sagittal images while subjects lay on the table in a supine position with arms placed beside their head. Sagittal images were used to identify lumbar vertebrae 2 (L2) and sacral vertebrae 3 (S3) as the landmarks for abdominal fat mass. Blocks of high-resolution axial HASTE TSE \(T_2\)-weighted images were acquired during two 16-second breath-holds to minimize breathing motion artifacts. Each acquisition block was comprised of ~20 axial image slices (5 mm thick, 5 mm gaps). Anatomical locations of the two blocks were noted in order to find the consecutive images for analysis and prevent duplication.
Segmentation and quantification of the axial images were performed with ImageJ [228, 229]. Briefly, a mask was applied to enhance edge definition and adipose and non-adipose tissues were segmented by k-means clustering using custom-designed ImageJ scripts. Subcutaneous and visceral adipose tissues were delineated within each slice and each segmented image was reviewed to correct misclassified pixels caused by blurring of tissue space. Subcutaneous and visceral adipose tissue areas for each image were calculated by summing the highlighted pixels and multiplying by the pixel surface area. The volumes (cm$^3$) were calculated by multiplying the tissue area (cm$^2$) by the slice thickness (5 mm). The adipose tissue volume in the 5.0 mm gap was estimated by averaging the results from the adjacent slices. Finally, the volumes were multiplied by 0.916 g/cm$^3$, the density of adipose tissue, to obtain total mass (g) of the visceral and subcutaneous adipose tissue. All scans were read by a single observer. The intra-rater reliability coefficients of variation for subcutaneous and visceral adipose tissue mass measurements were 0.3% and 5.0%, respectively, based on two repeated, blinded, analyses of five randomly-selected sets of MRI images by a single observer.

**Statistical Analyses**

Descriptive statistics are presented as mean values ± SD (Table 3.1). Women were recruited for the study based on their pre-pregnancy weight and height and placed into the two categories for comparison: normal-weight (18.5 – 24.9) and overweight/obese BMI (25-40). Differences between the two BMI categories were analyzed with independent t-test. In order to normalize the absolute value of abdominal fat mass to each subject’s size, each abdominal compartment of adipose was divided by the amount of total body adipose. Linear relationships were calculated between body composition and CD4$^+$ T cell cytokines with Pearson correlation.
Relationships between fat mass measurements and cytokines were further assessed by simple linear regression. Shapiro-Wilks test was used to test normality, with outcomes variables not normally distributed log-transformed prior to all analyses. Results of five cytokines (IL-3, EGF, PDGF-BB, TGF-α, and CCL5) were not included in analyses because concentrations were outside the range of the assay. All data were analyzed with IBM SPSS Statistics 20.0 software (SPSS, Chicago, IL). P values presented are two-tailed and significance was set at 0.05.

IV. Results

The majority of research on the impact of obesity in pregnancy uses pre-pregnancy BMI to compare inflammatory differences. Neither Pearson’s correlation nor t-tests revealed any association between the two BMI categories (healthy weight, overweight/obese) and CD4⁺ T cell-produced cytokines (data no shown). Total body fat mass was compared to CD4⁺ T cell cytokine production as well. Out of the thirty-five detectable cytokines measured only FGF-2 was related (inversely) to total body fat mass ($r^2 = 0.218$, $P = 0.009$). We found no relationships between abdominal subcutaneous fat mass and CD4⁺ T cell cytokines.

In contrast, abdominal visceral fat mass correlated with CD4⁺ T cell cytokine production. Nineteen cytokines were inversely related to visceral fat mass and two cytokines (IL-9 and IL-15) trended toward a significant inverse relationship (Table 3.2). Notably, IL-7 was negatively correlated with increased visceral fat mass ($r = -0.545$, $P = 0.016$) as well as IL-17A ($r = -0.570$, $P = 0.013$), IFN-α2 ($r = -0.499$, $P = 0.03$), TNF-β ($r^2 = 0.425$, $P = 0.002$), and soluble CD40 ligand (sCD40L) ($r = -0.618$, $P = 0.005$). Among the cytokines measured, five CD4⁺ Th1-related
cytokines had significant negative associations with visceral fat mass (Figure 3.1A): INF-γ ($r^2 = 0.400, P = 0.004$), TNF-α ($r^2 = 0.237, P = 0.035$), IL-12p40 ($r^2 = 0.289, P = 0.018$), and IL-12p70 ($r^2 = 0.328, P = 0.010$). Additionally, Th2-related cytokines were negatively associated with greater abdominal fat mass: IL-4 ($r^2 = 0.240, P = 0.039$), IL-10 ($r^2 = 0.461, P = 0.001$), and IL-13 ($r^2 = 0.248, P = 0.03$) (Figure 3.1B). Negative relationships were also observed between visceral fat mass and CD4⁺ T cell expressed chemokine concentrations: CCL3 ($r^2 = 0.263, P = 0.025$), IL-8 ($r^2 = 0.446, P = 0.002$), and CXCL10 ($r^2 = 0.227, P = 0.046$) (Figure 3.1C). Lastly, numerous growth factors were inversely related to visceral fat mass: FGF-2 ($r^2 = 0.308, P = 0.014$), G-CSF ($r^2 = 0.238, P = 0.003$), GM-CSF ($r^2 = 0.459, P = 0.012$), and FLT-3L ($r^2 = 0.461, P = 0.001$) (Figure 3.1D).

