Vitamin deficiency, infection and cytokine interactions in pregnant Panamanian women: impact on cortisol - IGF-1- fundal height pathway

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ABSTRACT

In developing countries, multiple micronutrient deficiencies and multiple infections co-occur during pregnancy, and may have adverse consequences on fetal growth outcome. The main objective of this study was to examine the impact of vitamin deficiencies, infection and cytokines on fetal growth. A previous cross-sectional study of pregnant indigenous women (n=184) from the Ngäbe-Buglé Comarca of western Panama provided data on maternal anthropometry, infections (severity of trichomoniasis, dental caries, vaginal candidiasis, bacterial vaginosis; presence of gonococcal, urinary tract, scabies and respiratory tract), and micronutrient status (vitamins A, D, B12 and folate). For the present study, remaining maternal serum was analyzed using Luminex for cortisol, IGF-1 and select cytokines. Fundal height was used as the indicator for fetal growth. Data was analyzed by t-tests and multiple regression analyses while controlling for gestational age. Through our analysis we found that none of the eight infections measured in this study were associated with fundal height, and only one of the four vitamins, vitamin D, was associated with fundal height, although negatively. In addition the T-regulatory cytokine IL-10 was also associated with fundal height and again this association was negative. Counter to what we had expected our regression model for fundal height show that it is negatively influenced by both vitamin D and IL-10, but not by infections.

ABRÉGÉ

Dans les pays en voie de développement, on constate qu'il y a présence de carences en micronutriments et de co-infections multiples lors de la grossesse. Il en résulte des conséquences négatives sur le développement du foetus. L'objet principal de l'étude est d'examiner l'impact des carences en vitamines, des infections et des cytokines sur le développement du foetus. Une étude transversale réalisée antérieurement auprès de femmes indigènes enceintes (n=184) de la comarca Ngöbe-Buglé dans l'ouest du Panama a recueilli des données, utilisées dans la présente étude, sur l'anthropométrie maternelle; les infections, dont la trichomonase, les caries dentaires, la candidose vaginale, la vaginose bactérienne; la gonorrhée, les infections des voies urinaires et respiratoires, la gale, de même que les niveaux de micronutriments, soit la vitamine A, D et B12 et le folate. Lors de la présente étude, le sérum maternel a été analysé avec la méthode Luminex afin de détecter les niveaux de cortisol, d'IGF-1 et de certaines cytokines. La hauteur utérine sert d'indicateur pour le développement du foetus, en fonction de l'âge gestationnel. Les tests T et l'analyse de régression multiple représentent les deux méthodes d'analyse des données dans cette étude. Grâce à cette analyse, aucun lien n'a été établi entre les huit infections susmentionnées et la hauteur utérine, et seulement une des quatre vitamines - la vitamine D - est liée à la hauteur utérine, par une association négative. Par ailleurs, une association négative est établie entre les lymphocytes T régulateurs producteurs d'IL-10 et la hauteur utérine. Contrairement aux hypothèses, les données démontrent une association négative entre la hauteur utérine et la vitamine D et l'IL-10, et non pas les infections.

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LIST OF ABBREVIATIONS

11β-HSD2 11β-hydroxysteroid dehydrogenase type 2
BV Bacterial vaginosis
CRP C-reactive protein
GH Growth hormone
GI Gastrointestinal
hPL Human placental lactogen
HPLC High Performance Liquid Chromatography
IFN- γ Interferon gamma
IGF-1 Insulin-like growth factor 1
IL Interleukin
IUGR Intrauterine growth restriction
LBW Low birth weight
LGA Large for gestational age
MCP-1 Monocyte chemoattractant protein 1
MINSA Ministry of Health
OR Odds ratio
PGE_2 Prostaglandin E_2
RR Relative Risk
RHC Regional Health Center
SD Standard deviation
SGA Small for gestational age
T reg T-regulatory subset
Th T-helper cell subtypes (1, 2, 17)
TNF-α Tumour necrosis factor alpha
UNICEF United Nations Children's Fund
UTI Urinary tract infection
VA Vitamin A
VAD Vitamin A deficiency
VB12 Vitamin B12
VDR Vitamin D receptors
VEGF Vascular endothelial growth factor
WHO World Health Organization

CONTRIBUTION OF AUTHORS

As stated by the McGill University Guidelines for a Manuscript-based thesis, this document includes a table of contents, abstracts in both English and French, an introduction, a comprehensive review of literature in the field of study, a general discussion, a bibliography and appropriate appendices. Furthermore, I attest to the originality of this thesis as being the primary author of this manuscript. This thesis includes one manuscript which is co-authored by my supervisors Drs. Kristine Koski and Marilyn Scott who provided guidance in the data analysis, writing and editing of this thesis.

For this thesis, I conducted laboratory analysis for retinol, cortisol, cytokines and IGF-1. A colleague and I received training for the analysis of retinol using HPLC, however due to limitations of sample amounts analysis was conducted in Panama by another technician. I completed multiple steps required in the analysis of cortisol and cytokines including the aliquoting, extraction, plating and sample analysis. I aided in the training of the IGF-1 assay to a lab member, including helping perform the assay as well as train on data extraction using Luminex technology.

I was responsible for the extraction and conversion of the data and entry into the database for the cytokines and cortisol assays and created variables for cytokine ratios. Furthermore, I performed data analysis obtaining the results presented in this thesis. I also wrote the entirety of this thesis.

I was also involved in the preparation, presentation and discussion of results obtained from this study with the collaborators and Indigenous communities at two, full-day symposia in Panama

This thesis was produced with the permission of all co-authors. Additional collaborators will be included as authors when portions of this thesis will be submitted for publication including Doris Gonzales-Dernandez, Odalis Sinisterra from the Ministry of Health of Panama and Professor Enrique Murillo from the University of

Panama who both contributed to the papers as co-applicants on the SENACYT grant that funded this study.

CHAPTER I: OVERVIEW

The Republic of Panama is the southernmost country in Central America, with a population of 3,517,000 people (WHO 2011). Thirty-seven percent of the Republic's population live in poverty (FAO 2010), and indigenous people are among the most economically disadvantaged. Approximately 9% of the population belongs to one of five indigenous groups: Kuna, Emberá and Wounaan, Ngäbe-Buglé (previously known as Guaymíes), Bokotas, and the Teribes (WHO 2001). The largest indigenous group in Panama is the Ngäbe, who live in the *comarca* Ngäbe-Buglé, a semi-autonomous indigenous area created from portions of the western provinces of Bocas del Toro, Chiriguí, and Verguas. While they have been granted institutional autonomy and political influence by the central administration, Ngäbe-Buglé and Panama's other *comarcas* are isolated, poor and have limited access to government health and education services (Damman 2007).

The women and children of the Ngäbe-Buglé *comarca* are more affected by food insecurity and multiple infections than the rest of the Panamanian population, resulting in anemia, chronic malnutrition (MINSA 2003) and growth faltering (Payne et al. 2007). Growth faltering reflects the well-being and health status at the level of individuals and the population as a whole, and is associated with reduced income, lower levels of schooling and subsequent decreased progeny birth weights (Victora et al. 2008). It is widely accepted that growth faltering begins postnatally, at 3 months of age; however mounting scientific literature indicates that the prenatal environment can influence growth as well (Shrimpton et al. 2001). Many maternal factors significantly influence growth during the prenatal environment, including maternal nutritional status and infection burden.

Pregnant women have an increased risk of macro and micronutrient deficiencies due to their higher requirements, which can exacerbate any pre-existing deficiencies (Black et al. 2008). Common micronutrient deficiencies known to negatively impact fetal development include iron, folic acid, iodine, zinc and

riboflavin, as well as vitamins B6, B12, A and D (Haider, Yakoob, et al. 2011). Infection burden can severely diminish the nutritional status of an individual by increasing nutrient requirements and decreasing the individual's ability to absorb nutrients, both of which affect fetal growth (Scrimshaw et al. 1968; Stephensen 2001). Infections that have been associated with poor growth outcomes include, but are not limited to, respiratory tract (Banhidy et al. 2008) and periodontal infections (Shub et al. 2006). Urogenital infections including bacterial vaginosis (Brotman 2011), vaginal candidiasis, trichomoniasis, gonococcal infection (Walker et al. 2011) and urinary tract infections (Mazor-Dray et al. 2009; Schnarr et al. 2008) have also been implicated in negatively impacting growth outcomes. How nutritional deficiencies and infections affect growth through remain to be elucidated. Growing research indicates that both nutritional deficiencies and infections may increase stress and inflammation levels which in turn maybe negatively impacting growth.

The steroid cortisol has been identified as contributing to poor linear growth by adversely affecting bone health (Mushtaq et al. 2002), and elevated cortisol concentrations have been observed in pregnant women with infections (Ruiz et al. 2001). Infections also elevate pro-inflammatory cytokine concentrations (Beigi et al. 2007). Both cortisol (Hofbauer et al. 1999; Weinstein et al. 1998) and pro-inflammatory cytokines have been associated with a decrease in insulin-like growth factor (IGF-1) (Hofbauer et al. 1999; Odiere et al. 2010; Weinstein et al. 1998), a main endocrine regulator of fetal growth. Furthermore, vitamin deficiencies, particular A and D, have been observed to increase these potentially detrimental pro-inflammatory cytokines (Bessler et al. 2007; Iwata et al. 2003; Khoo et al. 2011). Therefore, fetal growth may be impaired as a result of a reduction in IGF-1, associated with an increase in cortisol and pro-inflammatory cytokines in response to multiple infections and vitamin deficiencies. However, to the best of our knowledge, this framework of interactions has not been previously investigated in pregnant women, and given the importance of fetal growth exploration of these relationships is warranted.

The main objectives of this study were to examine the relationship between maternal cortisol, IGF-1 and fetal growth (as measured by fundal height) and to examine the effect of vitamin deficiencies, infections and cytokines on maternal cortisol, IGF-1, and fundal height. We hypothesized that maternal cortisol and pro-inflammatory cytokines would lead to a decrease in maternal IGF-1 which would cause low fundal height, and that maternal vitamin deficiency and infections would increase pro-inflammatory cytokines and cortisol, and thus decrease fundal height.

Figure 1 Conceptual Framework

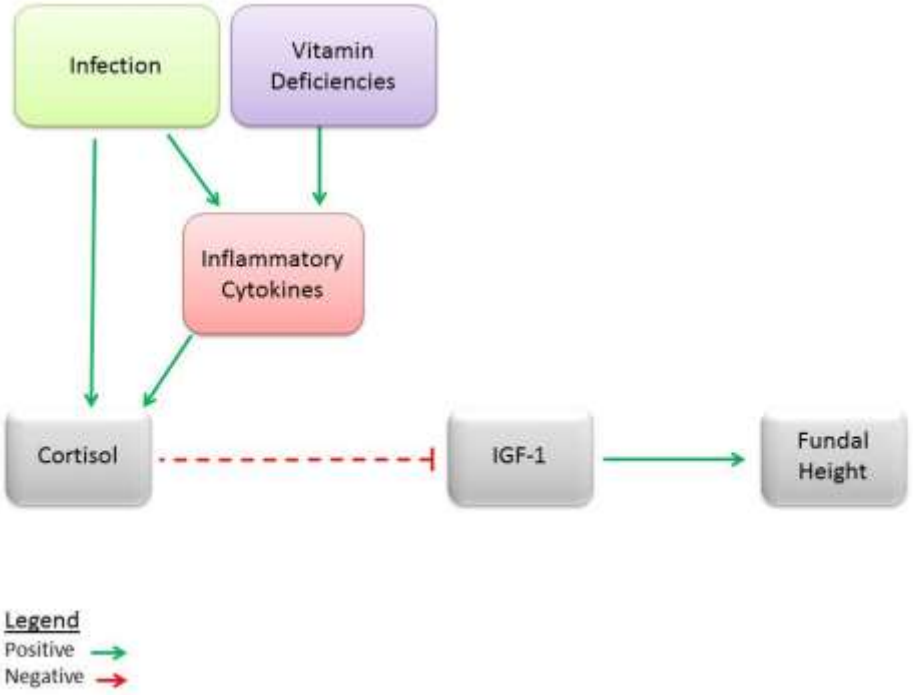

Diagram of the conceptual framework of how infection (green), vitamin deficiencies (purple) and inflammatory cytokines (pink) impact our proposed cortisol-IGF-1-fundal height pathway. Positive associations are represented by solid green lines, and negative associations are represented by red dotted lines.

CHAPTER II: LITERATURE REVIEW

A. Fetal Growth

Child malnutrition manifests in a variety of ways most obviously via poor growth outcomes including, but not limited to, stunting and wasting. Worldwide, stunting and wasting have together been estimated to be responsible for 2.2 million deaths and 21% of disability-adjusted life-years (DALYs) for children <5 y and are pervasive in low and middle income countries (Black et al. 2008).

Growth faltering reflects health status for both individual and population level well-being and is associated with increased morbidity and mortality (Harris et al. 2001; Prentice et al. 2006). Growth faltering of all types leads to shorter adult stature which is associated with reduced income, lower levels of schooling, and subsequent decreased progeny birth weights (Victora et al. 2008). For this reason, The Child Survival and Development Revolution, was put forth by the United Nations Children's Fund (UNICEF) during the 1980s, in an effort to increase awareness of the critical importance of monitoring growth in children (Cash 1987). Today, the United Nations has continued this effort with the establishment of the Millennium development goals, among which are goals aimed at reducing child mortality rates world-wide (WHO 2013). The establishment of these goals further reiterates the ongoing problem of child mortality despite decades of scientific research and government policy.

Under the umbrella of growth faltering exists poor growth outcomes related to both acute- and chronic- malnutrition. According to Waterlow, acutely malnourished children are wasted, they have adequate height-for-age but inadequate weight-for-height, whereas chronically malnourished children are stunted with inadequate height-for-age measurements (Waterlow 1973). Popular rhetoric is that growth faltering begins at around 3 months of age (Rogers et al. 1997; Waterlow et al. 1979). However these studies, in addition to not being population-based of national scale, only looked at weight gain and not height growth (Shrimpton et al. 2001). Recent literature

indicates that both the prenatal and the postnatal environments can have severe consequences on growth (Shrimpton et al. 2001) and that children at 3 months already present anthropometric deficits (Huttly et al. 1991; Rivera et al. 1997). Poor fetal growth has long-term consequences with regards to mortality and morbidity including increased risk of disease such as obesity, diabetes, and ischaemic heart disease (Barker 1998; Bernstein et al. 2000; Gluckman et al. 2003).

Low birth weight (LBW) is defined by the World Health Organization (WHO) as a birth weight <2500 g. In a seminal paper, Kramer et al. (1987) explained that LBW occurs by one of two processes, the first being duration of gestation and the second being fetal growth (Kramer 1987). Therefore, infants can be LBW because they are born early, (preterm birth) or are small for gestational age (SGA), which is a proxy for intra uterine growth restriction (IUGR). For further clarification, preterm birth is defined as gestation lengths less than 37 weeks and SGA as a birth weight below the 10th percentile for gestational age based on the sex of the infant (Kramer 2003). LBW can be measured with validity and precision whereas preterm birth and or IUGR requires an accurate gestational age, which in developing countries is difficult to achieve due to lack/infrequent access to prenatal care (Kramer 2003). Regardless of the difficulties, prevalence of IUGR (the more common of the two causes of LBW) has been observed globally to be between 30-55% of infants in South Central Asia, 15–25% in Africa, and 10–20% in Latin America (Kramer 2003).

Developing reliable methodologies for detecting growth restrictions is important to the health of the offspring and includes methodologies such as ultrasound biometry (Morse et al. 2009). However, in the context of a low income region where resources are limited, ultra sound technology may not be available and fundal height measurement may be the only useful tool for measuring gestational age of pregnancy (Rondó et al. 2003), height and growth of the fetus (Mongelli et al. 2004). Fundal height measurement is a simple tool requiring only a measuring tape and an accurate estimation of gestational age and is only appropriate for the second trimester onwards (Divon 2012). The fundal height measurement is obtained by identifying the upper

border of the symphysis pubis and the uterine fundus and measuring the distance between with a tape measure. If measured in centimetres (cm) a simple rule of thumb may be applied to ascertain growth. If the fetus is growing normally the fundal height measurements in cm, should correspond to gestation in weeks (28 cm for 28 week's gestation) for singleton pregnancy with an allowance of +/- 2 cm difference (Robert et al. 2012). Fundal height measurement has been found to be reproducible and reliable (Belizan et al. 1978; Challis et al. 2009; Grover et al. 1991). Fundal height is used in many countries on a large scale as it is low cost, easy to execute and requires little training (Robert et al. 2012). However the greatest use for this rapid, low cost and easy to use anthropometric methodology is in developing regions of the world, where access to hospitals and clinics may be limited.

Although *in utero* growth faltering is easily described, the factors comprising the etiology of this outcome are complex and varied. Various known factors that influence and impede growth include maternal factors, endocrine and metabolic systems, vitamin deficiencies as well as infection and compromised immunity.

B. Maternal Factors Affecting Fetal Growth Outcomes

In a seminal paper by Kramer et al (1987a) that reviewed risk factors for low birth weight, 71 modifiable risk factors were identified including maternal height and weight, age, social economic status (SES), education and smoking. Since this paper, many researchers have continued investigating these important maternal characteristics further validating them in populations worldwide.

Maternal height is among one of the strongest determinants for fetal growth (Heinrich 1992; Kramer 1987b; Thompson et al. 2001). Maternal height may affect fetal growth via genetic or physical mechanism. Regardless of etiology, deficits in maternal stature impair fetal growth by placing a physical limitation on growth of the uterus, placenta or fetus directly (Kramer 1987a).

Maternal weight, including pre-pregnancy weight (Clausson et al. 1998; Roland et al. 2012) and weight gain during pregnancy, is also an important determinants for fetal growth (McDonald et al. 2011). Like maternal height, pre-pregnancy weight comprises a genetic component which may be passed onto the fetus and will therefore influence the growth and weight of the fetus (Kramer 1987a). Furthermore, maternal weight may impact fetal growth as it reflects nutritional stores potentially available to the growing fetus. Maternal weight gain during pregnancy is also important determinant of fetal growth. Low maternal weight gain has been associated with SGA whereas high maternal weight gain has been associated with large for gestational age (LGA) (Dietz et al. 2009; Munim et al. 2012; Voldner et al. 2008).

The age of the mother at delivery has also been noted to impact birth outcomes especially pregnancies from teenage mothers (Clausson et al. 1998; Hayward et al. 2012; Janssen et al. 2007). Young mothers are at risk of adverse pregnancies because they have not yet finished growing and are more likely to have lower weight for height than older women (Kramer 1987a). A recent review by Gibbs at el (2012) investigating the impact of age on birth outcomes found that early age (<15 years or <2 years post-menarche) of the first childbirth was associated increased risk of poor pregnancy outcomes including low birthweight and preterm birth and was separate from poor socioeconomic and behavioural differences observed with teenage pregnancy.

