I, Anuradha Gundamaraju, hereby submit this original work as part of the requirements for the degree of:

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The relationship between vitamin D intake and markers of inflammation (TNF-a and IL-6) in overweight and obese pregnant women in third trimester

Student Signature: Anuradha Gundamaraju

This work and its defense approved by:

Committee Chair: Debra Ann Krummel, PhD

Debra Ann Krummel, PhD
The relationship between vitamin D intake and markers of inflammation (TNF-α and IL-6) in overweight and obese pregnant women in the third trimester

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By
Anu Gundamaraju
MSc, Applied Microbiology
Madras University, India

Committee Chair: Debra A. Krummel, PhD, RD
Abstract

Objectives. To evaluate the relationship of vitamin D intake and pro-inflammatory cytokines (TNF-α and IL-6) in overweight and obese pregnant women.

Subjects. Forty-eight overweight and obese pregnant subjects (pre-pregnancy body mass index (BMI) > 25 kg/m²) in their third trimester between 18 and 40 years old were recruited mainly through fliers and ads in magazines.

Methods. This cross-sectional study included 48 pregnant women in the third trimester (16 overweight and 32 obese). Dietary vitamin D intake (DD) measures of the subjects were obtained from 24 hr dietary recall conducted using NDSR. Total vitamin D intake (TD) was calculated by adding dietary vitamin D intake and supplemental vitamin D intake. Women were classified into low and high DD groups (< and ≥5 mcg of vitamin D intake) according to the dietary intake. They were also classified into low TD and high TD intake groups based 50th percentile frequency of total vitamin D intake. Plasma concentrations of pro-inflammatory cytokines (TNF-α and IL-6) were determined by enzyme-linked immunosorbent assay (ELISA) using MilliplexTM Multiplex kits (Millipore, Billerica, MA) according to manufacturer’s protocol. Associations between pre-pregnancy BMI, vitamin D intake, and inflammatory markers were evaluated by bivariate correlation analysis, two-way ANOVA, and non-parametric Mann-Whitney U test.

Results. There was no relationship between pre-pregnancy BMI and vitamin D intake. Pre-pregnancy BMI was positively correlated to IL-6 (r=+0.295, P<0.05). There was a trend for a relationship between total vitamin D intake and TNF-α. Dietary vitamin D intake was significantly different between low DD (n=38) and high DD (n=10) groups.
Total vitamin D intake (TD) was significantly different between low TD (n=24) and high TD (n=24) groups (P=0.000). The difference between the mean levels of TNF-α in low TD and high TD groups had a slight trend (P=0.08) with high TD having lower TNF-α levels. There were no significant main and interaction effects of pre-pregnancy BMI and vitamin D on the levels of inflammatory markers (TNF-α, IL-6).

**Conclusion.** There was no significant relationship between vitamin D intake and markers of inflammation. There were no interactive effects of pre-pregnancy BMI and vitamin D on inflammation. However, the result of a trend for a relationship between total vitamin D intake and TNF-α shows that further investigation is required to analyze the relationship between vitamin D (using serum 25-OHD concentrations) and markers of inflammation.
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Introduction

Vitamin D has an established role in bone metabolism (1) and emerging evidence also suggests a role for vitamin D in cardiovascular health (2). Vitamin D is available in two forms. Vitamin D2, or ergocalciferol, which is derived from irradiated plants or other plant forms, vitamin D3 or also known as cholecalciferol, and it is synthesized by the skin when exposed to ultraviolet (UV) light and from animal sources (3). The major dietary sources of vitamin D2 are raw white mushrooms (4), and the major sources of vitamin D3 are cod liver oil, salmon, dairy products and fortified cereals (3). These both forms of vitamin D are called prohormones. Hepatic hydroxylation of these prohormones takes place and they are converted to 25-hydroxyvitamin D2 (25OHD2) and 25-hydroxyvitamin D3 (25OHD3) accordingly. The enzyme, 1-\(\alpha\)-hydroxylase present in the kidney converts the serum 25-hydroxyvitamin D (25-OHD) to 1,25-hydroxyvitamin D (1,25-OHD) (3). The 25-OHD is called as calcidiol and the 1,25-OHD is calcitriol (5).

Vitamin D insufficiency (25-50 nmol/L) is a risk factor for a variety of chronic diseases including multiple sclerosis, type 1 and type 2 diabetes, cancer, osteoporosis, psychiatric illness, and cardiovascular disease (6). Heightened susceptibility to some of these disorders may originate in early life, with long-lasting structural and functional changes in developing organs or organ systems (6). Poor prenatal vitamin D status increases susceptibility to these diseases in later life via specific target organ effects and/or through changes in the developing immune system (6). The role of vitamin D as an immunosuppressive agent during inflammation is becoming clear (7). Therefore, Maternal vitamin D supplementation during pregnancy could be an important public health measure to decrease the risk of a range of chronic diseases (7).
Physiologically, vitamin D status has a wide range of possible health outcomes during pregnancy from preconception to birth (8). Maternal concentrations of serum calcidiol and calcitriol were determined in 40 pregnant Saudi women during pregnancy and at term. The calcidiol concentrations decreased significantly from 54 ± 10 nmol/l in the first trimester to 33 ± 8 nmol/l in the third trimester (P < 0.001) and remained decreased at term. Maternal serum calcitriol concentration increased during pregnancy, from 69 ±17 pmol/l in the first trimester to 333 +/- 83 pmol/l at term (P < 0.001) (8). Such increases could be explained by increased synthesis in the kidney and/or decreasing catabolism of 1,25-OHD (8). The level of serum 25-OHD in cord blood correlates highly with the maternal serum levels (9). Fetal 1,25-OHD derives mainly from the fetal kidney, possibly with some contribution from other sites such as the placenta (9). Therefore this suggests the significance of maternal intake of vitamin D in fetal stores.

Vitamin D deficiency defined as 25-OHD<20 ng/ml has been shown to be associated with increased risk of developing type 1 and type 2 diabetes mellitus and cardiovascular disease (1). The major mechanism contributing to vitamin D deficiency is that the skin pigment melanin absorbs UVB photons and can reduce vitamin D-3 synthesis by > 90% (10). Those populations with darker skin pigmentation, or those who cover-up with clothing when outside (some due to cultural reasons), and those who live in regions far from equator during the winter season are at particular risk for vitamin D insufficiency (serum 25-OHD ≤ 75 nmol/L) (11).

Serum 25-OHD readily crosses the placenta. Therefore, fetal and newborn vitamin D status is almost entirely dependent on vitamin D from the mother (11). A secondary analysis of the National Health and Nutrition Examination Survey (NHANES)
2001-2006 showed that adolescent and adult women of childbearing age have a high prevalence of vitamin D insufficiency (serum 25-OHD ≤ 75 nmol/L) (11). The mean 25-OHD level was found to be 65 nmol/L for pregnant women and 59 nmol/L for childbearing age women (11). Therefore, the prevalence of 25-OHD ≤ 75 nmol/L was 69% and 78%, respectively (11). Pregnant women in the first trimester had similar 25-OHD levels as nonpregnant women (55 vs 59 nmol/L), even with high proportion of the women taking vitamin D supplementation (61% vs 32%) (11).

Cashman et al. aimed to establish the amount of dietary vitamin D required to maintain the levels of serum 25-OHD. They found that a daily intake of 8.7 mcg/day of vitamin D intake would maintain serum 25-OHD levels of >25 nmol/L (12). Vitamin D intakes required to maintain serum 25-OHD concentrations of >37.5, >50, and >80 nmol/L were 19.9, 28.0, and 41.1 mcg/d, respectively (12). Current prenatal multivitamins of 400 IU (10 mcg of vitamin D) helped to raise serum 25-OHD levels, but higher doses for longer duration may be necessary (11).

**Review of Literature**

**Pre-Pregnancy Obesity and Pro-inflammatory Cytokines (TNF-α and IL-6)**

In the United States, the prevalence of obesity is growing among women of childbearing age (13). According to the NHANES, obesity among women in the age group of 20 to 39 years increased 33% in the interval between 1988 to 1994 and 1999 to 2000, from 21% to 28% (13, 14). Data from the Pregnancy Risk Assessment Monitoring System conducted in nine US states were analyzed to observe the trends in pre-pregnancy obesity (13). Interestingly, it was found that pre-pregnancy obesity increased 69% from 13.0% in 1993 - 1994 to 22.0% in 2002 - 2003 (13). The increase in the prevalence of
obesity ranged from 45% to 105% for individual states (13). Obesity is an inflammatory disease that is associated with several complications and increased risk of diseases such as metabolic syndrome, type 2 diabetes mellitus, or coronary heart disease.

