COMBINED IMPACT OF INFECTIONS AND NUTRITIONAL DEFICIENCIES ON MATERNAL HEALTH AND FETAL PARAMETERS IN A RURAL COMMUNITY IN PANAMA

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ABSTRACT

In a context of extreme poverty and food insecurity, 213 pregnant women belonging to the Ngäbe-Buglé indigenous community in rural Panama were assessed for respiratory, oral, skin, urogenital infections and intestinal parasites, and for iron, folic acid, vitamin B12, A and D deficiencies in a cross sectional study. Fetal wellbeing was assessed through simple measurements available in the field (fundal height -FH, fetal cardiac rate -FCR and fetal movements -FM as perceived by the mothers). Infections and micronutrient deficiencies were extremely prevalent, with all mothers affected by at least one condition and most affected by several. Inflammation indicated by high levels of C-reactive protein (CRP) was found in 16.5% of the mothers. CRP was significantly higher in women with oral infection and/or respiratory infections alone or in combination with genital infection or hookworm infection. CRP was significantly lower in women with lower levels of iron and vitamin B12, in women with vitamin D levels above 75 nmol/L and vitamin A levels above 20 μg/dL. Intra-uterine growth retardation was found in 7% of the fetuses, 27% of fetuses were categorized as large for gestational age, but the majority was found as adequate for gestational age (66%). Fetal cardiac rate and fetal movements were within normal limits. Indicators of infections, micronutrient deficiencies, and maternal inflammation were associated in a complex manner with our simple measures of fetal wellbeing. FH was negatively associated with higher percent of monocytes accompanied by higher mean corpuscular volume in the second trimester, and with vitamin D in the third trimester, while controlling for gestational age, maternal body mass index and wood smoke exposure. Indicators of infection also entered regression models for FCR and FM, indicating early fetal responses to mild-moderate extra-uterine maternal infections.

ABRÉGÉ

Une étude transversale ayant pour but d'évaluer l'état immunitaire et la présence d'infections respiratoires, orales, cutanées, urinaires et parasitaires, ainsi que l'état nutritionnel, notamment les déficiences en vitamines B12, A, D, en fer et folate. Cette étude fut menée auprès de 213 femmes enceintes de la communauté rurale du Ngäbe-Buglé au Panama vivant dans l'extrême pauvreté et l'insécurité alimentaire. L'état de santé du fœtus a été évalué usant des mesures disponibles lors des visites prénatales, incluant la hauteur utérine (HU), la fréquence cardiaque fœtale (FCF) et les mouvements fœtaux (MF) tel qu'estimés par la mère. La présence d'inflammation, signalée par une augmentation de la protéine-C réactive (PCR), fut observée chez 16.5% des mères. De plus, la PCR était significativement plus élevée chez les mères souffrant d'infections orales et/ou respiratoires, soit séparément, soit accompagnées d'infections génitales ou d'anchylostomes. Chez les femmes ayant une concentration plasmique abaissée en fer ou vitamine B12, en vitamine A au-dessus 20 μg/dL ou en vitamine D au-dessus de 75 nmol/L, la PCR était significativement inférieure. Un retard de croissance intra-utérin fut décelé chez 7% des fœtus, 27% naquirent gros pour leur âge gestationnel et 66% naquirent avec d'un poids normal. La FCF et les MF se révélèrent être dans les limites normales. Les marqueurs d'infections, de déficiences en micronutriments et d'inflammation maternelle étaient associés de façon complexe aux mesures de bien être fœtal. Après avoir contrôlé pour l'âge gestationnel, l'indice de masse corporelle de la mère et l'exposition à fumée de bois, nous avons observe que l'HU est négativement associée au pourcentage de monocytes et au volume corpusculaire moyen d'hémoglobine lors du deuxième trimestre, ainsi qu'a fœtus la vitamine D lors du troisième trimestre. Les marqueurs d'infection furent évalues usant des modèles de régression multiples pour la FCF et les MF, et ceux-ci indiquèrent une réponse fœtale précoce face aux infections maternelles extra-utérines légères a modérées.

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CONTRIBUTION OF AUTHORS

This thesis includes two manuscripts. In both cases, Professors M.E. Scott and K.G. Koski were my research supervisors. They provided guidance on the design of the research and assisted in the interpretation of the results. I was responsible for organization of all the field work, for data entry and analysis, and for preparing the manuscripts.

LIST OF ABBREVIATIONS

ACD: Anemia of chronic disease

BMI: Body mass index

BV: Bacterial vaginosis

CRP: C-Reactive protein

DBP: Diastolic blood pressure

DC: Dendritic cells

Epg: eggs per gram

FCR: Fetal cardiac rate

FH: Fundal height

FM: Fetal movements

GA: Gestational age

Hb: Hemoglobin

hCG: Human chorionic gonadotropin

Hcy: Homocysteine

Htc: Hematocrit

IDA: Iron deficiency anemia

IL: Interleukin

IOM: Institute of Medicine

IUGR: Intra-uterine growth retardation

LBW: Low birth weight

LGA: Large for gestational age

LPS: Lipopolysaccharide

MCH: Mean corpuscular hemoglobin

MCHC: Mean corpuscular hemoglobin concentration

MCR: Maternal cardiac rate

NRC: National Research Council

NK: Natural Killer (cells)

PD: Periodontal disease

PMN: Polymorphonuclear neutrophils

PROM: Premature rupture of membranes

PTD: Pre-term delivery

RBC: Red blood cells

RDW-SD: Red blood cells deviation width-Standard Deviation

ROS: Reactive oxygen species

SBP: Systolic blood pressure

SGA: Small for gestational age

sTfRs: Serum transferrin receptors

STH: Soil transmitted helminthes

UTIs: Urinary tract infections

AB/UTI: Asymptomatic Bacteriuria/Urinary tract infection

VDR: Vitamin D receptor

WBC: White blood cells

WHO: World Health Organization

Chapter I - INTRODUCTION

Improvement of maternal health and the reduction of child mortality are part of the WHO Millennium Development Goals, based on the fact that complications during pregnancy and delivery are the main cause of mortality of reproductive-age women in developing countries. In developed counties, maternal mortality rates are lower than 15 for every 100,000 live births (WHO, 2011). The current project was developed in Panama, where maternal mortality rates differ between 70 for every 100,000 live births in urban areas, and 283 per 100,000 live births in the Ngäbe-Buglé indigenous community in Panama, the second highest maternal mortality rate in the country (Gabinete Social de la República de Panamá and Sistema de Naciones Unidas, 2005), and has been the focus of several research projects in collaboration with Panamanian institutions and McGill University in Canada.

We have evidence of the impact of nutritional deficiencies and intestinal parasites on child growth in the region; previous studies revealed that 69% of pre-school children are affected by iron deficiency anemia, and rates of stunting are as high as 56% in the Ngäbe-Buglé community (Halpenny et al., 2012) where the majority of the population lives in extreme poverty, where daily nutrient intakes are far below internationally recommended daily requirements, and where the consumption of diluted-sweetened coffee is traditional (Lardeau et al., 2012, Payne et al., 2007).

It is clear from the literature that pregnancy outcomes and normal growth and development of the infant are negatively affected by micronutrient deficiencies and by a diversity of infections. Yet research to date focuses on these conditions in isolation, despite the high probability that individual women in vulnerable populations are concurrently infected with more than one pathogen and experience more than one micronutrient deficiency.

We hypothesize that infections and micronutrient deficiencies together impact maternal and fetal wellbeing.

In our research, we tried to capture the most complete picture of the health situation of pregnant women as possible, using resources available in the field during the follow up of normal pregnancies in 14 rural health centers. We took into account obstetric history and environmental factors known as risk factors during pregnancy, together with the evaluation of maternal skin, oral, intestinal, urinary, genital and respiratory tract infections through clinical and/or laboratory diagnosis. We also evaluated maternal serum concentrations of iron, folic acid, vitamin B12 vitamin A and vitamin D, given reported deficiencies in the children in the region. Finally we explored associations between infections and micronutrient deficiencies and the simple measures of fetal growth and development that were possible in the rural setting.

RATIONALE AND OBJECTIVES

Factors affecting maternal health in the Ngäbe-Bugle community in Panama are complex and start with the extreme poverty that affects food availability leading to micronutrient deficiencies and growth stunting, and poor sanitation and access to clean water that leads to frequent infection with multiple pathogens. Furthermore, geographical isolation also leads to decreased access to health care, and traditional beliefs make home delivery the first option for the majority of pregnant women, accounting for about 66% of deliveries in this community (Gabinete Social de la República de Panamá and Sistema de Naciones Unidas, 2005). Health centers run by nurses or health promoters are dispersed among the communities, often up to a 2 hour walk from the home of the beneficiaries along paths or unpaved roads (Halpenny et al., 2012). Some health centers have ocassional access to running water service but they rarely have electricity. Physicians visit the centers one or twice a week. If laboratory tests (including the routine pre-natal tests) or specialized care are needed, pregnant women are

asked to travel to the local hospital that has a basic laboratory. A gynecologist, not permanently available, does obstetric consultations and ultrasounds. Complicated cases requiring cesarean section are referred to the nearest city. Under such circumstances, health professionals in the field assess maternal and fetal wellbeing to the best of their ability with available tools. In this context, it is important to better understand how the complex set of overlapping conditions of multiple micronutrient deficiency and multiple infections may be associated with fetal wellbeing.

The goals of this cross-sectional study were to determine the prevalence of the different infections evaluated through clinical and basic laboratory analyses, to determine the prevalence of micronutrient deficiencies, to find the factors influencing infection and inflammation in the population and to assess the impact of infections and micronutrient deficiencies on simple fetal measurements. The research focused on five micronutrients (iron, FA, vitamins B12, A and D) and five groups of infection (respiratory, oral, cutaneous, urogenital and gastrointestinal) that are common in this indigenous community of Panama.

Chapter II -LITERATURE REVIEW

The fetal programming hypothesis or "Barker Hypothesis" proposes that adverse influences during pregnancy may result in permanent changes in metabolism, structure (vascular) and physiology (endocrine) in the developing fetus, that lead to phenotype adaptations and to an increased risk of disease in adulthood (Balci et al., 2010). Over the last two decades, this hypothesis has been supported by a number of articles showing the relationship between intra-uterine pathologies and multisystemic repercussions later in life. A recent review by Victora and collaborators of 28 articles from populations in low-income and middle-income countries shows that undernutrition leads to long-term impairment. Undernourished children are more likely to become short adults, to have lower educational achievement and to give birth to smaller infants. They also found that birth weight is positively associated with lung function and that there is some evidence that undernutrition might contribute to mental illness. On the other hand, there are studies suggesting a positive association between birth weight and the incidence of some cancers (Victora et al., 2008).

Understanding that any effort to improve pregnancy outcomes would lead to better conditions later in life at the individual and public health levels, this study aimed to better understand the interactions between infections and nutrition during pregnancy and their effects on the fetus in a context of poverty.

1 BIOLOGICAL CHANGES IN PREGNANCY AND ADVERSE PREGNANCY OUTCOMES

Pregnancy involves a series of anatomical, physiological and biochemical changes in women. During the first weeks of pregnancy, between the morula and blastocyst stages, the trophoblast is the first cell lineage to differentiate and to start placentation, which occurs in a relatively hypoxic environment. Hypoxia at this moment is essential for appropriate embryonic development, not only to protect the fetus against teratogen effects of reactive-oxygen-species (ROS)

during organogenesis, but also to favor placentation by promoting angiogenesis and by increasing trophoblast proliferation. Prior to the end of the first trimester, the trophoblast starts forming spiral arteries, the embryo obtains nourishment from endometrial glands and the oxygen tension within the placental inter-villous space increases (Burton, 2009).

Later in pregnancy, the trophoblast develops into the placenta and fetal membranes, while the blastocyst develops into the embryo, the umbilical cord and the placental mesenchyme (Huppertz, 2008). The outer part of the trophoblast, the syncytiotrophoblast, produces human chorionic gonadotropin (hCG), which promotes myometrial spiral artery angiogenesis. It is measured in pregnancy tests, being detectable only after implantation has occurred, approximately 5 days after missing menses or at 5 weeks of gestation (Cole, 2009). In contrast to what was understood a decade ago, the placenta is not a tissue containing paternal antigens that should be rejected under normal immunological conditions, but is a complex organ having synergistic interactions with the maternal immune system (Riley, 2008). For example, it has been found that the trophoblast, which promotes fetal acceptance under normal conditions, may be able to initiate signals promoting fetal rejection in the presence of infection. Infections may perturb the specific properties that each immune cell type has during implantation and placentation (Koga et al., 2009). Inadequate placental invasion or ischemic placental disease can lead to disorders such as preeclampsia, placental abruption, intrauterine growth retardation (IUGR) and recurrent pregnancy loss (Eastabrook et al., 2008).