Socio-economic status (SES) represents one of the strongest determinants in pregnancy outcomes including perinatal and infant mortality, LBW, IUGR and preterm birth (Kramer et al. 2000). Numerous epidemiological studies have found low SES to be particularly harmful (Ancel et al. 1999; Kramer 1987a; Raisanen et al. 2013); however SES itself does not directly affect growth of the fetus. Instead low SES is associated with behaviours and exposure to stresses that negatively impact fetal growth including lower levels of education and cigarette smoke for example. Low levels of education limits a person's ability to access jobs and other social resources needed to integrate within society and failure to do so increases risk for subsequent poverty (Kramer et al. 2000). Low SES generally implies low levels of education, however in a

retrospective study in Quebec, individual-level maternal education was independently and more strongly associated with risk of preterm birth, SGA and still births than average income of the mothers or families alone (Luo et al. 2006). Smoking is another important variable mediating SES disparity on poor fetal outcomes in developed countries (Kramer et al. 2000). Adverse consequences of smoking have been well documented including retarded fetal growth, reduced birth weight and increased risk of pregnancy loss (Esposito et al. 2008; Ong et al. 2002; Voigt et al. 2009). However the investigation of smoking on fetal growth has issues with confounding as mothers who continue to smoke during pregnancy are different from mothers who do not in a variety of ways including having less education and coming from lower income families (Maughan 2009).

C. Placental Insufficiency

The placenta is a structure formed during gestation that is essential in aiding the development of the fetus. It supplies nutrients, facilitates gas exchange, removes waste products, secretes pregnancy hormones and growth factors (Rossant et al. 2001). Placental insufficiency is a term referring to inadequate placental function or development, which in turn reduces transfer of oxygen and nutrients to the fetus (Gagnon 2003). As a consequence of reduced oxygen transfer, fetal hypoxemia may occur and fetal growth is reduced to compensate for the limited nutrients available (Lackman et al. 2001). A reduction in placental nutrient transport via reduced blood flow is linked to the pathophysiology of IUGR (Nardozza et al. 2012; Pardi et al. 2002; Roland et al. 2012).

Vascular endothelial growth factor (VEGF) is a positive regulator of angiogenesis and is considered one of the most important angiogenic factors involved in normal placentation (Barut et al. 2010). VEGF has a dual role in the placenta by aiding angiogenesis and the proliferation and migration of trophoblasts (Barut et al. 2010). Recently, it has been shown that VEGF concentrations are significantly higher in women with pre-eclampsia and IUGR (Ahmed et al. 2000; Brouillet et al. 2013).

The increase of VEGF during IUGR is believed to occur as a compensatory mechanism to aid in proper fetal and placental development, however further research is needed (Brouillet et al. 2013).

D. Endocrine Factors Affecting Growth Outcomes

A combination of hormones regulate fetal growth including insulin-like growth factors (IGF) (I and II), leptin, prolactin, and placental growth hormones (GH). Leptin is a hormone that affects both amino acid and fatty acid transport across the placenta, stimulates pancreatic growth and is important for modulating fetal body fat content (Hoggard et al. 2001). Human placental lactogen (hPL) and GH aid in maternal adaptation to pregnancy and have been found to correlate positively with fetal weight (Sankaran et al. 2009). Furthermore, growth restricted pregnancies have been observed in pregnancies with reduced fetal hPL and maternal GH concentrations (Freemark 2006).

i. Insulin Like Growth Factor

IGF-1 and IGF-2 are anabolic hormones functioning as the main endocrine regulators of fetal growth (Chiesa et al. 2008). Alterations in these hormones can have serious consequences either by severely diminishing (dwarfism) or exaggerating (acromegaly) growth (Heemskerk et al. 1999). Furthermore, consequences of IGF-1 deletion have been observed in one human patient who presented severe IUGR (Woods et al. 1997) further illustrating the importance of this hormone. However this condition is extremely rare and only 4 known cases have been recorded, with all of them being characterized by intrauterine and postnatal growth restriction (Netchine et al. 2011).

Whereas IGF-1 is necessary for fetal growth in later gestation, IGF-2 promotes embryonic growth and diminishes significantly after parturition (Chiesa et al. 2008; Glasscock et al. 1992). Maternal IGF-1 however, is predominantly stimulated in response to growth hormone. Growth hormone (GH) is a peptide hormone released

from the anterior pituitary which stimulates growth and repair of cells and tissues. Predominantly the liver, but other tissues as well respond to GH by releasing IGF-1. However the fetus does not respond to GH as highly as the mother does because GH receptors are expressed at low concentrations in fetal tissue. Expression remains low until surges in glucocorticoid concentrations accompanying parturition trigger the upregulation of GH receptors (Gluckman et al. 1992). Instead, fetal IGF-1 is stimulated from exposure of fetal insulin in response to glucose and amino acid (Gluckman et al. 2003).

ii. Glucocorticoids

Glucocorticoids (GC) are a class of steroid hormones produced by the adrenal gland in response to environmental stress or low blood sugar (Hochberg 2002). In humans, cortisol (hydrocortisone) is one of the major hormones belonging to the class of glucocorticoid steroids. Synthetic GC administration is commonly used as a therapeutic immunosuppressive agent, but in high concentrations can contribute to poor linear growth by adversely affecting bone health (Mushtaq et al. 2002) and impairing the growth hormone/insulin growth-factor 1 axis (GH-IGF-1) (Hochberg 2002).

Glucocorticoids increase the deterioration and loss of bone via suppression of osteoblastogenesis, decreasing new bone formation (Hochberg 2002) and increasing apoptotic activity of both osteocytes and osteoblasts (Weinstein et al. 1998). Additionally, GC increase bone resorption by extending the life span of osteoclasts (Hofbauer et al. 1999). Synthetic glucocorticoids like dexamethasone and prednisolone have been shown to impair growth and skeletal development in children (Ahmed et al. 2002).

Although GC are necessary for development of fetal tissue including liver, lungs, gut, skeletal muscle and adipose tissue (Fowden et al. 1998), excessive amounts have been observed to harm fetal growth. Administration of glucocorticoids to either

the mother or fetus during late gestation can cause fetal growth retardation (Fowden et al. 2009), with body weight of fetus being reduced in proportion to GC therapy (Fowden et al. 1998). This is believed to occur via impairment of the GH-IGF-1 axis (Giustina et al. 1990). Although it has been reported that short-term administration of GC stimulates GH and IGF-1 synthesis (Veldhuis et al. 1992), long-term high-dose GC therapy reduces both of these hormones (Hochberg 2002). Excess GC administration interferes with IGF-1 production at the hypothalamic, pituitary and target organ levels (Hochberg 2002). The result is a reduction not only in IGF-1 release, but also in IGF-1 receptor abundance and mRNA translation (Chen et al. 1991; Hochberg 2002).

The placenta has a natural defense mechanism against GC excess, the enzyme placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). This enzyme inactivates cortisol into cortisone protecting the fetus from negative effects of maternal GCs (McTernan et al. 2001). Reduced placental 11β-HSD2 activity has been observed with IUGR pregnancies in both animal and human studies (Harris et al. 2011; McTernan et al. 2001) supporting the deleterious effects of maternal glucocorticoids on fetal growth.

Several hormones play a crucial role in the growth and development of the fetus including aiding the adaption of the maternal system to the fetus, increasing placental nutrient transport as well as development of fetal tissue. Although glucocorticoids are necessary for proper fetal development, pharmacological concentrations impair fetal growth via interfering with the production of IGF-1, demonstrating a delicate balance which warrants further investigation.

E. Infection Factors Affecting Birth Outcomes

A number of maternal infections have been associated with poor growth outcomes, including urinary tract infections (Mazor-Dray et al. 2009; Schnarr et al. 2008), respiratory tract infections (Banhidy et al. 2008), and periodontal infections (Shub et al. 2006). Urogenital infections including bacterial vaginosis (Brotman 2011),

vaginal candidiasis, trichomoniasis, and gonococcal infection (Walker et al. 2011), HIV-AIDS (Grivell et al. 2009) and syphilis (Gilbert 2002) have also been observed to have negative impacts on birth outcomes. HIV-AIDS was not present in our study population and only 4 women were positive for VDRL test for syphilis, and therefore the literature review does not include them. Similarly, malaria is not endemic in the region and we did not test for toxoplasmosis, so these infections are not included below. Intestinal parasites are common and there has been considerable work on the impact of hookworm in particular on pregnancy outcomes (Dreyfuss et al. 2000). This infection is not considered in this thesis because stool samples were available for only a small proportion of the pregnant women.

i. Urinary Tract Infections

Urinary tract infections (UTI) represent one of the most common bacterial infections occurring in 2–10% of all pregnancies (Sharma et al. 2007). A meta-analysis that included 17 cohort studies of pregnant women with untreated aymptomatic UTI infections, (Schnarr et al. 2008) found an increased risk of LBW rates and preterm delivery associated with infection. However, methodological differences between studies, including classification of infection burden, limit the strength of the conclusion. A prospective study in the United Kingdom (n= 25,844) found that when adjusting for social and demographic variables, UTI infection was not associated with preterm delivery (OR=1.2; 95% CI= 0.9-1.5) (Meis et al. 1995). In yet another meta-analysis, antibiotic treatment of UTI was associated with reduction of LBW (RR=0.66; 95% CI=0.49, 0.89) but not a reduction in preterm delivery (RR=0.37; 95% CI=0.10, 1.36) (Smaill et al. 2007).

ii. Respiratory Tract Infection

Respiratory infections of the sinuses, throat airways or lungs commonly occur, but the literature regarding their impact during pregnancy is limited (Hartert et al. 2003). Studies examining prevalence of respiratory infections during pregnancy

have ranged from 0.2% in Hungary (Banhidy et al. 2008), 1.2% in Australia (Lain et al. 2011) and 1.1% to 1.9% in Canada (Dodds et al. 2007).

In a Hungarian study looking at maternal acute respiratory infections during pregnancy, researchers found that women with severe RTI including (bronchitis–bronchiolitis and particularly pneumonia) had a higher risk of preterm births (13.0%, OR=1.4, 95% CI=1.1, 1.8). Comparatively, women with mild infections (sinusitis, pharyngitis, tonsillitis, laryngitis–tracheitis) had a lower rate of preterm births (OR=5.5% 95% CI= 0.4, 0.7). This research suggests that the type of respiratory infection, especially severe RTI, were associated with increased risk of preterm birth (Banhidy et al. 2008).

iii. Periodontal Disease

Periodontal disease is a common infection associated with chronic inflammation of the periodontal tissues supporting the tooth and alveolar bone, resulting in destroyed connective tissue and later bone loss via bone resorption (Shub et al. 2006).

The prevalence of periodontal disease ranges between 10 and 60% depending on diagnostic criteria (Xiong et al. 2006) and has been associated with adverse pregnancy outcomes including Pre-eclampsia, low birthweight and preterm delivery (Paquette 2002; Xiong et al. 2006).

Adverse consequences may be a result of increased pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α which are produced within the periodontal pocket (Shub et al. 2006). Due to the high vascularity of the periodontium, cytokines travel to other locations in the body including amniotic fluid. This phenomenon was observed in a study of women with periodontal disease in the second trimester of pregnancy, where level of IL-1β in gingival crevicular fluid of the periodontium was strongly correlated with levels in amniotic fluid (Damare et al. 1997). Unfortunately pregnancy outcome

data were not available for this group of women. In another study, elevated IL-6 in amniotic fluid was observed in women with periodontal disease and was significantly associated with increased rates of preterm birth (Dörtbudak et al. 2005).

iv. *Scabies*

Scabies is a common skin infection caused by the mite *Sarcoptes scabiei var hominis*, and there are approximately 300 million cases of scabies worldwide per year (Hicks et al. 2009). This skin parasite is common in instances of overcrowding, poor hygiene, homelessness and poor nutritional status. Literature regarding outcomes or impacts on pregnancy is limited to the effects of treatments of scabies using drugs benzyl benzoate and permethrin during the second and third trimester (Mytton et al. 2007).

v. *Vaginal Infections*

Bacterial vaginosis (BV) is an infection which occurs when the normal lactobacillus-predominant flora of the vaginal tract is replaced with the harmful anaerobic bacteria *Mycoplasma hominis* or *Gardnerella vaginalis* (Morris et al. 2001). BV requires laboratory testing for diagnosis, and therefore estimates of prevalence vary based on the population studied (Brotman 2011). Rates of BV, diagnosed using Gram-stained smears, had an estimated prevalence of 9% (Lamont et al. 2000) and 18% (Morris et al. 2001) in studies from the United Kingdom. In a population based survey in the US, this rate was even higher at 29% (Koumans et al. 2007) and higher still in rural Ugandan villages with a prevalence of 50% (Wawer et al. 1999). A recent review by Brotman (2011) has concluded that this seemingly innocuous condition is of great consequence during pregnancy and has been linked to fetal loss including spontaneous abortion and miscarriage. The risk of preterm birth also increased as a result of BV; a series of studies have showed that 10%–30% of pregnant women with BV infections gave birth prematurely (Donders et al. 2000; Oakeshott et al. 2002).

Unfortunately there is a lack of evidence regarding whether treatment of BV indeed reduces risk of pre-term birth (Brotman 2011).

Low birthweight has also been observed in women with BV. In a study of pregnant Danish women (n=3262), mean birthweight was significantly lower in infants of women with BV than in infants from women without BV (3408 versus 3511 g, $P < 0.01$) and (OR=1.95, 95% CI 1.3, 2.9) (Svare et al. 2006). These findings were similar to an older study (n=10,397) where infection was detected in 16% of the pregnant women and multivariate analysis revealed that bacterial vaginosis was related to preterm delivery of a LBW infant (OR=1.4; 95%, CI=1.1, 1.8). These results were independent of other recognizable risk factors including smoking, SES and having previously delivered low-birth-weight infants (Hillier et al. 1995).

vi. Vaginal Candidiasis

Vaginal candidiasis, commonly referred to as vaginal yeast infection, is the second most common of vaginal infection next to bacteria vaginosis (Sobel 2007). This infection is caused by yeast of the genus *Candidia* and can colonize the skin, gastrointestinal tract as well as the reproductive tract (Sobel 2007). As reviewed by Sobel (2007) approximately 70-75% of women have candidiasis during their life time, with nearly 40-50% experiencing reoccurring infections. Furthermore, higher prevalence of candidiasis has been observed in pregnant women compared to those who are not pregnant (Nyirjesy et al. 2003). Although the incidence is high, research regarding the effects of infection on birth outcomes is limited. A recent study by Giraldo et al. (2012) found no association between infection and preterm delivery; a finding supported by an earlier study by Cotch et al. (1998) who also found no association with preterm delivery or low birthweight.

vii. Trichomoniasis Infection

Trichomoniasis is a sexually transmitted infection caused by the protozoan parasite *Trichomonas vaginalis* that infects the uterus and vaginal tract (Schwebke et al. 2004). Infection has been associated with premature rupture of membranes, premature delivery, low birth weight, stillbirth and neonatal death (Young 2006). Prevalence of infection has been reported to be between 4-35% in women across multiple studies (Anderson et al. 2004).

In a multicenter study, *T. vaginalis* infection was associated with low birth weight, premature rupture of membranes and preterm delivery (OR=1.3; 95% CI=1.1, 1.4) (Cotch et al. 1997). Subsequent studies have examined the effect of treating trichonomiasis during pregnancy on birth outcomes using drugs such as metronidazole with inconclusive results (Gulmezoglu et al. 2011).

viii. Gonococcal Infection

Gonorrhea (often referred to as gonococcal infection) is a sexually acquired bacterial infection caused by *Neisseria gonorrhoeae* and identified microbiologically by its Gram-negative intracellular diplococci *N. gonorrhoeae* (Walker et al. 2011). Gonorrhea infection occurs in mucosal sites of the lower genital tract including the urethra and cervix and may spread to the upper genital tract into the uterine tubes and abdominal cavity (Walker et al. 2011). There are more than 700,000 cases each year in the US alone, making it the second most commonly reported STI next to chlamydial infections (CDC 2010). Approximately 10%–20% of women with untreated gonorrhea develop pelvic inflammatory infection which can cause uterine tubal scarring. Consequences of uterine tubal scarring may result in infertility in 20% of women, ectopic pregnancy in 9%, and chronic pelvic pain in 18%. Often pelvic inflammation is asymptomatic resulting in delayed treatment of approximately 85% of women, greatly enhancing long term damage to uterine tubes and other sequelae (Walker et al. 2011). Therefore pregnant women living in endemic regions are advised to be tested for

gonorrhea and those who test positive should again be retested during the third trimester (Force 2005).

Various infections during pregnancy have been observed to have detrimental effects on birth outcomes, ranging from low birth weight to ectopic pregnancy and miscarriage. Although a large body of research supports these observations, the majority of these studies have examined the effects of single infections in isolation. The prevalence of multiple infections during pregnancy, particularly in rural areas of developing regions (Gonzalez-Fernandez 2012) suggests there is an urgent need for researchers to examine the effects of multiple infections during pregnancy, in addition to examining the implementation of treatment and prophylaxis.

F. Vitamin Status Affecting Birth Outcomes

Micronutrient deficiencies during pregnancy including vitamins A, D, B12 and folic acid have been observed to negatively impact pregnancy outcome (Haider, Yakoob, et al. 2011). Unfortunately data regarding these nutrients prenatally on fetal development is limited (Gluckman et al. 2003).

i. Vitamin A

Vitamin A (VA) is a generic term referring to a family of fat-soluble dietary compounds possessing biological activity of retinol. Dietary sources of VA are preformed retinyl esters from animal tissues including milk, liver, and eggs, and provitamin A carotenoids from plants, particularly dark green leafy vegetables, pumpkins, carrots, squash as well as yellow and orange non-citrus fruits (mangos, apricots, papayas) (Holden et al. 1999). Vitamin A deficiency (VAD) is defined by the International Vitamin A Consultative Group as having a blood serum values less than 0.7μmol/L (Arroyave 1989). For pregnant women, some authors argue that due to their

increased requirements of VA and risk of deficiency, marginal VA status is defined as having serum retinol less than <1.05µmol/L is appropriate (West 2002).

Approximately 19.8 million (18%) pregnant women world-wide are burdened with marginal vitamin A status (<1.05µmol/L), and of that 7.2 million (37%) are classified as having subclinical VAD (<0.7µmol/L) (West 2002). A more recent survey by the WHO has revealed that the prevalence of VAD in Panamanian pregnant women was 3 percent with the rest of the population ranging from 2 to 10 percent (WHO 2009). VAD is a major cause of morbidity and mortality among children in developing countries (da Silva Ribeiro et al. 2010), with approximately 250 million preschool children worldwide suffering from inadequate vitamin A intake (WHO 2009).