Basically, a cytokine is a soluble glycoprotein released by cells which acts non-enzymatically to regulate cellular and immunological function (15). In non-pregnant women, obesity is described as a low grade inflammatory condition associated with an increased production of pro-inflammatory cytokines that are released from the macrophages present in the adipose tissue (16).

Apart from its role in energy storage, adipose tissue could also be considered as an endocrine organ with various functions (17). The two kinds of adipose tissue (visceral and subcutaneous) are significantly different in their functions (17). Hormones secreted from the visceral tissue go through the portal circulation and exert a direct effect on metabolic processes in the liver (17). Substances secreted by the subcutaneous adipose tissue enter the systemic circulation (17). It has been found that synthesis of interleukin-6 (IL-6) is significantly higher in visceral adipose tissue and the synthesis of adiponectin and tumor necrosis factor-α (TNF-α) take place in subcutaneous adipose tissue (17).

The adipose tissue is made up of intercellular matrix consisting of collagen and elastic fibers (18). In obese individuals, the expansion of adipose tissue results in the formation of more blood vessels, more connective tissue fibroblasts, and particularly more macrophages. This leads to an enhanced secretion of the inflammatory cytokines (TNF-α, IL-6) (18). Current evidence suggests that the obesity induced expanded adipocytes are associated with increased secretion of macrophages, M1 (classically
activated) phenotype from the circulation (19). These macrophages are usually recruited in regions of tissue damage and are pro-inflammatory in nature (19).

Adipose tissue is involved in the synthesis of numerous, metabolically active proteins called adipokines (20). These proteins play an important role in the regulation of local and systemic metabolism, showing typical endocrine activity (20). Adiponectin is an adipokine secreted by adipocytes, which stimulates glucose uptake in skeletal muscle and reduces hepatic glucose production and hence adiponectin is considered as an endogenous insulin-sensitizing hormone (21).

Adipocytokines such as IL-6 are secreted by stromal cells in adipose tissue. They were found to play a significant role in down regulation of insulin sensitivity (21). Recent evidence suggests that macrophages from the stromal vascular fraction are the primary source of TNF-α and that the increased levels of this cytokine in obesity are due to the increased infiltration of adipose tissue with M1 macrophages. It has been demonstrated that TNF-α and IL-6 independently down regulate adiponectin gene expression and consequently depress insulin sensitivity (22) (23). A higher level of adiposity is correlated with the secretion of pro-inflammatory cytokines from adipose tissue, suggesting that these cytokines may play an important role in the availability of energy resources during future obese pregnancy (21). Inflammatory cytokines such as TNF-α and IL-6 have been found to be highly expressed in adipose tissue in obese mouse models and in overweight humans (24). They have the ability to disrupt insulin signaling and contribute to the development of metabolic dysfunction and eventually insulin resistance (25) (24).
Inflammation is characterized by the infiltration of inflamed tissues by immune cells, such as macrophages (24). Increased amounts of macrophages have been found in the adipose tissue of both obese mice and human subjects (24). The percentage of macrophages in adipose tissue is strongly and positively correlated with adiposity (24). Also, it has been found that macrophages derived from the adipose tissue contribute significantly to elevated levels of inflammatory cytokines, including TNF-α and IL-6. Therefore, it is suggested that the chronic inflammation associated with obesity-related insulin resistance would be initiated in adipose tissue (24). (See figure 2)

Figure 2 Obesity and inflammation (figure adapted from Galic et al. 2010)
In animal model studies it has been shown that obese mice lacking the cytokine TNF-α have higher rate of protection from obesity-related insulin resistance in muscle and fat tissues (24). It has been demonstrated that TNF-α acts by inhibiting tyrosine kinase activity present in the insulin receptor in adipocytes, and reduces the phosphorylation and activation of insulin receptor substrate-1 (IRS-1) (25). Hence, TNF-α inhibits the insulin signaling pathway and may be responsible for insulin resistance (25).

Research strongly suggests that TNF-α is produced in higher amounts in adipose tissue derived from obese human subjects (26). Furthermore, there was a significant increase in adipose tissue derived TNF mRNA levels with increasing adiposity. There was a significant correlation between TNF mRNA and percent body fat ($r = +0.46$, $P < 0.05$, $n = 23$) (26). At the same time, loss of weight was found to reduce TNF-α levels (26).

Adipose tissue alone contributes to 15–35% of the body’s basal IL-6 synthesis (24). Evidence based research suggests that IL-6 is a key participant in metabolic diseases, such as diabetes (24). Because of its pleiotropic nature, cytokine IL-6 is known to regulate diverse functions in different cells and tissues, including the proliferation and differentiation of hematopoietic cells, acute phase response induction in liver cells, and inflammation at sites of tissue injury (24). Chronically higher levels of IL-6 are linked to its role in insulin resistance through pathways related to insulin action (24).

Suppressor of cytokine signaling proteins (SOCSs) play an important role in pathogenesis of the metabolic syndrome by the way of modulating insulin and cytokine signaling (27), thereby playing a significant role in the pathogenesis of insulin resistance.
Specifically, the expression of SOCS-3 is increased by inflammatory cytokine such as IL-6 (24). It was observed that the overexpression of SOCS-3 repressed insulin-induced glycogen synthase activity and glucose uptake in adipocytes, while hepatocyte-specific SOCS-3 deletion improved insulin sensitivity in the liver (24). The c-Jun N-terminal protein kinase (JNK1) is a protein kinase which is reactive to stress stimuli, such as cytokines and ultraviolet irradiation (28). This stress reactive protein, JNK1 acts when specifically stimulated by inflammatory cytokine TNF-α (28).

In a research study, adipocytes containing free fatty acids (FFAs) were utilized to examine the molecular mechanisms underlying fat-induced insulin resistance (29). It was observed that FFAs repressed insulin receptor-mediated signal transduction and reduced insulin-mediated glucose transporter (GLUT)-4 translocation and glucose transport (29). This action was mediated by FFAs activating the stress/inflammatory kinases c-Jun N-terminal kinase (JNK) and the SOCS 3, along with the increased secretion of the TNF-α (29). It was therefore concluded that TNF-α using this mechanism could positively regulate JNK pathway and mediate insulin resistance (29).

In another experiment conducted in human fat cells from insulin resistant subjects, IL-6 had long term inhibitory effects on the gene transcription of the insulin-receptor substrate (IRS)-1, GLUT-4 (30). Also TNF-α strongly increased the expression of IL-6 mRNA as well (30). Since higher levels of IL-6 have been positively correlated with increased adiposity (24), it is therefore concluded that IL-6 can trigger insulin resistance in liver (24).

Obesity is associated with the development of diseases resulting from the excessive stores of adipose tissue (20). Gnacinska et al. compared the subjects (N = 55)
with BMI > 25 kg/m² to those of normal BMI (18.0 – 24.9 kg/m²) (N = 23) and found that the overweight/obese group had a significantly higher concentration of IL-6 than the normal BMI group (P = 0.03) (20). Plasma concentrations of IL-6 were significantly and positively correlated with waist circumference (r = +0.38, P ≤ 0.0001) and BMI (r = +0.36, P ≤ 0.0001) of the overweight BMI group (20). Even though TNF-α was not significantly different in both the groups, it was about 15% higher in the overweight group (20).

In a study, overweight or obese subjects (with or without metabolic syndrome) were analyzed for the levels of TNF-α and IL-6. Subjects with metabolic syndrome found to have significantly higher concentrations of IL-6 (P = 0.012) when compared to (17). Interestingly though, there was no significant difference between the two groups in the levels of TNF-α (17).

The main role of TNF-α on insulin action in human subcutaneous adipose tissue was investigated in 42 obese women (BMI 39 ± 19 kg/m²) was analyzed (31). Lofgren et al. found a strong negative correlation between adipose tissue secretion of TNF-α and maximum insulin stimulated glucose transport in adipocytes. This was independent of fat cell volume, age, and BMI (r = -0.58, P < 0.001) (31). Therefore it was concluded that TNF-α released from adipose tissue may have a role in insulin resistance in obese women (31).