Around week 16, fetal growth leads to expansion of the amniotic cavity and fusion of membranes that together form the chorioamnion. Fetal membranes surround the fetus acting as a barrier to the external environment and containing the fetus and the amniotic fluid (Chua and Oyen, 2009). The chorioamnion only ruptures during or just before labour at 37-42 weeks. Rupture

of membranes before term (preterm rupture of membranes –PROM), results in preterm delivery (PTD) and significant perinatal morbidity (Chua and Oyen, 2009). Physiopathology of PROM includes the disturbance of the fibroblast-collagenolytic activity, ROS-induced damage and the presence of infection, particularly urogenital infections such as cervicitis and/or vaginitis (Benedetto et al., 2004), asymptomatic *Escherichia coli* bacteriuria (Sheiner et al., 2009), or bacteria that produce metalloproteinases (Woods, 2001). PROM complicates around one third of PTD, and is a cause of increased perinatal infection (ACOG, 2007). PTD, defined as delivery occurring before week 37 of pregnancy, can be triggered by factors such as multiple gestations, placental disorders (placental abruption or placenta previa), extremes in the volume of amniotic fluid (polyhydramnios or oligohydramnios), maternal medical disorders, maternal psychological conditions (stress, depression), smoking and intra-uterine infections that alone, account for 25-40% of PTDs (Goldenberg et al., 2008).

Intrauterine growth restriction (IUGR), defined as an infant falling below the 10^{th} percentile for gestational age for the reference population, is a pathologic condition where the fetus does not reach its genetic growth potential due to an event or events that occur *in utero* (Bamberg and Kalache, 2004); the other extreme, fetuses large for gestational age (LGA), are those who fall above the 90^{th} percentile for gestational age (Ota et al., 2011) indicating macrosomia, which has been associates with both, maternal and fetal/neonatal complications such as peri-partum infections (chorioamnionitis, endometritis and neonatal infection), labor dystocya leading to perineal laceration and newborn trauma, neonatal meconium aspiration syndrome and hypoglycemia (King et al., 2012); in spite of this, no information about LGA impact on developing countries is found in the litarature. Instead, research focuses on IUGR as the main adverse pregnancy outcome. Pre-existing maternal disease including macro/micronutrient malnutrition, acquired blood borne infections and abnormal placental position are risk factors for the development of IUGR. These

conditions share a common placental phenotype commonly called “placental insufficiency” (Cetin and Alvino, 2009, Christian, 2009). Common determinants of IUGR among developed and developing countries include low weight gain, low body mass index [BMI= weight in kg/(height in m)^2] and short stature; in developed countries, cigarette smoking and primiparity are also major factors causing IUGR, whereas in developing countries additional factors include malaria and pregnancy-induced hypertension (WHO, 2002).

Birthweight provides a summary of fetal growth. Low birth weight (LBW), defined by The World Health Organization (WHO) as birthweight less than 2500 g, is a significant contributor to neonatal morbidity often associated with neonatal respiratory distress syndrome, meconium aspiration, hypoglycemia, hypothermia and hypocalcemia. LBW also increases mortality up to 15 years of age (Murphy et al., 2006) and has been associated with cardiovascular diseases, specifically ischemic heart disease in adults (Kaijser et al., 2008), psychiatric symptoms and disorders especially attention deficit, anxiety symptoms and relational problems (Indredavik et al., 2004), and chronic kidney disease (White et al., 2009) to cite some examples.

LBW must be differentiated from small for gestational age (SGA). The terms SGA and IUGR are often used interchangeably, but they are not synonymous. SGA is described as weight below the 10th percentile, a definition that can be applied only after birth since the knowledge of gestational age (GA) is required, and includes some small but normal babies (Groom et al., 2007). Consequences of SGA include persistent short stature, low lean body mass and increased central adiposity (risk factor for insulin resistance and metabolic disease) and alteration in pubertal development (Labarta et al., 2009).

2 IMMUNOLOGICAL CHANGES IN PREGNANCY

From the immunological point of view, pregnancy has been classically considered as a Th-2 state where imbalance towards Th-1 responses could lead to abortion

or pregnancy complications (Challis et al., 2009) but this concept is the subject of debate. It is accepted that pregnancy can be divided into three distinct immunological phases, characterized by different biological processes, which are related with placental formation. The placenta is an important endocrine organ that produces numerous hormones including estrogens, progesterone, hCG and human placental lactogen, some of which play a role in the regulation of fetal growth (Murphy et al., 2006). During the first immunological phase, implantation needs strong pro-inflammatory processes for the trophoblast to be able to break the epithelium of the uterus and invade endometrial tissue. The second phase, the "anti-inflammatory" state is characterized by mother, placenta and fetus symbiosis that coincides with rapid fetal growth and development and the induction of an anti-inflammatory state resulting in cessation of initial symptoms of pregnancy. In the pre-delivery stage, contraction of the uterus, delivery of the baby and expulsion of the placenta requires a pro-inflammatory environment (Koga et al., 2009). In general, it is accepted that the adaptive immune response is down regulated during pregnancy and the innate immune response is enhanced. Innate immune cells play a critical role in the fetal-maternal immune adjustment and in successful placentation, supporting the fetus in a harmonious fetal-maternal immunological relationship. These responses are reflected in the white blood cell volume in the form of increased number of mature of polymorphonuclear neutrophils (PMN) or neutrophilia up to 15,000/mm^3, a slight increase in eosinophils, a slight decrease in basophils but no change in monocytes or lymphocytes (Torgersen and Curran, 2006). Not only the number of peripheral PMNs increases, but they experience transformations similar to those observed during sepsis, as part of the pregnancy-induced inflammatory changes; there is evidence of activation of PMN from placental debries, and it is believed that persistent inflammation during pregnancy might be favored by normal delayed apoptosis of neutrophils, which might explain pregnancy-associated neutrophilia (Muller et al., 2009, Hahn et al., 2006).

3 FETAL EVALUATION

One of the most important aspects during prenatal care is the detection of SGA or IUGR fetuses that have higher risk of morbidity or mortality. Various methods are currently available, however the most widely used is the measurement of the symphysis fundal height (FH). It consists of the measurement of the abdominal curvature from the top of the symphysis to the top of the uterine fundus with a non-elastic tape measure calibrated in centimeters while the woman lies supine on the examination couch (Griffiths et al., 2008).

Although the method has not been widely confirmed as reliable, its accuracy has been documented to be similar to sonographic measurement of fetal abdominal circumference in the prediction of birth weight (Kayem et al., 2009). When assessing low-risk populations, this method is simple, safe, quick to perform and inexpensive, and is widely used to support important clinical decisions in developing countries, where access to sophisticated technological resources for assessment of fetal growth is not available (Freire et al., 2010).

Antenatal fetal cardiac rate (FCR) auscultation has been one of the first direct methods of prenatal diagnosis and is an extensively used to assessment tool of the fetal condition (Smith, 2008). The FCR is controlled by intrinsic and extrinsic factors, indirectly indicating fetal oxygenation. Heart rate is controlled by sympathetic and parasympathetic systems. Sympathetic nerves (through norepinephrine) increase FCR. Parasympathetic influence, mediated primarily by the vagus nerve (with acetylcholine as neurotransmitter), decreases the heart rate; parasympathetic maturation from mid-gestation causes the gradual decrease in baseline FCR. FCR is also influenced by the action of baroreceptors (located in the aortic arch and between internal and external carotid arteries) and chemoreceptors (localized in the carotid bodies, aortic arch and in the medulla), that respectively detect the blood pressure and oxygen levels. Barorreceptors decrease FCR in response to increased blood pressure, and

chemoreceptors increase FCR in response to decreased oxygen (Fassett, 2000). The combination of effects results in FCR variability, which together with the presence of accelerations and decelerations, is clinically more useful than the basal FCR, and is measured using ultrasound, continuous heart-rate monitoring and fetal electrocardiography among others (Martin, 2008). In absence of those techniques in rural areas, the auscultation of FCR determining if it is above or below normal values (>160 beats/min and <120 beats/min respectively) (WHO, 1996), helps clinicians to detect fetuses at risk.

Independently of health professional advice, mothers use the perception of fetal movements (FM) as a screening tool for fetal wellbeing. Their perception usually starts around week 20 of pregnancy, the frequency of FM progressively increases with a peak at week 32 and decreasing during the last month of gestation due to the decreased amount of amniotic fluid, the progressive maturity of the central nervous system and the elongation of sleeping periods of the fetus (Sergent et al., 2005). FMs are included them as candidates for diagnostic purposes, since they are useful for interpretation of other clinical measurements such as FCR by revealing fetal central nervous system and motor development (Visser et al., 2010). Although definitions of reduced FM need to be better assessed, there is significant evidence that reduced fetal movements are a significant marker of a vulnerable fetus. The reduction of FM has been associated with fetal hypoxia, increased incidence of stillbirth and IUGR (Heazell and Froen, 2008). The increased perception of fetal movements is the focus of very recent research, that associates unusually vigorous FMs with a greater risk of late stillbirth (Stacey et al., 2011).

4 INFECTION

The described response of the immune system during pregnancy makes women more susceptible to parasitic infections when pregnant than in non-pregnant state; also, bacterial, viral and parasitic maternal infections are more severe in

the first pregnancy, and infections occurring during the first trimester are associated with more severe fetal and placental consequences than when occurring later in pregnancy (Dotters-Katz et al., 2011). Considerable information is available about intra-uterine infections and pathogens that are able to cross the placenta (Gilbert, 2002), but little is known about the effect of common maternal infections on the offspring. Next, the most frequent infections observed in outpatient clinics in rural settings in Panama are reviewed.

4.1 Respiratory infections

Viral respiratory infections such as influenza virus rarely cross the placenta, but they can have an effect on prematurity mediated through inflammatory pathways (Omer et al., 2011). Recently, a Norwegian study found that ear-nose-throat infections occurring before gestational week 17, but not later in pregnancy, were associated with spontaneous PTD (Morken et al., 2011). Pneumonia, usually community acquired and produced by *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Mycoplasma pneumoniae*, has been associated with LBW and increased risk of PTD (Goodnight and Soper, 2005). Severe acute respiratory infection (bronchitis, bronchiolitis and pneumonia) was found to be associated with a higher rate of PTD (Banhidy et al., 2008). In another study by Deutscher and collaborators, pregnant women had a 2-fold increased incidence of group *β Streptococcus* respiratory disease, compared with non-pregnant women of similar age. The incidence was also higher than the one found in adults aged ≥65 years (Deutscher et al., 2011).

4.2 Oral infections

Another infection that is currently a focus of interest in research is periodontal disease (PD) during pregnancy. It is believed that either low-grade bacteremia and/or cytokines generated within the oral tissues can trigger inflammatory responses leading to adverse perinatal outcomes such as late miscarriages, preterm birth and neonatal morbidity (Matevosyan, 2011). Reviewed by

Wimmer & Pihlstrom, several PD causative microorganisms have been linked to PTD, including *Streptococcus* spp, *Fusobacterium nucleatum*, *Campilobacter rectus* and *Porpyyromonas gingivalis.* This review also considers the influence of pro-inflammatory mediators generated within the periodontal tissue and particular gene polymorphisms as possible causes of pregnancy complications such as IUGR, SPTD and PROM (Wimmer and Pihlstrom, 2008).

4.3 Skin infections

Scabies is a common skin infection in developing countries and is associated with poverty and overcrowded living conditions. Scabies is caused by the arthropod *Sarcoptes scabiei var humanus*, an obligate parasite of human skin. Fertilized females excavate tunnels in the epidermis and lay their eggs; resulting larvae migrate to the skin surface to complete their entire life cycle (Orion et al., 2004). The larval migration plus mite excrements are responsible for majority of the symptoms in the host, mainly intense, intractable, generalized pruritus, which is worse at night. There is usually an erythematous, symmetrical rash in areas such as interdigital webs, nipples or in the genitals, but clinical presentation depends on host's immune response. The pathognomonic lesions are the skin burrows (serpinginous gray line corresponding to the tunnel made by females mites) and scabietic nodules (Hicks and Elston, 2009). Little information is found about clinical features during pregnancy. Only two drugs are available for treatment (benzyl benzoate and permethrin), during the second and third trimesters but no medication is available for use during the first trimester of pregnancy (Mytton et al., 2007).

Other skin infections share similar epidemiologic conditions. Bacterial soft tissue infections are caused mainly by *Staphylococcus aureus* and *β hemolytic streptococci.* The former usually manifests as localized pus-producing lesions, and the latter produces rapidly spreading infections (Dryden, 2009). Pregnant women are susceptible to the same fungal infections as non-pregnant women,

but certain fungal infections occur with higher frequency or severity during pregnancy (Moudgal and Sobel, 2003). The literature is scarce regarding the incidence and impact of those infections during pregnancy, particularly in developing countries.

4.4 Genital infections

Bacterial vaginosis (BV) is a condition in which the normally protective *Lactobacillus* (hydrogen peroxide-producing species, making the vaginal environment acidic) is replaced by high quantities of commensal anaerobes, resulting in symptomatic vaginitis (Marrazzo, 2011). Among those bacteria, *Gardnerella vaginalis* is the most prevalent cause of vaginal discharge in developing countries (Mullick et al., 2005), and one of the most studied. The prevalence of BV varies significantly in populations from different countries; black women have been found to be more colonized (23%) than white women (9%) (Tolosa et al., 2006). Interestingly, BV has been associated with low serum concentrations of vitamin D, which could contribute to the strong racial disparity in the prevalence of BV (Bodnar et al., 2009). BV has been implicated as a cause of PTD, LBW, PROM, postpartum sepsis and spontaneous miscarriage in developing countries and the United States (Mullick et al., 2005, Denney and Culhane, 2009). Clinical diagnosis is based on vaginal pH>4.5, homogeneous vaginal discharge, detection of fishy odor on addition of potassium hydroxide to vaginal fluid and the presence of clue cells that particularly characterize BV. Another way of diagnosing BV is applying the Nugent criteria to Gram stains of vaginal fluid, which quantifies the number of lactobacilli relative to BV-associated bacteria morphotypes to create a scale of flora abnormality ranging from normal through intermediate to frank BV. The Nugent score is widely accepted as the gold standard for the diagnosis of BV in research studies (Marrazzo, 2011).