V. Discussion

This study was designed to measure peripheral blood CD4⁺ T cell cytokine profiles in relationship to several measures of fat mass in healthy pregnant women who were recruited based on their pre-pregnancy BMI. To our knowledge, no one has assessed the nature of peripheral CD4⁺ T cells in relationship to obesity in pregnancy even though obese individuals have increased CD4⁺ T cell populations [45, 46]. We hypothesized that we would find relationships between the production of CD4⁺ T cell cytokines and body composition. Additionally, we hypothesized that whole body fat mass or abdominal fat mass (ie, visceral or subcutaneous) would be better related to cytokine production than would BMI. Indeed, BMI was not related to any cytokine response. Likewise, total body fat mass (with one exception) and abdominal subcutaneous fat mass were not related to any cytokine response. Only abdominal
visceral fat mass was associated with peripheral blood CD4$^+$ T cell cytokine production with lower production of Th1- and Th2-related cytokines, growth factors and chemokines.

While CD4$^+$ T cell populations increase in obesity, the increase is from the Th2 and Treg cell populations rather than Th1 or Th17 when insulin resistance is not present [44, 45]. Women in this study experienced pregnancies without gestational or other types of diabetes so their CD4$^+$ T cell responses should have favored a less inflammatory profile than had they been insulin resistant. We did see a pattern of cytokines suggesting a decreased inflammatory response in relation to visceral adiposity, but not in relation to total body fat or BMI. We find the decreased CD4$^+$ T cell secretion of G-CSF and GM-CSF associated with visceral fat mass particularly interesting as these growth factors are typically sent to the bone marrow to increase hematopoiesis for granulocytes and macrophages. In regards to the innate immune response in obesity, women with pre-pregnancy obese BMI have increased PBMC mRNA expression of CD14, CD68, TNF-$\alpha$, and IL-6, indicating increased monocyte activation [40]. Our data suggest that CD4$^+$ T cells in late pregnancy may be less responsive, which could potentially dampen the innate inflammatory response in women with greater visceral adiposity.

Perhaps as important as finding a relationship between abdominal visceral fat mass and CD4$^+$ T cell responses, we did not find a relationship between a commonly used indicator of body fatness, i.e., BMI, and cytokine production. Our results reinforce the ideas that a) visceral fat is an important measurement of fat mass, as opposed to weight status, and b) that abdominal visceral fat mass is a health risk. We note that there was virtually no association of total body fat mass to CD4$^+$ T cell production of cytokines even though there was to abdominal visceral fat mass, direct evidence for the importance of location of body fat.
A limitation of our study is that we did not measure the peripheral CD4$^+$ T cell populations responsible for the cytokine expression. However, cytokine expression is typically used in exploratory studies to categorize the response of immune cells. Additionally, *ex vivo* stimulation with PMA induces a broad spectrum of cytokine production and may not represent the true nature of the subjects’ CD4$^+$ T cell profile. Strengths include quantification of total body fat mass and abdominal visceral and subcutaneous fat masses instead of using BMI as an indicator of fat mass. To our knowledge, this is the first time visceral fat mass in pregnancy has been measured and compared to CD4$^+$ T cell cytokine profile. By quantifying CD4$^+$ T cell cytokine production, we obtained information about the CD4$^+$ T cell populations and gained insight into the adaptive immune response to fat mass in pregnancy. The 41-cytokine multiplex assay allowed us to identify cytokines with associations to increased visceral adiposity that have not been identified until now. Additionally, the response of CD4$^+$ T cell IL-2 expression indicates overall cell viability. Future studies should include measuring the circulating inflammatory markers elevated in obese pregnant women categorized by pre-pregnancy BMI throughout pregnancy in comparison to their abdominal adiposity will provide insight as to the source of obesity-induced inflammation.