Studies of non-pregnant obese individuals have indicated that adipose tissue can recruit macrophages and promote inflammation, undergo necrosis, and express high levels of pro-inflammatory cytokines, including TNF-α and IL-6 (32). Therefore, research suggests a role for increased adiposity in inflammation (33, 34). Interest in
vitamin D is increasing because research suggests that it might be related to obesity and inflammation.

**Pregnancy and the Effects of Markers of Inflammation (TNF-α and IL-6)**

Inflammation during pregnancy leads to an increased synthesis of pro-inflammatory cytokines (IL-6 and TNF-α) (35). There are two different kinds of T helper cells (Th) that are responsible for cytokine secretion (36). They are the Th1 and Th2 cells (36). Th1 cells support cell-mediated immunity and they are responsible for inflammation and cytotoxicity; whereas Th2 cells synthesize cytokines inducing humoral immunity and down regulate the inflammatory actions of Th1 cells and provide a balance to the environment (36). Th1 cells secrete mainly TNF-α and promote strong cell-mediated responses. It is also a significant fact that Th1 mediated inflammatory response is predominant during late gestation (35). This may be the key factor in initiating an intense cascade of inflammatory cytokine production leading to adverse pregnancy outcomes (35). Th2-cells produce IL-10, IL-13, and granulocyte macrophage colony stimulating factor (GM-CSF) (35). Therefore, Th1 mediated pro-inflammatory response increases the levels of inflammatory prostaglandins which is one of the factors responsible for softening and dilation of the cervix (35). The release of prostaglandins also leads to the weakening and the rupture of the membranes (35) and this enhances myometrial contractility (35). These inflammatory markers are similar in the functioning of the hormone oxytocin and positively regulate the untimely production of prostaglandins and oxytocin receptor and stimulate uterine contraction (35).

Human pregnancy could be characterized as a period of significant metabolic changes. There are specific changes in carbohydrate and lipid metabolism which is
mainly to provide a continuous supply of nutrients to the growing fetus (21). Early stage
of pregnancy is considered an anabolic condition as there is an increase in maternal fat
stores and the reduction in free fatty acid concentration (21). Also, during early trimester,
insulin secretion increases and status of insulin sensitivity may vary accordingly (21).
Insulin loses its ability to block lipolysis in late pregnancy and hence contributes to
greater increases in free fatty acids (FFAs), increased hepatic glucose production (21). As
discussed earlier, this as a result leads to severe insulin resistance (21). Therefore, in late
pregnancy, maternal adipose tissue stores are broken down, additionally postprandial free
fatty acid (FFA) levels increase and as a result insulin-mediated glucose uptake is
reduced by 40–60% compared with pre-pregnancy (21).

Normal pregnancy could be considered as a “diabetogenic state” because of
various factors such as a ~50% decrease in insulin-mediated glucose clearance action,
and a 200–250% increase in insulin secretion to maintain euglycemia in the pregnant
women (21). Therefore, insulin resistance becomes an evident characteristic of normal
pregnancy and is further increased in the third trimester as the fetus grows (37). The
notable fact is that the levels of substances such as TNF-α, triglycerides, and LDL-c (low
density lipoprotein cholesterol) increase during normal pregnancy when insulin resistance
is elevated (37). These elevations could also be a result of the hormonal and metabolic
changes, such as elevations in levels of placental lactogen, progesterone, cortisol, and
estradiol (37).

In particular, late pregnancy is specifically characterized as a period of insulin
resistance in all species (21). Also, pregnancy shows a large shift in maternal metabolism,
which enables the provision of appropriate nutrients to the developing fetus as well as
providing for physiological maintenance of the mother and preparation for lactation (21).

Placental-derived substances are believed to be a major factor in reprogramming maternal physiology to achieve an insulin-resistant state (21).

Cytokines are not only synthesized by cells of the immune system and macrophages in response to any foreign stimulus (38). In addition, the mother’s utero-placental tissues regulate cytokine production as well (38). It is therefore understood that maternal and placental adipose tissue both contribute to the release of these inflammatory molecules and this in turn is markedly associated with obesity and insulin resistance (39). Most placental cytokines are similar to the ones secreted by adipose tissue (38).

Cytokines were later identified to be secreted by the Hofbauer cells which are considered as the placental macrophages, as well as by the syncytiotrophoblast cells in placenta (38). Even in this environment the pro-inflammatory factors such as TNF-α and IL-6, negatively regulate glucose and lipid metabolism and inhibit insulin action in insulin sensitive tissues (38). It has been demonstrated that macrophages originate from fetal hematopoietic cells as they were later detected in the placenta during early developmental stages (16). They perform the similar function of secreting pro-inflammatory cytokines and immunoregulatory molecules (16).

The interplay between maternal and placental tissues elicits an interesting immunological response (40). Macrophages are abundant in the decidua and their numbers remain constant throughout gestation in normal pregnancy (40). They are secreted into the decidua by both stromal cells and trophoblast cells, where they assist in various functions in decidual homeostasis and placental development (40). Abnormal behavior of these macrophages can affect placental development, potentially leading to
increased secretion of pro-inflammatory cytokines causing adverse pregnancy outcomes ranging from pre-eclampsia to fetal demise (40).

Production and distribution of cytokines during pregnancy varies with gestational age and may depend on the specific function of each cytokine (15). Several reports suggest that concentrations of inflammatory cytokines increase with increasing gestational age (15). At term, mRNA for TNF-α and IL-6 were both identified in trophoblast and decidual tissues. This corroborates that these cytokines were secreted in the placental tissues (15). To be particular, IL-6 was found in cytotrophoblast and endothelial cells (21) (38). IL-6 gets released into the fetal and maternal systemic circulation and exerts endocrine action by acting in places away from the source of production (21). This could be a potential mechanism by which the placental inflammatory response stimulates increased availability of fatty acids for fetal fat stores, in addition to increased maternal supply (21).

The placenta responds to a variety of inflammatory stimuli (38). Many signaling molecules responsible for the stimulation of inflammatory response have been identified in placental cells (38). Cytokine production is significantly affected by several immunological regulators such as lipopolysaccharide (LPS) (25). This study investigated the effect of stimulation on the release of cytokines from placental and maternal adipose tissue obtained from 22 pregnant women (10 normal subjects and 12 with gestational diabetes mellitus (GDM)) with singleton infants delivered at ≥37 wk gestation by elective cesarean section (25). Placenta and adipose tissue, were incubated either in the absence (control), presence of lipopolysaccharide (LPS 10 µg/ml), TNF-α (10ng/ml), or IL-6 (10 ng/ml) (25). Interesting fact is that there was no difference in the release of TNF-α and
IL-6 from placenta and adipose tissue obtained from normal pregnant women and women with GDM in the control group with no stimulation (25). However, LPS and TNF-α stimulation of placenta and adipose tissue resulted in greater release of IL-6. LPS-stimulation alone significantly increased TNF-α in placenta and adipose tissue (25).

Altinova et al. investigated the levels of TNF-α in 34 pregnant women with GDM and 31 pregnant women with normal glucose tolerance (NGT) with pre-pregnancy BMI of 26.7 ± 3.3, 25.4 ± 4.2 (kg/m²) respectively at the beginning of the 3rd trimester (41). Even though the serum TNF-a levels were significantly elevated in GDM when compared to NGT (P = 0.042), there was a significant positive correlation between TNF-a levels and pre-pregnancy BMI, as well as the current BMI in GDM as well as in NGT (r = +0.37, P = 0.04) (41). This suggests that heavier body weight is positively associated with TNF-α levels irrespective of the presence of GDM in pregnant women (41). The only limitation in this study is that current BMI is not a standard procedure to measure body composition in pregnant women.