Candida spp. can be present as a commensal organism or a pathogen in the vagina. Changes in the host vaginal environment lead to germination of yeast

producing pseudohyphae and/or hyphae, which are responsible for symptoms that include vulvar pruritus, burning, soreness, irritation, dyspareunia and dysuria (Ilkit and Guzel, 2011). The prevalence of vaginal candidiosis is 2-fold higher in pregnant women than in non-pregnant women, and although it has not been directly linked to adverse pregnancy outcomes such as PTD, there is increasing evidence that its treatment during pregnancy is associated with significantly higher mean gestational age and reduction in the prevalence of PTD (Banhidy et al., 2009).

According to WHO, the annual incidence of the common sexually transmitted diseases in 2005 were 88 million for *Neisseria gonorrhoeae*, 11 million new cases of syphilis and 248 million for *Trichomonas vaginalis* (WHO, 2011).

T. vaginalis infection produces green-yellow frothy vaginal discharge, dyspareunia, vulvar and utethral irritation with itching and dysuria. Diagnosis is reliable when made on clinical basis combined with wet mount smear through microscopic visualization of motile protozoa; also, diagnostic techniques such as culture, RNA and rapid antigen and nucleic acid amplification are available in developed countries (Harp and Chowdhury, 2011). *T. vaginalis* infection has been related with LBW and PTD, and its role in increasing the transmission of HIV infection has been postulated, mediated by the damage that *T.vaginalis* produces to the vaginal mucosal barrier (Gulmezoglu and Azhar, 2011).

Gonorrhea, produced by sexually-acquired Gram-negative intracellular diplococcus *Neisseria gonorrhoeae*, has been reported to have higher prevalence among black women, adolescent females and those with low education (Walker and Sweet, 2011). Clinical presentation varies. Early infection may be asymptomatic or subclinical, with symptoms that include discharge and mild irritation. Infections can also affect the upper reproductive tract of women, manifesting as cervicitis and development of pelvic inflammatory disease with complications such as tubal scaring that leads to infertility, ectopic pregnancy

and chronic pelvic pain; gonococcal infection can be vertically transmitted during childbirth and is a leading cause of infectious neonatal blindness (Johnson and Criss, 2011). Gonococcal infection has other critical implications for maternal health, including 5-fold increased risk of HIV transmission, first trimester abortion and neonatal complications such as severe neonatal eye infection (WHO, 2011).

Another prevalent sexually transmitted infection is syphilis, caused by *Treponema pallidum*. It is responsible for severe adverse pregnancy outcomes such as abortion, stillbirth, PTD, newborns with clinical signs of congenital disease and often, apparently healthy children who later develop clinical signs. About 40% of pregnancies with the disease end in spontaneous abortion, stillbirth and perinatal death (Casal et al., 2011).

4.5 Urinary tract infection

A number of changes occur in the urinary tract of pregnant women such as dilation of renal pelvis and ureters, bladder displacement, mechanical compression on ureters and smooth muscle relaxation induced by progesterone, and differences in urine pH and osmolality that together facilitate bacterial growth (Schnarr and Smaill, 2008). In the general population, urinary tract infections (UTIs) are classified as uncomplicated or complicated. The majority of UTIs are uncomplicated and are caused by *Escherichia coli* (Nielubowicz and Mobley, 2010). In pregnant women, UTIs are classified as asymptomatic or symptomatic. Asymptomatic bacteriuria (bacteria in urine without symptoms) occurs in 2-10% of all pregnancies and, if untreated can lead to maternal pyelonephritis and LBW in infants. Maternal urinary tract infection has been independently associated with PTD, pre-eclampsia and IUGR, and this is the reason why screening and treatment of asymptomatic bacteriuria during pregnancy is now a standard of care (Mazor-Dray et al., 2009).

4.6 Intestinal parasites

Although parasitic infections have different pathophysiologic mechanisms, almost every parasite causes anemia and malnutrition either directly or indirectly (Dotters-Katz et al., 2011). Poverty, tropical climate and lack of adequate water-sanitation systems are some of the common conditions that favor intestinal parasitism in humans. About 2 billion people are infected with soil-transmitted helminths (STH), the most common parasitic infection in humans. In 2004, it was estimated that there were 1450 million people infected with *Ascaris lumbricoides*, 1300 million with hookworm and 1050 million with *Trichuris trichiura*. Hookworm is responsible for the highest annual mortality (65,000 deaths), followed by ascariasis (60,000) and by trichuriasis (10,000) (Savioli and Albonico, 2004).

In pregnancy, the most important known impact of STH is probably anemia, due to its known link with adverse pregnancy outcomes such as stillbirth, LBW and PTD (Haider et al., 2011). Hookworm (*Ancylostoma duodenale* and *Necator americanus*) infection in particular produces anemia though direct small intestine mucosa damage. About 0.825 mg of hemoglobin per gram feces are lost for every increment of 1000 *N. americanus* eggs per gram (epg) of feces (Larocque et al., 2005). In pregnancy, it has been well established that high intensity of hookworm (≥ 4,000 epg) is associated with lower hemoglobin levels when compared with low intensity (1-1,999 epg) (Brooker, 2008).

Trichuris trichiura inhabits the human cecum and proximal large bowel, where the anterior part of adult worms penetrates the intestinal mucosa. When parasitic load is high (≥ 10,000 epg) (Montresor et al., 1998), it causes mucoid diarrhea, rectal bleeding, rectal prolapse and iron deficiency (Khuroo and Khuroo, 2010).

Ascaris is also a parasite of the human small intestine. Although it has not been directly implicated as a cause of anemia during pregnancy, high intensity of

infections (≥ 50,000 epg) reduce food consumption, interferes with the absorption of fat and protein and produce intestinal damage, leading to reduction of mucosal lactase activity (Crompton and Nesheim, 2002).

In addition to the role of intestinal parasites in producing anemia during pregnancy and the associated adverse pregnancy outcomes related to anemia, recent research is oriented towards the impact of helminths on the modulation of the maternal and fetal immune systems. It has been stated, for example, that helminthic infections in pregnant women are able to sensitize fetal parasite-specific responses through the activation of Th-1 and Th2-type cytokines; furthermore, that the post-partum predominance of one or the other response will depend on postnatal exposure to environmental antigens (Pit et al., 2000). Helminths have also been reported to decrease fetal length, through the decreasing of serum leptin and insulin-like growth factor-1 in experimental studies (Odiere et al., 2010).

Giardia lamblia is a protozoan found primarily in mammals including humans, transmitted by the ingestion of its cyst form in contaminated water, food or by direct fecal-oral contact; clinical presentation varies from the asymptomatic carrier state to a severe disease form (Gardner and Hill, 2001). Both innate and adaptive immune responses are necessary for the control of the infection.The immune response towards *G. lamblia* is mainly characterized by the production of immunoglobulin a (IgA), but interferon-γ (IF-γ), IL-6 and IL-5 are also liberated in response to infection (Solaymani-Mohammadi and Singer, 2010); still, host-parasite interactions remain incompletely understood. There is very little information on giardiasis during pregnancy and even less during pregnancy in humans. In goats, the excretion of *Giardia* cysts in the peri-parturient period was found to be 7-10 times more than the excretion of cysts occurring during the three weeks before and after this period (Castro-Hermida et al., 2005).

5 C-REACTIVE PROTEIN

C-Reactive protein (CRP) is used in clinics as a measurement of inflammation. It is a component of the acute-phase inflammatory response, first identified in patients with *Streptococcus pneumoniae* infection and named this way because of its ability to precipitate the C-fraction of pneumococcal cell wall. It is synthetized in the hepatocytes in reaction to infection, inflammation or tissue injury. It acts as an opsonin, by coating microbial particles and dying cells to promote bacterial killing and inducing phagocytosis via the early stages of the classical complement cascade (Rhodes et al., 2011). During pregnancy, CRP concentrations increase in early gestation, and respond to different stimuli according to different stages of pregnancy, such as to IL-6 production by monocyte and macrophages at implantation, and to the necrotic process of placenta aging and progressive increments of estrogen levels. CRP is also affected by factors such as maternal age, weight and possibly by smoking (Mendall et al., 1996, Julius et al., 2011). In most population studies, 95% of people have levels <10 mg/L, higher levels are associated with infection, inflammatory diseases or malignancy (Windgassen et al., 2011). Very limited data is found on normal ranges of CRP during pregnancy. Initial investigations with CRP levels measured turbidimetrically estimated the 95th percentile was 20 mg/L, and this value has been considered as the upper limit of normal values (Nielsen et al., 1990). A more recent study in a diverse population of healthy pregnant women using a high sensitivity assay found a median value of 4.8 mg/L, with an interquartile range of 0.63-15.7 mg/L (Picklesimer et al., 2008). Belo and collaborators demonstrated consistently elevated CRP values throughout pregnancy with a wide variation among subjects (Belo et al., 2005). The normal values published by the American College of Obstetricians and Gynecologists are different for the second (20 mg/L) and for the third trimester (8.1 mg/L) (Abbassi-Ghanavati et al., 2009). CRP has been found to be elevated at early stages of pregnancy in women who further developed pre-eclampsia compared

with controls (levels of 8.7 ± 5.5 and 5.3 ± 4.3 mg/dL respectively) (Garcia et al., 2007), and also in early stages of pregnancy of women delivering preterm when compared with women delivering at term with odds ratios between 1.7 and 2 depending on the CRP cut-off value (Hvilsom et al., 2002). The value of CRP in the prediction of chorioamnionitis in women with PROM has been studied but is still controversial (van de Laar et al., 2009)

6 NUTRITION

Maternal diet, caloric intake and metabolic function are important for supply of nutrients to the fetus. The fetus responds and adapts to undernutrition, but adaptations may lead to permanent changes in the structure and function of the body (Martin-Gronert and Ozanne, 2006). Poor nutritional status is associated with poor fetal growth due either to decreased nutrient availability or increased demand, whereas a high proportion of protein as part of the total energy intake in early pregnancy has a positive impact on birth weight, placental weight and ponderal index [ratio of body weight (g) to length (cm), (Mandy, 2011)]. On the other hand, carbohydrate intake in early pregnancy is inversely related to ponderal index at birth (Moore et al., 2004, Murphy et al., 2006, Moore et al., 2004). Also, the incidence of PTD, LBW and neonate mortality is higher in neonates whose mothers have low BMI when compared with those from mothers of healthy weight (Kalk et al., 2009).

The last recommended total weight gain ranges for women with singleton pregnancies have been defined by the Institute of Medicine (IOM) and the National Research Council (NRC) as follows: For underweight women (BMI<18.5 kg/m^2), 12.5-18 kg; for normal weight women (BMI between 18.5-24.9 kg/m^2), 11.5-16 kg; for overweight women (BMI between 25.0-29.9 kg/m^2), 7-11.5 kg, and for obese women (BMI ≥30 kg/m^2), 5-9 kg. (NRC, 2009). Besides BMI, maternal height itself represents the uterine capacity and the potential for fetal growth and is a major determinant of fetal size (Murphy et al., 2006).

6.1 Coffee

Of special interest for our study is coffee consumption during pregnancy, due to its traditional consumption among the indigenous community (Lardeau et al., 2012). The effects of caffeine on pregnancy are still the subject of research, and the results are variable. A study done in The Netherlands found short-term effects of maternal coffee ingestion on the fetus: increased fetal movements, fetal cardiac rate accelerations and variation in mothers who were non-habitiual drinkers (Mulder et al., 2010). Previous studies had also shown a 2-fold increase in fetal breathing activity and a significant fall in basal fetal cardiac rate in mothers who regularly consumed coffee (Salvador and Koos, 1989). According to the Cochrane review of 2009 on the effects of caffeine ingestion during pregnancy, excess consumption (more than eight cups per day) would theoretically produce increased catecholamine levels in the mother and in the fetus, which would lead to uterus-placental vasoconstriction, increased fetal heart rate and fetal arrhythmia, with a consequent decrease in fetal oxygenation. However, several studies have shown no association between caffeine consumption, birthweight, gestational age/birthweight rate, or between caffeine consumption during the first and third trimester and IUGR, LBW or PTD. Other studies even show beneficial effects of caffeine during pregnancy (Jahanfar and Sharifah, 2009). Moderate consumption may have a protective role in the development of gestational diabetes (Adeney et al., 2007).