Visceral fat is an ectopic fat store and hypothesized to play a greater role in the metabolic dysfunction in obesity due to increased secretion of inflammatory cytokines into circulation [48]. Interestingly, we found a dampened CD4$^+$ T-cell cytokine response in pregnant women with increased abdominal visceral fat mass. We conclude that abdominal visceral fat plays an important role in immune response in pregnancy and is a better indicator of impaired peripheral CD4$^+$ T cell immune function than BMI.
Acknowledgments

The authors wish to acknowledge the nursing staffs at our participating clinics and hospital whose support was essential to recruitment and availability of blood samples; Hoglund Brain Imaging Center for the creation of the MRI adipose protocol and financial support; Flow Cytometry Core Laboratory for technical support; and our subjects for their time.
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<td>Abdominal visceral fat mass (g)</td>
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<td>Gestational weight gain (kg)</td>
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</tbody>
</table>

<sup>a</sup>n = 37; <sup>b</sup>n=19 for abdominal fat mass measurements.
Table 3.2 Pearson’s correlation of CD4⁺ T cell cytokines to visceral fat mass (g) / total body fat mass (kg)

<table>
<thead>
<tr>
<th>Categories</th>
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<td>TNF-α* IL-12p40* IL-12p70† IFN-γ† TNF-β†</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-6 IL-5</td>
<td>IL-4* IL-13* IL-10†</td>
</tr>
<tr>
<td>Growth factor</td>
<td>VEGF PDGF-AA</td>
<td>FGF-2* GM-CSF* G-CSF† FLT-3L†</td>
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<tr>
<td>Chemokine</td>
<td>CCL22 CCL7 CCL2 CCL4</td>
<td>CXCL10* CCL3* IL-8†</td>
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<td></td>
<td>CCL11 CXCL3 CXCL1</td>
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</tr>
<tr>
<td>Not categorized</td>
<td>IL-2 IL-9# IL-15#</td>
<td>IFN-α2* IL-7* IL-17A† sCD40L†</td>
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</tbody>
</table>

* P ≤ 0.05; † P ≤ 0.01; ‡ P ≤ 0.001; # P = 0.052.
Figure 3.1 Significant attenuation of CD4$^+$ T cell cytokines by abdominal visceral fat mass

A

B
**Figure 3.1 Significant attenuation of CD4+ T cell cytokines by abdominal visceral fat mass.**

Data were analyzed with simple linear regression, values log-transformed when normal distribution was not present, n = 19. Only significant associations are shown. See Results section for specific *P* values (range from 0.001 to 0.046). All graphs are the comparison of cytokine relationship to visceral fat mass (g)/ total body fat (kg). (A) CD4+ Th1 cytokines: INF-γ (*r*² = 0.400), TNF-α (*r*² = 0.237), IL-12p40 (*r*² = 0.289), and IL-12p70 (*r*² = 0.328). (B) CD4+ Th2 cytokines: IL-4 (*r*² = 0.240), IL-10 (*r*² = 0.461), and IL-13 (*r*² = 0.248). (C) CD4+ T cell produced chemokines: CCL3 (*r*² = 0.263), IL-8 (*r*² = 0.446), and CXCL10 (*r*² = 0.227). (D) CD4+ T cell produced growth factors: FGF-2 (*r*² = 0.308), G-CSF (*r*² = 0.238), GM-CSF (*r*² = 0.459), and FLT-3L (*r*² = 0.461).
CHAPTER 4

DISCUSSION AND CONCLUSIONS
I. Summary of Findings

This study aimed to (1) quantify abdominal subcutaneous and visceral fat mass in healthy pregnant women, designed to capture a spectrum of maternal body composition (pre-pregnancy BMI 18.5 – 40), (2) measure third trimester, circulating adipokines leptin, resistin, visfatin, and adiponectin and assess for relationships to maternal body composition including abdominal fat compartments, and (3) measure peripheral CD4+ T cell cytokine production and assess for relationships to maternal body composition including abdominal fat compartments.

Overall, our results suggest that abdominal visceral fat is highly variable within BMI categories and more influential than overall body fatness concerning immune function in pregnancy. Additionally, resistin correlated with abdominal visceral fat and has the potential to be a biomarker in pregnant women. Finally, our results indicate the need for future research to consider the effect of women’s abdominal visceral fat in pregnancy complications and long-term health consequences.