From early developmental stages onward, the secretory activity of placental cells clearly contributes to increased local as well as systemic levels of cytokines and inflammatory molecules (38). It is believed that the placental cytokines released in the maternal systemic circulation contribute to maternal metabolic changes which culminate during the third trimester of pregnancy to accommodate increased energy needs of the fetus (38). As discussed earlier, TNF-α has a direct negative effect on components of the insulin signaling and induces the release of increased amounts of free fatty acids via stimulation of lipolysis (25). Therefore TNF-α has been linked to insulin resistance and diabetes mellitus (DM) (41).
Maternal Obesity and Markers of Inflammation (TNF-α and IL-6)

Obesity is an increasingly common condition amongst both sexes, but even more in pregnant women. It is estimated that more than one in five pregnant women are obese (42, 43). Maternal obesity is associated with increased morbidity and mortality for both mother and offspring (44). Inflammation is an imbalance between pro and anti-inflammatory cytokines tilting towards pro-inflammatory cytokines leading to the development of diseases (44). Excess adipose tissue in obese population acts as an endocrine and inflammatory organ (44). The expansion of adipose tissue is associated with inflammation, and there is evidence that this inflammation is causally linked both to insulin resistance and to other obesity related morbidities such as cardiovascular disease (44).

Pregnancy is considered as a natural inflammatory condition leading to the stimulation of maternal leucocytes and increased systemic concentration of pro-inflammatory cytokines (16). The physiological inflammatory state may be involved in mediating adverse clinical outcomes during pregnancy, especially if the pregnant women develop overweight, obesity, diabetes and/or pre-eclampsia (44) and returns to baseline levels after delivery (16). Obese women are also more likely to have pregnancy-induced hypertension, neural tube defects, and macrosomia (45). More specifically, obesity in late pregnancy is associated with conditions such as chronic hypertension, gestational and pregestational diabetes, intrauterine fetal death, urinary tract infection, thromboembolism post-date delivery, asthma, obstructive sleep apnea, and gallbladder disease (27) (45).

Population-based studies based on maternal obesity have identified complications of increased risk of antenatal, intrapartum, and postpartum complications, including pre-
eclampsia, cesarean delivery, shoulder dystocia, and neonatal intensive care unit admissions (46-49). The complications of obesity in pregnancy may lead to lifelong maternal as well as neonatal complications such as cardiovascular disease (50, 51). Consequently, the fetus of an overweight mother also has an increased risk of developing insulin resistance which may lead to abnormal blood glucose and fetal macrosomia. Fetal macrosomia is usually defined as a birth weight of 4000 g or more, regardless of gestational age (52). It is suggested that maternal hyperglycemia may contribute to fetal macrosomia (52). This may increase the risks of obstetric complications, and in some cases, may increase the risk of stillbirth (53, 45).

It was shown in a population based cohort study that the risk of preterm birth was lowered by ~10% in overweight (RR = 0.89, CI 95%) and obese women (RR = 0.90, 95% CI) (54). Overweight (RR = 1.17, 95% CI) and obese (RR = 1.35, 95% CI) women had significantly higher risk of post-term birth compared to normal women (54). The risk of fetal macrosomia and operative delivery increased with BMI. Morbidly obese women (BMI > 40 kg/m²) were at greatest risk of both macrosomia (RR = 4.78, 95% CI) and caesarean section (RR = 1.66, 95% CI) (54). A study of obese pregnant women reported that women with class III obesity (BMI > 40 kg/m²) experienced higher rate of cesarean delivery and large for gestational age (LGA) births, than women with class I (BMI 30.0-34.9 kg/m²) or class II (35.0-39.9 kg/m²) obesity (55).

The significance of macrophages in obese pregnancy related inflammation is depicted in this research study group where Challier et al. examined the accumulation of macrophages along with the increased secretion of pro-inflammatory cytokines in the placenta of lean and obese women (16). Significantly increased concentrations of IL-6
were found in the plasma of obese women compared to lean women (P < 0.05) (16). It was also observed that the chronic inflammatory condition of pre-pregnancy obesity strongly affects the fetus by the increased levels of macrophage accumulation and eventually higher levels of pro-inflammatory cytokine expression (TNF-α and IL-6) in the placenta (16). Therefore it was proposed that this inflammatory condition in which the fetus grows may have critical risks of obesity and cardiovascular disease (16).

It is considered that insulin resistance induces inflammation and cytokine secretion (53). Pro-inflammatory cytokine, TNF-α is known to be produced in the placenta may have higher levels in obese pregnant women (53). In this research study, longitudinal changes of levels of TNF-α were observed in lean, obese (NGT), and obese (GDM) during late pregnancy (53). It was identified that the plasma concentrations of maternal TNF-α and fat mass were significantly increased in late pregnancy in all the three groups (P < 0.001) (53). It was reported that 94% of in-vitro measured placental TNF-α was released into the maternal circulation and 6% was released to the fetal side (53). During late pregnancy, the plasma concentrations of TNF-α (pg/ml) were found to be very similar between obese (NGT) women (2.80±0.72) and obese (GDM) women (2.84±0.17) (53). Overall, TNF-α was found to be negatively correlated with insulin sensitivity (r = -0.58, P < 0.02) (53). And therefore TNF-α was identified as a significant predictor of insulin resistance during pregnancy (53).

The placenta may work together with the adipose tissue during normal pregnancy to produce an inflammatory response and this integration becomes severe in maternal obesity and further enhances the secretion of pro-inflammatory cytokines. It is believed that the abnormal metabolic environment generates stimuli within the adipose cell to first
increase the production of inflammatory cytokines (38). In pregnancy complicated with obesity or diabetes mellitus, continuous adverse stimulus is associated with dysregulation of metabolic, vascular and inflammatory pathways supported by increased circulating concentration of pro-inflammatory cytokines such as IL-6 and TNF-α (38, 39).

In a study, maternal TNF-α concentrations were found to have a strongest correlation with the changes in maternal insulin resistance when measured by euglycemic clamps (39). It was noted that TNF-α and IL-6, inversely influence glucose and lipid metabolism and inhibit insulin action in insulin sensitive tissues (38). TNF-α in the adipose tissue may also function in a paracrine fashion to increase the secretion of IL-6 that has been correlated to increased insulin resistance and obesity (39).

In a study a total of 19 women with GDM and 19 BMI-matched healthy pregnant women in the third trimester of pregnancy were studied to measure insulin sensitivity and insulin secretion, and TNF-α (56). Of the circulating factors, TNF-α correlated inversely with insulin secretion in pregnancy ($r = -0.35$, $P = 0.03$) and was significantly higher in the GDM group ($2.62 \pm 0.3$ vs $1.88 \pm 0.3$ pg/mL, $p = 0.01$) (56). Therefore it is established that TNF-α may exert an inhibitory effect on insulin secretion in GDM, contributing to the associated hyperglycaemia (56). The only limitation in this study was that even though they matched the subjects based on BMI, both GDM [mean BMI 31.6 ± 1.3 (range 24.8–43.0)] and healthy [mean BMI 31.5 ± 1.3 (range 24.2–41.6)] groups had a wide range of BMI and they were not grouped according to the Centers for Disease Control and Prevention (CDC) stated BMI categories.

In a study, 47 healthy women (24 lean pregnant women and 23 obese pregnant women) in the third trimester of pregnancy were observed for the differences in the
inflammatory parameters. It was shown that plasma concentrations of IL-6 was significantly elevated in obese pregnant women ($P = 0.003$) (17). In a research study the subjects were grouped based on the total neonatal fat mass outcome such as T1 (309 ±25 g), T2 (478 ±40 g), and T3 (529 ±39 g) (39). The maternal plasma IL-6 (pg/ml) were significantly elevated in T3 subjects and T2 subjects ($P < 0.05$) than in the T1 subjects (39). This shows a link between heavier fetal fat mass and pro-inflammatory cytokine, IL-6.

Therefore, it has been well illustrated in various studies that overweight and obese women have elevated serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α (57). Maternal and placental hormones regulate the production of inflammatory cytokines. Maternal obesity is associated with elevated placental pro-inflammatory cytokine release. There is also substantial evidence that IL-6 and TNF-α induced cytokines may threaten pregnancy outcome (58, 59). It has been analyzed that TNF-α induces expression of itself and IL-6 which are all pro-inflammatory and these may threaten pregnancy outcome (59). There is a need to alleviate the pro-inflammatory effect of these cytokines in overweight and obese pregnant women.