Caffeine has been implicated with higher risk of osteoporosis by decreasing bone mineral density and negatively influences calcium absorption (Zhou et al., 2009). In this sense, there is recent evidence that caffeine is able to decrease vitamin D receptor (VDR) protein expression on osteoblasts. This effect was evident at doses of caffeine as low as 1 and 10 mM, which led to 50 and 70% reduction in VDR protein expression respectively (Rapuri et al., 2007).

Interestingly, caffeine has been described to protect *in vitro* against erytrhocytary suicidal death, when apoptosis is triggered by stress conditions that lead to increased intracellular calcium. This protective effect requires caffeine concentrations equivalent to plasma levels of 100 μM (Floride et al., 2008).

Coffee consumption has been found to interfere with the utilization of supplemental iron in toddlers in Guatemala; changes in plasma ferritin were significantly lower in children who consumed coffee compared with children who did not (Dewey et al., 1997). Although coffee consumption in Ngäbe-Buglé indigenous community in Panama is high, caffeine concentration is low due to the diluted traditional way of preparation (Lardeau et al., 2012). Despite this, Lardeau found that maternal coffee consumption was a negative predictor of both maternal hemoglobin and serum ferritin concentrations.

6.2 Anemia

Very briefly, anemia is defined as a decrease in red blood cell (RBC) mass that can be detected in hemoglobin (Hb) concentration, hematocrit (Hct) and RBC count, and is defined in pregnancy as Hb <11 gr/dL by the WHO (Milman, 2008). Hemoglobin levels are physiologically decreased during gestation due to the expansion of plasma volume; this allows better circulation for the placenta (Ervasti et al., 2009). Anemia is an important cause of adverse pregnancy outcomes. The risk of PTD and LBW increase in proportion to the severity of maternal anemia (Kidanto et al., 2009).

There are many causes of anemia and they often overlap; the main and most studied cause of anemia is iron deficiency, but other micronutrient deficiencies are able to produce anemia and also, acute and chronic infection and inflammation are associated with anemia (Milman, 2011). The average volume of a RBC (mean corpuscular volume, MCV) is used to determine possible causes of anemia and permits its classification into microcytic (MCV<80 fL), macrocytic

(MCV>100 fL) or normocytic (MCV=80-100 fL) anemia. RDW represents the coefficient of variation of the RBC volume distribution (size) and may be the first parameter indicating an abnormality in RBC production, even before anemia appears (Sarma, 1990). Defective hemoglobin synthesis as in iron-deficiency anemia or chronic inflammation, is expressed as small cells, leading to normocytic or microcytic anemia (Sarma, 1990, Moreno Chulilla et al., 2009); defects in nuclear maturation like in folate or vitamin B12 deficiency result in larger erythrocytes with a normal hemoglobin content (macrocytic anemia) (Chandra, 2010).

For the purpose of this review, we refer only to micronutrients related with nutritional anemia. Other causes of anemia such as hemoglobinopathies, congenital diseases, malignant disorders, endocrine disorders or toxicant-related are not discussed.

6.2.1 Iron

In addition to binding and transport of oxygen, iron is essential for regulation of cell proliferation and differentiation in the functioning of immune system; it participates in cytokine production and function. It also participates in the process of killing bacteria by its interaction with myeloperoxidase activity (Curry et al., 2009). During pregnancy iron requirements increase from 0.8 mg/d in the first trimester to 7.5 mg/d in the third trimester due to increased maternal RBC production and the growth of the fetus and the placenta. The average requirement in the entire gestation period is around 4.4 mg/d (around 1240 mg during the whole pregnancy). Postpartum, erythrocyte mass goes back to pre-pregnancy levels, and the hemoglobin is recycled to body iron reserves. Thus, the net iron loss associated with pregnancy is approximately 630 mg (Milman, 2008). Iron is also crucial for the adequate formation of the fetus. It is not only required for hemoglobin synthesis but also for central nervous system development by participating in neuronal synapse formation, myelination and synthesis of certain

neurotransmitters (Hernandez-Martinez et al., 2011). Such is the importance of iron for fetal development that its transportation at the placental level leads to a balance in favor of the fetus at the expense of maternal iron stores and hematocrit (Gambling et al., 2011).

Iron is incorporated into multiple proteins that have important functions such as gas transport (hemoglobin, myoglobin), electron transfer in the respiratory chain, DNA replication and repair, and cell signaling, where iron switches between the ferrous and ferric states. In its free state, iron participates in redox reactions leading to uncontrolled production of free radicals and to cell membrane, protein and DNA damage (Evstatiev and Gasche, 2011). Cellular iron is therefore compartmentalized to control reactions with oxygen, and ferritin is crucial for this regulation; most of the iron store is located in the spleen, liver and bone marrow in the form of ferritin. Small quantities of ferritin are secreted into the circulation, making serum ferritin the most convenient indicator of the size of the iron store in the absence of liver disease and infectious and inflammatory disorders (Lynch, 2011). The second edition of "Assessing the iron status of populations" report (WHO, 2007) cites the IOM recommendation of identifying iron deficiency during the first and second trimesters of pregnancy with the combination of hemoglobin values <11 gr/dL and ferritin concentration < 20 ng/mL and suggests in a separate chapter that detection of iron deficiency in acute or chronic disease uses a threshold of 30-50 µg/L of ferritin in order to distinguish between the presence and absence of iron storage. The same document presents a review of indicators of iron status during an acute phase response and suggests levels of ferritin between 30-100 ng/mL to detect depleted iron stores under such situation. A more recent review by Weeler (2008) describes how ferritin cut-off at 30 ng/mL during pregnancy has a sensitivity of 90% and specificity of 85.1% for the detection of iron deficiency, compared with 37.5% sensitivity and 93.7% specificity for cut-off levels at 12 ng/mL (Wheeler, 2008).

In the plasma, iron is transported by transferrin that donates iron to cells through specific membrane receptor interactions. Transferrin receptors are glycoproteins contained by almost all cells in their surface (except for mature red cells) and expressed in response to iron deprivation, indicating the iron status of the cell. The circulating form of transferrin receptors, serum transferrin receptor (sTfR), is a soluble truncated version of transferrin receptors and is useful to quantify tissue iron deficiency and erythropoietic activity (Beguin, 2003). It is estimated that levels of sTfRs above 8.5 μg/mL may indicate iron depletion (Coad and Conlon, 2011). sTfRs are released into the circulation by transferrin receptor-expressing cells, they inversely correlate with total body iron content and do not increase with inflammation. Therefore sTfRs are of high sensitivity and specificity when diagnosing iron deficiency during pregnancy (Lee and Okam, 2011).

Viral, bacterial or fungal infections can be associated anemia of inflammation (also termed anemia of chronic disease, ACD) (Skikne et al., 2011), which has similar characteristics of IDA in that both produce normocytic or microcytic anemia. ACD is the “hypoferremic response to infection” described in the classic paper by Weinberg, as one of the strategies to withhold growth-essential iron from microorganisms, since pathogens require iron and other micronutrients for replication and survival. The strategies include the presence of iron-binding proteins at potential sites of invasion, decreased intestinal absorption and movement of plasma iron to the liver (Weinberg, 1984). On the other hand, to overcome iron unavailability, microorganisms such as gram-negative bacteria produce small organic molecules called siderophores, which have high affinity specifically for ferric ions, chelating them and transporting them across the cytoplasm through a complex system that includes an outer membrane receptor, a periplasmic binding protein and a transporter (Chakraborty et al., 2007). Therefore it is difficult to diagnose ACD especially when it coexists with IDA. During an inflammatory response, serum ferritin might be falsely normal or

elevated, as it reacts as an acute phase protein, and plasma CRP should be measured (Breymann et al., 2010, Wheeler, 2008).

In spite of intense research, there is no consensus on cut-off values of iron indicators during pregnancy in populations with high prevalence of infections. As a rule, determining the serum ferritin is sufficient for the diagnosis of IDA; if ferritin is <30 ng/mL, there is 90% certainty, even if there is no evidence yet of anemia. The measurement of CRP is recommended to detect false-normal or -elevated ferritin (Breymann et al., 2010); complementary, the use of sTfRs is also recommended (Lee and Okam, 2011).

6.2.2 Folic acid (FA) and Cobalamin (Vitamin B12)

Folic acid (FA, also called vitamin B9) and cobalamin (vitamin B12) are metabolically linked. Both vitamins participate in an enzyme reaction for the synthesis of the essential amino acid methionine from homocysteine (Hcy). In this reaction the active form of folate, tetrahydrofolic acid, acts as cosubstrate providing a methyl group, and vitamin B12 acts as a cofactor of the enzyme (methionine synthase). Deficiency of either vitamin impairs DNA synthesis (because of lack of thymidine), leading to megaloblastic anemia and impaired methylation which affects the nervous system and other organs (Green, 2011). Beyond the hematological and neurological effects, maternal supply of folate and vitamin B12 maintain the synthesis of nucleic acid and proteins during fetal development. Inadequate folate metabolism is a cause of neural tube defects, and has been related to the development of oral clefts, congenital heart disease, urinary and limb defects. Vitamin B12 deficiency has been independently associated with neural tube defects, preterm delivery, IUGR and recurrent pregnancy loss (Hure et al., 2011). FA and cobalamin deficiencies increase levels of Hcy, an amino acid linked to the pathogenesis of cardiovascular diseases, which has been associated with pregnancy complications such as IUGR, gestational hypertension and PTD (Furness et al., 2011, Kaymaz et al., 2011).

Impaired folate status, defined as serum levels <10 nmol/L (de Benoist, 2008), can result either from dietary deficiency, from genetic variations that affect folate absorption, transport, processing, retention or degradation, or from secondary nutritional deficiencies linked to folate metabolism, specifically vitamin B12 and choline (Beaudin and Stover, 2009). Dietary intake of meat, liver, fish, eggs and milk is the main source of vitamin B12 (Petrus et al., 2009), which is absorbed in the ileum via the intrinsic factor, a glycoprotein secreted by parietal cells in the stomach (Petrus et al., 2009). The loss of parietal cells, gastritis including that produced by *Helicobacter pylori* infection or decreased dietary intake can lead to vitamin B12 deficiency (Kaferle and Strzoda, 2009), defined as serum cobalamin levels <148 pmol/L, and can be accompanied by hematologic or neurological symptoms. In subclinical deficiency, symptoms may be absent; a marginal status of vitamin B12 is considered as values between 148-221 pmol/L (Carmel et al., 2003, Allen, 2009).

FA supplementation in the periconceptional period has been found to significantly reduce Hcy levels, to prevent neural tube defects, to possibly have additional benefits in preventing preeclampsia and decreasing the risk of PTD, LBW, SGA and PROM (Timmermans et al., 2009, Gupta et al., 2007, Bukowski et al., 2009, Siega-Riz et al., 2004).

6.3 Vitamin D

According to the natural selection hypothesis, human skin pigmentation evolved to optimize vitamin D synthesis (Jablonski and Chaplin, 2010, Yuen and Jablonski, 2010). Skin exposed to sunlight is the major source of vitamin D, which is better addressed by modern authors as a pro-hormone (WHO, 2004).

In a process that occurs in the epidermis, the immediate precursor of cholesterol, 7-dehydrocholesterol is able to absorb ultraviolet B radiation during exposure to sunlight, generating previtamin D3. Within the plasma membrane, through a heat-dependent process, previtamin D3 is rearranged to form vitamin

D3, which, still inactive, diffuses through dermal capillaries to the liver, where it is bound by an α_2-globulin. There it undergoes further metabolism to produce 25-hydroxyvitamin D (25OHD), which is the main circulating form of vitamin D and used for measuring vitamin D status. Bound to vitamin D binding protein, 25OHD is transported to the kidney where it undergoes hydroxylation to produce a variety of metabolites including 1,25-dihydorixyvitamin D [$1,25(OH)_2D$, or calcitriol], the most biologically active. Calcitriol plays a major role in calcium and phosphorus metabolism promoting mineralization of bones and manteining neuromuscular function. Humans also obtain vitamin D to a much smaller extent from dietary sources, where Vitamin D2 and D3 are incorporated into chylomicrons and are transported by the lymphatic system into the venous circulation to be stored in fat cells (Holick, 2008, Salle et al., 2000). Most tissues in the body have receptors for calcitriol, named vitamin D receptors (VDRs); furthermore, many of these tissues also contain the enzyme 25-hydroxyvitamin D-1αhydroxylase (CYP27B1), which is able to convert 25OHD into calcitriol under a process that is regulated differently from the kidney production of calcitriol (Bikle, 2009).

Melanin is thought to limit penetration of ultra-violet radiation resulting in decreased production of vitamin D in darker skin types. African-American women have lower vitamin D levels than white women, but have higher bone density and fewer fractures. A study in the UK with Caucasian females looked at the differences of 25OHD levels according to skin type: light skin type 1 and 2 had significantly lower levels (mean 71 nmol/L) compared to darker skin types 3 and 4 (mean 82 nmol/L), demonstrating higher risk of vitamin D depletion in less dark skin types (Glass et al., 2009).