Supplementation trials have been routinely conducted to determine if VA supplementation is beneficial in decreasing adverse birth outcomes. Meta-analyses regarding vitamin A supplementation during pregnancy have been conducted by Gogia et al. (2010), van den Broek et al. (2010), Haider and Bhutta (2011) and (Thorne-Lyman et al. 2012a). In all four of these reviews, it was found that VA supplementation had little or no impact on improving adverse effects of birth outcomes. The most recent meta-analysis of 17 studies conducted by Thorne-Lyman et al. (2012a) found that VA supplementation during pregnancy did not have an overall effect on birth weight, preterm birth, stillbirth, miscarriage or fetal loss. However, among HIV positive women supplementation was protective against LBW (<2.5 kg) (RR= 0.79, 95% CI=0.64, 0.99). A similar finding was found in a review by van den Broek and colleagues (2010) who observed that VA supplementation reduced the risk of low birth weight in Nepal, Indonesia and Tanzania (RR=0.67, 95% CI= 0.47, 0.96).

Yakoob et al. (2009) conducted a meta-analysis investigating the effects of nutritional and behavioural interventions on incidence of stillbirths. A total of 3 Cochrane reviews were included for vitamin A supplementation (Rumbold et al. 2011; van den Broek et al. 2010; Wiysonge et al. 2005) of 12 different interventions and found no effect of vitamin A in reducing risk of stillbirths. However the lack of dosage

information or the use of VA in combination with other micronutrients warrants further investigation into this association (Ulbricht et al. 2012).

A study examining maternal vitamin A status, as indicated by maternal night blindness, found a strong relationship between VA status and growth outcomes in offspring. Researchers found there was an associated increased risk of poor growth at 6 months including being underweight (RR=1.14, 95% CI=1.02, 1.26), and stunted (RR=1.19, 95% CI=1.05, 1.34) in women who had night blindness (Tielsch et al. 2008). The manifestation of night blindness occurs from severe long term VAD which is different from what the meta-analysis was investigating which was the effect of supplementation. Therefore it may be that VAD plays an important role on growth outcomes, and future research may need to consider deficiency in addition to supplementation.

ii. *Vitamin D*

Vitamin D is a fat soluble vitamin, derived from cholesterol, synthesised via skin exposure to ultraviolet B-light, or from the diet including cod-liver oil, egg yolk, cow's milk and fortified beverages including juices (IOM 2011). Two common forms of vitamin D found in the body are of 25(OH)D known as D2, and $1,25(OH)_2D$ known as D3. Although they are different in structure they are considered to be bioequivalent as they are both metabolized to the active form of vitamin D, calcitrol (IOM Institute of Medicine 2011; Simpson et al. 2011; Thacher et al. 2011).

Measurements of vitamin D2 is considered the best indicator of vitamin D status as concentrations reflect vitamin D obtained through diet as well as vitamin D produced cutaneously (IOM 2011). Additionally D2 has a half-life of 15 days compared to D3, which has a half-life of only 15 hours, providing a longer term assessment of vitamin D status (Jones 2008).

Cutoffs for vitamin D are controversial and there is a lack of consensus when defining optimal vitamin D concentrations. The 2011 American College of Obstetrics and Gynecology (ACOG) Practice Bulletin 'vitamin D: screening and supplementation' defined vitamin D deficiency 25(OH)D concentration <50nmol/L (ACOG 2011) which is supported by other experts in the field (Holick 2007). However, some argue this cutoff is not high enough, and should be set at having 25(OH)D concentrations <75nmol/L (Thacher et al. 2011). The Institute of Medicine broadens this definition to define vitamin D deficiency as having serum values <50 nmol/L and insufficiency between 50 and 75 nmol/L (IOM 2011), however specific values remain to be set for pregnant women.

Vitamin D deficiency is emerging as a global health problem existing not only in northern countries, but tropical regions as well (Arabi et al. 2010; Mithal et al. 2009). Prevalence's of vitamin D insufficiency of women in developing countries have been reported as high as 87% in Argentina, 42% in Brazil, 50% in Chile, 67% in Mexico and 96% in Guatemala (Mithal et al. 2009). These findings are surprising given the high amounts of sunlight exposure in these areas. Until recently, the data regarding vitamin D status in pregnant women has been limited. A study from Belgian observed that 74.1% of pregnant women were vitamin D insufficient (<75nmol/L), 44.6% were vitamin D deficient (<50nmol/L), and 12.1% were severely vitamin D deficient (<25nmol/L) (Vandevijvere et al. 2012). Recently, it was observed that Indigenous women from rural Panama had rates of deficiency as high as 65% (<50nmol/L), and a further 32% were insufficient (50-75nmol/L) (Suissa 2013).

Reviews of vitamin D supplementation and the effects on birth outcomes have been conducted by Thorne-Lyman et al. (2012b), Urrutia et al. (2012), Dror (2011), and Lewis et al. (2010).

A meta-analysis by Thorne-Lyman et al. (2012b) was conducted by using observational studies of vitamin D supplementation, as well studies observing intake or status during pregnancy on perninatal and infant health outcomes. Pooled analysis of

trials using fixed-effects models found a protective effect of vitamin D supplementation on LBW (RR = 0.40, 95 % CI= 0.23, 0.71) and a non-significant association for SGA (RR = 0.67, 95 % CI=0.40, 1.11) and preterm delivery (<37 weeks) (RR = 0.77, 95 % CI= 0.35, 1.66). Pre-eclampsia was also investigated by Thorne-Lyman and colleagues, however results were inconsistent. In one study from Pittsburgh, vitamin D dosages found that for every 50 nmol/L decline in 25(OH)D concentrations, the risk of pre-eclampsia more than doubled (OR= 2.4 95% CI=1.1, 5.4) (Bodnar et al. 2007) and similar results were observed in a study from North Carolina, where vitamin D deficiency was associated with increased risk (OR=5.41 95% CI=2.02, 14.52) (Baker et al. 2010). On the contrary, a nested case control study conducted in Massachusetts did not observe an impact of vitamin D concentrations <37.5 nmol/L (OR=1.35 95% CI= 0.40, 4.50) (Powe et al. 2010) and similar findings were also reported in Canada (Shand et al. 2010).

Whereas some studies examining the impact of vitamin D status on mean or low birthweight found no association (Farrant et al. 2009; Gale et al. 2008; Morley et al. 2006), two large studies did identify a relationship. A multi-ethnic cohort of mothers in the Netherlands (n = 3730) measured serum vitamin D during early pregnancy at 13 weeks (Leffelaar et al. 2010). They found that when compared with mothers having adequate vitamin D status, those who were deficient tended to have infants with lower birth weights and a higher risk of being small for gestational age (OR= 2.4, 95 % CI=1.9, 3.2). Another study in Australia looked at vitamin D status of mothers (n =374) in relationship with length of gestation and height (Morley et al. 2006). Gestation length was 0.7 weeks shorter and length measured by knee-heel length, was 4.3 mm smaller in infants from mothers who were vitamin D deficient Knee heel length was later adjusted for gestational age, but was still -2.7 mm, suggesting a relationship between maternal vitamin D status and growth of offspring.

Another meta-analysis by Urrutia et al. (2012) found that vitamin D supplementation had conflicting results on LBW, preterm labour and pre-eclampsia and lacked statistical power. Lack of conclusive evidence may be due to the

heterogeneity of populations, small sample sizes and poor adjustment for confounders (Urrutia et al. 2012).

Recently, it has been demonstrated that vitamin D receptor (VDR) polymorphisms, which affect vitamin D concentrations and calcium homeostasis, have been associated with both insulin resistance and reduced birth size (Bodnar et al. 2010; Chiu et al. 2001; Keen et al. 1997; Swamy et al. 2011). In a prospective cohort study of pregnant women by Bodnar et al. (2010) maternal VDR genotype was significantly associated with risk of SGA. One single nucleotide polymorphism (SNP) was observed in the VDR gene among white women and 3 SNPs were found in black women. In another prospective cohort of pregnant women (n=615) a total of 38 SNPs were examined with one SNP in particular (rs7975232) being associated with decreased birth weight in non-Hispanic black women but not in non-Hispanic white women (Swamy et al. 2011). These results suggest that with future research genetic screenings may be developed to aid in detecting abnormalities related to vitamin D and adverse birth outcomes.

iii. *Vitamin B12*

Vitamin B12 (VB12), is a water soluble vitamin found animal source foods including fish, eggs, milk, meat and poultry (Saravanan et al. 2010). The cutoff for plasma vitamin B12 deficiency is < 150 pmol/L (203 pg/mL) (WHO 2008). Deficiency in this vitamin has been associated with megaloblastic anaemia, impaired DNA synthesis, neurological abnormalities and low birth weight (Hovdenak et al. 2012).

Vitamin B12 is emerging as a public health concern among children and pregnant/lactating women (McLean et al. 2008). Deficiency is especially high in developing countries where the intakes of animal protein are low (Allen 2009). In India, preschool children had VB12 deficiency of 36% for breastfed and 9% for non-breast fed children (Taneja et al. 2007), and deficiency was 47% in adults (Refsum et al. 2001). A review by (Allen 2009) found that across Latin America, approximately

20% of children and adults were vitamin B12 deficient. This included women and children in the 1999 Mexican National Nutrition Survey who had prevalence rates of 18% (Allen 2004). In a recent study in Guatemala, B12 deficiency was observed in 35% of mothers (Deegan et al. 2012). These findings demononstrate a global trend of high rates of B12 deficiency.

A recent review by Hovdenak et al. (2012) examined the relationship between VB12 deficiency and the impacts on birth outcomes. They observed an increased risk of neural tube defects; IUGR, preterm births, poor brain and cognitive development and miscarriages in offspring belonging to VB12 deficient mothers.

The relationship between VB12 deficiency and pre-eclampsia is unclear. Pre-eclampsia is a syndrome occurring after 20 weeks of pregnancy with an unknown etiology. It is a serious condition which increases the risk of morbidity and mortality for both the mother and the fetus and has been characterized by maternal endothelial cell dysfunction, poor trophoblastic implantation and excessive inflammation (Saito et al. 2003). Diagnostics are made based on symptoms including proteinuria, elevated blood pressure and edema (Parikh et al. 2008). Sanchez et al. (2001) found no evidence of an increased risk of pre-eclampsia from VB12 deficiency; however Cotter et al. (2001) found a significant increase in pre-eclampsia in mothers with high homocysteine concentrations, a bi-product of general B vitamin deficiencies. Therefore, it may not be B12 specifically that plays a role in pre-eclampsia, but B vitamins in general.

The impacts of VB12 deficiency on birth outcomes during pregnancy (n=80) was investigated by Yajnik, Deshpande et al. (2005). They found that elevated plasma homocysteine, along with VB12 deficiencies, was a significant negative predictor of birth weight (Yajnik et al. 2005). A cohort study of pregnant women in India (n=478) measured serum VB12 concentrations during each trimester (Muthayya et al. 2006) and found that infants who were born to mothers in the lowest tertile of VB12 concentration in the 1st and 2nd trimester had an odds ratio of 5.98 and 9.28 of LBW,

compared to the highest tertile (p 0.006, <0.001, for 1st, 2nd trimester respectively) (Muthayya et al. 2006). These findings were significant even after controlling for maternal age education, parity and baseline weight. Another study found the risk of preterm birth decreased by 60% in women with VB12 status greater than or equal to 258 pmol/L versus women who were classified as deficient (OR=0.4, 95% CI= 0.2, 0.9) (Ronnenberg et al. 2002).

iv. *Folate*

Folate (or vitamin B9) exists in either folic acid or folate form depending on source. Where folate derives from foods such as meat, fish, dairy and green leafy vegetables, folic acid is the oxidized form of folate found in fortified foods as well as dietary supplements and is the more stable of the two forms (Pitkin 2007). Folate deficiency is classified as having < 10 nmol/L (4 ng/mL) (WHO/FAO 2004) and is associated with megaloblastic macrocytic anemia (Aslinia et al. 2006; Metz 2008) and neural tube defects (Pitkin 2007).

Globally, folate deficiency remains a health concern especially in women of childbearing age. A recent cross sectional study in the North of Portugal reported folate deficiency rates in pre-pregnant women 58% and 91% in pregnant women (Pinto et al. 2009). In the US, the National Health and Nutrition Examination Survey (NHANES III; 1988–1994) observed women of childbearing age to have folate deficiency rates of 21% (Pfeiffer et al. 2007). In Latin America, deficiency has been reported as high as 36% in pregnant women (Garcia-Casal et al. 2005). A review by Lee et al. (2012) of studies surveying folate intake of pregnant women in developing regions including Asia, Africa and Latin America, found that in every instance either the median or mean intake of folate was below the recommended EAR of 480μg dietary folate equivalents/day.

Well known manifestations of maternal folate deficiency include congenital malformations (neural tube damage, orofacial clefts, cardiac anomalies), and lesser

known outcomes include preterm birth, LBW and IUGR (Abu-Saad et al. 2010; Phiri 2008). Studies investigating the link between folate deficiencies and preterm birth (<37 weeks of gestation) have been conducted. A study of pregnant women (n=832) in New Jersey, USA observed that women with a low mean daily intake of folate (<240 mg/d) had an approximately two fold greater risk of preterm delivery and LBW. Findings were significant after controlling for maternal characteristics and energy intake (Scholl et al. 1996). A more recent study by Bodnar et al. (2010) observed that in pregnant women (n=313) women who had higher concentrations of folate there was a reduction in risk of preterm birth by 67% (RR=0.33, 95% CI= 0.11, 0.97). Similar findings were observed by Siega-Riz et al. (2004) who found that women who delivered at term had higher mean serum folate concentrations, versus women who had preterm deliveries (21.3 and 20.1 ng/mL, respectively, $P = 0.04$) (Siega-Riz et al. 2004).

A study in Pakistan observed fetal growth via ultra sound starting from the 12th week of pregnancy (n=128). A total of 46 newborns were IUGR and the risk was reduced in women who had folate concentrations in the highest quartile (OR=0.31, 95% CI=0.10, 0.84). There was no association between vitamin B12 and IUGR in this study (Lindblad et al. 2005). However, another study in India did find VB12, in addition to folate, to be significantly associated with IUGR. Analysis of maternal serum (n=180) revealed that women with IUGR pregnancies had significantly lower concentrations of folate (10.24 +/- 3.91 ng/mL) compared to controls (15.20 +/- 3.41 ng/mL). Additionally, vitamin B12 concentrations were also lower (146.99 +/- 43.51 pg/mL) compared to controls (171.96 +/- 25.75 pg/mL). As anticipated, both lowered vitamin concentrations were accompanied with higher concentrations of serum homocysteine (Gadhok et al. 2011).

In conclusion a vast majority of studies looking at either supplementation of vitamins, or vitamin status on birth outcomes have been conducted with varying results. With the exception of vitamin D, many of these studies do not go beyond associative relationship, into possible mechanistic pathways of how vitamins may be

influencing birth outcomes. Furthermore, no studies investigated the relationship between vitamin status on birth length or fetal length.

G. Cytokines and Growth Outcomes

i. T Helper Lymphocyte Subsets

Cytokines are small soluble proteins that are expressed by various cells and tissue types that act as immune mediators (Diaconu et al. 2010). There is a diverse family of cytokines, all of which aid in determining the nature of an immune response. The CD4+ T helper (Th) cells are the predominant producers of cytokines and are divided into subsets: Th1, Th2, Th17 and regulatory T (Treg) (Diaconu et al. 2010). During T cell activation, the types and amounts of cytokines present in the local microenvironment are a major determining factor for the development of CD4+ T cell subsets (Dawson et al. 2006). For example, interleukin 12 (IL-12) and interferon (IFN)-γ directly induce naïve T helper cell differentiation into Th1 cells (Seder et al. 1998), whereas IL-4 induces differentiation into Th2 cells (Murphy et al. 2000). Furthermore, T helper subset cytokines are antagonistic to the differentiation and activity of one another (Mosmann et al. 1996). For example the Th2 cytokine IL-4 represses IL-12 signaling decreasing Th1 expression (Szabo et al. 1997).

Th1 cells are responsible for cell-mediated immunity against viral infections, bacterial, protozoal and intracellular parasites (Murphy et al. 2000). Th1 cells secrete IL-2, IL-12, IFN-γ, tumour necrosis factor (TNF)-α and TNF-β cytokines. This subset is implicated in the development of inflammatory delayed-type hypersensitivity reactions. The Th17 subsets play a role in clearing extracellular bacteria and fungi, particularly at mucosal surfaces. This subtype produces IL-17A (or IL-17), IL-17F, and IL-21, and IL-22 cytokines (McGeachy et al. 2007; Zhou et al. 2009). In the presence of IL-17 and IL-12, Th17 cells have been seen to produce IFN-γ, suggesting a close relationship with Th1 cells (Romagnani et al. 2009). The Th17 response is pro-

inflammatory and is involved in the pathogenesis of various autoimmune and inflammatory diseases such as rheumatoid arthritis (Crome et al. 2010). Classic Th2 cytokines are IL-4, IL-5 and IL-13 and mediate antibody dependent immunity against parasitic helminth infections and are also responsible for the development of allergic reactions (Murphy et al. 2000; Seder et al. 1998).

Sometimes referred to T helper 3 (Th3), Tregs are responisble for maintaining immunological balance and self-tolerance and produce IL-10 (Sakaguchi 2005). Concentrations of IL-10 increase markedly in early pregancy and reamin high until labour (Thaxton et al. 2010). IL-10's role in self-tolerence is particularly important in allowing acceptence of the fetal allograft by the maternal immune system, and is thought of as being an integral key player in the development of normal pregnancy (Thaxton et al. 2010).

ii. The Cytokine Milieu During Pregnancy

The immune system of a woman during pregnancy has been noted to change, or be modulated in order to have a healthy pregnancy (Denney et al. 2011). This immunomodulation occurs in order to tolerate the growing semiallogenic fetus, containing both maternal and paternal genes. Under normal circumstances the presence of non-self-cellular components triggers an immune response, a process which does not occur during normal healthy pregnancies. Various cellular paradigms have been proposed to explain the immunomodulation which occurs during pregnancy necessary to carry a healthy fetus to term. The theory of immune suppression explains that tolerance is achieved during pregnancy by suppression of the mother's immune system, characterizing the pregnancy as a time of diminished immune responses (Wegmann 1984). However the theory of immunosubversion remains unpopular as continued research finds pregnancy to be a time of increased or heightened immune functioning which is carefully controlled (Mor et al. 2011).

The next, and perhaps best known theory, is that pregnancy induces a shift towards Th2 cytokines, or has a Th2 immunologic bias. This was first proposed by Wegmann et al. (1993) suggesting that an anti-inflammatory response is beneficial for the viability of the pregnancy, as one of the supporting mechanisms to prevent maternal immune response against the semiallogenic fetus. A number of studies support the theory that an increased anti-inflammatory Th2 milieu during pregnancy is protective for the fetus against the deleterious Th1 response (Challis et al. 2009; Mor et al. 2011; Saito et al. 2010). This theory is further strengthened with the observation that inappropriate shifts towards Th1 cytokines have been observed with recurrent spontaneous abortion (Saito et al. 2010) and pre-eclampsia (Saito et al. 2003). However, the reported Th2 dominated immune response may be localized only to the feto-maternal interface rather than a systemic maternal immune response (Saito 2000) and therefore may not be an adequate description of the immunomodulation occurring during pregnancy.