**Relationship between Vitamin D, Maternal Obesity, and Markers of Inflammation (TNF-α and IL-6)**

Obesity is a primary risk factor for vitamin D deficiency, but this relationship has not been analyzed in pregnant women, who have to maintain their own vitamin D levels as well as those of their fetuses (10). Obesity or the presence of increased amounts of visceral fat is found to have lower vitamin D concentrations because vitamin D might get absorbed by fat-tissue and not get released in the blood stream (60). Obesity does not
affect the skin’s ability to synthesize vitamin D, but larger amounts of subcutaneous fat sequester more of the vitamin and alter its release into the circulation (61). Additionally, low prenatal vitamin D status increases susceptibility to obesity related diseases in later life via specific target organ effects and/or through changes to the developing immune system (10). Maternal vitamin D supplementation during pregnancy could be an important public health measure to decrease risk of a range of chronic diseases (10).

Serum concentrations of 25-OHD were measured at 4-21 wk gestation and pre-delivery in 200 Caucasian and 200 African American pregnant women. Women were either classified as vitamin D deficient (25-OHD <37.5 nmol/L), insufficient (25-OHD 37.5-80 nmol/L), or sufficient (25-OHD>80 nmol/L) (62). At delivery, vitamin D deficiency and insufficiency were observed in 29.2% and 54.1% of African American women respectively. And 5% and 42.1% of Caucasian women were vitamin D deficient and insufficient, respectively (62). This was also prevalent in mothers consuming prenatal vitamins. Accordingly, it was concluded that higher dosage of vitamin D supplementation is needed to improve vitamin D status in this population (62).

Obese pregnant women had lower mean serum 25-OHD concentrations at 4-22 wk gestation when compared to normal pregnant women (56.5 vs. 62.7 nmol/L, P < 0.05) and a therefore higher prevalence of vitamin D deficiency was observed in obese population (P < 0.01) (10). It was also shown that as the BMI increased from an average of 22 to 34, there was an increase in the odds of mid-pregnancy vitamin D deficiency (95% CI: 1.2, 3.6) (10). It was later analyzed that the vitamin D status of neonates born to the obese mothers was lower than neonates of lean mothers (50.1 vs. 56.3 nmol/L; P < 0.05) (10).
The role of 1,25-OHD is primarily affected by the presence of the vitamin D receptor (VDR) (7). It has been studied that VDR is found in significantly high levels in T cells, monocytes, and macrophages (7). The enzyme, 25-OHD-1-alpha-hydroxylase is responsible for the final hydroxylation step in the synthesis of active vitamin D (7). This enzyme is found to be expressed by activated macrophages, allowing these phagocytic cells to synthesize and secrete 1,25-OHD in a regulated fashion (7). All of these findings suggest an important role for vitamin D in the modulation of immune system (7).

It was studied in seven patients with 25-OHD insufficiency (25-OHD <25 ng/ml) to observe if cholecalciferol supplementation alters calcitriol-responsive monocyte proteins and decreases serum levels of inflammatory cytokines (63). It was reported that there was a four-fold increase in serum 25-OHD levels and a threefold increase in monocyte vitamin D receptor expression in cholecalciferol therapy (63). There was also a 30% reduction in IL-6 levels (P = 0.05), and a 60% decrease in TNF-α levels (P<0.05) after cholecalciferol therapy (63).

In an observational study, 69 healthy women were recruited and randomized into high UVB exposure and low UVB exposure groups (7). Women with a high UVB exposure (High vit-D) had significantly higher serum 25-OHD concentrations (P < 0.0001) than women exposed to the lower levels of UVB (Lo-vit D) (7). Markers of inflammation such as TNF-a, and IL-6 were analyzed (7). Mean serum TNF-α was significantly low in the Hi-vit D than the Lo-vit D women (1.22 ± 0.11 vs. 0.79 ± 0.11, P = 0.02) (7). IL-6 did not significantly differ between groups (7).

Vitamin D directly and indirectly regulates the dendritic cells (64). Dendritic cells are professional antigen-presenting cells (APC) that have essential role in the initiation
and maintenance of T-cell-dependent immune responses (64). In vivo 1,25-OHD has a direct immunosuppressive effect on dendritic cells, monocytes, and macrophages. It inhibits dendritic cell maturation. It reduces cytokine production of TNF-α in monocytes and macrophages and reduces IL-12-mediated Th1 response (64). Therefore, Systemic administration of calcitriol elicits strong immunosuppressive effects, attenuating the production of pro-inflammatory cytokines (64).

Evans et al. used primary cultures of human decidual cells from first and third trimester pregnancies to demonstrate expression and activity of the enzyme that catalyzes synthesis of 1,25-OHD, 1alpha-hydroxylase (65). The synthesis of 1,25-OHD was significantly higher in first trimester decidual cells than in third trimester cells (P < 0.05) (65). Furthermore, Decidual Natural Killer cells treated with 1,25-OHD for 28 h showed decreased synthesis of cytokines such as TNF-α and IL-6 (65). The only disadvantage observed in this study is that the human decidual cells are able to synthesize 1,25-OHD particularly in early gestation and not during the third trimester.

In a clinical study, associations between monocyte/macrophages, 1,25-OHD, and TNF-α levels were analyzed in 18 women with normal pregnancies in their third trimesters (66). It was therefore reported that higher levels of 1,25-OHD was found to directly suppress TNF-α via the functioning of monocytes/macrophages (66). Therefore, the researchers demonstrate that by reducing the levels of TNF-α, vitamin D could play a major role in reducing inflammation and insulin resistance.

In a study, forty-four obese women (BMI 36.7±4.9kg/m²) and 25 controls (BMI 22.9±1.5 kg/m²) were examined (67). It was observed that the serum 25-OHD levels were significantly lower in obese compared with control subjects (P< 0.001) (67). This
emphasizes the fact that vitamin D levels need to be increased to improve the pregnancy outcome in obese women.

**Purpose and Null Hypotheses**

Balance between pro and anti-inflammatory status in obese pregnant is important for the health of the mother and fetus. The purpose of this sub-study was to evaluate the relationship between vitamin D intake and pro-inflammatory cytokines (TNF-α and IL-6) in overweight and obese pregnant women in the third trimester.

The following null hypotheses will be measured.

1. There is no relationship between vitamin D intake and pre-pregnancy BMI in pregnant women in their third trimester

2. There is no relationship between pre-pregnancy BMI and markers of inflammation (TNF-α and IL-6)

3. There is no relationship between vitamin D intake and markers of inflammation (TNF-α and IL-6)

4. There is no interactive effect of pre-pregnancy BMI and vitamin D intake on markers of inflammation (TNF-α and IL-6) in overweight and obese pregnant women.

5. There is no difference in TNF-α and IL-6 levels in women with high versus low vitamin D intakes among the subject population.
Methods

This is a cross-sectional sub-study, is part of a larger intervention study, “DHA, Inflammation, and Insulin Sensitivity in Obese Pregnant Women” (NIH, R21 HL093532-0231, PI Dr. Debra Krummel).

Inclusion and Exclusion Criteria

Inclusion criteria of the sub-study are pregnant women of all races with BMI $\geq 25$ kg/m², between the ages of 18-40 years, singleton pregnancy, 26-27 weeks of gestation, English speaking. Exclusion criteria of the study include concurrent inflammatory, vascular or metabolic disease; current or previous use of tobacco, street drugs, or medications such as corticosteroids that affect inflammatory markers; and inability to travel to the GCRC (General Clinical Research Center) for study visits. Forty-eight subjects were eligible and had complete data for the sub-study.

Study Procedures

All the study procedures were approved by the IRB at the University of Cincinnati and Cincinnati Children’s Hospital Medical Center. All the research study coordinators completed the yearly CITI (Collaborative Institutional Training Initiative) training procedure prior to the beginning of the study.

Subject Recruitment and Screening

Subjects were recruited from the greater Cincinnati area by placing fliers and postcards in physician’s offices and clinics. They were also placed at participating hospitals, health care clinics, and universities such as the University Hospital, the Christ Hospital, Mercy Fairfield, Elm Street Clinic, and University of Cincinnati, East Campus) and WIC clinics around Cincinnati; through the Children’s Hospital Research Study
Boards; through the use of magazine ads; and through the study’s website. Screening of eligible subjects is performed through phone/web site screening. Individuals who meet the screening criteria were invited to participate in the study, and were scheduled for their first study visit. This research was supported in part by the office of Research in Women’s Health, USPHS Grant #UL1 RR026314 from the National Center for Research Resources, NIH and NIH, R21 HL093532-0231.

**Study Visit 1**

Data for this sub-study were taken from the study subjects who completed the first study visit of the larger DHA supplementation research study.