The first objective of this study was to quantify abdominal subcutaneous and visceral fat mass in pregnancy. The majority of research on obesity in pregnancy uses pre-pregnancy BMI to determine obesity. BMI uses weight and height to estimate general body fatness but does not allow for determination of the location or amount of body fat. Additionally, BMI is not strongly associated to inflammation and cardiometabolic health risks compared to abdominal adiposity. To our knowledge, this is the first study to measure abdominal subcutaneous and visceral fat mass in pregnancy using MRI, a validated technique for measuring abdominal fat compartments. We compared body fat mass (total, abdominal subcutaneous, abdominal visceral) in twenty of...
our subjects, divided into two BMI categories: normal weight and overweight/obese. As pregnancy makes it difficult to correctly measure abdominal adiposity, we chose to measure abdominal subcutaneous and visceral fat mass using MRI within 3 weeks postpartum to characterize abdominal adiposity during pregnancy.

We hypothesized that while pre-pregnancy BMI could predict total body fat, it may not a reliable indicator of abdominal fat compartments. Surprisingly, women with an overweight or obese pre-pregnancy BMI did have greater amounts of both abdominal subcutaneous and visceral fat mass than women with normal-weight BMI (1503 ± 508 g vs. 685 ± 180 g abdominal subcutaneous fat; 214 ± 160 vs. 94 ± 34 g abdominal visceral fat in overweight/obese BMI and normal-weight BMI, respectively). However, in visceral fat there were large variances within the two groups and the groups’ medians were closer in amount, 134 g vs. 87 g in overweight/obese and normal-weight BMI, respectively. The two BMI groups appear to have similar visceral fat mass with the exception of three subjects who had > 300 g of abdominal visceral fat (Figure 4.1).

A confounder in measuring women’s visceral adiposity is race. Kanaley et al. [83] reported significantly less visceral fat mass in postmenopausal African-American women compared to Caucasian women. In this present study, we consented women without concern for their race. Out of the twenty women who had abdominal adiposity measured, four were African-American. Our results are in agreement with Kanaley as we saw much smaller amounts of visceral adiposity in our African-American women regardless of BMI. The Caucasian women in the overweight/obese BMI group averaged 312 ± 159 g vs. African-American women’s 78 ± 17 g (Figure 4.2).
Figure 4.1 Pregnant women’s abdominal visceral fat mass. Scatterplot of visceral fat mass (g) relative to pre-pregnancy BMI (kg/m²) shows similar fat mass across the BMI groups. Note the lines of demarcation, dividing the women into normal weight (18.5 – 24.9), overweight (25.0 – 29.9), and obese (30 – 40) BMI categories. The dashed circle highlights the three subjects with extreme amounts of visceral fat compared to the other 17 subjects.
Figure 4.2 Racial differences in women’s abdominal visceral fat mass. Women’s visceral fat mass within each BMI category varied greatly for each race. African-American women in the overweight/obese pre-pregnancy BMI had lower amounts of abdominal visceral fat, comparable to women with a normal-weight BMI. It is difficult to compare Hispanic women to other races because of small sample size.
The second objective of this study was to ascertain whether the adipokines leptin, resistin, visfatin, and adiponectin had relationships to BMI, total body fat mass, abdominal subcutaneous fat mass or visceral fat mass in pregnant women in the third trimester. We believe this is the first study to assess the potential relationships between adipokines and abdominal adiposity during pregnancy.

We hypothesized that we would find more relationships between abdominal adiposity and adipokines than with BMI. We found resistin to be the only adipokine to correlate with abdominal visceral fat mass. No associations occurred between abdominal subcutaneous fat mass and any adipokines. Additionally, adiponectin and visfatin did not correlate to any body composition measure. Finally, leptin correlated with total body fat mass and was the only adipokine to be significantly different between the normal weight and overweight/obese BMI categories.

The third objective of this study was to determine the peripheral CD4$^+$ T cell cytokine responses in the third trimester and look for any associations with BMI, total body fat mass, abdominal subcutaneous fat mass or visceral fat mass. The majority of research on the impact of obesity in pregnancy focuses on macrophage-initiated inflammation [40, 218] but CD3$^+$ cytotoxic and T helper cells are also involved in the etiology of obesity-induced inflammation and interact with adipose tissue macrophages in response to expanding adiposity. To our knowledge, measuring the adaptive immune response to body fat compartments in pregnancy has not occurred. We hypothesized abdominal fat mass to be a more important physiologic indicator than BMI or total body fat in pregnancy. We found significant correlations between abdominal visceral fat mass and CD4$^+$ T cell cytokines and no associations were found with pre-pregnancy
BMI, abdominal subcutaneous fat mass, or total body fat (with one exception). Nineteen of the 36 CD4+ T cell expressed cytokines exhibited decreased concentration to increased visceral fat mass.