Calcitriol not only regulates calcium homeostasis, but also participates in the modulation of immune responses, because many cells within the immune system express the nuclear VDR including dendritic cells (DC), activated macrophages,

monocytes, T and B cells and natural killer (NK) cells (Bellia et al., 2011); calcitriol also enhances the antimicrobial responses of chemotaxis, phagocytosis, cathelicidin production, defensin β-4, and reactive oxygen species (Velagapudi et al., 2011). Furthermore, calcitriol has shown in experimental studies its capacity to enhance macrophage bacterial killing, to suppress NK cell function, to inhibit DC maturation, to inhibit T-cell proliferation, to modulate T-cell phenotype and to promote cellular differentiation (Evans et al., 2006).

The role of D3 as an immunomodulatory agent emerged from studies showing that its deficiency favored *Mycobacterium tuberculosis* infection and was associated with more grave disease, due to insufficient levels of the antimicrobial protein cathelicidin (Selvaraj, 2011). This protein increases killing of mycobacteria by macrophages in presence of vitamin D (Liu et al., 2006). Hansdottir et al (2008) found that respiratory epithelial cells activate vitamin D, creating a high level microenvironment with high levels of active vitamin and increased cathelicidin mRNA, in a viral infection model (Hansdottir et al., 2008).

Vitamin D has been recognized to play a role during pregnancy. Serum levels in the mother are increased due to renal synthesis and to placental contribution (Evans et al., 2006), but calcitriol concentrations in the fetus are low. It is believed that the fetus might get necessary vitamin D through placental production plus contribution of the fetal kidney (Lucas et al., 2008). Vitamin D has an important role in the regulation of the decidualization and implantation process; it also regulates placental lactogen expression, progesterone and estradiol secretion as evidenced in cultured human syncytiotrophoblasts (Barrera et al., 2008). Increased levels of calcitriol ensure the availability of extra calcium destined to fetal skeletal growth (Lucas et al., 2008). The literature indicates that newborns from mothers with deficient vitamin D intake or low 25(OH)D levels may have shorter gestation, lower birthweight, and less intrauterine bone growth (Lucas et al., 2008).

The last systematic review of the American Journal of Obstetricians and Gynecologists concludes that there is no clear definition of vitamin D deficiency in pregnancy (Nassar et al., 2011). Current papers on vitamin D and pregnancy base their finding on levels of >75 nmol/L as sufficient, between 75-50 nmol/L as insufficient, <50 nmol/L as deficiency and <25 nmol/L as severe deficiency of vitamin D (Dror and Allen, 2010, Lucas et al., 2008, Holick, 2008). The Institute of Medicine (IOM) has recently set dietary reference intakes (RDI) for adequacy of vitamin D, using bone health as the main outcome to define current recommendations. It states that the level of 50 nmol/L of 25OHD appears to cover the needs of 95.7% of the population; levels of 40 nmol/L are consistent with the median requirement and the lower end of the requirement range is 30 nmol/L, where deficiency symptoms may appear. It also sets the recommended dietary allowance for pregnant women the same as that for non-pregnant and adolescent women (600 international units -IU of vitamin D per day), because of insufficient evidence on association of serum 25OHD level with maternal bone mineral density during pregnancy and the available evidence that maternal 25OHD level has no effect on fetal calcium homeostasis or skeletal outcomes (Ross et al., 2011).

6.4 Vitamin A

Vitamin A or retinol is a fat-soluble vitamin, necessary for growth and epithelial integrity, red blood cell production, immunity, reproduction and the normal functioning of the eye (WHO, 2009) that can be ingested in form of retinal or preformed vitamin A, contained in animal source foods (liver, fish oils, sardines, tuna and diary products), or from carotenoids (pro-vitamin A) contained in vegetable foods such as carrots, yellow squash, dark leafy vegetables, corn, tomatoes, oranges, papayas and mangoes (van den Broek et al., 2002). Vitamin A absorption takes place in the small intestine facilitated by a process of micellar solubilization that allows the passage of the vitamin through the intestinal mucosal cells in form of retinyl esters, which are transported in chylomicrons and

stored in the liver (Jaensson-Gyllenback et al., 2011). Besides being the largest storage deposit of vitamin A in the body, the liver is also where vitamin A hydrolyzes into retinol for release into circulation, in complex with the retinol-binding protein (Blomhoff and Blomhoff, 2006). Of the many functions of vitamin A, it is important to highlight the critical role of retinol in the generation of IgA, mediated by the synthesis of retinoic acid by dendritic cells (Hall et al., 2011) and the association that has been found between vitamin A stores and higher circulating concentrations of natural killer cells, higher production of ROS by monocytes and lower serum IL-6 and IL-17 concentrations (Ahmad et al., 2009). All this indicates an important role of vitamin A in immune-modulation.

Vitamin A is also required for reproduction starting with the implantation process, following by placental formation and maintenance and fetal development (Clagett-Dame and DeLuca, 2002). It has been demonstrated through experimental studies that vitamin A deficiency (VAD) produces a number of congenital malformations affecting a variety of systems, including ocular, cardiac, respiratory and urogenital, which are collectively referred as fetal VAD syndrome (Mark et al., 2009), but also, toxicity due to vitamin A intake can occur at doses ≥30.000 IU/day, increasing the teratogenic risk, which can lead to abnormalities such as ear, limb and craniofacial malformations (Gutierrez-Mazariegos et al., 2011). Therefore, plasma concentrations of vitamin A are strictly regulated despite fluctuations in the daily dietary intake (Blomhoff and Blomhoff, 2006) and the placenta is able to control vitamin A exchange between the mother and the fetus, protecting the latter when maternal intake is deficient (Wang et al., 2009).

Requirements of vitamin A during pregnancy are higher, especially during the second and third trimester, since it is needed for fetal lung development and maturation (Strobel et al., 2007). The WHO suggest the intake of 800 µg RE/day (but no more than 3,000 µg RE/day) for pregnant women and recommends a

cut-off of 0.7 μmol/L (20 μg/dL) for deficiency in the general population (WHO, 2004).

7 SUMMARY

Physiological adaptations during pregnancy include an increase in plasma volume that leads to hemodilutional anemia, which is necessary for the adequate perfusion of the placenta and oxygenation of the fetus, and a shift from Th1 to Th2 immune responses during the second and most part of the third trimester, returning towards a Th1 response close to delivery time. Immune changes are expressed in the increased number of neutrophils and the alteration of markers such as CRP levels that are also physiologically elevated during pregnancy. Factors that generate a shift towards a Th1 response may lead to adverse pregnancy outcomes. This is the case for infections that, due to the above-mentioned physiological changes, are more frequent or severe during pregnancy. The effects of mild-moderate extra-uterine infections found on outpatient clinics in developing countries on maternal and fetal health have not been well established. Micronutrient deficiencies including iron, folate, vitamin B12 and D deficiencies have been studied independently as important cause of adverse pregnancy outcomes, but their combined impact on maternal and fetal health has not been reviewed before. The coalition of adverse conditions such as infections and micronutrient deficiencies affecting both, the mother and the fetus, is more possible to occure in poor settings where fetal monitoring, eventhough it has become a very important part of pre-natal sourveillance and is based on advanced technologies, might not be available. The challenge of fetal evaluation in rural settings could be surmounted through the use of simple measurements such as FH, FCR and FM, which serve as assessment tools in order to detect fetal adverse outcomes, but very little recent information is available abut their usefulnes and accuracy.

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Chapter III - PREGNANT WOMEN IN A RURAL INDIGENOUS COMMUNITY OF PANAMA EXPERIENCE UROGENITAL, INTESTINAL, SKIN, ORAL AND RESPIRATORY INFECTIONS ASSOCIATED WITH SEVERAL MICRONUTRIENT DEFICIENCIES

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ABSTRACT

This cross-sectional study documents the diversity of respiratory, oral, skin, gastrointestinal and urogenital infections in 213 pregnant Ngäbe-Buglé indigenous women living in extreme poverty in rural Panama, together with a range of micronutrient deficiencies (iron, folic acid, vitamins B12, A and D), and identifies relationships among them. Co-infections of two or more systems occurred in 87% of the pregnant women, and only 2% of women had no infections. One or more urogenital infections (urinary tract infection, bacterial vaginosis (BV), vaginal candidiasis, vaginal trichomoniasis or *Diplococcus*) were found in 95% of women; 23% had oral infections (caries or gingivitis) and 20% had skin infections (scabies, bacterial or fungal). Intestinal parasites (*Ascaris*, hookworm, *Trichuris* or *Giardia*) were detected in 62% of the 120 stool samples collected. Ferritin levels were below 30 ng/mL in 82.6% of the mothers, 65% had vitamin D levels below 50 nmol/L, vitamin B12 deficiency was present in 85% of the mothers, folate deficiency in 23% and vitamin A deficiency in 5.7%. Anemia (hemoglobin <11 gr/dL), diagnosed in 38%, co-occurred with multiple deficiencies and with elevated CRP. Lower iron status was associated with more severe dental caries but with less prevalence of urinary tract infection, less severity of gonococcal infection and vaginal candidiasis and lower *Ascaris* epg. Folic acid was negatively associated with severity of BV and hookworm infection; higher vitamin B12 was associated with more severe vaginal trichomoniasis but with less severe vaginal candidiasis. When both deficiencies were combined, they positively related with the severity of gonococcal infection. Severe vitamin D deficiency was associated with more severe BV, gonococcal infection and *Ascaris* epg, whereas high levels of vitamin D were linked with caries, more severe vaginal trichomoniasis and candidiasis and with higher *Trichuris* epg. Vitamin A was positively related with the presence of scabies and with the severity of BV. On the other hand, women with vitamin D levels above 75

nmol/L, vitamin A sufficiency, and iron and vitamin B12 deficiencies had significantly lower CRP. Despite this range of infection and micronutrient deficiencies, inflammation detected through C-Reactive protein (CRP) levels was elevated in only 13.6% of the mothers where it was significantly higher in women with one or more infections when compared with women without infections, particularly respiratory and oral infections and in women with the combination of one or the other of these two infections with skin, genital or hookworm infections. Our findings highlight the extent of infection burden and micronutrient deficiency and the complexity of interactions.

INTRODUCTION

In impoverished rural areas in developing countries, wellbeing during pregnancy is determined by social and environmental conditions that include limited access to health care, clean water and sanitation that increase frequency, duration and severity of infections and poor nutritional status. Women in such populations are also at increased risk of pregnancy complications due to agricultural labor and indoor exposure to wood smoke from cooking. Practices driven by traditional and cultural beliefs, such as pica, may also be detrimental to their health (Basnyat, 2011). This is likely the case in the Ngäbe-Buglé indigenous community in rural Panama, where more than 90% of the population lives in extreme poverty (Gabinete Social de la República de Panamá and Sistema de Naciones Unidas, 2005), and where intestinal parasites and micronutrient deficiencies are widespread in mothers and their preschool children (Lardeau, 2009, Payne *et al.*, 2007, Halpenny *et al.*, 2012, Sinisterra, 2006).

During pregnancy, most studied infections are those involving congenitally transmitted pathogens such as HIV and toxoplasmosis, but women with low access to health services may present a variety of infections, including bacterial vaginosis (BV), vaginal candidiasis, trichomoniasis (Ilkit and Guzel, 2011, Anderson *et al.*, 2004), asymptomatic bacteriuria/urinary tract infections

(AB/UTI), most often caused by *Escherichia coli* (Schnarr and Smaill, 2008), intestinal nematode infections (*Ascaris*, *Trichuris* and hookworm) and oral infections (Matevosyan, 2011). Little information is available regarding skin infections such as scabies, dermatomycosis and bacterial skin infections during pregnancy, even though they are very common under conditions of poor hygiene, nutritional status and overcrowding (Afsar, 2010). Unfortunately, limited access to routine screening and technologies for targeted diagnoses in impoverished rural settings as well as restrictions on use of many drugs during pregnancy hinder the ability of health professionals to diagnose and treat many of these infections.

Most research to date focuses on a limited set of infections (Romoren *et al.*, 2007, Adegnika *et al.*, 2010) or individual micronutrient deficiences (Coad and Conlon, 2011, Dror, 2011), despite the high probability that individual women in vulnerable populations are concurrently infected with more than one pathogen and experience more than one micronutrient deficiency. The few studies that are more comprehensive typically consider either to multiple micronutrient deficiencies (Asemi *et al.*, 2010, Mariela *et al.*, 2010) or multiple infections (Goldberg *et al.*, 2012, Abruzzi and Fried, 2011, Ortashi *et al.*, 2004) but rarely both (van den Broek and Letsky, 2000, Dunlop *et al.*, 2011, Quintero *et al.*, 2011). Moreover, there is evidence that adverse pregnancy outcomes such as intra-uterine growth retardation (IUGR) and pre-term delivery (PTD) are associated not only with many of these maternal infections but also with maternal micronutrient deficiencies that occur in these population. Of particular note is the increasing evidence that both infections and micronutrient deficiencies exert their negative effects through inflammation (Challis *et al.*, 2009, Mestan *et al.*, 2010, Romero *et al.*, 2007). Given the cross-sectional nature of the study, we did not measured adverse pregnancy outcomes, but we took CRP levels as a measurement of the impact of infections and micronutrient deficiencies on maternal health. Thus, the objectives of this cross-sectional survey were to

record the diversity and co-occurrence of respiratory, oral, skin, urogenital and intestinal infections and indicators of inflammation in pregnant women belonging to the Ngäbe-Buglé indigenous community in rural Panama, and to explore maternal, environmental and nutritional factors that were associated with increased risk of the more common infections and of inflammation.