Moreover, Th2 cytokines have also seen to be elevated in recurrent spontaneous abortion (Chaouat et al. 2003) further complicating this theory. The current theory that pregnancy is predominantly a Th2 response may be inadequate in explaining the vastly complex maternal immunity during pregnancy (Mor et al. 2011).

iii. Pregnancy as a Dynamic Process

Lack of a cohesive theory regarding the behaviour of the maternal immune system during pregnancy may arise from the diversity of studies conducted, ranging from murine and mouse models to women with reproductive pathology that are typically limited to cross sectional design (Denney et al. 2011). The idea of using cross sectional data to elucidate immune responses during pregnancy may be inherently flawed argues Mor et al. (2011) who see pregnancy as not a single event but three separate immunological phases. The first trimester is a time of pro-inflammation during implantation and placentation, noted by increased concentrations of pro-inflammatory mediators such as prostaglandin E_2(PGE_2), IL-1, IL-6, and TNF-α (Mor

et al. 2011). The presence of these mediators contributes to vascular remodeling and placental invasion, as well as aid in repairing damaged cells of the uterine epithelium ensuring proper placentation necessary for fetal growth (Mor et al. 2011). The second trimester is an immunological phase of pregnancy and involves a decrease in inflammatory markers as the placenta reaches its full size at approximately 20 weeks of gestation. These mediators remain low until the third trimester, as the onset of labour is accompanied by an increase in PGE_2 and pro-inflammatory cytokines to aid in partition (Yuan et al. 2009).

Another recognized component of healthy pregnancies is cytokine IL-10, believed to be involved in adequate placental formation by aiding in trophoblastic invasion (Agarwal et al. 2000). IL-10 induces the production of vascular endothelial growth factor C and the aquaporin system from trophoblastic cells, thus stimulating placental angiogenesis (Thaxton et al. 2010). Towards the onset of labour, IL-10 production is decreased allowing the necessary rise in inflammatory cytokines to induce labour (Hanna et al. 2000).

Although there is changing discourse regarding what cytokine response is appropriate for pregnancy, there is evidence that perturbations in cytokine profiles, particularly increased maternal inflammation, can result in adverse pregnancy outcomes (Von Dadelszen et al. 2002).

iv. Adverse Consequences of Inflammation During Pregnancy

Pro-inflammatory cytokines, particularly, TNF-α, IL-1β and IL-6 are associated with negative birth outcomes including recurrent pre-eclampsia (Saito et al. 2003) and preterm birth (Saito et al. 2010).

One of the defining characteristics of pre-eclampsia is excessive maternal inflammation during pregnancy (Saito et al. 2003). A review by Ramma et al. (2011) explains that in pre-eclamptic women the decidual lymphocytes as well as peripheral

blood mononuclear cells synthesize higher concentrations of Th1 cytokines while exhibiting low expression of Th2 cytokines. Particular cytokines of noted increase have been IL-6, TNF-α and chemokine and monocyte chemoattractant protein 1 (MCP-1) (Jonsson et al. 2006; Szarka et al. 2010).

Elevated concentrations of cytokines have also been observed in women with spontaneous preterm deliveries resulting from histologic chorioamnionitis, a condition associated with infection of the fetal membranes. It was observed that women who had preterm deliveries <35 weeks with histologic chorioamnionitis had elevated concentrations of IL-1β, IL-4, IL-6, IL-12 and TNF-α compared to women who had preterm deliveries without histologic chorioamnionitis (Gargano et al. 2008). In addition, a more recent study observed elevated concentrations of IL-17 in women undergoing preterm delivery with histologic chorioamnionitis. From their observations, these authors concluded that IL-17 was responsible for promoting inflammation at the feto-maternal interface during preterm delivery (Ito et al. 2010).

Pro-inflammatory cytokines have also been implicated in the sequelea of growth faltering; however the majority of literature has focused on the postnatal environment. A review by Briana et al. (2009) found that the literature regarding cytokines and IUGR is inconclusive. Both decreases (Ødegård et al. 2001) and increases (Street et al. 2006) of fetal IL-6 have been observed in IUGR as have normal (Opsjon et al. 1995) and decreased (Schiff et al. 1994) concentrations of fetal TNF-α. In a review by MacRae, Wong, et al. (2006) cytokines IL-1β, IL-6 and TNF-α were key inflammatory mediators implicated in growth faltering. This is most likely occurring via inhibition of proper bone growth and development, as TNF-α and IL-1β have been reported to inhibit growth plate chondrocyte dynamics (MacRae, Farquharson, et al. 2006; Mårtensson et al. 2004) and IL-6 modulates skeletal development via directly affecting osteoclast maturation and activity (Kamimura et al. 2003; Tamura et al. 1993).

In addition to hindering bone development, pro-inflammatory cytokines impair the GH-IGF-1 axis (De Benedetti et al. 1997), although it is not well understood if inflammatory cytokines exhibit their effect on the production of IGF-1 or the downstream effects of IGF-1. In another study by De Benedetti et al. (2001), IL-6 transgenic mice had significantly lower concentrations of IGF-I compared to their wild-type littermates. However, concentrations of growth hormone production remained unaltered, suggesting the effects of IL-6 were not mediated via GH, but rather on IGF-1 production itself. These findings are supported by previous research which found that IL-6 inhibited IGF-1 concentrations in rats (Lazarus et al. 1993). In a study using rat cells, exposure to TNF-α led to an 85% decrease in IGF-1 production, as measured by mRNA concentrations (Anwar et al. 2002). A study using human cells, reported that TNF-α, IL-1β, and IL-6 dose-dependently inhibit IGF-I-promoted DNA synthesis but not IGF-1 itself (Shen et al. 2002). Recent studies have examined this phenomenon in prenatal environments.

Cord blood samples from fetal growth restricted neonates had higher concentrations of IGFBP-1, IGFBP-2, and IL-6 contents compared with controls (Street et al., 2006). These findings are supported by a previous study by Marchini et al. (2005) who found increases in both IL-6 and IGFBP-1 was associated with decreased IGF-1 in cord blood of infants from complicated pregnancies (Marchini et al., 2005).

The immune response during pregnancy is complex and not well understood. Further research into the characterization of immune responses during this time will continue to aid our understanding of the etiology of adverse growth outcomes occurring from abnormal immune responses and cytokine expressions. Furthermore, greater understanding of key cytokines will be beneficial in creating effective therapies as well as screening tools of abnormal fetal growth.

H. Vitamin and Cytokine Interactions

The Th1 and Th2 (pro- and anti- inflammatory) subtypes are kept in delicate balance to one another, as imbalances or improper expression of one subset can lead to an inappropriate immune response which can cause damage to the host and result in failure to overcome pathogens (Bour-Jordan et al. 2009). The possible role of nutritional deficiencies in skewing this polarization has been under investigation as part of a larger effort to better understand the relationship between nutrition and immunity.

Vitamin A and related compounds have been found to be potent modifiers of Th1 and Th2 responses; however the mechanisms of action are poorly understood (Carman et al. 1991; Cui et al. 2000; Ikeda et al. 1994; Wiedermann et al. 1996; Wiedermann et al. 1993). Studies using vitamin A supplementation have shown a decrease in Th1 pro-inflammatory cytokines, and an increase in Th2 cytokines (Wintergerst et al. 2007). In a study by Wiedermann et al. (1996) vitamin A deficient rats were observed to have two times greater IFN-γ, compared to control. Similar findings have been reported in other animal studies (Cantorna et al. 1994; Carman et al. 1991). In addition suppression of the Th2 phenotype has been observed in VAD mice, marked by a reduced expression of cytokine IL-4 (Carman et al. 1992). Dawson et al. (2006) using human T cells treated with either all-trans-retinoic-acid or 9-*cis*-retinoic acid, observed an induction in the expression of Th2 cytokines: IL-4, IL-5 and IL-13 and inhibition of IFN-γ.

Vitamin D was first recognized for its antimicrobial properties (Rook et al. 1986) is now widely accepted as an immune modulator, exerting its effect via regulation of gene transcription. Studies have observed an increase in Th1 cytokines, particularly of IL-17 and TNF-α, in vitamin D deficient patients (Milovanovic et al. 2012) and a decrease in inflammatory Th1 responses with vitamin D supplementation, via the decreased production of pro-inflammatory cytokines (Lemire et al. 1991; Lemire et al. 1992; Mathieu et al. 1994; Tsuji et al. 1994). Tang et al. (2009) has

shown that vitamin D directly suppressed IL-17 production by $CD4^+$ T cells and also inhibited the production of cytokines IL-1β, IL-6, TNF-α and IL-12 in mice (Tang et al. 2009). Interestingly, both vitamin A and D have been shown to act synergistically in inhibiting the formation of inflammatory Th17 cells (Ikeda et al. 2010).

Despite the growing literature regarding the impacts of vitamin A and D on the immune system, little research exists regarding folate and cytokines, and to the best of our knowledge, no investigations have previously observed a relationship between vitamin B12 and cytokines. Recently, it has been observed that lower concentrations of folic acid in pregnant women were associated with increases in pro-inflammatory cytokines versus anti-inflammatory cytokines (Simhan et al. 2011). Furthermore, an earlier study had observed that folic-acid supplementations in a porcine pregnancy model decreased concentrations of granulocyte/macrophage colony-stimulating factor (Guay et al. 2004), a cytokine that stimulates the production of white blood cells, and high amounts this cytokine have been associated with rheumatoid arthritis (Li et al. 2012).

Vitamins A, D and folate appear to be modulating cytokine expression in a similar fashion by elevating Th2 cytokines and supressing Th1 cytokines. Furthermore, deficiencies in these vitamins have been linked with increases in pro-inflammatory Th1 cytokines therefore skewing the immune system towards a pro-inflammatory state. As we mentioned earlier, there is a growing body of literature linking pro-inflammatory cytokines to adverse fetal outcomes, therefore the impact of vitamin deficiencies on maternal cytokine expression during pregnancy warrants further investigation.

I. Conclusion

In conclusion there are many factors influencing fetal growth and development ranging from maternal anthropometrics to vitamin deficiency, hormones, infections and immune responses. Despite this, the majority of research to date has focussed on one or two of these aspects - that is to say most studies have failed to bring these

concepts together. Furthermore, the majority of literature investigating growth outcomes has only measured the postnatal anthropometrics and by doing so has failed to capture what is occurring *in utero.* Therefore there is a need for future research to look at multiple concurrent infections alongside multiple nutrient deficiencies *in utero* to help broaden our understanding of the physiological mechanisms which impact fetal growth.

CHAPTER III: RATIONAL AND OBJECTIVES

The indigenous populations in Panama are vulnerable to health problems and growth faltering, particularly the Ngöbe-Buglé, who have reportedly high rates of infection, chronic malnutrition and stunting (MINSA 2003; Payne et al. 2007). In our cohort of pregnant women, we observed high rates of infection: 23% had dental caries, 17% had scabies skin infection, and 6% had respiratory tract infection. Urogenital infections were also examined including vaginosis, yeast infection, gonococcal and trichomoniasis with infection rates of 81%, 24%, 9%, 17% and 60% respectively (Gonzalez-Fernandez 2012; Suissa 2013). Additionally, micronutrient deficiencies have known to be a long standing issue in this population, particularly vitamin A (Payne et al. 2007). Recent data however reveals that while vitamin A concentrations are adequate, with only 6% of women classified as subclinical (0.35 to 0.7μmol/L) and none to found to be clinically deficient (<0.35μmol/L), vitamins D, B12 and folate concentrations are a problem. In fact, 65% of women were vitamin D deficient (<50nmol/L), and a further 32% of women were vitamin D insufficient (50 to 74.9nmol/L) leaving only 3% who having adequate serum concentrations (≥75nmol/L) (Gonzalez-Fernandez 2012; Suissa 2013). Vitamin B12 deficiency (< 150pmol/L) was present in 85% and folate deficiency (<10 nmol/L) was present in 24% of these pregnant women (Willams 2012). The combination of multiple infections and micronutrient deficiencies presumably has adverse consequences on health and in pregnant women may adversely affect the growth and the fetus.

Maternal vitamin deficiencies and infections have been associated with negative birth outcomes including low birth weights occurring from both IUGR and preterm deliveries (Giraldo et al. 2012; Kramer 1987a; Leffelaar et al. 2010; Morley et al. 2006; Shroff et al. 2011; Tielsch et al. 2008). The mechanism by which multiple infections and micronutrient deficiencies affect growth have not been fully elucidated, though there is evidence that deficiency of vitamins A and D may be implicated via modulating inflammatory processes particularly that of cytokines.

Vitamin A and D play functional roles in modulating inflammatory responses of the immune system particularly T helper (Th) cells. Numerous *in vitro* studies have demonstrated that deficiencies in both of these fat-soluble vitamins shift the cytokine milieu from Th2 and Treg (anti-inflammatory) towards the Th1 and Th17 (pro-inflammatory) profile (Cantorna et al. 2000; Cantorna et al. 1994). Maintenance of an anti-inflammatory cytokine milieu is particularly important because it has been shown, along with the hormone cortisol (Hofbauer et al. 1999; Weinstein et al. 1998) that pro-inflammatory cytokines impair the growth hormone (GH) insulin-like growth factor (IGF-1) axis, resulting in lower concentrations of IGF-1 (Odiere et al. 2010). IGF-1 is a main endocrine regulator of fetal growth and alterations in this hormone have been observed to negatively impact growth and development (Chiesa et al. 2008). From this framework we can hypothesize that vitamin status maybe influencing inflammatory responses which in turn can affect fetal growth via decreased concentrations of IGF-1.

In contrast to vitamins A and D, few studies have examined the relationship between vitamins B12 and folate on modulating cytokine profiles. However, given the importance of these B vitamins on fetal growth and development, with deficiencies being observed with increases risk for low birthweight pregnancies (Yajnik et al. 2005), exploration of this potentially novel relationship is warranted. Therefore, we hypothesize that vitamin status (A, D, B12 and folate) will modulate maternal cytokines during pregnancy. Our secondary hypothesis is that cortisol and pro-inflammatory cytokines will be negatively associated with fetal growth via downregulation of maternal IGF-1.

The goal of this proposed cross-sectional study is to determine the relationship between maternal cortisol, IGF-1, and fundal height, as well as the effect of vitamin deficiencies, infections and cytokines on the maternal cortisol-IGF-1-fundal height pathway. The specific objectives are:

- To examine the relationship between vitamin status (A, D, B12 and folate) on serum cytokine concentrations

- Determine the relationship between maternal cortisol, IGF-1 and fundal height
- To examine the effect of vitamin deficiencies, infection and cytokines on the maternal cortisol, IGF-1 and fundal height pathway

CHAPTER IV: PRENATAL PAPER

Title: Vitamin deficiency, infection and cytokine interactions in pregnant Panamanian women: *impact on cortisol-IGF-1-fundal height pathway.*

Short title: Factors influencing fetal growth.

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ABSTRACT

In developing countries, multiple micronutrient deficiencies and multiple infections co-occur in pregnant women. Laboratory studies provide evidence that both nutrient deficiencies and infection modulate cortisol and inflammatory cytokines resulting in lower concentrations of IGF-1, a hormone essential for *in utero* growth. However, these interactions have not been explored in human populations. The objective of this study was to determine how vitamin deficiencies, infection and cytokine interactions influence the cortisol-IGF-1–fundal height pathway. Specifically we examined the consequences of vitamin deficiencies (vitamin A, D, B12 and folate), infections (oral, urogenital, skin and periodontal) as well as cytokines (TNF-α, IFN-γ, IL-1b, IL-4, IL-6, IL-10, IL-12p40, IL-13, IL-17) and one chemokine (MCP-1) on fundal height. A previous cross-sectional study from pregnant indigenous women (n=184) from the Ngäbe-Buglé Comarca of western Panama provided data on maternal anthropometry, infections, and micronutrient status. Fundal height was used as the indicator for fetal growth and was corrected for gestational age. Data was analyzed by t-test and multiple regression analyses.

A positive relationship between cortisol and IGF-1 emerged, and IGF-1 was positively associated with fundal height. However, none of the eight infections measured in this study were directly associated with fundal height. Moreover, of the four vitamins, only vitamin D entered as a predictor of fundal height but it was negatively associated. In addition, of the 10 cytokines included in our study, only the T-regulatory cytokine IL-10 and was negatively associated with fundal height. Interestingly, trichomoniasis infection increased concentrations of IL-10, and through this mechanism may have been associated with impaired fundal height. Our exploration of the impact of vitamins and infections on maternal cytokines, cortisol and IGF-1 revealed that vitamins and infections modulated the cytokines, cortisol and IGF-1 differently. Whereas dental caries increased cortisol, vitamin D lowered concentrations of cortisol. In addition, bacterial vaginosis decreased concentrations of IGF-1, whereas vitamin A increased concentrations of IGF-1. Higher concentrations of vitamin B12 increased cytokine concentrations (IL-6, IL-10, IL-13, IFN-γ, TNF-α). Together our data demonstrate that fundal height is influenced via the cortisol-IGF-1

pathway, which is modulated directly by both vitamins and infections, and indirectly via cytokines. Fundal height is both positively and negatively influenced by infections, by vitamin concentrations and by both pro- and ant- inflammatory cytokines. Our findings demonstrate the importance of examining the impacts of both micronutrient deficiencies and multiple infections on maternal health to better understand of the mechanisms which impact fetal growth.

INTRODUCTION

Rural women and children in Panama are prone to growth faltering due to high rates of malnutrition and co-infection. This is particularly true for the indigenous populations of the Ngäbe-Buglé Comarca, where stunting rates of up to 61% of preschool children have been observed (MINSA 2003; Payne et al. 2007). Additionally, recent data from pregnant women in this region have revealed multiple micronutrient deficiencies of iron, folic acid, and vitamins A, D and B12, as well as high incidences of vaginal (92%), urinary tract (52%), oral (23%) and skin (20%) infections (Gonzalez-Fernandez 2012).

The consequences of growth faltering are long-term and far-reaching: reduced quality of life due to lower income and lower levels of schooling, as well as lower birth weights in subsequent generations, which further perpetuates the cycle of reduced living standards (Victora et al. 2008). It is widely accepted that growth faltering begins at 3 months of age (Shrimpton, Victora, de Onis, Lima, Blossner, et al. 2001); however, mounting scientific evidence indicates that the prenatal environment is indeed a crucial period of development and that *in utero* interventions may be critical to prevent growth failure later on in life (Reyes et al. 2005). The etiology of *in utero* growth faltering is complex and may involve multiple factors, including vitamin deficiencies, infection, and inflammation.