**Data Collection**

**Pre-pregnancy BMI**

The pre-pregnancy BMI was used to classify pregnant women into overweight and obese groups (68). Measures of height and pre-pregnancy weight obtained during the study visit 1 were used to calculate the body mass index (BMI) by the study coordinators using the website as given here: [http://www.nhlbisupport.com/bmi/bmicalc.htm](http://www.nhlbisupport.com/bmi/bmicalc.htm). The criteria of overweight = 25-29.9 kg/m² and obesity = ≥ 30 kg/m² were used to classify the pregnant women into overweight and obese groups.

**Dietary Recall Procedure**

A nutritional professional conducted a nutrition analysis (24-hour diet recall and survey) during the study visit 1 at GCRC. The training manual from the training workshop at the University of Minnesota School of Public Health Nutrition Coordinating Center (NCC), and the Nutrition Data Systems for Research software (for dietary data analysis) was used to train research study staff in dietary recall collection procedures. The
tutorial provided by the makers of the NDSR software was completed by the members of the research team who will be conducting dietary interviews. Standardized recall menus were given to the research team to enter into NDSR and checked for accuracy by the study coordinator.

The participants each received a Food Portion Estimating Tool Book to help assist them in showing the appropriate size and portion of food they consumed. The book consisted of pictures of common meats and foods, along with serving spoons, glasses, measuring cups, bowls, and other shapes to help accurately decide how much each individual consumed. The 24 hour recall uses the multiple pass method while interviewing the subjects. The multiple pass method consists of four steps: briefly list the foods, review the list, query on brand, amount, preparation, and read back to the participant. At the first step, the subject will just briefly list the foods they consumed the previous 24 hours. The study staff will put the information into the NDSR computer program as a quick list. The second step would be for the study staff to review the list with the subject. The third step is to have the subject tell us the amount and type of the food they consumed and how they prepared their meals. The last step is to review the information one more time to make sure the subject did not forget anything they have eaten. The multiple pass method is a great way to ensure that the participant does not forget any of the foods they have consumed in the previous 24 hours.

**Dietary Vitamin D Measures**

The dietary vitamin D measures were analyzed using the nutrient per food reports option available in the NDSR for each participant of the study. This allows the NDSR to list all the foods the subject consumed along with the amount of vitamin D in each food.
The report consists of the total dietary vitamin D (mcg) per meal (breakfast, lunch, mid-day snack, dinner etc). Finally at the end of the list, the total dietary vitamin D for the day is provided. The total dietary vitamin D for the day data is collected for all the subjects included in the study and further used for calculating the total vitamin D intake.

**Supplemental Vitamin D Measures**

The information on the kind of vitamin/mineral supplement (prenatal, regular, specific, or children’s), brand name, and the serving size were obtained from each subject during the diet recall. After collecting the supplement information, the amount of vitamin D was obtained from the nutrient label of each supplement bottle given by the subjects (generic prenatal vitamins from Meijer, Foltab prenatal vitamin from Walgreens) from the local pharmacy stores such as Meijer, Walgreens, Kroger, and CVS.

**Total vitamin D Intake Measurement**

The supplemental vitamin D amounts were recorded from the supplement labels. The amounts were given in International Units (IU) format. The amounts were converted to micrograms (mcg) (400IU=10 mcg). The dietary and supplemental vitamin D levels were added together to obtain the total vitamin D intake for one day of all the subjects included in the study.

**Markers of Inflammation (TNF-α and IL-6)**

At the first study visit, 2 mL of fasting venous blood sample was taken in a lavender (EDTA) tube. The samples were gently inverted three to five times to allow the anti-coagulant to thoroughly mix with the blood. Then the samples were allowed to sit upright at room temperature for 10-15 minutes. The tubes were centrifuged at 3500 rpm
for 10 -15 minutes. Then plasma from the cell pellet sample was carefully pipetted. A small amount (0.5 mL) of plasma was aliquoted into tall plastic freezer tubes. Tubes were labeled with patient ID number, study visit number (1), date and time of specimen collection, and “cytokines- lab”. High sensitivity assay of IL-6, and TNF-α were performed and measured in the Research Lab at S location, CCHMC.

Cytokines (TNF-α and IL-6) concentrations in the sample supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using MilliplexTM Multiplex kits (Millipore, Billerica, MA) according to manufacturer’s protocol. Briefly, in a 96 well multiscreen filter plate, 25µL sample in duplicate was incubated with 25µL antibody coated beads overnight at 4°C on a plate shaker. Plates were then washed 2 times on a vacuum apparatus and 25µL of secondary antibody was added and incubated at room temperature for 1 hour on while shaking. Finally, 25µL of strept-avidin-RPE was added directly to the secondary antibody and incubated for 30 minutes at room temperature with shaking. Plates were then washed 2 more times and 150µL of sheath fluid was added. Plates were shaken for 5 minutes and then read using luminex technology on the Bio-PlexTM (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml. The cytokine analysis was conducted by the Cytokine and Mediator Measurement Core laboratory run by Dr. Marsha Wills-Karp.

Statistical Analyses

The distributions of all variables were tested with the use of Kolmogorov-Smirnov tests. Tests of normality (Kolmogorov-Smirnov) indicated significant results for IL-6, which suggests violation of the assumption of normality. Therefore, we used log-
transformed values to normalize the data. Descriptive statistics were calculated for the entire sample of N=48. Mean differences between experimental groups (overweight and obese groups) were determined by Independent samples t-test and mean differences between experimental groups (low and high vitamin D intake groups) were determined by one-way ANOVA procedures. The relationship between pre-pregnancy BMI, dietary vitamin D, total vitamin D, TNF-α and IL-6 were assessed using bivariate correlation analyses. Two-way ANOVA was used to determine the interaction effect of pre-pregnancy BMI and vitamin D (diet and total) on the markers of inflammation. The main effects included pre-pregnancy BMI (overweight and obese groups), dietary vitamin D intake (low dietary vitamin D intake group = <5mcg/day, high dietary vitamin D intake group=≥5mcg/day), and total vitamin D intake (low total vitamin D intake group= <50th percentile of intake, high total vitamin D intake group = >50th percentile of intake). The log-transformed IL-6 (IL-6 Ln) was used to perform the two-way ANOVA analysis. The non-parametric Mann-Whitney U test was used to analyze the difference in levels of TNF-α and IL-6 in high versus low total vitamin D intake groups. Significance levels were set at 5%. Data was analyzed using Statistical Package for the Social Sciences (version 17.0, 2008, SPSS, Inc, Chicago, IL).

**Results**

Data are reported for 48 pregnant women whose data were collected.

**Table 1** The clinical characteristics of study sample

<table>
<thead>
<tr>
<th>Study visit 1</th>
<th>Total (N = 48)</th>
<th>BMI 25 - 29.9 (N = 16)</th>
<th>BMI ≥ 30 (N = 32)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td>Mean±SD</td>
<td></td>
</tr>
</tbody>
</table>
Descriptive statistics are provided in table 1. There was no significant difference in (mean±standard deviation, SD) age between the two groups. As expected, there was a significant difference in pre-pregnancy BMI between the groups (P=0.000). There was a trend observed for TNF-α between the two groups (P = 0.093).

Table 2 Vitamin D intake groups among study subjects (N = 48)

<table>
<thead>
<tr>
<th>Dietary vitamin D intake (DD) groups</th>
<th>Low DD (n = 38)</th>
<th>High DD (n = 10)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SD</td>
<td>2.19±1.55</td>
<td>6.72±1.78</td>
<td>0.000*</td>
</tr>
<tr>
<td>Total vitamin D intake (TD) groups</td>
<td>Low TD (n = 24)</td>
<td>High TD (n = 24)</td>
<td>ANOVA</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>9.52±1.97</td>
<td>14.56±1.95</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

SD Standard Deviation; P<0.05*; a - Total vitamin D intake – diet+supplement
Low DD <5mcg/day, High DD≥5mcg/day; Low TD<50th percentile of intake, High TD>50th percentile of intake

The dietary vitamin D intake revealed that there was a significant difference between the amounts of vitamin D intake among low DD (< 5mcg) and high DD (≥5mcg) groups (P=0.000). The total vitamin D intake (diet + supplements) was found to be significantly different between low TD (<50th percentile of frequency) and high TD (>50th percentile of frequency) groups (P = 0.000).