MATERIALS AND METHODS

Study Population

The Ngäbe-Buglé indigenous population, located in a wide mountainous territory in Western Panama, comprises 110,080 inhabitants living in extreme poverty in the provinces of Chiriquí, Veraguas and Bocas del Toro. Households rely on small-scale agriculture (corn, tubers and pifá), or seasonal labor (coffee, sugar cane, livestock). Untreated river or stream water is delivered to homes by gravity through plastic tubes. They use latrines, they lack electricity, they cook on wood stoves and they use organic waste as fertilizer (Halpenny *et al.*, 2012).

Our study was conducted within the "*Comarca*", an indigenous administrative area of Chiriquí. We targeted all pregnant women within a 2 hr walk of 14 health centers accessible by car from the Hospital in San Felix in communities of Chamí, Alto Caballero, Oma, Soloy, Quebrada Hacha, Hato July, Kuerima, Hato Pilón, Lajero, Quebrada Guabo, Quebrada Loro, Corotú, Emplanada de Chorcha and Chichica. Pregnant women, identified by staff at the health centers, community health workers and traditional midwives, were invited to attend information sessions at the health centers where the study was explained and where fully informed consent was obtained. The inclusion criterion was pregnancy as diagnosed by the local health personnel including positive pregnancy test in women with amenorrhea > 5 wks. Exclusion criteria were twin pregnancies or critical illness, of which none were identified. Of the 217 women approached between August and October 2010, 214 agreed to participate. One participant

was excluded because of abnormal pregnancy (hydatidiform mole), providing a participant population of 213 pregnant women.

Ethical Considerations

Ethical approval was obtained from McGill University in Canada and from Panamanian authorities (Gorgas Memorial Institute Ethics Board, the Panamanian Ministry of Health -MINSA, provincial and local health authorities, and indigenous authorities). Mothers interested in participating in the study were approached individually, and research purposes, procedures and confidentiality were explained. They were made aware of their right to refuse to participate and to withdraw at any point during the study. They signed a consent form or used a witness when they were not able to read and write. Mothers received no financial compensation, but the results from laboratory tests processed on site were delivered within a week to the clinician responsible for prescribing treatment when needed.

Study Procedures and Methods

Participants received a complete medical consultation conducted by a physician. They responded to a questionnaire on obstetric history and the following factors known to affect maternal/fetal health: number of times in the past 7 days they consumed animal source foods, yellow fruits or vegetables, and green vegetables, micronutrient supplementation, coffee consumption, wood smoke exposure, hours of field work per day, symptoms of infection and the practice of pica (yes/no) defined as ingestion of non-food materials including earth, soap or ice. The physical examination included anthropometry, maternal body mass index [BMI = Weight (kg)/height $(m)^2$], vital signs, and obstetric and genital examination, as well as clinical diagnosis of oral (caries, gingivitis), respiratory, skin (impetigo, scabies, dermatomycosis) and urinary and genital infections. Women provided a 10 ml blood sample, a urine sample, a stool sample and a

vaginal smear. Community health workers randomly asked 28 pregnant women to bring a sample of the coffee they consumed at home for caffeine analysis.

Clinical information, vaginal smears and blood samples were obtained from all 213 participants, and 208 provided urine samples. Only 120 women were able to provide a stool sample at the time of the clinical exam; all samples were examined using a direct smear, 105 were processed using Kato-Katz and 72 were examined using FLOTAC.

The vaginal smear was taken by the physician at the end of the medical consultation. While lying in gynecological position, a disposable speculum was placed into the vagina. Direct visual exam allowed the clinical diagnosis of vaginitis and/or cervicitis. A sterile swab was used to take a sample of cervical and vaginal discharge. A slide was prepared for Gram staining, and the swab was then placed in a tube with 1 ml of sterile saline solution, immediately refrigerated for transportation to the laboratory for direct microscopic examination (wet mount). BV was diagnosed using Gram-stain-based scoring system (Nugent, 1991) using the abundance of three bacterial cell morphotypes: *Lactobacillus* (large Gram-positive rods), *Gardnerella* and *Bacteroides* (small Gram-variable or Gram-negative rods) and *Mobiluncus* spp. (curved Gram-variable rods). BV was diagnosed when the Nugent score was 7 or higher. A score of 4-6 represented intermediate vaginal microflora and score of 0-3 corresponded to normal vaginal microflora (Verstraelen, 2009). Severity of *Diplococcus* (gonococcus) (Gram stain), hyphae (vaginal candidiasis) (wet mount or Gram stain), and trichomoniasis (wet mount) was recorded as few (1+), moderate (2+) or abundant (3+). We also had access to results of VDRL test (latex agglutination test indicator of syphilis infection) for 172 of our participants, conducted during routine pregnancy follow-up.

Urine samples were analyzed using dipstick URISCAN® strips including reagents for semi-quantitative measurement of urinary pH and specific gravity, leukocyte

esterase, nitrites, blood, proteins and glucose, on a Miditron-M semi-automated reflectance photometer, and microscopic analysis where a fresh sample of 15 ml of urine was centrifuged at 3000 rpm for five min; the supernatant was decanted and the sediment re-suspended in the remaining liquid. A drop of the sediment was placed onto a clean glass slide, protected with a cover slip and examined using low- and high-power field (hpf) magnification. AB/UTI was diagnosed when one or more of the following conditions was present: leukocyte esterase ≥2+ on a scale of negative, trace, small (+), moderate (2+) or large (3+); detection of nitrites; hemoglobin from dipstick analysis ≥1+ on a scale of negative, 1+ to 4 +; leukocytes ≥ 5/hpf; bacteria ≥5/hpf; red blood cells ≥2/hpf (Simerville, 2005). Severity of AB/UTI was assessed based on urinary leukocyte esterase, given its negative predictive value of 99% at bacterial colony counts of 10^3 (Semeniuk and Church, 1999). AB/UTI was ruled out in samples with high amounts of mucus and epithelial cells. Urine culture was not possible at the study location.

Stool samples were examined using a direct smear for protozoan infections by a skilled laboratory technician trained in distinguishing *Entamoeba coli* from *E. histolytica.* When a sample of sufficient volume and consistency was available, nematode intensity (eggs per gram, epg) was recorded using Kato-Katz (Peters *et al.*, 1980) and FLOTAC (Cringoli *et al.*, 2010) techniques. A positive result from any of the three methods was used to calculate the prevalence of nematode infections, and FLOTAC results were used to determine the epg, given the higher sensitivity when compared with Kato-Katz technique found in various studies including ours (Knopp *et al.*, 2011, Halpenny C.M., 2012).

Blood samples were analyzed for complete blood cell count using a BC-5500 Mindray Auto Hematology Analyzer, and HIV processed through ELISA antibody test at the San Felix hospital laboratory. We also had access to 178 sera random glucose (processed with spectrophotometry) conducted locally as part of routine pregnancy follow up. Gorgas Memorial Institute in Panama City processed the

213 serum samples for iron status using Ferritin Enzyme Immunoassay, based on the principle of a solid phase enzyme-linked immunosorbent assay with a minimum detectable concentration of the Ferritin ELISA assay estimated to be at least 5.0 ng/mL (MP Biomedicals, Orangeburg, NY); Ramco's serum Transferrin Receptor (sTfR) enzyme immunoassay based upon the double antibody sandwich method, with a lower limit value of 1 ug/mL; and high sensitivity enzyme immunoassay for quantitative determination of C-Reactive Protein (CRP) concentration, based on the principle of a solid phase enzyme-linked immunosorbent assay, which minimum detectable concentration of the CRP ELISA assay is estimated to be 0.1 mg/L, and the upper limit is 10 mg/L (MP Biomedicals, Orangeburg, NY). Vitamin A levels in plasma were determined using high-performance liquid chromatographic technique (HPLC) (Gundersen *et al.*, 1997) at the Institute of Scientific Research and High Technology Services – INDICASAT in Panama City. The remaining sera were stored at -20° for later processing in Montreal, Canada where the LIAISON 25 OH Vitamin D assay, a direct competitive chemiluminescence immunoassay (CLIA), was used for quantitative determination of total 25 OH vitamin D in serum, and immuno electro-chemiluminescence (with a Roche Modular E170) was used to determine folic acid and vitamin B_{12} concentrations. Caffeine was measured using high resolution reverse phase liquid chromatography at the University of Panama (Hewlett Packard, Model 1050) as described previously (Warner C, 1983, Lardeau, 2012).

Cut-offs for iron deficiency were defined as ferritin < 30 ng/mL according to recommendations of WHO for populations with high rates of inflammation (WHO, 2007, de Benoist, 2008). Folic acid deficiency was < 10 nmol/L and vitamin B12 was defined as < 150 pmol/L (de Benoist, 2008). Vitamin A deficiency was defined as < 20 µg/dL (WHO, 2004), and vitamin D as <50 nmol/L

(Ross *et al.*, 2011), but since precise cut-offs for vitamin D during pregnancy have not been well established, we also took in account levels below 75 nmol/L (inadequacy) and below 25 nmol/L (severe deficiency) (Dror and Allen, 2010, Lucas *et al.*, 2008). CRP cut-off of > 3.0 mg/L for the first trimester was based on recommendations for non-pregnant women, given the absence of other recommendations; during the second trimester the cut-off of > 20.3 mg/L and third trimester > 8.1 mg/L were used . (Abbassi-Ghanavati *et al.*, 2009)Anemia was defined as hemoglobin <11 gr/dL, microcytosis as mean corpuscular volume (MCV) < 80 fL and macrocytosis as MCV >100 fL (WHO, 2007).

Data Analysis

All data were analyzed using STATA 10. Summary statistics were calculated for all prevalences and scores of severity. Chi-square tests were used to determine whether infections occurred together more frequently than expected by chance, multiple logistic regression or ordered logistic regression analyses were used to explore factors associated with common infections, which for analysis were divided into absence of presence (0= no present, and 1= present) or severity of infection (0=no present, 1= mild, 2= moderate, 3= severe).

Depending on the nature of the dependent variable, we selected either simple or stepwise multiple regression (continuous variables), logistic regression (binomial variables) or ordered logistic regression (ordinal variables). For each dependent variable, we then ran a series of exploratory analyses examining groups of independent variables separately (maternal and environmental factors, red, white and platelets cell lines counts, nutrients). We then obtained a final composite model where significant variables from all of the exploratory models were entered into the analysis. Results are presented only for models where explanatory model explained more than 10% of the variability of the dependent variable and that identified significantly related variables. Final models include

only those variables with a $p < 0.15$. Gestational age was included in all analyses that included maternal BMI, to control for increasing BMI with gestational age.

We followed a similar sequence for the analysis of inflammation. Two-tailed T-test was used to determine which infections and micronutrient deficiencies were associated with CRP levels, and logistic regression was used to determine factors related with elevated CRP.

Non-normal independent variables were normalized using logarithmic conversion (serum ferritin, transferrin, vitamin B12, folic acid, vitamin A and CRP). All results are presented as mean ± SEM or 95% confidence intervals unless otherwise indicated. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

Characteristics of Study Participants including Nutritional Status

Mothers ranged from 13 and 44 yrs; 29.1% were adolescents (≤ 19 yr) and 13.1% were ≥35 yr. Parity ranged from 1 (28.1%) to five or more (32.4%). Of participants, 11.3% were in the first trimester, 37.6% in the second trimester, and 51.2% in the third trimester.

Women used wood for cooking and were exposed to wood smoke 2.3 ± 0.1 hr/d. Almost half the women (45.5%) reported working in the field during their pregnancy (21% for 2-4 hr/d, 21% for 5-8 hr/d, 3% for > 8 hr/d), mainly harvesting vegetables, collecting coffee or dry wood and carrying water.

Women reported consuming 3.2 ± 0.2 portions of animal protein, 2.0 ± 0.2 portions of yellow fruits or vegetables and 1.7 ± 0.16 portions of green leaves or vegetables per week, usually accompanying rice, which was eaten alone when other food was not available. They traditionally drank coffee 1.6 ± 0.07 cups/d, containing an average of 6.4 ± 1.2 mg of caffeine /100 mL. At the time of the study, 62% of the women were receiving iron or multi -micronutrient supplementation. Several women (6.6%) reported pica.

Micronutrient deficiencies were common among the pregnant women: 64.8% of the mothers were Vitamin D deficient, 82.6% were iron deficient, 85% had Vitamin B12 deficiency, 24% had folic acid deficiency and 5.6% had vitamin A deficiency. Furthermore, 90% of the women were deficient in at least two of these micronutrients (Fig.1a) and 2% were deficient in all five micronutrients; only 3% of women had none of these micronutrient deficiencies. Among the 38% of mothers with anemia, 92.6% had ferritin levels below 30 ng/mL, 89% were vitamin B12 deficient, and 32.5% were FA deficient. Microcytosis was only found in anemic mothers (11%); macrocytosis was found in 13% of the non-anemic population and in 3% of anemic mothers.

Two thirds of women (67.1%) had a normal weight per height according to their gestational age, 9.8 % were underweight and 23% were overweight. None of the women was positive for gestational diabetes, defined as >140 mg glucose/dL from random blood sample and/or positive glucosuria.