Maternal vitamin deficiencies have been associated with poor growth outcomes in offspring, including lower birth weights and stunting (Leffelaar et al. 2010; Morley et al. 2006; Shroff et al. 2011; Tielsch et al. 2008). Vitamin A deficiency during pregnancy has been linked with an increased risk of low birth weight and low length of offspring (Rondo et al. 2001; Semba et al. 1997; Shah et al. 1984; Tielsch et al. 2008; Tolba et al. 1998). Similarly, maternal vitamin D deficiency has been shown to have a negative impact on newborn weight and length in both human and animal studies (Brooke et al. 1980; Brooke et al. 1981; Sabour et al. 2006; Scholl et al. 2009). Vitamins B12 and folate deficiencies have been linked to adverse growth outcomes,

particularly with regard to intra-uterine growth restriction (IUGR) (Lindblad et al. 2005; Muthayya et al. 2006).

A number of infections have been associated with poor *in utero* growth outcomes, including vaginosis (Thorsen et al. 2006) urinary tract infections (Mazor-Dray et al. 2009; Schnarr et al. 2008), respiratory tract infections (Banhidy et al. 2008), and sexually transmitted diseases such as HIV-AIDS (Grivell et al. 2009) and syphilis (Gilbert 2002).

The mechanisms through which vitamin deficiencies and infection affect neonatal growth remain to be elucidated, though there is mounting evidence to suggest that these poor growth outcomes are caused by increases in inflammation, shown by several markers including higher concentrations of cortisol (Harris et al. 2011) and pro-inflammatory cytokines (Saito et al. 2010).

Deficiencies in vitamins A and D (Hall et al. 2011) and folate (Simhan et al. 2011) have been observed to increase pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNF-α. Furthermore, increased concentrations of both pro-inflammatory cytokines (Beigi et al. 2007) and cortisol (Ruiz et al. 2001) have been observed to be elevated from infections during pregnancy. Consequently, both elevations of pro-inflammatory cytokines (Anwar et al. 2002; De Benedetti et al. 1997; Lazarus et al. 1993) and cortisol (Chen et al. 1991; Hochberg 2002) have been observed with a decrease in IGF-1 concentrations, an anabolic hormone crucial for proper growth and development in both the pre- and post-natal environments (Chiesa et al. 2008). Therefore, it may be that nutrient deficiencies, along with multiple infections may create a pro-inflammatory environment which in turn adversely affects growth by decreasing concentrations of IGF-1.

The main objectives of this study were to examine the relationship between maternal cortisol, IGF-1, and fundal height, as well as the effect of vitamin deficiencies, infections and cytokines on maternal cortisol- IGF-1-fundal height

pathway. We hypothesized that maternal vitamin deficiency and infections would decrease fetal growth through increased pro-inflammatory cytokines and cortisol which would lead to a decrease in maternal IGF-1, causing low fundal height.

METHODOLOGY

This study is part of a large cross-sectional study focused on anemia, infection and fetal growth conducted in Panama by McGill University, in collaboration with University of Panama as well as the Panamanian Ministry of Health (MINSA). Bio-banked blood samples were used to measure our biochemical markers of interest including cytokines IL1-β, IL-4, IL-6, IL-10, IL-12p40, IL13, IL17, TNF-α, IFN-γ and chemokine MCP-1 as well as cortisol and IGF-1. Our data were integrated with existing data from this larger study to address our objectives.

Our study population was pregnant women who lived in the Ngäbe-Buglé Comarca, in Western Panama. The country of Panama has one of the highest inequality disparities in the world with Indigenous Panamanian groups faced with the most severe cases of poverty (WHO 2011). This hardship correlates with child malnutrition due to the food insecurity faced by these groups, with the highest incidence occurring among the Ngäbe-Buglé (WHO 2011). In addition, the Ngäbe-Buglé are known to suffer from a variety of health issues including parasitic infections, anemia, tuberculosis and stunting (MINSA 2003). Pregnant women and women of childbearing age are at increased risk of multiple micronutrient deficiencies and malnutrition because their intake requirements are high (Haider, Yakoob, et al. 2011) further exacerbating their situation.

During the months between August and November of 2010, women from the Ngäbe-Buglé Comarca were recruited from Rural Health Centres (RHC) in the following 14 locations: Chami, Alto Caballero, Oma, Soloy, Quebrada Hacha, Hato July, Kuerima, Hato Pilon, Lajero, Quebrada Guabo, Quebrada Loro, Corotu, Emplanada De Chorcha and Chichica. RHCs were chosen based on proximity to the Hospital laboratory in San

Felix, as well as accessibility by car. Individuals were made aware of the study in a preliminary introduction to the community by community health workers, midwives, and nurses in the region. Participants who responded to this word-of-mouth advertisement were recruited for the study, as well as women who presented themselves to the health centers for antenatal care or pregnancy testing. Both the recruiting and this study were conducted at the RHC.

Participants were included based on the following criterion: (1) indigenous pregnant women who lived with in a 2 hour walk of a RHC and who reported to the RHC during the study. However, women were excluded from the study if they were (1) pregnant with twins, (2) required regular medication for major chronic diseases or (3) were critically ill at the time of the study. A total of 217 women were approached to participate in this study and 214 agreed to participate; one woman did not meet the inclusion criteria and was excluded from the study. The final 213 women consented to the personal interview and subsequent sample collections. However, for our analysis the first trimester of pregnancy was excluded, resulting in a final total of 184 women for this study.

ETHICAL CONSIDERATION

This study received approval from the Institutional Review Board of McGill University, the formal Ethics Review Board for research in Panama at the Gorgas Institute, the Panamanian Ministry of Health and Provincial, Local and indigenous health authorities.

Interested women were approached individually and informed about the study and research purposes. Study procedures, confidentiality and the request for permission to review medical records were explained in detail. Women were informed they had the right to refuse to participate and to withdraw from the study at any time. Consent forms were provided and signed in the presence of a witness; individuals who could

not read or write had a witness sign on their behalf. There was no financial compensation for participation in this study. Additionally, clinicians caring for these women were sent results in order to provide care or prescribe treatment for infection or illness.

Data Collection

Field Procedures: Data was collected over the period of July to December 2010. Most of the data was collected via medical consultations but some information was also gathered from medical records.

Data collection included: anthropometric measurements, assessment of skin type, measurement of vitals, systemic search for infections, obstetric examination, clinical/obstetric history, and an interview regarding factors that may affect maternal and infant health such as food sources of key micronutrients, micronutrient supplementation, coffee consumption, wood smoke exposure, and hours of daily field work. Urine, stool, venous blood (10 ml) and vaginal samples were also collected from the women; all methods were previously described by Gonzalez-Fernandez (2012)

Fundal height, a measure of fetal growth and size, was the fetal outcome of this study. Fundal height was obtained by measuring the length between the pubic symphysis to the top of the uterine fundus via a measuring tape (Divon, 2012). The tool of measuring fundal height as an indicator for fetal growth is only appropriate for the 2nd and 3rd trimester (Divon, 2012) and so participants from the first trimester were excluded from all analysis.

Prior Analyses of Samples

Diagnoses of infection are previously described in Gonzalez-Fernandez (2012). Oral, respiratory and skin infections and vaginal infections were diagnosed by a clinician and also through laboratory analyses.

Urine samples: The diagnosis of urinary infection (n=209).was performed using microscope examination as well as dipstick strips (URISCAN®) testing for urinary blood and leukocytes as indicators of infection. See Gonzalez-Fernandez (2012) for more details.

Vaginal smears: Vaginal infections were diagnosed based on clinical observations and/or microscopic examination of stained vaginal smears. A sterile swab was used by the physician to take a vaginal smear, which was then plated on a slide, air-dryed and Gram stained for the detection of *Diplococcus,* vaginal candidiasis, gonococcal and trichomoniasis infection. Bacterial vaginosis was diagnosed by presence of clue cells. See Gonzalez-Fernandez (2012) for more details.

Blood samples: Methods used to assay concentrations of C-reactive protein (CRP), vitamins A, D, B2 and folate were reported previously by Gonzalez-Fernandez (2012), Suissa (2013) and Williams (2012).

Clinical vitamin A deficiency is defined as having a serum retinol concentration below 0.35μmol/L and subclinical deficiency is below 0.7μmol/L, normal or adequate status being above 0.7μmol/L (Sommer et al., 2002; WHO, 2009b). Some researchers argue that an additional cutoff of 1.05μmol/L be made for pregnant populations to identify those at risk of deficiency (Baeten et al., 2004; West, 2002). In this study both marginal vitamin A (<1.05 μmol/L) and subclinical deficiency (<0.7μmol/L) were used.

We defined vitamin D deficiency as having serum 25(OH)D <50nmol/L. Due to the high frequency of deficiency in our population we further distinguished severe deficiency as <25nmol/L.

Classification of serum vitamin B12 deficiency was set at <150 pmol/L and folate at <10nmol/L or 4ng/mL as recommended by WHO (2008).

PROTOCOLS FOR ADDITIONAL ASSAYS

As part of the present study, the concentrations of cytokines and chemokines (n=211), insulin human growth factor (IGF)-1 (n=210), and cortisol (n=212) were determined via Luminex machine (Luminex Corp., U.S.A.) using the Human 10-plex Cytokine/Chemokine Magnetic Bead Panel (Cat. HYCTOMAG-60K), the Human IGF-1 single plex (Cat. HIGF1-52K-01) and the Steroid/Thyroid Hormone Magnetic Bead Panel (Cat. STTHMAG-21K) respectively (Millipore Corporation Canada). For each assay, all samples, standards and quality controls were conducted in duplicate and quality controls were within accepted ranges. All assays were performed in our laboratory in accordance to instructions provided by the Millipore Company and under similar conditions to minimise environmental differences.

The analysis plates were washed with 200 µL of wash buffer and placed on a shaker for ten minutes and then decanted. 25 µL of standard and controls were added in duplicate to appropriate wells. Assay buffer and matrix solution was then added to every well on the plate. 25µL of our unknown serum samples were added in duplicate to each of the appropriate wells. Finally 25µL of light sensitive magnetic detection beads were added to each of the wells and incubated overnight in the refrigerator (-8°C). The following day, plates were placed on a powerful magnet and contents gently decanted. Unnecessary solutions, analytes and debris were carefully washed out using 200µL of wash buffer while the plate remained on the magnet to ensure all contents except magnetic beads were decanted. Failure to properly do this would result in loss of magnetic beads. After this was done twice, 25µL of detection antibodies were added to each of the wells and the plate was incubated for 1 hour. 25µL Streptavidin-phycoerthrin was added to each of the wells and plates were incubated for 30 minutes at room temperature. Plates were again washed using the procedure mentioned above and finally 150µL of sheath fluid was added to each well. Each plate was read on the Luminex machine using 100µL sheath fluid reading at 100 beads per bead set.

STATISTICAL METHODS

Data analysis was done using SAS software version 9.2 (SAS Institute Inc.). Data from the first trimester was excluded from our analysis as fundal height measurement is only validated in the 2nd and 3rd trimester. Kolmogorov-Smirnov goodness-of-fit test revealed that none of the cytokines were normally distributed. Log and natural transformations were performed in attempt to normalize the data but failed to do so. Therefore non-parametric procedures were used for cytokine analysis including single cytokines and cytokine ratios. Wilcoxon Rank Sum non-parametric tests were conducted to determine if the median cytokine concentrations differed between mothers classified as sufficient versus insufficient with regard to vitamin B12 and folate concentrations. As concentrations of both vitamins A and D were separated into 3 groups, the Kruskal-Wallis non-parametric ANOVA in conjunction with the Bonferroni post-hoc test was used to compare median cytokine concentrations for these fat-soluble vitamins. Data are reported as mean ± SE. Median is also included when differences were significant.

We were interested in examining the impact of infections, vitamins and cytokines on cortisol, IGF-1 and fundal height, our major dependent variables. Therefore we performed a series of stepwise regression models beginning with hierarchal exploratory stepwise regressions. For each dependent variable (cortisol, IFG-1 and fundal height), two sets of exploratory models were created, the first which included infections and the second which included vitamins. In this population all cytokines were highly correlated with the exception of chemokine MCP-1. To overcome this, in each set of models, we made multiple models, the first set consisting of entering cytokines individually, and the second set was done by including all cytokines together in the exploratory regression models. Regardless of methodology, a consistent pattern of cytokine significance in the models emerged, signifying that correlation among cytokines was not impacting model outcomes. Each set of model also included CRP as well as control variables maternal height, maternal weight and trimester. Variables that entered the exploratory models with a p value <0.15 were then

entered into a final multiple regression model, along with our control variables of maternal height, maternal weight, and trimester. For any cytokine that emerged as significant ($P < 0.05$), a similar exploratory process was performed to create regression models for each significant cytokine. This provided insight as to which infections and vitamins, mediated through cytokines, were also impacting our main dependent variables.

RESULTS

Population characteristics have been previously reported by Gonzalez-Fernandez (2012), Williams (2012) and Suissa (2013). Gestational age ranged from 5 to 42 weeks.

Cortisol: For this pregnant population the mean value for cortisol was 71.9 ± 3.5 ng/ml.

Insulin-like Growth Factor: The mean value for IGF-1 was 4.4 ± 0.43 ng/mL. IGF-1 concentration did not differ according to vitamins A, D, B12 or folate status, although IGF-1 was positively correlated with vitamin A ($r=0.225$, $p=0.0011$). A dichotomous variable for IGF-1 was created using the mean values in this population (4.418 ng/mL) as a cutoff between higher and lower concentrations. Individuals with lower IGF-1 concentrations had significantly lower concentrations of cortisol (59.80 ± 3.3 ng/mL vs. 101.0 ± 5.9 ng/mL, $p<.0001$) and also had lower fundal height (25.5 ± 0.79 cm vs. 28.2±1.5 cm, $p=0.0289$).

Cytokines: Histogram representations of the measured cytokines and chemokines revealed a high amount of skewing (see Appendix), suggesting that cytokine distributions were non-normal, a finding that was statistically confirmed by goodness-of-fit tests. Cytokines were all positively correlated with all other cytokines with the exception of the chemokine MCP-1 which was not correlated with any of the other nine cytokines.

Cytokine and Vitamins: Vitamin A was categorized in three separate categories: deficient (<0.7μmol/L), marginal (0.7μmol/L and 1.05μmol/L) and sufficient (1.05μmol/L). No difference between cytokine means and ratios were observed from women with deficient, marginal or sufficient vitamin A status (Table 1).

Vitamin D was also grouped into three categories: deficient (<25nmol/L), marginal (25nmol/L-50nmol/L) and sufficient (>50nmol/L). Pro-inflammatory cytokine, TNF- α, was significantly lower in women with who were vitamin D deficient compared to women who had marginal vitamin D status (p=0.01), but not compared to women with sufficient vitamin D status (Table 2).

Vitamin B12 was set as dichotomous variable, with women being either deficient (<150pmol/L) or sufficient (>150pmol/L). Mothers who were B12 sufficient had higher concentrations of IL-6 (p=0.018), IFN-γ (p=0.019) and TNF-α (p=0.022) when compared women who were classified as deficient (Table 3).

Folate was also set as a dichotomous variable, with women being either deficient (<10nmol/L) or sufficient (>10nmol/L). Compared to women who were folate deficient, folate sufficient women had lower concentrations of IL-10 (p=0.0151). These results were reflected by cytokine ratios which included IL-10. We observed that women with sufficient folate status had higher concentrations of IFN-γ:IL-10 (p=0.0190), TNF-α:IL-10 (p= 0.0003), and IL-17:IL-10 (p=0.0024) compared to folate insufficient women. Furthermore, the cytokine ratio TNF-α:IL-13 (p=0.029) was increased in folate sufficient groups, suggesting folate sufficiency is increasing TNF-α and decreasing IL-13 (Table 4).

With the exception of vitamin A, vitamin status in our population of pregnant women was observed to have an impact on cytokine concentrations and ratios. TNF- α was the only cytokine to have been modulated by two nutrients (vitamin D and B12), with other cytokines and ratios being affected by only one vitamin.

Multiple Linear Regression Models

Factors associated with Cortisol

In our final multiple regression model we were able to predict 43.5% of the variability of cortisol (Table 5a) with the strongest mediators of cortisol being cytokines. A variety of cytokines entered the model, including anti-inflammatory cytokines IL-4 and IL-13 which had opposing effects on cortisol, with IL-4 negatively predicting cortisol and IL-13 positively predicting cortisol. Similarly, pro-inflammatory chemokine MCP-1 positively predicted cortisol, while pro-inflammatory cytokine IL-6 negatively predicted cortisol. The only infection to enter the final cortisol model was severity of dental caries, which was positive predictor, and the only vitamin to enter the model was vitamin D as a negative predictor.

Factors affecting cortisol via mediation of IL-4, IL-6, IL-13 and MCP-1 were explored. The regression model for IL-4 (Table 5b) predicted 11.5% of the variability and included respiratory, gonococcal infection and scabies as positive predictors. In contrast severity of vaginosis was a negative predictor.

Our linear regression model predicted 8.67% of the variability for IL-6 (Table 5c). Vitamin B12 was a strong positive predictor for IL-6. Vaginosis was the only infection to appear and was a negative predictor of IL-6.

The regression model for IL-13 included only vitamin B12 as positive predictor, and accounted for 12.30% of the variability (Table 5c).

Regression model for MCP-1 predicted 7.55% of the variability with the negative predictors being wood smoke exposure and severity of trichomoniasis infection (Table 5e). No vitamins entered this model.