In summary, our results suggest that while an overweight/obese pre-pregnant BMI can indicate greater body fatness compared to a normal-weight pre-pregnant BMI but there is great variation in the amount of abdominal visceral fat mass within groups. With the exception of leptin, there are no clear associations between BMI and circulating adipokines or the peripheral CD4+ T cell cytokine profile. Subcutaneous abdominal adiposity had no relationships to adipokines or CD4+ T cell cytokines while abdominal visceral adiposity had a positive correlation with resistin and a dampening effect on the cytokine expression of peripheral CD4+ T cells.

II. Clinical Implications

Our results suggest that visceral fat mass, an ectopic fat pool linked to cardiometabolic risk in non-pregnant individuals, may be an important cause of immunosuppression in pregnancy. The true implications of this cannot be determined or understood without more targeted studies of fat mass in pregnant women that are linked to physiological and pathological outcomes. The implications of our findings are that some women may have a normal-weight BMI but large amount of visceral fat mass that could potentially increase their risk of pregnancy complications while some overweight/obese women may not have a high risk of adverse pregnancy outcomes because they have very small visceral fat pool. Because over half of the
childbearing women in the US are overweight or obese and 40% of pregnancies are unplanned, it is not practical to intervene with weight loss strategies before conception for all overweight/obese women, however, it is conceivable that additional research in this area might allow us to determine which women are most at risk for adverse pregnancy outcomes, both at delivery and, potential long term programming effects on the offspring that have been linked to weight status.

Resistin is a reliable indicator of abdominal visceral adiposity in pregnancy. It has the potential to be a circulating marker of a woman’s visceral adiposity that may increase her risk of pregnancy complications. As noted above, resistin’s potential needs to be evaluated with larger studies that link resistin to pregnancy outcomes. While resistin’s role in pregnancy complications remains inconclusive [118, 119], it is known that its secretion from the placenta and subcutaneous adipose does not differ between women with and without GDM [173]. Therefore, increased visceral adiposity may be the source of increased circulation of resistin and in certain subsets of women, contribute to pregnancy complications.

As previously shown, circulating leptin is highly correlated with total body fat mass. Hyperleptinemia is associated with macrosomia [109, 174], preeclampsia [34, 99, 115] and GDM [113], yet most of these relationships are independent of pre-pregnancy BMI. Lappas et al. [173] reported leptin metabolism dysregulation from muscle and adipose tissue in women with GDM compared to women with healthy pregnancies, also independent of BMI. Our study involved women in healthy pregnancies yet our overweight/obese pregnant women had a ~ 2-fold increase in circulating leptin compared to pregnant women with a normal-weight BMI (66 ± 34 vs. 36 ± 19 ng/mL, respectively). It seems plausible that leptin may be less predictive of
adverse pregnancy outcomes than peripheral tissue leptin dysregulation that occurs in a subset of pregnancies. Finally, while adiponectin and visfatin have important physiologic roles in glucose homeostasis and in pregnancy, their concentration was not related to body mass or fat mass (total or abdominal compartments).

Because chronic, low-grade inflammation occurs in obesity, obesity-induced inflammation is potentially involved in the etiology of pregnancy complications. Differences in innate inflammation between obese and normal weight pregnant women occur at delivery [40, 218]. The response of adaptive immunity to the incidence of obesity in pregnancy is potentially important and relatively unstudied. Consequently, we chose to study the effect of weight status and body fat mass and location on peripheral CD4+ T cell cytokine production in pregnancy. Our findings demonstrate that pre-pregnancy BMI or total body fat does not influence the nature of CD4+ T cells while visceral adiposity potentially attenuates their ability to express cytokines. This immunosuppression may occur in order to dampen the macrophage-created inflammation in women with greater visceral adiposity. How this is involved in pregnancy complications and long-term health outcomes remains to be determined.

These results are significant because of the high rate of overweight and obese childbearing women in the United States and the obesity-related pregnancy complications affecting both mother and infant. While BMI can assess general fatness, it is not accurate enough to indicate physiologic, obesity-related responses. However, abdominal visceral adiposity does appear to have a greater impact on circulating adipokines and adaptive immunity and may potentially be the key to elucidating obesity-related pregnancy complications. Understanding
how inflammatory mediators like adipokines and CD4\(^+\) T cells respond to excess visceral adiposity in pregnancy will help provide useful evidence needed for interventions.

III. Limitations

Limitations of the study include using the cross-sectional design. Measuring body composition and adipokines at various time points in pregnancy would have given us a greater scope to the relationship between body fat compartments and adipokines and the adaptive immune response. Another limitation is the small number of women used for the MRI analysis. Increasing the number of women could provide insight as to what has the potential to influence the amount of abdominal visceral fat during pregnancy. Additionally, we did not measure any typical macrophage-related inflammation markers in our women. We hypothesize that the attenuation of the CD4\(^+\) T cell cytokines in relation to increased visceral fat mass could be in response to macrophage-driven inflammation. Measuring the interaction between macrophages and CD4\(^+\) T cells would provide insight to how the immune system responds to greater visceral adiposity during pregnancy.