Table 3 Supplemental form of vitamin D intake (for a day)
<table>
<thead>
<tr>
<th>Supplemental vitamin D (mcg)</th>
<th>Percent</th>
<th>Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1</td>
<td>1</td>
</tr>
<tr>
<td>2.50</td>
<td>6.3</td>
<td>3</td>
</tr>
<tr>
<td>5.0</td>
<td>8.3</td>
<td>4</td>
</tr>
<tr>
<td>10.0</td>
<td>83.0</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>48</td>
</tr>
</tbody>
</table>

**Figure 1** Percentage of pregnant women using vitamin D supplements

As indicated in the figure 1, most subjects (83%) consumed 10mcg of vitamin D supplement on the day of the 24-hour diet recall. There were around ~6% consumed who 2.50 mcg/day, ~9% of the subjects consumed 5.0 mcg/day of vitamin D via supplements and ~2% who did not consume any form of vitamin D supplements.

**Table 4** Pearson correlation analysis of pre-pregnancy BMI with vitamin D intake (diet and total) and inflammatory markers

<table>
<thead>
<tr>
<th></th>
<th>Pre-pregnancy BMI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary vitamin D intake</strong></td>
<td>-0.153</td>
</tr>
<tr>
<td><strong>Total vitamin D intake</strong></td>
<td>-0.001</td>
</tr>
</tbody>
</table>
The bivariate correlation analysis was performed to understand the relationship of pre-pregnancy BMI with vitamin D intake and inflammatory markers (Table 6). Pre-pregnancy BMI was significantly correlated to IL-6 with $r = +0.295$ ($P<0.05$).

**Table 5** Pearson correlation analysis between vitamin D intake (diet and total) and inflammatory markers

<table>
<thead>
<tr>
<th></th>
<th>Dietary vitamin D intake</th>
<th>Total vitamin D intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>-0.049</td>
<td>-0.254</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.184</td>
<td>-0.131</td>
</tr>
</tbody>
</table>

The bivariate analysis was conducted to analyze the relationship between vitamin D intake (diet and total) and TNF-α and IL-6 levels among overweight and obese pregnant women. There was a trend ($r = -0.254$, $p = 0.081$) observed between TNF-α and total vitamin D. There was no relationship between vitamin D intake and IL-6 levels.

**Table 6** Mean plasma concentrations of TNF-α (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>Pre-pregnancy BMI (25≤29.9)</th>
<th>Pre-pregnancy BMI (≥30)</th>
<th>Total (N)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BMI vitamin D interaction</td>
</tr>
<tr>
<td>Low DD group</td>
<td>4.10±1.80 (n = 12)</td>
<td>4.80±1.67 (n = 26)</td>
<td>38</td>
<td>0.083† 0.814 0.504</td>
</tr>
<tr>
<td>High DD group</td>
<td>3.53±1.32 (n = 4)</td>
<td>5.08±2.01 (n = 6)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total (N)</td>
<td>16</td>
<td>32</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

Two-way ANOVA; Values are means±SD (n); $P < 0.05$; $P$(trend)$<0.10$; Dietary vitamin D intake - DD.
The mean plasma TNF-α concentrations are shown in the table 6. There was no significant effect of pre-pregnancy BMI and dietary vitamin D on TNF-α levels. There was no significant interaction effect as well. However, there was a trend observed with P=0.083 under BMI explaining that the TNF-α did not differ in terms of low and high vitamin D levels but it may differ based on overweight and obese BMI categories.

The mean plasma TNF-α concentrations are provided in the table 7. Two-way ANOVA showed no significant effects of BMI and total vitamin D on TNF-α levels. There was no effect of interaction on the TNF-α levels. However there was a trend of P = 0.080 for an effect of vitamin D (low and high total vitamin D intake). This suggests that the TNF-α levels did not differ in terms of BMI status, but shows a possibility to differ based on total vitamin D intake.

Two-way ANOVA was performed for mean IL-6 levels and there were no significant effects of BMI and dietary vitamin D on IL-6 concentrations. There was no significant interaction effect on IL-6 concentrations.

**Table 7** Mean plasma concentrations of TNF-α (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>Pre-pregnancy BMI (25≤29.9)</th>
<th>Pre-pregnancy BMI (≥30)</th>
<th>Total (N)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BMI</td>
</tr>
<tr>
<td>Low TD group</td>
<td>4.60±1.22 (n = 7)</td>
<td>5.1±1.50 (n = 17)</td>
<td>24</td>
<td>0.129</td>
</tr>
<tr>
<td>High TD group</td>
<td>3.46±1.87 (n = 9)</td>
<td>4.4±1.89 (n = 15)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Total (N)</td>
<td>16</td>
<td>32</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

Two-way ANOVA; Values are means±SD (n); P < 0.05*; P(trend)<0.10†; Total vitamin D intake – TD.
Two-way ANOVA analysis resulted in no significant effects of BMI and total vitamin D on IL-6 levels. No significant interaction effects were observed on IL-6.

For all the above two-way ANOVA analyses, the Levene’s test of equality of error variances was not significant indicating that the variance of the dependent variable is equal across the groups and we have not violated the homogeneity of variances assumption.

**Difference in TNF-α and IL-6 levels in high versus low total vitamin D**

A Mann-Whitney U test was performed to identify any differences in TNF-α levels among low and high total vitamin D (TD). The test revealed no significant difference in the TNF-α levels among low total vitamin D (≤11.861 mcg) intake group (Median (Md) = 5.10 pg/ml, N = 24) and high total vitamin D (≥11.874 mcg) intake group (Md = 4.54 pg/ml, N = 24), U = 222, Z = -1.361, P = 0.174. However, the results of the test were in the expected direction (high TD group with lower mean TNF-α levels).

A Mann-Whitney U test was performed to identify any significant differences in IL-6 levels among low and high total vitamin D intake groups. The test revealed no significant difference in the IL-6 levels among low total vitamin D (≤11.861 mcg) intake group (Md = 4.725 pg/ml, N = 24) and high total vitamin D (≥11.874 mcg) intake group (Md = 5.795 pg/ml, N = 24), U = 278, Z = -0.196, P = 0.845.

**Discussion**

The present study examined the relationship between vitamin D intake and markers of inflammation (TNF-α and IL-6) in overweight and obese pregnant subjects. There were a total of 16 overweight pregnant subjects and 32 obese pregnant subjects in
the study. According to Gnacinska et al., obese or overweight patients were found to have higher concentrations of TNF-α and IL-6 when compared to the normal BMI controls (20). In another study by Kirwan et al., significant increases in TNF-α and fat mass were found in late pregnancy (P < 0.001) (53). Madan et al. analyzed the levels of pro-inflammatory cytokines such as TNF-α in a cross-sectional study to evaluate whether obesity brings any change in inflammation during the second trimester of pregnancy in 80 subjects with BMI categories (<26.5 kg/m² as normal; 26.5–31 kg/m² as overweight; >31–41 kg/m² as obese and >41 kg/m² as morbidly obese) (69). The BMI categories were adjusted to expected weight gain in early second trimester (69). There were no significant changes in the mean concentrations of TNF-α (P = 0.77) (69). In the present study, a trend was observed in TNF-α concentrations (P = 0.093) when compared between overweight and obese subjects with higher levels in obese pregnant subjects. The mean IL-6 concentrations were not significant between the two BMI groups. Larger sample size might give us the significant differences in the mean concentrations of inflammatory markers among the pregnant subjects.

Schleithoff et al. evaluated the effect of vitamin D supplementation on the cytokine profiles in heart failure patients in a double-blind randomized, placebo controlled trial (70). They assessed the dietary vitamin D intake levels among D+ (vitamin D of 50 mcg) and D- (no supplementation) groups at baseline and at the endpoint of the study (70). No significant difference was observed in median daily intake of dietary vitamin D in D+ group (1.35 mcg) and D- group (1.20 mcg) (70). In the present study, the vitamin D intake (diet and total) data of the pregnant subjects was obtained at one point (study visit 1) during the larger DHA supplementation study. The mean dietary vitamin D
intake (DD) was found to be significantly different between low (< 5mcg) and high (≥5mcg) DD groups (P=0.000). There were 38 subjects who had not met the recommended levels of vitamin D intake of 5 mcg/day by means of diet. The total vitamin D intake (diet+supplements) was assessed in our subjects. The total vitamin D intake (TD) was found to be significantly different among low (<50th percentile of frequency) and high (>50th percentile of frequency) TD groups (P=0.000).