Factors Associated with IGF-1

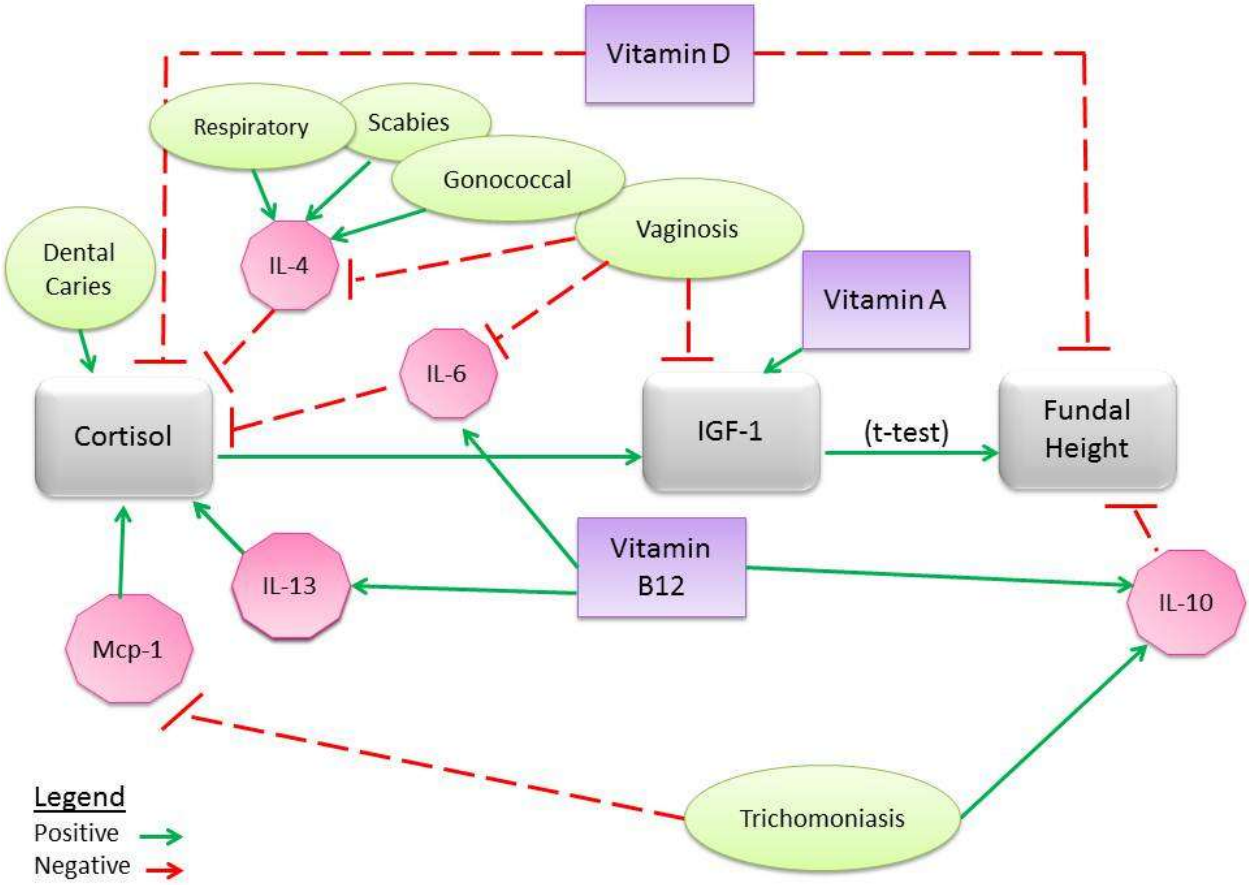
In a multiple regression model of IGF-1 (Table 6a) we were able to predict 19.84% of the variability. After controlling for trimester, positive predictors of IGF-1 included cortisol and vitamin A. Severity of vaginosis infection was the only infection to enter this model and was a negative predictor. No cytokines significantly entered the model for IGF-1.

Factors Associated with Fundal Height

Multiple regression models predicted 81.23% of the variability for fundal height, explained primarily by gestational age, maternal weight and height (Table 7a). Vitamin D was a negative predictor and no other vitamins or infections entered this model. IL-10 was the only significant cytokine that entered and was a negative predictor of fundal height.

To better illustrate our findings, we have provided a diagram with all the interactions between vitamins, infections and cytokines with cortisol, IGF-1 and fundal height.

Figure 2 Overview of results



Diagrammatic representation of our regression models for the major outcome variables cortisol, IGF-1 and fundal height, shown in grey. Interactions are shown between vitamins (purple) and infections (green) on our pathway as well as cytokines (pink). Positive associations are represented by solid green lines, and negative associations are represented by red dotted lines.

DISCUSSION

This study allowed us to consider the mechanisms by which multiple micronutrient deficiencies as well as multiple infections impact fetal growth of pregnant women living in abject poverty. Despite the growing body of literature implicating both infections and micronutrient deficiencies with adverse growth outcomes (Brotman 2011; Haider, Yakoob, et al. 2011; Shub et al. 2006) these factors are often studied in isolation of one another. In reality, micronutrient deficiencies and multiple infections often exist concurrently, as we found in our population of pregnant Ngäbe-Buglé women (Gonzalez-Fernandez, 2012). Therefore, there exists a need to study concurrent infections and nutrient deficiencies to fully elucidate the full effects on growth outcomes, especially during gestation. To the best of our knowledge, no other studies have previously examined the impacts of both micronutrient deficiencies and multiple infections on fundal height in women living in rural developing regions of the world. A major strength of our study was the large selection of variables including infections, cytokines/chemokines, hormones as well as physical and nutritional indices all of which allowed us to adequately conduct our research while taking necessary controls.

For our research, we hypothesized that maternal cortisol and pro-inflammatory cytokines would lead to a decrease in maternal IGF-1, which would cause low fundal height, and that maternal vitamin deficiency and infections would increase pro-inflammatory cytokines and cortisol, and decrease fundal height. We began our analysis with an examination between vitamins and cytokines only and observed that vitamin A did not appear in any of our analysis as a modulator of cytokines or cytokine ratios and furthermore vitamin D and folate were both observed to increase pro-inflammatory cytokines. A potentially novel role for vitamin B12 emerged, as it was observed to increase cytokine concentrations regardless of T helper subset, in both our t-test analysis and our regression models.

From our regression models we observed that cortisol increased maternal IGF-1 which lead to increased fundal height, and that there was no impact of pro-inflammatory cytokines on IGF-1. None of the eight infections measured in this study were directly associated with fundal height. Moreover, of the four vitamins, only vitamin D entered as a predictor of fundal height and it was negatively associated. In addition, we observed that vitamins and infections had varying impact on the cortisol–IGF-1–fundal height pathway. Whereas dental caries increased cortisol, vitamin D lowered concentrations of cortisol. Bacterial vaginosis decreased concentrations of IGF-1, whereas vitamin A increased concentrations of IGF-1. Our pathway was also impacted by a series of cytokines, which were in turn were predicted by both vitamin B12 and infections. The only cytokine to impact fundal height was T regulatory cytokine IL-10. Furthermore cytokines IL-4 and IL-6 decreased cortisol while IL-13 and MCP-1 increased cortisol. In our regression models, vitamin B12 was the only vitamin to impact cytokines, and was observed with increased concentrations of IL-6, IL-10 and IL-13. Bacterial vaginosis was observed to decrease cytokines IL-4 and IL-6, and IL-4 was increased by respiratory, gonococcal and scabies infections. Lastly, trichomoniasis infection was observed to increase concentrations of IL-10 and decrease concentrations of MCP-1.

MATERNAL CORTISOL AND IGF-1

Our observation that cortisol was associated with an increase in IGF-1 concentrations was contrary to our hypothesis as cortisol has been previously observed to decrease concentrations of IGF-1 (Fowden et al. 2009; Giustina et al. 1990; Hochberg 2002). The disparity between results may be because the majority of studies examining the effects of GC and growth faltering have looked at GC at supraphysiological concentrations as part of therapies for lupus, irritable bowel syndrome, or other inflammatory diseases (Jensen et al. 2010). Furthermore, it has been reported that short-term administration of GC stimulates GH and IGF-1 concentrations (Veldhuis et al. 1992), whereas long-term high-dose GC therapy reduces concentrations of both of these hormones, suggesting that the relationship

between GC and IGF-1 is dependent not only on dosage of GC, but also on duration of exposure. Recent studies in ewes investigating the relationship between cortisol and IGF-1, have demonstrated that the pregnancy-associated elevation in maternal cortisol was necessary for fetal development (Jensen et al. 2002, 2003) and when increases of cortisol concentrations were blocked, growth restriction occurred (Jensen et al. 2005). Furthermore the same researchers observed that in experimentally adrenalectomised ewes given low dosages of cortisol, there was a lower concentration of fetal IGF-I when compared with ewes in the control group (Jensen et al. 2011). Therefore, long-term mega doses of steroids, although necessary for amelioration of inflammatory pathologies, may not accurately reflect the naturally occurring physiological relationship between cortisol and IGF-1 during pregnancy, and caution should be used when extrapolating results from these studies.

Maternal IGF-1 is a hormone necessary for proper growth and development of the fetus during pregnancy (Akman et al. 2006; Chiesa et al. 2008; Holmes et al. 1997; Larsen et al. 1996; Sifakis et al. 2012). Although it appeared in our model for fundal height, it was not a significant predictor, and was only significant via t-test analysis showing that individuals with lower concentrations of IGF-1 had significantly lower fundal height. Our inability to capture an association suggests that there may have also been other variables which were responsible for predicting fundal height, one of which being IGF-2. Whereas IGF-1 is necessary for fetal growth in later gestation, IGF-2 is primary in aiding embryonic growth and diminishes significantly after parturition (Chiesa et al. 2008; Glasscock et al. 1992). Therefore it is possible that IGF-2 may also be aiding to fetal growth, however our limitation to measure this hormone prevents us from verifying this at the present time.

The Impact of Infections on the Cortisol-IGF-1-Fundal Height Pathway

In our study on pregnant women, infections had varying impacts on the cortisol-IGF-1-fundal height pathway both directly and through cytokines. Dental caries were associated with increased concentrations of cortisol while BV infection

was associated with decreased concentrations of IGF-1, IL-6 and IL-4. Respiratory, scabies and gonococcal infections increased concentrations of IL-4 while trichomoniasis increased IL-10 while decreasing MCP-1. None of the eight infections included in this study appeared in our model for fundal height.

Dental caries is an infectious disease where organic acids, produced by bacterial fermentation of dietary carbohydrates, erode the mineralization of teeth (Selwitz et al. 2007). In our study, the severity of dental caries infection increased concentrations of maternal cortisol, which has been supported by a number of studies in children (Boyce et al. 2010; Rai et al. 2010; Shub et al. 2006) and to our knowledge has not been previously demonstrated in pregnant women. Through this mechanism of increasing concentrations of cortisol, in our study, dental caries was associated with improved fundal height.

Bacterial vaginosis is an infection which occurs when the normal lactobacillus-predominant flora of the vaginal tract is replaced with the harmful anaerobic bacteria *Mycoplasma hominis* or *Gardnerella vaginalis,* and in our study was associated with decreased concentrations of IGF-1, and thus decreasing fundal height. Previous literature has observed an association between LBW of offspring from mothers with bacterial vaginosis infections (Hillier et al. 1995; Svare et al. 2006), however no studies have examined the relationship between BV on concentrations of IGF-1 specifically. In our study BV was also observed to decrease cytokines IL-4 and IL-6, cytokines which were observed to decrease cortisol and through this mechanism BV was observed to increase fundal height, therefore making the impact of this infection on fundal height inconclusive. Our finding that IL-4 was decreased by BV is supported by one study (Fan et al. 2008); however BV decreasing IL-6 is counter to what we had expected and what is found in the literature (Friese 2003).

In our study, respiratory, scabies and gonococcal infections were negatively impacting growth by increasing concentrations of IL-4, which was decreasing concentrations of cortisol. All of these infections, except for scabies, have been

observed with adverse pregnancy out comes ranging from pre-term births (respiratory) (Banhidy et al. 2008) to ectopic pregnancy (gonococcal) (Walker et al. 2011), although no mechanism has been provided for either. Research regarding these infections and IL-4 is limited; presence of scabies infection in mice have shown elevated levels of IL-4 (Lalli et al. 2004), however no literature is available to the best of our knowledge regarding the relationship between diplococcal or RTI and IL-4.

As previously mentioned no infections emerged in the final multiple regression model of fundal height, however through further investigation a relationship did emerge between infection and fundal height via cytokine IL-10. In our study elevated concentrations of IL-10 were observed to have a negative impact on fundal height. This finding was surprising given the numerous studies linking the occurrence of healthy pregnancies with higher concentrations of IL-10 (Denney et al. 2011) with some authors arguing it is one of the most crucial cytokines for ensuring a healthy pregnancy (Szarka et al. 2010). However, in the context of infection, increase in IL-10 concentrations are not necessarily indicative of a healthy pregnancy as further analysis revealed that trichomoniasis infection was associated with an increase of IL-10. Trichomoniasis is a sexually transmitted infection caused by the parasite *Trichomonas vaginalis* that infects the uterus and vaginal (Schwebke et al. 2004) and has been previously associated with preterm birth and low birth weight infants (Gulmezoglu et al. 2011). Although there are no previous studies examining the relationship between trichomoniasis and IL-10, one study has observed an increase in IL-10 during an infection with *Plasmodium vivax,* another protozoal parasite (Jangpatarapongsa et al. 2008). Therefore it is conceivable that the negative relationship we observed between IL-10 and fundal height was due to elevation of this cytokine in response to trichomoniasis infection.

The Impact of Vitamin A and D on the Cortisol-IGF-1-Fundal Height Pathway

Vitamin A and D are fat soluble vitamins necessary for proper growth and development and deficiencies have been linked with poor growth outcomes such as

low birth length, LBW and SGA (Leffelaar et al. 2010; Morley et al. 2006; Thacher et al. 2011; Tielsch et al. 2008) suggesting that these vitamins could also have an impact on *in utero* growth. Although in our analysis vitamin A was not associated with impacting cytokines or cytokine ratios, vitamin D was observed to increase concentrations of pro-inflammatory cytokine TNF-α. From our regression analysis we observed that higher IGF-1 concentrations were associated with higher vitamin A concentrations, and that better fetal growth was associated with lower vitamin D concentrations.

Our finding that vitamin A is associated with increased concentrations of IGF-1 is supported by numerous animal studies (Bartlett et al. 1990; Fu et al. 2001; Holmes et al. 2002; Oka et al. 2004) and one human study (Holmes et al 2002). However a mechanism by which vitamin A is exerting this effect on IGF-1 has not been provided and requires further investigation. Although we were able to detect a positive relationship between vitamin A and IGF-1, we were not able to detect significant relationships between vitamin A and cytokines. Vitamin A and related compounds have been found to be potent modifiers of both Th1 and Th2 responses with deficiencies upregulating pro-inflammatory Th1 cytokines (Carman et al. 1991; Cui et al. 2000; Ikeda et al. 1994; Wiedermann et al. 1996; Wiedermann et al. 1993). For this reason we expected deficiencies in vitamin A to increase pro-inflammatory cytokines, however we observed no such relationship throughout our analysis. This phenomenon may possibly be explained by the limited number of deficient women in our population limiting our statistical power to observe a difference as many were receiving vitamin A supplementation.

Our findings that higher vitamin D concentrations were associated with impaired fetal growth was counter to what we had expected given the important role of vitamin D in the growth and development of bone and calcium homeostasis (Thacher et al. 2011). However, within the context of multiple infections during pregnancy it is possible that lower vitamin D concentrations are having a protective effect via

decreases of pro-inflammatory cytokines and infection burden, meanwhile increasing concentrations of cortisol.

In our study we observed that vitamin D deficient pregnant women had significantly lower concentrations of TNF-α compared to women who were classified as having marginal vitamin D status, by non-parametric ANOVA. TNF-α is a cytokine previously observed to decrease longitudinal growth in rats by inhibiting bone growth (Mårtensson et al. 2004). However, our finding is counter to the majority of literature showing vitamin D as a nutrient that decreases pro-inflammatory cytokines (Khoo et al. 2011; Thota et al. 2013; Wintergerst et al. 2007). However, in a recent study during pregnancy, vitamin D concentrations were positively correlated with another pro-inflammatory cytokine, IFN-γ (Chi et al. 2011). Taken together, these results suggest that the effects of vitamin D on the development of cytokine responses *in utero* may differ from the postnatal environment (Chi et al. 2011). Therefore during pregnancy vitamin D deficiency may have a protective effect on fetal growth by downregulating pro-inflammatory cytokines, in our case TNF- α. However, neither TNF- α, nor any other pro-inflammatory cytokines appeared in our model as significant a predictor of fundal height so this theory is strictly speculative.

Another mechanism by which vitamin D may be negatively impacting fundal height is by increasing infection burden. Indeed, in our population it was previously observed that there was a greater incidence of trichomoniasis and candidiasis in women with higher serum concentrations of vitamin D (Gonzalez-Fernandez 2012). Furthermore, vitamin D concentration has been observed to increase risk of eczema, a common skin inflammatory condition (Gale et al. 2008); as well as increasing host susceptibility to *Citrobacter rodentium,* an enteric bacterial pathogen responsible for causing intestinal inflammation via suppression of mucosal Th17 responses (Christakos 2012). Therefore, it is conceivable that within the context of multiple infections, lower vitamin D concentration is protective for fetal growth due to higher levels being associated with increases of infection burden.

Due to the finding that increased cortisol was beneficial for fundal height, via increased concentrations of IGF-1, vitamin D decreasing cortisol presented another mechanism by which vitamin D could be having a negative impact on fundal height. In the literature an inverse relationship has been observed between the administration of GC on vitamin D concentrations (Searing et al. 2010; Toloza et al. 2010); however the administration of vitamin D not been observed to lower GC concentrations, but rather be protective against their deleterious effects. In a rat study administration of dexamethasone, a synthetic analogue of cortisol, inhibited IGF-1 production by osteoblast-like cells. However when combined with vitamin D this inhibitory effect was diminished suggesting a protective effect of vitamin D on IGF-1 synthesis (Chen et al. 1991). Furthermore, other studies have observed that supplementation of vitamin D was protective for rats against the effects of GC excess on cardiovascular responses. Rats with vitamin D supplementation had better systolic, diastolic and mean arterial blood pressures and heart rate compared to control or rats only receiving dexamethasone (Ahmed 2013). In a cohort of asthmatic boys aged 5-12 years, vitamin D was protective against the demineralizing effects of glucocorticoids and those with higher amounts of vitamin D had better bone mineral accretion (Tse et al. 2012). Although there is evidence that vitamin D plays a protective role against the deleterious effects of GCs, further research is required to elucidate whether this phenomenon is occurring due to a direct inhibition of GCs or by another mechanism occurring downstream of production.

The Impact of Folate and Vitamin B12 on Cytokines

The relationship between vitamins and cytokines were tested in our study by two separate statistical methods. The first being comparing means of cytokines and cytokine ratios in vitamin sufficient versus deficient populations (or sufficient, marginal or deficient in the case of vitamin A and D). The second was by entering vitamins into our regression models for cytokines. Through this we observed that vitamins D, B12 and folate impacted means of cytokines and cytokine ratios, and that

vitamin B12 was the only vitamin to significantly predict cytokines in our regression models.

In our study folate sufficient women had increased concentrations of pro-inflammatory cytokines compared to folate deficient women. We observed lower concentrations of IL-10, further supported by the Th1:Th2 cytokine ratios (IFN-γ:IL-10, TNF-α:IL-10, and IL-17:IL-10), which were higher in folate sufficient women. Furthermore, the cytokine ratio TNF-α:IL-13 was increased in folate sufficient groups, suggesting folate was increasing TNF-α and decreasing IL-13. Studies regarding the impact of folate status on cytokines are limited however a relatively new study has investigated the relationship between cytokines and folate status in pregnant women (Simhan et al. 2011). These researchers grouped pro-inflammatory cytokines (IL-1β, IL-6, IL-8, MCP-1) and anti-inflammatory cytokines (IL-4, IL-10, IL-13) using factor analysis in pregnant women (n=417) at <16 weeks gestation. Although the study does not provide information on specific cytokines, the general trend was that anti-inflammatory cytokines were decreased with increased concentrations of folate, contrary to what our findings suggest. However, our research is focused only on the 2nd and 3rd trimester which research which is immunologically distinct from the first 1st trimester when this study was conducted. Although folate was observed with decreased concentrations of IL-10, suggesting that folate may be protective for fetal growth, we are not able to make this assumption because folate was not a significant predictor in our regression models.