This study did not assess the impact of abdominal adiposity on fetal outcomes. Previous studies have found inflammatory and physiologic changes to the placentas of obese women categorized by pre-pregnancy BMI. Assessing the placenta for these similar alterations in relationship to abdominal visceral fat mass would have revealed if the changes seen in maternal circulation also occur in the placenta. Additionally, measuring the impact of abdominal adiposity on offspring outcomes would help link obesity in pregnancy to impact on offspring. Measuring
inflammation in the cord blood and assessing infant body composition are parameters used to gauge the impact of maternal obesity on offspring.

Finally, the use of the cytokine multiplex technique allowed us to characterize the CD4\(^+\) T cells by their expression of 41 cytokines. However, it remains unknown how CD4\(^+\) T cells respond to adiposity throughout pregnancy. Defining the CD4\(^+\) T cell population would have given us a detailed explanation of how adaptive immunity responds to varying amounts of adiposity in pregnancy. Because this was an exploratory study, our cytokine results provide quick and useful insight into the nature of the CD4\(^+\) T cell cytokine profile in which future studies can follow-up. Additionally, measuring the known innate markers that change in obese pregnant women would have been provided insight in regards to their relationship to abdominal adiposity and the CD4\(^+\) T cell cytokine profile.

IV. Future Directions

A current area of research seeks to elucidate the phenotypic and genotypic differences in obese people who are otherwise metabolically healthy. Termed “metabolically healthy obese”, up to 30% of United States adults with an obese BMI do not have the clinical markers for metabolic syndrome [52, 232]. These individuals typically do not have the low-grade inflammation associated with obesity [233], have less abdominal visceral fat [234], and a lower waist circumferences than their metabolically unhealthy obese counterparts [235]. It is plausible to assume up to 30% of pregnant women who are obese are also metabolically healthy. If we rely only on pre-pregnancy BMI, we are introducing error into our study models. Future studies on
the effect of obesity in pregnancy should involve a new category of “metabolically healthy obese” women by either measuring inflammation as an exclusion criterion or assessing the amount of visceral adiposity.

Assessment of the relationship between the innate and adaptive immune response in women with excessive visceral adiposity will provide insight as to how this ectopic fat mass influences circulating inflammation in pregnant women. Research showing macrophage-driven inflammation use pre-pregnancy BMI to characterize obesity yet abdominal visceral adiposity is a greater inducer of metabolic dysfunction in the non-pregnant population. Measuring the impact of abdominal visceral fat mass on macrophage-related markers will provide insight on the role visceral fat has in obesity-related pregnancy complications in comparison to total body fat as well as in relationship to the adaptive immune response.

Future studies on the impact of obesity in pregnancy should assess the relationship of abdominal visceral adiposity to the placenta in order to further our knowledge on the effects of excess adiposity on the in utero environment. It has recently been shown that women with a pre-pregnancy obese BMI have reduced placental proliferation [236], altered vascular function [237], and increased nitrative and oxidative stress [215]. While increased placental macrophages [40] and neutrophils [218] have been observed in obese pregnant women, to our knowledge, no study has measured the impact of abdominal adiposity on placental changes. Because we saw noticeable relationships between circulating markers of inflammation and visceral adiposity, relating this ectopic fat compartment to placental changes is necessary to understand the impact of obesity on fetal development and long-term outcomes. Because innate and adaptive immune systems work in cohesion to protect the body from pathogens, addressing the role adaptive
immunity plays in the inflammation observed is integral to understand the etiology and progression of the immune response to excess energy. Future studies should assess the impact of visceral adiposity on both innate and adaptive immune changes in the placenta.

Excess maternal adiposity affects birth weight [174]. There are limited data on the association of offspring body composition and maternal circulating adipokines [111, 117], as most reports compare adipokines to infant birth weight. Another research component of this study measured body composition in the offspring of our subjects at birth and at 4 months. Body composition measurements included skin fold thickness and body fat percentage measured by ADP. Potential relationships could be found by assessing the maternal circulating adipokines and offspring body composition.