The rate of overweight and obese adults with low vitamin D levels is increasing (Tzotzas 2010) (67). In a study by Parikh et al., 154 obese subjects were compared with those of 148 normal subjects for serum vitamin D concentrations. Serum 25-OHD and 1,25-OHD were significantly high in normal BMI subjects (P<0.0001) than in obese subjects (71). Bodnar et al. showed that the maternal pre-pregnancy obesity was associated with lower serum 25-OHD concentrations and higher odds of vitamin D deficiency (25-OHD 50 nmol/L) among mothers in mid-gestation and neonates at birth (10). It was also shown that overweight and obese women were more likely than normal women to have vitamin D deficiency at 4–22 wk (P<0.01) (10). In our study, as expected, there were no significant differences in vitamin D intake (diet and total) among overweight and obese groups (data not shown).

Vilarassa et al. analyzed the relationship of serum 25-OHD concentrations with anthropometric and adiposity parameters in 43 morbidly obese, 28 obese, and 50 normal Caucasian women (72). In their bivariate correlation analysis 25-OHD was strongly and inversely associated with BMI (r=-0.432, P=0.001) (72). In a study by Parikh et al., serum 25-OHD was strongly and inversely correlated with BMI (r = -0.4; P < 0.0001) and body fat mass (r = -0.41; P < 0.0001). Serum 1,25-OHD was also negatively correlated with
BMI (r = -0.26; P < 0.0001) and body fat mass (r = -0.25; P = 0.0001) \(^{(71)}\). They state that the apparent significant and inverse relationship between 1,25-OHD and BMI was found over an inclusion of a wide range of BMI (from 18 to 56 kg/m²) in the study sample \(^{(71)}\). In the present study, an inverse relationship between dietary and total vitamin D intake with pre-pregnancy BMI was observed. However, the relationship was not significant. The results of our study could be improved by the inclusion of serum 25-OHD and dietary variables. Inclusion of subjects with wide range of BMI would also show the strength and the direction of the relationship between pre-pregnancy BMI and vitamin D status.

Pro-inflammatory cytokine, TNF-\(\alpha\) is known to be secreted by many cell types including macrophages, monocytes, T-cells, adipocytes, and fibroblasts \(^{7}\). According to Yudkin et al., the production of IL-6 increases with adiposity and it has been suggested that a third of total circulating concentrations of IL-6 are derived from adipose tissue \(^{(73)}\). Pro inflammatory cytokine, IL-6, similar to TNF-\(\alpha\) is found to be expressed in adipose tissue \(^{(73)}\). In the present study, bivariate analysis of pre-pregnancy BMI with the inflammatory markers (TNF-\(\alpha\) and IL-6) was performed to analyze the relationship between these variables. There was a positive relationship between pre-pregnancy BMI and the inflammatory markers. The relationship between pre-pregnancy BMI and TNF-\(\alpha\) was not significant. Pre-pregnancy BMI and IL-6 were strongly and positively correlated to each other (P<0.05). Notably the sample size for this study may have been insufficient to detect a significant relationship between pre-pregnancy BMI and TNF-\(\alpha\).

Peterson et al. reported that high TNF-\(\alpha\) levels are associated with advancement of heart disease condition \(^{(7)}\). Therefore vitamin D supplementation might be a possible
adjunct to anti-TNF therapy (7). They reported significant inverse relationship between serum 25-OHD and TNF-α (P=0.0463) (7). In the present study, our bivariate correlation analysis indicated a trend for a negative relationship between total vitamin D and TNF-α levels (P=0.081). Peterson et al. also found a slight trend towards negative relationship between serum 25-OHD and IL-6 levels (P = 0.0909) (7). In the present study, there was negative relationship between vitamin D and IL-6 levels but no significance was observed. In the studies that had significant correlations between serum 25-OHD and inflammatory markers could be because they used the serum concentrations of biologically stable form of vitamin D for comparisons. It could also be a result of a larger sample size.

In the present study, a two-way ANOVA analysis was conducted to observe any significant differences and interaction effects in TNF-α concentrations by pre-pregnancy BMI or dietary vitamin D intake (DD). There was a slight trend for a possible effect on mean TNF-α levels based on pre-pregnancy BMI (P = 0.083) with higher levels of TNF-α in obese subjects. There were no significant effects on TNF-α levels based on DD or interaction (pre-pregnancy BMI and DD). This is the first study that tried to evaluate whether there are any interactive effects of pre-pregnancy BMI and vitamin D intake on the markers of inflammation (TNF-α and IL-6).

In the study conducted by Peterson et al., the mean serum TNF-α were found to be significantly lower in High-vitD subjects than in low-vitD subjects (P = 0.0200) (7). It has been suggested that vitamin D can suppress TNF-α production (7). Schleithoff et al. reported that vitamin D supplementation (D+ of 50 mcg) caused TNF-α levels to remain stable whereas the levels increased in unsupplemented group (D-) (70). According to their
study, the TNF-α levels did not differ significantly in D+ and D- groups after 9 months of supplementation (70). However, there was a 12% increase in TNF-α levels in D- group during the period of the study (70). In the present study, the mean TNF-α concentrations were found to be low in the high TD intake group than in low TD intake group with a slight trend of P = 0.080. There were no significant effects of pre-pregnancy BMI and no interaction effect of pre-pregnancy BMI and total vitamin D intake on mean TNF-α levels.

It has been reported that 1,25-OHD could inhibit the secretion of IL-6 in several cell types (7). Turk et al. showed that oral and intravenous supplementation of 1,25-OHD caused a significant decrease in IL-6 levels (P = 0.02 and P < 0.001, respectively) at the end of 6 month treatment in hemodialysis patients (74). In our study, the mean concentrations of IL-6 were not significantly different either between low and high vitamin D (diet and total) or between pre-pregnancy BMI categories. Our study could be improved by randomly assigning subjects into vitamin D supplementation and placebo groups and analyzing the relationship of oral vitamin D intake levels, serum concentrations of 25-OHD levels, and 1,25-OHD with the inflammatory markers such as TNF-α and IL-6.

The non-parametric analyses were conducted to analyze whether there are any differences in the levels of the markers of inflammation in low TD and high TD groups. Similar to the parametric analyses, there were no significant differences among the two groups in TNF-α and IL-6 levels. More clinical and randomized control studies are required to analyze the effects of vitamin D on TNF-α and IL-6.
The strengths of the study were that the dietary data was obtained from each subject via a 24hr dietary recall and dietary vitamin D levels were assessed using Nutrition Data System for Research (NDSR). The supplemental vitamin D intake was obtained by dietary recall procedure from which the total vitamin D levels (diet+supplements) were calculated. Cytokines (TNF-α and IL-6) were assessed by high sensitivity assays using MilliplexTM Multiplex kits (Millipore, Billerica, MA). The study sample with overweight and obese pregnant subjects was one of the major strengths of the study.

The limitations of the study were that the analysis of the relationship between vitamin D and inflammation depended on the oral vitamin D intake alone. The inclusion of serum measures of 25-OHD would improve the results of the study. Inclusion of normal BMI pregnant women may improve the sample size of the study.

**Conclusion**

There was no significant relationship between pre-pregnancy BMI and vitamin D intake. However, there was a non-significant inverse relationship of pre-pregnancy BMI with dietary and total vitamin D intake. There was no relationship between pre-pregnancy BMI and TNF-α. There was a positive significant relationship between pre-pregnancy BMI and IL-6. There was no significant relationship between vitamin D intake (diet and total) and pro-inflammatory cytokines (TNF-α and IL-6). However, there was a trend with a relationship between TNF-α and total vitamin D intake. There were no significant effects (pre-pregnancy, vitamin D intake, and interaction) on the markers of inflammation (TNF-α and IL-6).
For future studies, the relationship between vitamin D and pre-pregnancy BMI could be further investigated using overweight, and obese pregnant subjects with a larger sample size. The relationship between vitamin D and inflammatory markers should be investigated using dietary and serum 25-OHD in pregnant subjects in their third trimester. The analysis of the interaction effects of pre-pregnancy BMI and serum 25-OHD on the inflammatory markers would prove to be beneficial in understanding the relationship between vitamin D and inflammation during the third trimester of pregnancy.
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