Unlike folate which we observed to increase pro-inflammatory cytokines, vitamin B12 was observed to increase cytokines from both T-helper subsets, including IL-6, IL-10, IL-13, IFN-γ and TNF-α. Previous literature has indicated an impact of vitamin B12 on cellular immunity although with regards to T cell lymphocyte populations and not cytokines. In two separate studies, it was observed that individuals with B12 deficiency had depressed natural killer cell functioning and decreased number of both CD4+ and CD8+ lymphocytes. Furthermore, there was a smaller proportion of $CD8^+$ compared to CD4+ cells resulting in an abnormally high

CD4+/CD8+ ratio when compared to healthy controls (Erkurt, Aydogdu, Dikilitas, Kuku, Kaya et al., 2008; J. Tamura, Kubota, Murakami, Sawamura, Matsushima et al., 1999). Therefore it is possible that through these increases of T helper cell populations, concentrations of cytokines are also increasing. In addition our observation may be explained by the integral role of vitamin B12 in cellular replication (Bohnsack et al. 2004), however as our results our novel the mechanism of how this phenomenon is occurring is only speculative and requires further investigation.

In conclusion, our findings suggest that both folate and vitamin B12 have impacts on cellular immunity which may not have been previously considered. This is especially important for marginalized women living in low income countries whose intakes of these vitamins are low (Willams 2012).

Unique Impacts of Infections and Vitamins on Cytokines

The majority of literature regarding the relationship between cytokines and their effect on growth, suggest a strong dichotomy between pro- and anti-inflammatory cytokines (whether it be from the Th1, Th2, Th17 or Treg subsets) and that pro-inflammatory cytokines negatively impact growth (MacRae, Farquharson, et al. 2006; Mårtensson et al. 2004). We created cytokine ratios to help determine if vitamin status was impacting the Th1:Th2 ratio, however the only vitamin-related difference was seen between folate deficient and sufficient mothers and folate did not enter our models for cortisol–IGF-1–fundal height pathway. Furthermore, we found that both pro- and anti-inflammatory cytokines were implicated for both increases and decreases of fundal height. For example, although Th2 cytokines IL-4 and IL-13 are considered to have redundant physiological effects (Mosmann et al. 1996), they had opposing interactions on cortisol, with IL-4 decreasing and IL-13 increasing cortisol. Similarly pro-inflammatory cytokine IL-6 and chemokine MCP-1 had contrasting effects on cortisol with IL-6 decreasing and MCP-1 increasing cortisol. Furthermore the only cytokine that emerged in our model for fundal height was the T regulatory cytokine IL-10, and was observed to be negatively predicting fundal height. Therefore in our study

there was no predominant indication that a pro- or anti- inflammatory immune response was responsible for impaired fetal growth.

However, the differences in cytokine behaviours may be explained by variables which impacted them. For example, IL-6 concentrations were increased by vitamin B12, but IL-6 was decreased BV. IL-4 concentrations were increased by respiratory tract, scabies and gonococcal infection, but IL-4 was decreased by BV. Trichomoniasis was observed to increase concentrations of IL-10, however decrease concentrations of MCP-1. In light of these results, we conclude that the importance of the cytokine milieu during pregnancy needs further investigation and that the established paradigm of pro- versus anti-inflammatory responses may be an inadequate description of the immune response during pregnancy, especially in the context of multiple infections and vitamin deficiencies which are uniquely impacting these cytokines.

LIMITATIONS

Several limitations of our study pertained to the measurement biochemical indices which would have further provided us with insight into the impacts of vitamin deficiencies and infections on fetal growth. At the time of the study we were not able to measure IGF-2 vitamin D3 ($1,25(OH)_2D$) because the assays had not been developed yet by the company whose machinery we were using. Analysis of vitamin D3 would be beneficial as it is the active metabolite that interacts with cytokines (Khoo et al. 2011) and would have provided a more in depth look at the immunomodulatory effect of vitamin D in our study. Furthermore, we did not measure homocysteine, a sensitive indicator of vitamin B12 and folate status, which has previously been observed to be associated with adverse birth outcomes like LBW and pre-eclampsia. Another limitation was that few of the women were folate and vitamin A deficient as they had received supplementation both of these nutrients. Although this has a positive impact on maternal health, it decreased our ability to adequately test for effects of deficiency of these vitamins on fetal growth. Lastly, the majority of pregnant women in our study were infected with multiple infections limiting our ability to differentiate the effects of particular infections on maternal health and fetal growth.

CONCLUSION

Through our research we examined the impact of multiple micronutrient deficiencies as well as multiple infections on fetal growth in pregnant Ngäbe-Buglé women. In examining the relationship between vitamins and cytokines we obtained results which were counter to what we had expected. First of all, vitamin A did not appear in any of our analysis as a modulator of cytokines and secondly, vitamin D and folate were both observed to increase pro-inflammatory cytokines. Lastly, we did not expect vitamin B12 to have any impact on cytokines however in our study it was consistently associated with increased concentrations.

Through our work, we observed a complex network of interactions occurring between the cortisol–IGF-1–fundal height pathway and cytokines, vitamins deficiencies and infections. Counter to what we had anticipated, cortisol was observed to be increasing fetal growth by increasing concentrations of IGF-1. Although IGF-1 was not significant in our model for fundal height, it was significant through t-test analysis. We observed that higher vitamin D concentrations were negatively associated with fundal height via decreases in both fundal height directly, and through decreased concentrations of cortisol. Higher vitamin A concentrations were associated with higher concentrations of IGF-1, and vitamin B12 was observed to increase a variety of cytokines. Folate did not enter into any of the final regression models as a significant predictor.

None of the eight infections included in this study were observed in our model for fundal height however were observed to have impacts on cytokines, cortisol and IGF-1. What is perhaps the most interesting finding is the range of types on infections that impacted on growth including skin, respiratory, dental and urogenital infections, suggesting that any infection burden has the potential to negatively impact growth. We anticipated that an increase in pro-inflammatory cytokines would negatively impact fundal height, instead we found that cytokines from both the pro- and anti-inflammatory subsets were implicated in both the increase and decrease of fundal

height via the cortisol–IGF-1–fundal height pathway. This phenomenon may be explained by the presence of multiple vitamins and infections which were observed to have unique impacts on cytokines, therefore influencing our pathway and fundal height.

To conclude, our findings demonstrate the importance of examining environmental influences on maternal health in all their complexity, especially in the developing world, where individuals are often burdened with multiple infections and nutrient deficiencies. A network of complex interactions are occurring between infections, nutrients, hormones and the immune system and therefore research examining only one aspect will undoubtedly arrive at only a partial understanding of the mechanisms which impact fetal growth.

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Table 1: Maternal cytokines (pg/mL) and ratios according to deficient, marginal or sufficient Vitamin A status in 2nd and 3rd trimester (n=183).

Vitamin A

Maternal Cytokines	Deficient <0.7µmol/L	Marginal 0.7µmol/L-1.05µmol/L	Sufficient >1.05µmol/L
N=	12	67	104
IL-1β	3.2 ±1.1	5.9 ± 1.5	5.6 ± 0.7
IL-4	19.7 ± 7.2	19.1 ± 3.2	17.0 ± 2.2
IL-6	10.6 ± 3.6	10.6 ± 2.3	9.3 ±1.3
IL-10	5.6 ±2.2	3.6 ± 0.9	4.6 ± 0.7
IL-12p40	10.7 ± 4.2	19.1 ± 5.9	14.9 ± 2.0
IL-13	4.9 ± 1.4	5.0 ± 1.0	4.1 ±0.6
IL-17	6.0 ±2.3	7.7 ± 1.5	6.5 ± 0.8
IFN-γ	6.5 ± 1.8	7.5 ± 1.0	8.2 ± 0.8
TNF-α	8.6 ± 2.5	8.6 ± 1.2	9.2 ± 1.1
MCP-1	190.6 ± 35.5	201.4 ± 15.6	208.1 ± 11.1
IFN-γ:IL-4	1.2 ± 0.7	19.3 ± 9.5	25.1 ± 13.2
IFN-γ:IL-6	1.6 ±0.5	4.4 ± 1.3	4.1 ± 0.8
IFN-γ:IL-10	32.4 ± 22.0	80.4 ± 31.4	215.9 ± 101.7
IFN-γ:IL-13	10.1 ± 6.5	16.6 ± 5.3	22.8 ± 4.9
TNF-α:IL-4	2.0 ± 1.8	6.9 ± 4.8	8.0 ± 2.8
TNF-α:IL-6	2.0 ± 1.2	2.7 ± 0.8	4.2 ± 1.0
TNF-α:IL-10	34.2 ±16.6	36.2 ± 8.7	48.9 ± 9.5
TNF-α:IL-13	11.2 ± 10.1	25.1 ± 7.2	31.7 ± 5.9
IL-17:IL-10	6.0 ± 2.9	82.5 ± 35.7	141.2 ± 70.6

Table 2: Maternal cytokines (pg/mL) and ratios according to deficient, marginal or sufficient Vitamin D status in 2nd and 3rd trimester (n=184).

	Vitamin D		
Maternal Cytokines	**Deficient <25nmol/L**	**Marginal 25nmol/L-50nmol/L**	**Sufficient >50nmol/L**
N=	14	104	66
IL-1β	1.5 ± 0.6	6.7 ± 1.1	4.8 ± 0.6
IL-4	9.6 ± 3.0	18.2 ±2.6	19.3 ± 2.6
IL-6	3.8 ± 2.1	12.1 ±1.8	7.5 ± 1.3
IL-10	5.5 ± 3.1	4.4 ± 0.8	3.8 ± 0.7
IL-12p40	5.6 ± 3.7	20.2 ± 4.1	11.9 ± 1.9
IL-13	1.4 ±0.5	5.0 ± 0.8	4.4 ± 0.7
IL-17	4.2 ±1.9	7.8 ± 1.1	6.0 ± 0.8
IFN-γ	2.5 ±1.2	8.9 ± 0.9	7.2 ± 0.8
TNF-α	6.0 ±2.1	9.4 ± 1.0	8.9 ± 1.4
(Median)	[a](0.029)	[b](0.8)	[ab](0.79)
MCP-1	197.7 ±33.4	206.7 ± 11.1	202.3 ± 15.7
IFN-γ:IL-4	34.6 ±30.6	26.2 ±13.7	11.2 ± 5.2
IFN-γ:IL-6	6.2 ±3.2	3.4 ± 0.8	4.6 ± 1.2
IFN-γ:IL-10	14.5 ±12.1	168.7 ± 96.8	162.3 ± 63.6
IFN-γ:IL-13	14.6 ±5.9	22.6 ± 5.4	17.2 ± 3.9
TNF-α:IL-4	24.2 ±23.0	6.2 ± 2.6	5.2 ± 1.9
TNF-α:IL-6	1.7 ±0.8	3.4 ± 0.8	4.1 ± 1.2
TNF-α:IL-10	3.4 ±2.7	46.0 ± 8.7	49.0 ± 11.1
TNF-α:IL-13	8.8 ±7.3	32.9 ± 5.9	25.7 ±7.4
IL-17:IL-10	3.9 ±3.2	109.7 ± 67.9	139.4 ± 50.0

* Kruskal-Wallis non-parametric ANOVA

Table 3: Maternal cytokines (pg/mL) and ratios according to deficient, marginal or sufficient Vitamin B12 status in 2nd and 3rd trimester (n=184).

Vitamin B12

Maternal Cytokines	Deficient <150pmol/L	Sufficient > 150pmol/L
N=	21	163
IL-1β	5.3 ±0.7	8.2 ± 1.7
IL-4	16.6 ±1.7	25.8 ± 7.0
IL-6	9.0 ±1.2	16.2 ± 3.8
(Median)	[a](1.6)	[b](11.9)
IL-10	3.8 ± 0.5	7.9 ± 2.6
IL-12p40	13.8 ± 2.4	34.2 ±10.4
IL-13	3.9 ± 0.4	9.1 ± 2.8
IL-17	6.2 ± 0.6	12.5 ± 3.5
IFN-γ	7.2 ± 0.6	13.0 ± 2.4
(Median)	[a](6.14)	[b](14.0)
TNF-α	8.2 ± 0.8	14.3 ± 3.1
(Median)	[a](3.45)	[b](8.0)
MCP-1	200.1 ± 9.2	238.1 ± 27.4
IFN-γ:IL-4	23.3 ± 9.3	7.9 ± 4.2
IFN-γ:IL-6	4.3 ± 0.7	1.8 ± 0.4
IFN-γ:IL-10	153.3 ± 64.3	164.5 ± 140.9
IFN-γ:IL-13	19.0 ± 3.5	27.9 ± 12.3
TNF-α:IL-4	7.9 ± 2.7	1.8 ± 0.9
TNF-α:IL-6	3.8 ± 0.7	1.6 ± 0.7
TNF-α:IL-10	44.7 ± 6.9	37.0 ±16.7
TNF-α:IL-13	27.5 ± 4.5	35.7 ± 15.8
IL-17:IL-10	114.9 ± 47.0	92.4 ± 61.2

*Wilcoxon rank sum test for non-parametric t-test

Table 4: Maternal cytokines (pg/mL) and ratios according to deficient, marginal or sufficient folate status in 2nd and 3rd trimester (n=183).

Folate

Maternal Cytokines	Deficient <10nmol/L	Sufficient >10nmol/L
N=	47	136
IL-1β	7.8 ± 2.3	4.9 ± 0.5
IL-4	13.3 ± 3.1	19.6 ± 2.0
IL-6	8.2 ± 1.8	10.4 ± 1.4
IL-10	5.3 ± 1.1	3.9 ± 0.6
(Median)	[b](1.8)	[a](0.72)
IL-12p40	15.3 ± 3.0	16.4 ± 3.1
IL-13	5.0 ± 0.9	4.3 ± 0.6
IL-17	8.1 ± 1.6	6.5± 0.8
IFN-γ	7.2 ± 1.3	8.1 ±0.7
TNF-α	10.3 ±2.1	8.4 ±0.8
MCP-1	194.3 ± 17.2	207.9 ± 10.2
IFN-γ:IL-4	12.6 ± 6.4	24.5 ± 10.9
IFN-γ:IL-6	2.4 ± 0.4	4.6 ± 0.9
IFN-γ:IL-10	108.1 ± 75.3	170.7 ±75.1
(Median)	[a](1.6)	[b](2.6)
IFN-γ:IL-13	14.8 ± 4.1	21.9 ± 4.4
TNF-α:IL-4	5.7 ± 2.0	7.7 ±3.1
TNF-α:IL-6	1.7 ± 0.7	4.2 ±0.8
TNF-α:IL-10	21.4 ± 10.6	51.6 ±7.7
(Median)	[a](1.3)	[b](3.3)
TNF-α:IL-13	11.9 ± 5.9	34.2 ± 5.4
(Median)	[a](0.9)	[b](1.6)
IL-17:IL-10	108.7 ± 59.5	113.6 ± 53.0
(Median)	[a](0.93)	[b](2.6)

*Wilcoxon rank sum test

Table 5: Predictors of maternal cortisol (a) and related cytokines (b-e) during 2nd and 3rd trimester of pregnancy (multiple linear regression n=182, n=184).

a) Predictors maternal cortisol **(n=182)**					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Trimester, 2nd or 3rd	0.108	0.24	±	6.14	0.0003
Serum 25(OH)D, nmol/L x10^3	0.022	-0.18	±	0.18	0.0027
Serum retinol, µmol/L x10^2	0.018	0.11	±	7.09	0.0607
Severity of dental caries 0,1,2	0.078	0.20	±	5.27	0.0009
IL-4, pg/mL	0.058	-0.27	±	0.13	<.0001
IL-6, pg/mL	0.022	-0.24	±	0.19	<.0001
IL-13, pg/mL	0.063	0.31	±	0.44	<.0001
MCP-1, pg/mL	0.082	0.29	±	0.02	<.0001
Model R-squared		**0.435**			
Model p-value		**<0.001**			

Controlled for by: maternal height, maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Wood smoke exposure (hours per day), severity of *Trichomoniasis* infection, presence of scabies infection, presence of respiratory tract infection, presence of urinary tract infection, severity of vaginal candidiasis, severity of vaginosis, presence of gonococcal infection, cortisol, CRP, IL-1β, IL-10, IL-12, IL-17, TNF-α, IFN-γ, Folate, Vitamin B12

b) Predictors of maternal IL-4 **(n=184)**					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Presence of respiratory infection 0=no, 1=yes	0.036	0.18	±	7.00	0.0096
Presence of gonococcal infection 0=no, 1=yes	0.028	0.17	±	5.97	0.0179
Presence of scabies 0=no, 1=yes	0.041	0.17	±	4.68	0.0188
Severity of vaginosis 0,1,2,3,4	0.019	-0.14	±	1.33	0.0478
Model R-squared		**0.115**			
Model p-value		**0.0002**			

Controlled for by: maternal height, maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Wood smoke exposure (hours per day), severity of *Trichomoniasis* infection, severity of dental caries, severity of vaginal candidiasis presence of urinary tract infection, CRP, Folate, Vitamin B12

c) Predictors of maternal IL-6 **(n=182)**					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Serum folate, nmol/L	0.026	0.14	±	0.14	0.0588
Serum vitamin B12, pmol/L	0.042	0.16	±	0.03	0.0289
Severity of vaginosis 0,1,2,3,4	0.049	-0.20	±	0.88	0.0048
Model R-squared		**0.0867**			
Model p-value		**0.0071**			

Controlled for by: maternal height, maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Wood smoke exposure (hours per day), severity of *Trichomoniasis* infection, presence of scabies infection, presence of respiratory tract infection, presence of urinary tract infection, severity of vaginal candidiasis, presence of gonococcal, cortisol, CRP, Vitamin A

*d) **Predictors of maternal IL-13*** **(n=184)**					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Wood Smoke Exposure (hours/day)	0.012	0.11	±	0.32	0.1109
Serum vitamin B12 pmol/L	0.126	0.26	±	0.01	0.0006
Presence of respiratory infection 0=no, 1=yes	0.006	0.13	±	2.10	0.0664
Severity of vaginosis 0,1,2,3,4	0.023	-0.12	±	0.41	0.1054
Severity of Trichomoniasis 0,1,2,3	0.008	0.10	±	0.85	0.1704
Model R-squared		**0.1230**			
Model p-value		**0.0001**			

Controlled for by: maternal height and maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Wood smoke exposure (hours per day), presence of scabies infection, presence of respiratory tract infection, presence of dental caries, presence of urinary tract infection, severity of vaginal candidiasis, presence of gonococcal infection, CRP, Folate, Vitamin A, Vitamin D