Interventions for women at risk of obesity-related pregnancy complications are ongoing. Currently, the Maternal Obesity Management trial is designed to help overweight and obese pregnant women gain the appropriate amount of weight during pregnancy through a diet and exercise intervention in order to prevent offspring obesity [238]. This and other pregnancy studies focus on healthy gestational weight gain, addressing the impact of caloric intake and exercise throughout pregnancy for healthier outcomes [239-241]. However, a meta-analysis by Tanentsapf et al. [242] points out that while these intervention trials can decrease the amount of gestational weight gained and lower the incidence of cesarean section deliveries, no effect has seen on the incidence of GDM, preeclampsia, preterm birth, and birth weight. Addressing the underlying inflammation and genetic predisposition of women in regards to these outcomes may help explain why lifestyle interventions of diet and exercise cannot alone prevent obesity-related
pregnancy complications. Drug or nutrient interventions may be able to help women who are at a greater risk.

In order to reduce fetal injury, women are often excluded from intervention studies on drugs and nutrients. However, because of the importance of health in pregnancy, intervention trials of drugs (metformin, insulin, misoprostol, and progesterone are the top four pharmaceutical drugs) and numerous types of vitamins and essential nutrients are occurring at a greater rate in the pregnancy population [243]. Metformin, though currently contraindicated in pregnancy, has been shown in DIO-rats to reduce fetal plasma TNF-α and CCL2 but not IL-6 while maternal plasma levels of these inflammatory markers were not reduced [244]. Supplementation with omega-3 fatty acids may also attenuate inflammation in pregnancy. Long-chain omega-3 fatty acids are important for neurodevelopment [245, 246] and it has been recommended that pregnant women consume at least 200 mg/day of docosahexaenoic acid (DHA) [247] and up to 600 mg/day can be consumed without adverse events [248]. That study also found a significant reduction in early preterm birth with DHA supplementation. The omega-3 fatty acid-derived resolvins and protections actively resolve inflammation [249]. Intraperitoneal injection of 2 μg/kg body weight of resolvin D1 improves glucose tolerance as well as reduces adipose tissue macrophage infiltration in mice [250]. Future interventions should include using DHA to see if it can reduce inflammation in pregnancy in relationship to excess adiposity.
V. Conclusions

In conclusion, the results of the present study suggest abdominal visceral adipose tissue mass in pregnancy is better linked to immune function than body mass. As abdominal adiposity has a known role in increasing cardiometabolic risk in obese individuals in general, this finding in pregnancy suggests a potential risk factor that could be targeted to reduce adverse pregnancy outcomes. More studies are needed to determine if this is so, and, if it is found to be, the determinants of increased visceral fat mass in pregnancy that might be targeted by lifestyle or other interventions.
REFERENCES


APPENDIX I

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APPENDIX II

PROCEDURE FOR QUANTIFICATION OF ABDOMINAL ADIPOSITY BY MRI
1. Open **syngo FastView** to orient yourself to the files and figure out where scans started and stopped and orient yourself to L2 and S3 (abdominal parameters)
   a. Click on “autorun” with head icon
   b. Source, click to open files of subject
   c. Click on “localizer” scans
      i. On right hand side, click Tools Stripe and 4x4 icon to see all localizer images, click on those needed to find organ location to top of L2

2. Open **ImageJ** software
3. File
   a. Import

4. Open folder containing subject of interest (folder 1 is typically localizer, folder 2 is the upper scan, folder 3 is the lower scan)
   a. DICOM folder (snapshots shows you want the images look like on one image)
      i. Click any file
5. Window pops open “Sequence Options”, click “ok”
   a. One have screen open labeled “DICOM” and you can scroll through all images
6. Run program to separate and calculate subcutaneous fat from rest of body
   a. Plugins
      i. Macros
         1. Run
2. Open text file “fat segmentation a_1” (created by Ping-Chang on 7/26/2012)

b. **Edit first mask**: Need to go through top screen “result of origin1-1 to make sure the body cavity is correctly segmented from subcutaneous fat

7. Compare with “origin1” images

a. Handy controls:
   i. CRTL + + to enlarge; CRTL + - to decrease size of image
   ii. CRTL + Z undo
   iii. CRTL + SHIFT + A unselect
   iv. Double click on pencil to get options (thickness and color)

8. Use pencil option to edit cavity boundary

   a. Draw boundaries of abdominal cavity that reflect the respective slice image from “origin1”

9. To fill holes: Process, Binary, Fill Holes, click yes to perform on all images

10. Run Macro B; Plugins, Macro, Run, open text file “fat segmentation b”

11. Calculation of subcutaneous fat quantity results will pop up to save to desktop, click yes (change file name later to prevent file override)

12. Histogram Lister: click ok

13. Visceral fat images now available to edit

   a. Use pencil to cut out grey area in “visceral fat stack” screen that represent the visceral fat portions (bright white spots) in “origin1” or “result of origin1” screen
14. After finished isolating visceral fat areas, click on “wand” tool, hover cursor over visceral fat area and click, then SHIFT + click to highlight numerous areas.
15. When finished highlighting appropriate areas, click CTRL + M to measure.
16. Amount of area measured will pop up in Results screen
17. After:

18. Go through all slides, measuring highlighted area in each slide when done.

19. Results, File, Save as: MRI_subject#_results_upper_visceral_fat

20. Save Image J files to new folder “subject #_Image J upper images”

21. ANALYSIS

   a. need information about the session? Click on one of the Image J screens, then go to Image, click on “show info”
APPENDIX III

PROCEDURE FOR ISOLATION AND EX VIVO

STIMULATION OF CD4+ T CELLS
Lymphocyte Isolation

1. Collect heparinized, green-topped tubes of blood by venipuncture.

2. Transfer the blood to a 50 ml conical tube. Fill each 50 ml tube with no more than 25 ml blood. Add 1:2 dilution sterile PBS.

3. Layer 15-25 mL blood onto each of the 9-13 mL aliquots of room temperature Histopaque. DO NOT MIX. Instead, slowly layer the diluted blood onto the surface of the Histopaque. Centrifuge for 30 minutes at room temperature, 400 x g with no brake.

4. Discard the topmost layer above the cells at the interface, which contains PBS and serum and discard. Be careful not to disturb the interface.

5. Collect the cells at the interface, about 5-7 ml per tube. Place interface cells into new 50 mL conical. Wash cells by diluting 3:1 in complete RPMI.

6. Centrifuge at 330 x g for 10 minutes. Resuspend the pellet in 10 mL medium.

7. Centrifuge a second time, and again resuspend in 10 mL medium.

8. Resuspend the cells in an appropriate volume of medium and determine yield and viability by hemacytometer counting (1:10 dilution in trypan blue).

9. Use the cells in your assay or freeze them in 90% FCS/10% DMSO at a density of 5-10 x 10^6/ ml/cryovial.

Thawing PMBCs

1. Raise temperature of cryovial rapidly to between 25 – 37° C.

2. Add 1 mL warm complete RPMI to the cells in the tube.

3. Transfer the cells from cryovial to 50 mL conical.
4. Very slowly, add complete RPMI (warmed to 37°C), diluting the total to 5x the original volume (4 ml to 1 ml cells). Subsequently, add somewhat faster an equal amount of warm RPMI. The gradual dilution of DMSO avoids the osmotic shock and the warm temperature ensures that the cells can actively compensate the osmotic pressure.

5. Centrifuge the cells at 330 x g for 10 min with rapid acceleration and brake on.

6. Aspirate supernatant and resuspend the cell pellet by tapping and add 10mL warm RPMI.

7. Centrifuge at 330 x g for 10 min.

8. Aspirate supernatant, then resuspend cells in desired medium (RPMI) to 2 x 10^6/mL.

9. Count live cells using a hemacytometer and microscope if desired.

**CD4^+ Isolation**

1. Centrifuge cell suspension at 300 x g for 10 min. Aspirate supernatant completely.

2. Resuspend cell pellet in 80 μL of buffer per 10^7 cells.

3. Add 20 μL CD4^+ microbeads per 10^7 cells. Mix well and incubate for 15 min at 2-8° C.

4. Wash cells by adding 1-2 ml buffer per 10^7 cells and centrifuge 300 x g for 10 min. Aspirate supernatant.

5. Resuspend up to 10^8 cells in 500 μL buffer.

6. Place column in magnetic field and Pre-Separation Filters on top on column, rinse with 500 μL buffer.

7. Pipette cell suspension into reservoir and let suspension run through.

8. Collect unlabeled cells. Wash filter with 3x500 μL of buffer. Only add buffer when reservoir is empty.

9. Remove column from separator and place it on a suitable collection tube.
10. Pipette 1 mL buffer onto column, immediately flush out magnetically-labeled cells by firmly pushing the plunger into the column.

11. Count on the hemacytometer.

12. Let rest at 37°C for at least a few hours to overnight (~12 hours).

**Intracellular Stimulation and Labeling**

1. Prepare 200 μL cells in duplicate at a concentration of 2 x 10⁶/ mL in RPMI (4-5 x 10⁵ cells in each well).

2. Add 10 μL PMA/Ionomycin mix of cells and incubate for 72 hours at 37°C in 5% CO₂ (final concentration: 50 ng/mL PMA/250 ng/mL A23187).

3. Harvest cells on ice and centrifuge at 18,400 x g x 10 min.

4. The supernatant samples were apportioned into 0.5 mL aliquots and stored at -20°C until just prior to the analysis.