Infections and Combination of Infections

Through a combination of clinical criteria and simple laboratory tests, pregnant women were diagnosed with 12 infectious diseases (Table 1). Our population inhabits a non-endemic area for malaria and no HIV positive women were identified.

Clinical exams revealed that vaginal infections (primarily vaginitis) were extremely prevalent, affecting over 90% of pregnant women. The vast majority of vaginal smears were positive for pathogens (95.7%) including BV, *Trichomonas vaginalis, Candida,* and *Gonococcus*. Only 5 women (6.1%) had clinical symptoms that were not confirmed by a Nugent score ≥ 7, but in all these women, the Nugent score was between 4 and 6, indicating intermediate vaginal microflora. VDRL tests for syphilis were reactive in 5 (2.9%) of the 172 women tested (2 at 1:1 dilution, 1 each at 1:4, 1:8 and 1:32 dilutions). Four of the VDRL reactive women had clinical vaginitis and the fifth had dermal lesions, all mentioned partner promiscuity. As no confirmatory tests were available, these 5 women were treated for syphilis.

The prevalence of AB/UTI (56.2%) detected through urine analysis was similar across the trimesters, but no symptoms or signs of complicated UTI were found in any of the participants.

None of the women spoke of gastrointestinal symptoms, but lab analyses revealed that 67.5% were infected with at least one intestinal pathogen (*Ascaris, Trichuris*, hookworm, *Giardia*). In addition, non-pathogenic *E. coli* was detected

in 10.8% of women. Neither prevalence nor intensity of the three intestinal nematodes differed among trimesters.

Skin and oral infections were observed in about 20% of women but respiratory infections were low (6.1%). Comparison across trimesters revealed that only three infections differed among trimesters. Gonococcal infection was significantly more prevalent during the third trimester (P=0.03), gingivitis was only found in women in their third trimester and *Trichuris* was not found in the first trimester.

Over half the women were infected with infections of three of more systems, one woman was currently infected with urinary, genital, oral, skin, respiratory and intestinal infections, and only 2% of women had no infectious involvement of any of these systems (Fig. 1.b). Moreover, several pathogens occurred together more frequently than expected ($P < 0.05$): *Trichomonas* and positive VDRL (observed 1.7%; expected 0.5%); *Trichomonas* and *Gonococcus* (observed 3.2%; expected 1.5%)*; Trichomonas* and AB/UTI (observed 12.5%; expected 9.8%); *Gonococcus* and scabies (observed 3.3%; expected 1.5%); *Gonococcus* and gingivitis (observed 1.4%; expected 0.4%); hookworm and *Ascaris* (observed 23.3%; expected 18.4%); hookworm and *Trichuris* (observed 11.7%; expected 7.1%).

Vaginal Infection Is a Risk Factor for Respiratory infection, Caries, Scabies and AB/UTI but Micronutrient Deficiencies are Protective

Despite the low prevalence of respiratory infection in these pregnant women, three factors emerged as risk factors for respiratory infection. Women who did not take iron or micronutrient supplements were at increased risk of respiratory infection (OR = 7.3). In addition, respiratory infections were more likely in those who consumed more coffee (OR = 1.8) and who had higher levels of CRP (OR = 2.3) (Table 2.a) although elevated CRP is likely a consequence of respiratory infection.

When controlling from maternal weight category, the model for scabies showed increased risk of scabies in women with severe impetigo (OR= 11.1) and severe clinical vaginitis (OR = 2.1), as well as in those with higher % lymphocytes (OR =1.1). Higher serum retinol concentrations also increased risk of scabies (OR = 4.9) whereas mothers who more frequently consumed animal source foods were at lower risk (OR = 0.8) (Table 2b).

As with scabies, the model for dental caries included both infections and micronutrients. Dental caries occurred in women with more severe vaginal infections (BV (OR = 1.4); vaginal candidiasis (OR = 1.6)) and in those with elevated CRP (OR = 1.5). Furthermore, poor iron status (higher sTfRs) also increased the risk of caries (OR = 2.5) (Table 2c).

The risk of AB/UTI increased with severity of clinical vaginitis (OR = 1.4) and in mothers with iron sufficiency (OR = 2.8). The combination of vitamin B12 and vitamin D deficiencies reduced the risk of AB/UTI, as did the practice of pica (OR = 0.1) (Table 2d).

Multiple Ordered Logistic Regression Analysis of Severity of Cervico-Vaginal Infections

Factors related to severity of cervico-vaginal infections are shown in Table 3. In ordered logistic regression models we found more complex interactions among different vaginal pathogens. BV showed antagonism with trichomoniasis and gonococcal infection, whereas severity of vaginal candidiasis positively related with trichomoniasis, which in turn was positively related with gonococcal infection.

With respect to severity of BV based on clue cell counts, two equally strong but distinct models emerged, both which showed an inverse relationship with severity of vaginal trichomoniasis (Model 1 OR = 0.44; Model 2 OR = 0.49) and that mothers who did not take supplements had more severe BV (Model 1 OR =

2.22; Model 2 OR = 2.36) (Table 3a). The models differed with regard to the role of micronutrient status. In the first model (Table 3a, Model 1), severity of BV was higher in mothers with combined anemia and folic acid deficiency (OR = 2.5) and in those with severe Vitamin D deficiency (OR = 2.9) whereas Model 2 shows that Vitamin A entered positively (OR = 2.23) when mean corpuscular hemoglobin concentration (MCHC) (OR = 0.61) (or one of the other haemoglobin-related variables) was included.

Trichomoniasis severity was elevated in mothers with high levels of vitamin D (OR = 7.3), vitamin B12 (OR = 3.1) and more severe vaginal candidiasis (OR = 1.9) but with less severe BV (OR = 0.7) (Table 3b; Model 1). When variables from the urinalysis were included, severity of trichomoniasis was elevated in those with urinary leukocyte esterase (an indicator of AB/UTI) (OR = 2.27) and those with higher MCHC (OR = 1.5) (Table 3b; Model 2).

The model for severity of vaginal candidiasis did not reveal any associations with other infections. Candidiasis was more severe in mothers with low Vitamin B12 (OR = 0.4) and who had the combination of anemia and folic acid deficiency (OR = 2.4). Interestingly, it was more severe in those with good iron stores (ferritin > 15 ng/mL) (OR = 2.73) even we added CRP to the model (OR = 2.0; model not shown). When measures of anemia were excluded, a separate model revealed that Vitamin D levels > 75 nmol/L increased the risk of vaginal candidiasis (OR = 8.6), as did higher urinary pH (OR = 1.5). Mothers in their first pregnancy were at lower risk (OR = 0.4) (Table 3c).

Gonococcal infection was related not only with vaginal infections (positively related with trichomoniasis (OR = 6.2) and negatively related with BV (OR = 0.5)), but also with the presence of gingivitis (OR = 30.8) and severity of scabies (OR = 3.1). Micronutrients also entered the model. Both the combination of folic acid and Vitamin B12 deficiencies (OR = 5.9) increased gonoccocal severity (Table 3d).

Stepwise Multiple Regression of Nematode Intensity

As both hookworm and Trichuris are causes of anemia and iron deficiency, we excluded variables related to these deficiencies in our models of nematode epg (Table 4). Trichuris epg was higher in mothers with higher hookworm epg, Vitamin D levels above 75 nmol/L, those who were less than 35 years old (Table 4a). Hookworm epg was higher in mothers with low concentrations of folic acid, with vitamin D > 75 nmol/L, and with a higher percentage of basophils (Table 4b). *Ascaris* epg was positively influenced by the presence of four different infections: gingivitis, dermatomycosis, vaginal candidiasis and AB/UTI. The only micronutrient that entered the model was vitamin D levels <50 nmol/L. Mothers with low vitamin D levels had higher *Ascaris* epg (Table 4c).

Inflammation and Associated Factors

The mean CRP levels through the three trimesters were 3.5 ± 0.6, 6.3 ± 0.7, and 4.7 ± 0.4 mg/L, respectively. Abnormally high CRP values were detected in 46% of the mothers in the first trimester (>3.0 mg/L), 6% of the mothers in the second trimester (>20.3 mg/L), and 17% in the third trimester (> 8.1 mg/L).

CRP was significantly elevated only in few of the infections we studied. Oral infection alone or combined with genital infection had mean CRP values between 6.5 - 7 mg/L, whereas respiratory infection alone or in combination with oral, skin or genital infections had CRP values between 11 and 17 mg/L. Women infected simultaneously with respiratory and hookworm had the highest values (mean 20.3 mg/L). As the number of infections increased in women, the CRP increased (Fig 2). In contrast, *Ascaris*-infected women had significantly lower CRP levels than those without *Ascaris* (Table 5).

We compared CRP concentrations between mothers with and without deficiency of each micronutrient (Table 5). CRP values were significantly higher in those women with vitamin A deficiency, in women with vitamin D lower than 75

nmol/L and in the subset who were also deficient in both, vitamin B12 and vitamin D.

Through multiple logistic regression we were able to elucidate which factors might influence on CRP concentrations, our measure of the inflammatory response. CRP was elevated in mothers with increased exposure to wood smoke (OR =1.35) and in those with low serum vitamin D (OR = 3.27) and higher MHC (OR = 1.47). In contrast, folic acid concentrations and combined iron and vitamin B12 deficiency were related with lower CRP concentrations (OR = 0.28 and 0.18 respectively) (Table 6).

DISCUSSION

Our comprehensive evaluation of a diverse range of infections in 213 indigenous women living under conditions of extreme poverty in rural Panama has demonstrated that virtually every pregnant woman was infected or had at least one micronutrient deficiency, and that most were infected with more than one pathogen and experience multiple micronutrient deficiencies and models generated for each of the common infections reveal almost invariably that both micronutrients and other infections are predictors. Our data indicate that bacterial colonization of the vagina may be protective against gonoccocal infection and trichomononiasis. Furthermore, we found a higher frequency of co-occurrence of gonoccocal infection with gingivitis and with scabies than expected by chance. Scabies is recognized as a sexually transmitted disease (Orion *et al.*, 2004, Currier *et al.*, 2011), but a study done in Spain did not find any significant relationship of scabies with other sexually transmitted diseases (Otero *et al.*, 2004). The observed co-occurrence of bacterial, fungal and ectoparasitic skin infections has been reported in the general population (Hay *et al.*, 2012, Andrews *et al.*, 2009), but has not been reported during pregnancy, and is likely facilitated by breakdown of the epithelial barrier (Dryden, 2010). Many micronutrient deficiencies were associated with more severe infections.

For example, iron deficiency was associated with dental caries as has been reported in children (Shaoul *et al.*, 2012), but not during pregnancy. Vitamin D deficiency increased the severity of BV but reduced the severity of trichomoniasis, vaginal candidiasis, *Trichuris* and hookworm. Furthermore the combined deficiency of vitamin B12 and vitamin D reduced the likelihood of AB/UTI and vitamin B12 deficiency was associated with reduced severity of trichomoniasis. Micronutrient supplementation exerted a beneficial effect on prevalence of respiratory infections and severity of BV. Together, these observations highlight not only the complex set of interactions among infections of the urogenital, intestinal, skin, oral and respiratory systems but also that micronutrient deficiencies may be risk factors for some infections but protective against others.

Range of Infections – Single / Co-Infections

Vaginal infections were the most common and most diverse among all organ systems examined. The most prevalent infection was BV, mainly produced by *Gardnerella vaginalis.* We observed a negative association between BV and more aggressive vaginal infections such as trichomoniasis and gonococcal infection that cause PTD and PROM (WHO, 2011, Harp and Chowdhury, 2011). BV has been reported to induce local production of IgA (Cauci, 2004), which has been suggested to exert a protective effect against these more serious vaginal pathogens (Witkin *et al.*, 2007) via the mucosal dendritic cells (Soloff and Barratt-Boyes, 2010). In contrast to BV, vaginal candidiasis was positively associated with trichomoniasis and gonococcal infections. Vaginal candidiasis is considered a "benign" vaginal condition (Roberts *et al.*, 2011) which elicits mainly cellular immune responses and no study has demonstrated a significant difference between systemic or local levels of IgA in women with recurrent vaginal candidiasis and controls (Fidel, 2007). Therefore, its presence may not be protective against these more severe vaginal pathogens (trichomoniasis and gonococcal infection) but rather might increase susceptibility.

The various soil-transmitted nematodes have frequently been shown to co-occur in children (Sorensen *et al.*, 2011) and in pregnant women (Yatich *et al.*, 2010), particularly those living in malaria endemic contexts (Adegnika *et al.*, 2010). Our data extend these findings by showing higher than expected co-occurrence of *Ascaris* together with hookworm and of *Trichuris* together with hookworm in pregnant women in this non-malaria endemic population. Co-infection with these soil-transmitted nematodes is typically explained by common exposure routes, common immune response mechanisms, or facilitated establishment in the presence of the other pathogen (Bethony *et al.*, 2006). Surprisingly, we did not detect a positive association between *Ascaris* and *Trichuris* even though both are transmitted by ingestion of soil-borne eggs.