*e) **Predictors of maternal MCP-1***					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Wood Smoke Exposure (hours/day)	0.037	-0.22	±	5.56	0.0023
Severity of trichomoniasis 0,1,2,3	0.021	-0.17	±	14.71	0.0183
Severity of vaginosis 0,1,2,3,4	0.023	0.14	±	7.18	0.1077
Model R-squared		**0.0755**			
Model p-value		**0.0028**			

Controlled for by: maternal height and maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Presence of scabies infection, presence of respiratory tract infection, severity dental caries, presence of urinary tract infection, severity of vaginal candidiasis, severity of vaginosis, presence of gonococcal infection, cortisol, Folate, Vitamin B12, Vitamin A, Vitamin D

Table 6: Predictors of maternal insulin-like growth factor (IGF-1) during 2nd and 3rd trimester of pregnancy (multiple linear regression, n=182)

a) Predictors of maternal IGF-1					
Variable	**Partial R-Square**	**β**	**±**	**SE**	**p-value**
Trimester, 2nd or 3rd	0.021	0.14	±	0.930	0.0845
Cortisol	0.124	0.26	±	0.009	0.0005
Maternal serum retinol, μmol/L x10^2	0.042	0.21	±	1.044	0.0022
Severity of vaginosis 0,1,2,3,4	0.023	-0.15	±	0.321	0.0373
IL-4, pg/mL	0.015	-0.12	±	0.017	0.0956
Model R-squared		**0.1984**			
Model p-value		**<0.0001**			

Controlled for by: maternal height, maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Wood smoke exposure (hours per day), severity of trichomoniasis infection, presence of scabies infection, presence of respiratory tract infection, severity of dental caries, presence of urinary tract infection, severity of vaginal candidiasis, presence of gonococcal infection, CRP, IL-1β, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-α, IFN-γ, MCP-1, Folate, Vitamin B12, Vitamin D

Table 7: Predictors of fundal height (a) and IL-10 (b) during 2nd and 3rd trimester of pregnancy (multiple linear regression, n=184).

a) Fundal Height

Variable	Partial R-Square	β	±	SE	p-value
Gestational Age	0.781	0.82	±	0.034	<.0001
Maternal height, cm x10^3	0.006	-0.11	±	0.057	0.0022
Maternal weight, kg x10^3	0.011	0.12	±	0.041	0.002
Insulin growth factor-1	0.002	0.05	±	0.043	0.1375
Wood Smoke Exposure (hours/day)	0.003	-0.05	±	0.169	0.1132
Maternal serum 25(OH)D, nmol/L x10^3	0.005	-0.07	±	0.016	0.0304
IL-10, pg/mL	0.003	-0.10	±	0.046	0.0208
IL-17, pg/mL	0.006	0.06	±	0.035	0.1566
MCP-1, pg/mL	0.004	-0.06	±	0.002	0.0916
Model R-squared		**0.8086**			
Model p-value		**<0.0001**			

Fundal height per gestational age in centimeters per week

Controlled for by: maternal height, maternal weight
Variables included in previous stepwise model with a p> 0.15: Severity of *Trichomoniasis* infection, presence of scabies infection, presence of respiratory tract infection, severity of dental caries, presence of urinary tract infection, severity of vaginal candidiasis, severity of vaginosis, presence of gonococcal infection, cortisol, IGF-1, CRP, IL-1β, IL-4, IL-6, IL-12, IL-13, TNF-α, IFN-γ, Folate, Vitamin B12, Vitamin A

b) IL-10

Variable	Partial R-Square	B	±	SE	p-value
Vitamin A	0.003	-0.03	±	1.43	0.7104
Serum folate, nmol/L	0.003	-0.09	±	0.07	0.2266
Serum vitamin B12, pmol/L	0.077	0.29	±	0.01	0.0001
Severity of trichomoniasis 0,1,2,3	0.029	0.19	±	0.95	0.0091
Model R-squared		**0.0937**			
Model p-value		**0.0010**			

Controlled for by: maternal height, maternal weight and trimester

Variables included in previous stepwise model with a p> 0.15: Presence of scabies infection, presence of respiratory tract infection, presence of dental caries, presence of urinary tract infection, severity of vaginal candidiasis, presence of gonococcal infection, cortisol, CRP, Vitamin D

CHAPTER V: GENERAL DISCUSSION

Our study investigated the impact of multiple micronutrient deficiencies and multiple infections on fetal growth from pregnant women living in abject poverty. Our study was part of a larger effort that examined a variety of maternal factors including anemia and infections on fetal growth. Our contribution to the previous work was the addition of data on cytokines/chemokines, cortisol and IGF-1, allowing us to examine the impact of stress and inflammation on fetal growth. Our study differs from majority of literature because we examined *in utero* growth by measuring fundal height during pregnancy, as opposed to measuring fetal growth by calculating infant weight at the time of birth. In addition, we investigated fetal growth in a context where multiple nutrient deficiencies and multiple infections are present, supported by a number of biochemical and laboratory measurements.

Our analysis of the relationship between vitamins on cytokines revealed no impact of vitamin A; however we did observe that vitamin D and folate increased concentrations of pro-inflammatory cytokines, whereas vitamin B12 was observed to increase both pro- and anti-inflammatory cytokine concentrations. These findings demonstrate a unique impact of vitamins on cytokine expression that requires further investigation. In addition we observed how multiple infections and vitamin deficiencies impact fetal growth via the cortisol–IGF-1–fundal height pathway, however these relationships were far more complex than we had anticipated.

Originally we had anticipated that increases in cortisol and pro-inflammatory cytokines in response to multiple infections and vitamin deficiencies would lead to a decrease in IGF-1 concentrations and therefore fundal height. Instead, we observed that cortisol was increasing IGF-1, which increased fundal height, and that infections and vitamins both had positive and negative impacts on fetal growth through this pathway. Each of our vitamins, excluding folate, exerted a unique effect on the cortisol–IGF-1–fundal height pathway. Of the four vitamins, only vitamin D predicted fundal height,

and unexpectedly was associated with decreased fundal height. Vitamin A, although not directly linked to fundal height, was observed to increase concentrations of IGF-1 and through this mechanism was positively impacting fetal growth. Vitamin B12 impacted fundal height via increasing both pro- and anti-inflammatory cytokines, and as a result the net effect of vitamin B12 on fundal height is inconclusive, as the cytokines had both positive and negative impacts on growth.

None of the eight maternal infections included in this study appeared in our model for fundal height; however through modulation of the cortisol–IGF-1–fundal height pathway, we observed infections to have both positive and negative impacts on fundal height. Given the diversity of infections which appeared in our study, it is important to take into consideration the impacts of all infection types on pregnancy outcomes, many of which may not be considered severe such as skin or oral infections.

As previously mentioned, we observed that higher concentrations of vitamin D negatively impacted fetal growth. This effect was previously observed by colleagues Suissa et al. (2013), although we had hoped that the addition of cytokines/chemokines to the data set might clarify the mechanism behind this unexpected result. However, we were not able to find any association between vitamin D concentrations and cytokine/chemokine levels with the exception of TNF-α, which was lower in vitamin D deficient women. Our inability to detect associations between vitamin D and cytokines in our regression models may be due to a limitation in vitamin D analysis available to us at the time of the study. We measured vitamin D2 (25(OH)2), however the metabolically active metabolite of vitamin D which has also been observed to interact with cytokines is vitamin D3 ($1,25(OH)_2D$) (Khoo et al., 2011), which we were not able to analyse. Therefore future work of this study may include re-analysis of our samples for this vitamin D metabolite, to further investigate the possible mechanism between nutrients, cytokines and fetal growth.

Counter to what we had expected, Th1 pro-inflammatory cytokines did not emerge as a consistent contributor to impaired fetal growth. In fact the only cytokine to be implicated as a direct predictor of fundal height was T-regulatory cytokine IL-10. A possible explanation for this phenomenon may be due to the presence of GI nematode infections (*Ascaris* and hookworm) in our population of pregnant women. The immune response to a GI nematode is a Th2 mediated antibody response (Murphy et al., 2000; Seder & Mosmann, 1998). Due to the antagonistic relationship between Th1 and Th2 cytokines, polarization of the cytokine milieu towards Th2, would suppress Th1 cytokines. It is possible then, that the combination of infections, including those that induce Th1 and those that induce Th2 responses had varying effect on the immune system, resulting in neither subset gaining dominance. Furthermore, it is possible that in an environment without GI nematode infections, we may be able to observe a relationship between Th1 cytokines and fundal height, but in the context of multiple infections it is difficult to predict what will be the resultant immune response. In addition, CRP, a clinical marker of inflammation (Steel et al. 1994) was entered into our models but did not emerge as a significant predictor. In addition levels of CRP, as previously described by Gonzalez-Fernandez (2012), were elevated in approximately only 13% of pregnant mothers, suggesting that in the face of multiple infections and micronutrient deficiencies, these women were not experiencing strong pro-inflammatory physiological responses.

A major strength of our study was the large set of variables including the range of urogenital, respiratory, skin and oral infections, cytokines/chemokines, hormones, as well as physical and vitamin status. We had a wide range of cytokines and chemokine measurements, allowing us to examine a full spectrum of T helper cell responses including Th1, 2, 17 and regulatory. Furthermore we were fortunate enough to have data not only pertaining to presence or absence, but also severity of infection. This allowed us an in depth exploration of the relationship between infection and fundal height. In addition, we had a relatively large number of women from a homogenous population, allowing us to fully explore various components and determinants of maternal health on fetal growth, with theoretically limited impacts of genetic diversity.

For statistical analysis the high number of variables was beneficial, as it allowed us to construct best fit multiple linear regression models for our outcome variables of cortisol, IGF-1 and fundal height, while controlling for confounding variables. An additional strength of this study was the validity of our analytical measurements. Despite the remoteness of the study population, all samples were collected and analyzed using sterile and rigorous laboratory methodology. When we could not conduct certain analyses in Panama, we carried them out in appropriate laboratories in Montreal.

Fortunately, due to the low cost, reproducibility and reliability of the fundal height measurement we were able to confidently include this anthropometric into our study, despite a recent Cochrane review having found insufficient evidence to determine if fundal height is effective in detecting intrauterine growth restriction (Robert et al. 2012). This review article was only able to include one study (n=1639) done in a hospital setting in a developed country (Lindhard et al. 1990), which demonstrated that there was no difference between findings based on fundal height and ultrasound. Obviously this developed country hospital setting is in sharp contrast to our impoverished rural community setting in a developing country. However our data shows that use of fundal height in conditions of extreme poverty where ultrasound is not available can provide insight into early fetal growth. Further investigation of fundal height measurements should be conducted focusing on developing regions of the world, where this technique would best be adapted due to its low cost, ease of implementation reproducibility and reliability (Belizan et al. 1978; Challis et al. 2009; Grover et al. 1991).

Limitations to the study include the lack of repeated measures, which is inherent to the design of a cross sectional study. Repeated measurements and follow-up data would have increased our understanding of the impacts of maternal nutrition and infection status on growth of the fetus. However, follow-up data may have realistically been very difficult to obtain, as the majority of participants live in rural areas that require long walking distances to reach the RHC, the site of recruitment. Also, women

do not routinely travel to the rural health centres for routine pre-natal care, and most women deliver their babies at home. Therefore, it is conceivable that loss to follow up may have been high. We were limited in that we were not able to measure IGF-2 and vitamin D3 because the assays had not been developed yet by the company whose machinery we were using. Another limitation was that relatively few women were folate or vitamin A deficient, as they had previously received sporadic government supplementation with both of these nutrients, thus decreasing our ability to adequately test for effects of deficiency on fetal growth. In addition, the majority of pregnant women in our study were infected with multiple infections, limiting our ability to differentiate the effects individual infections on maternal health and fetal growth. Lastly, cross sectional measurements do not allow us to determine causal pathways, but only to observe relationships between variables. Despite these limitations, we found interesting and novel relationships that require future research, possibly using a smaller selection of variables, and repeat measurements to better understand mechanisms at hand.

Fortunately, many of our limitations are also opportunities for future research. We began our analysis by looking at the relationship between vitamin status on cytokine and cytokine ratio. Only a limited number of cytokines and ratios were affected by vitamin status. However, additional cytokine ratios may be affected by vitamin status but were not captured in our analysis. Therefore, it would be beneficial to create and test additional cytokine ratios to further explore these relationships. For our study, it would be beneficial if we measured IGF-2 to help further understand the relationship between maternal cortisol and IGF-1 and fundal height. In addition, reanalysis of our serum samples for vitamin D3 ($1,25(OH)_2D$), which would aid in capturing additional associations between vitamin D and cytokines thus providing us with a fuller understanding of the immunomodulatory effect of this vitamin. Measuring concentrations of homocysteine would be invaluable, as it is sensitive indicator of vitamin B12 and folate status, and previous research has identified it as a crucial component in the etiology of adverse pregnancy outcomes such as LBW and pre-eclampsia (Yajnik et al. 2005). Lastly, we conducted our research as an overview of

pregnancy. Although we controlled for trimester, it would be interesting to investigate our relationships by individual trimester, to observe if our findings exist are restricted to one trimester versus another, and furthermore to see if these relationships change from trimester to trimester.

Growth faltering is a harmful and pervasive outcome of poor development *in utero.* Although the physical manifestations are obvious and easy to identify, elucidating the mechanisms of which contributed to this outcome is complex and multi-factorial. Various factors are implicated in the etiology of growth faltering including maternal characteristics, endocrine hormones, vitamins, infections and immunity. Although majority of research still examines growth faltering postnatally, a paradigm shift is occurring as researchers are recognizing the importance of the prenatal environment and are focusing on *in utero* development. Our data supports the need to examine maternal health as an important contribute to early *in utero* growth.

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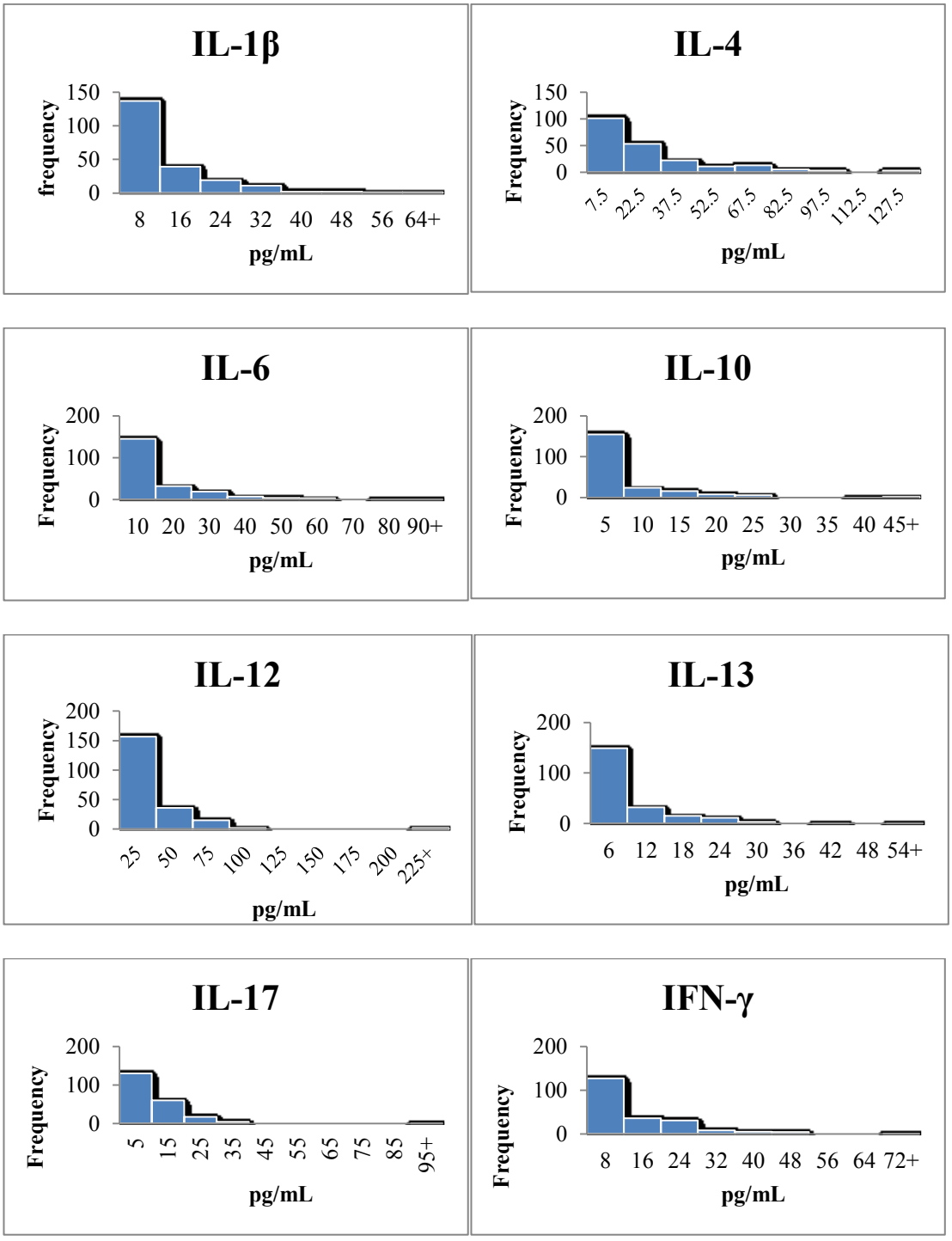
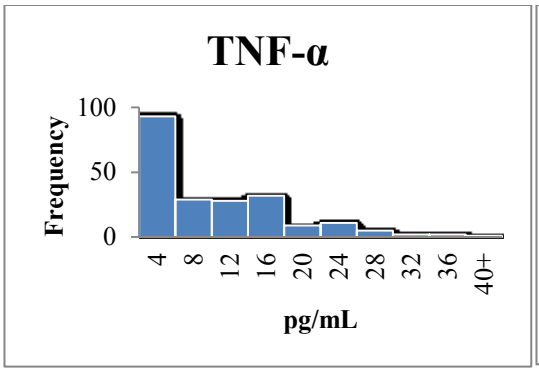
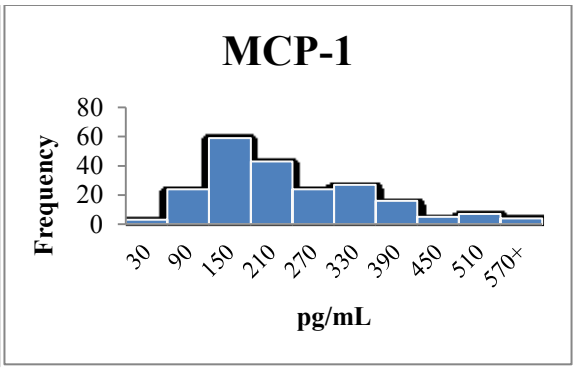
APPENDIX

A. Histograms of cytokines and chemokine (pg/mL) (n=184)




TNF-α
Frequency
100
50
0
4
8
12
16
20
24
28
32
36
40+
pg/mL


MCP-1
Frequency
80
60
40
20
0
30
90
150
210
270
330
390
450
510
570+
pg/mL