Infection-Nutrition Interactions

A wide variety of micronutrient deficiencies were present in this population, and most anemic mothers had iron deficiency and/or vitamin B12 deficiency and a third were folate deficient. Furthermore vitamin D deficiency was extremely common. The dominant factors emerging in our regression models for infection were a combination of other infections and micronutrient status.

Among the range of red blood cell and iron status markers, MCHC was most frequently associated with infection outcomes including BV and trichomoniasis. Iron deficiency has been reported as a strong predictor of BV in early pregnancy (Verstraelen *et al.*, 2005) and our data indicate that the relationship extends throughout pregnancy in our population. Severity of vaginal trichomoniasis was consistently associated with higher levels of MCHC and ferritin and lower MCHC appeared to be protective against severe AB/UTI. *Trichomonas vaginalis* requires iron for its growth and protein production (Torres-Romero and Arroyo, 2009, Lehker and Alderete, 1992, Lama *et al.*, 2009), and for enzyme activity involved in energy metabolism, growth and expression of immunogenic proteins, resistance to complement lysis, virulence and levels of cytoadherence (Torres-

Romero and Arroyo, 2009). Also, iron is an essential micronutrient for adhesion of *Candida albicans* to host tissues (Yan *et al.*, 1998), for survival of *N. gonorrheae* within epithelial cells (Hagen and Cornelissen, 2006) and for the virulence of UTI-producing bacteria (Nielubowicz and Mobley, 2010). The positive relationship between iron status and the aforementioned infections may therefore be explained by the beneficial effect of accessible iron for the pathogens, and may also help to explain why trichomoniasis and AB/UTI and trichomoniasis and gonococcal infection occurred together more frequently than expected by chance.

The relationships among genital infections showed interesting interactions in the vaginal microenvironment, where decreased iron and vitamin B12 availability favours the colonization by bacteria and fungus respectively, but is protective against trichomoniasis whereas folic acid deficiency increases the risk of all the vaginal infections studied, including BV. A recent study in Texas with non-Hispanic black and white population found increased occurrence of BV during pregnancy in women with maternal vitamin D below 30 nmol/L (12 ng/mL) and folate below 12.5 nmmol/L (5 μg/mL) (Dunlop *et al.*, 2011), which is in agreement with our findings; in an African-American population of non-pregnant women, higher intake of other nutrients including folate, vitamin A and calcium was associated with decreased risk of BV (Neggers *et al.*, 2007).

Trichuris and hookworm occurred together more frequently than expected by chance (14 of the 15 samples that were positive for *Trichuris* were also positive for hookworm); this strong relationship was confirmed in regression models, where additionally, hookworm epg was strongly related with lower levels of folate and trichuriasis with higher levels of vitamin D and with maternal and multiparity. With respect to these two variables, Adegnika and collaborators (2010) did not find a relationship of intestinal helminths with age or parity, but studies done in mammals have shown an increased parasitic load of intestinal

parasites during the periparturient period (Chartier *et al.*, 1998). We found no studies addressing the relationship between intestinal parasites and vitamin D, and protozoan infections have seldom been related with folate (Olivares *et al.*, 2002, Boeke *et al.*, 2010). The negative association between anemia and *Ascaris* prevalence parallels the observation in a Peruvian population of pregnant women (Larocque *et al.*, 2005) and a recent Ethiopian study of non-pregnant women that shows a lower prevalence of intestinal parasites in women with iron deficiency and folate deficiency anemia (Haidar, 2010).

Beyond the diversity and magnitude of concurrent multiple infections and multiple micronutrient deficiencies, perhaps the most intriguing result from this study was the relationship between vitamin D and infection. There has been considerable focus on vitamin D in recent years, both from an epidemiological and an immunological perspective. Low vitamin D concentrations have been reported in tropical and subtropical regions (van Schoor and Lips, 2011) where vitamin D deficiency had previously not been considered as a possible problem, because of year-round exposure to sunlight. We confirmed that vitamin D deficiency is extremely common in this indigenous population of pregnant women in rural Panama. Furthermore, our results indicate that both high and low vitamin D concentrations can pose risk factors, but for different infections. Higher vitamin D concentrations increased the risk of dental caries, AB/UTI and were associated with more severe trichomoniasis, vaginal candidiasis, and extra-genital infections such as trichuriasis and hookworm infection. Vitamin D supplementation has been reported to increase the risk of urinary infection in infants (Katikaneni *et al.*, 2009) but there is virtually no literature on the risk of high levels of vitamin D during pregnancy. In contrast, mothers with vitamin D deficiency had higher *Ascaris* epg, more severe gonococcal infection and were at greater risk of BV. Another study showed increased risk of BV in pregnancy at Vitamin D levels below 75 nmol/L (Hensel *et al.*, 2011) but we were not able to detect a significant risk of BV at higher cut-offs of vitamin D.

Inflammation

The literature indicates that many of the pathogens observed in this study induce a pro-inflammatory state and our data are consistent with this. In particular, CRP was elevated in mothers with respiratory and/or oral infections. Higher CRP levels have been documented in pregnant women with periodontal disease (Horton *et al.*, 2008, Sharma *et al.*, 2009), with trichomoniasis (Anderson *et al.*, 2007) and the association of BV and elevated CRP in the pathogenesis of PTD have been described (Goffinet *et al.*, 2003). With regard to intestinal nematodes, we observed lower CRP in women infected by *Ascaris* in simple binary comparisons. Elevation of CRP was described in hookworm infection of healthy volunteers at days 20-34 after an experimental primary infection (Wright and Bickle, 2005) but no relationship was found between CRP and ascariasis in Zanzibarian children (Kung'u *et al.*, 2009). An association between higher levels of CRP, trichuriasis and lower vitamin A was reported in a study done with Bangladeshi children (Kongsbak *et al.*, 2006); we were not able to determine whether intestinal nematodes entered the regression model for CRP due to the smaller number of women for whom stool samples were collected, but we found that CRP was significantly higher in pregnant women with lower vitamin A concentrations and that several of the micronutrients influenced CRP levels. Both folic acid and vitamin D deficiency increased CRP levels whereas the combination of iron deficiency and B12 deficiency lowered CRP concentrations. Our findings support other research on folate associations with lower inflammatory status during pregnancy (Kim *et al.*, 2011, Simhan *et al.*, 2011, Kaestel *et al.*, 2012), but the mechanistic pathways have not been clarified.

The usefulness of CRP in predicting adverse pregnancy outcomes such as preeclampsia, PTD, PROM, neonatal complications is still being debated (Arikan *et al.*, 2012, Lohsoonthorn *et al.*, 2007, Ernst *et al.*, 2011). Our data shows that it is possible to find elevated levels of CRP in a context of normal pregnancy follow up, and that factors that increase CRP might be in turn, a risk factor for adverse

pregnancy outcomes. This is the case with environmental wood smoke, which induces a pro-inflammatory state, and that has been associated with LBW and stillbirth (Pope *et al.*, 2010). To our knowledge, the relationship between wood smoke exposure in pregnancy and higher levels of CRP has not been reported before, even though it is known that second hand smoking is related with elevated CRP in non-pregnant subjects (Jefferis *et al.*, 2010); similarly, grand-multiparity is a known risk factor for adverse pregnancy outcomes (Al, 2012) but has not been specifically linked to elevated CRP. Less surprising were the findings of close relationship between platelets cell line and inflammation, since platelets are known as pro-inflammatory cells (Margetic, 2012), and the decreased CRP with higher number of lymphocytes, typical cells of Th-2 response (Warning *et al.*, 2011), even though there's no reference in the literature on number of lymphocytes and CRP during pregnancy.

The cut-off *stipulated for vitamin D deficiency based on bone requirements is < 50 nmol/L (Ross* et al*., 2011). We found that values above a cut-off of 75 nmol/L* were not only associated with more severe trichomoniasis, vaginal candidiasis and trichuriasis, but at the same time with significantly lower levels of CRP. Recent studies show a negative relationship between vitamin D and CRP in non-pregnant populations (Hypponen *et al.*, 2010, Bellia *et al.*, 2011), which is in agreement with our findings. Liu and collaborators (Liu *et al.*, 2011) found in an experimental model with mice that the placental vitamin D system is highly sensitive to immune regulators; vitamin D responds to immune challenge during pregnancy through a localized intracrine or paracrine mechanism, showed by the parallel induction of vitamin D activating enzyme 1α-hydroxilase (Cyp27b1) and vitamin D receptor after exposure to lipopolysaccharide (LPS); also, Cyp27b1 is expressed in vivo by LPS, indicating the placental synthesis of vitamin D induced by pathogen-associated molecular patterns (Liu *et al.*, 2011). A more in depth study of inflammatory factors would be necessary to determine the role of vitamins specifically in the infections we mentioned.

Considering that pregnant women in our study were infected with such a variety of pathogens, had such a diversity of micronutrient deficiencies and high exposure to indoor wood smoke, the CRP levels were not excessively high. CRP showed a pattern not far from other studies with normal pregnancies (Belo *et al.*, 2005, Picklesimer *et al.*, 2008), meaning that CRP is being modulated by a combination of maternal factors (wood smoke exposure, parity, number of platelets and lymphocytes), micronutrient sufficiency (vitamin D and A) and withholding of iron and vitamin B12.

Limitations

Field research often relies on diagnosis of infectious diseases based on a clinical exam rather than using more precise diagnostic tools. In spite of the limited technical facilities, our findings showed that clinical diagnoses were still useful in a rural setting. We may have missed subclinical infections, particularly oral infections that would have been detected by a dentist. The wet-mount and Gram stain of vaginal smear were valuable, but we were not able to rule out infections such as Chlamydia trachomatis, detectable only throughout culture that was not possible in this setting.

The lack of consensus on cut-offs for micronutrient deficiencies such as vitamin D and ferritin in populations with a high prevalence of infections poses a challenge. Also, recent research suggests that cut-off values for folate, vitamin B12 and vitamin A during pregnancy should differ among trimesters (Duerbeck and Dowling, 2012, Green, 2011), in which case, the prevalence of micronutrient deficiencies in our population would be even higher. We tried to overcome this issue by controlling for the continuous variable of the micronutrient and trimester/gestational age, and by using more than one cut-off as in the case of ferritin and vitamin D.

CONCLUSION

Our comprehensive examination of infections of multiple organ systems and of multiple micronutrients indicates the complexity of interactions. Presence and severity of infections vary according to micronutrient levels, and with them, the inflammatory response seems to be modulated. Many of the infections we detected are chronic and often sub-clinical conditions that are largely neglected both by the women and by health professionals in these rural settings. Together, however, they may have a large impact on women's health. It would be important to consider how this maternal burden especially when superimposed on multiple micronutrient deficiencies affects fetal and infant growth and development. Our study has the value of study those conditions under a non-HIV / non-malarial setting. Of particular interest are the observations where higher levels of micronutrients, particularly vitamin A and D, have a positive impact on infections. More research is needed to better understand the mechanistic pathways of those interactions.

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TABLES AND FIGURES

Table 1. *Prevalence of clinically detected infection and prevalence and intensity of laboratory diagnosed infections in pregnant Ngäbe women from Western Panama.*

System	Clinical Diagnosis	Pooled data	Trimester		
			First	Second	Third
Respiratory (n=213)	Cold, sinusitis	2.3%	4.1%	6.2%	1.8%
	Bronchitis	4.0%	4.1%	2.5%	1.8%
Skin (n=213)	Scabies	17.4%	12.5%	20%	16.5%
	Dermatomycosis	1.8%	0%	1.25%	2.7%
	Bacterial impetigo	1.8%	0%	2.5%	1.8%
Oral (n=213)	Caries	19.7%	16.6%	18.75%	21.1%
	Gingivitis	4.2%	0%	0%	8.3%
Genital (n=213)	Vaginitis	89.2%	91.7%	83.7%	92.6%
	Cervicitis	33.3%	37.5%	32.5%	33%
	Lab Diagnosis				
Cervico-vaginal (n=213)	Bacterial vaginosis	91.1%	91.6%	90%	91.7%
	Trichomonas vaginalis	17.4%	12.5%	22.5%	14.7%
	Candida sp	24.9%	33.3%	28.7%	20.2%
	Gonococcus sp	8.9%	0.0%	5%	13.8%
	Reactive VDRL (n=172)	2.9%	5.0%	2.8%	2.5%
Urinary (n=208)	Undetermined bacteria	56.2%	58.3%	60%	50.5%
Intestinal (n=120 for prevalence, n= 74 for intensity)	*Ascaris*				
	Prevalence	32.5%	36.3%	28%	35%
	Intensity, epg ± SE	1143±399	750±748	962±457	1298±627
	Hookworm				
	Prevalence	56.6%	18.2%	58%	62%
	Intensity, epg ± SE	893±277	280±278	669±304	1099±439
	Trichuris				
	Prevalence	12.5%	0%	11.6%	15.2%
	Intensity, epg ± SE	193±132	–	98±63	273±225
	Entamoeba coli	10.8%	18.2%	18.6%	4.5%
	Giardia	2.5%	0%	4.6%	1.5%

Table 2. *Multiple logistic regression models for presence of respiratory infection, scabies, caries and oral and AB/UTI in 213 pregnant Ngäbe women from Western Panama using data pooled across all trimesters.*

a. PRESENCE OF RESPIRATORY INFECTION[1]				
Independent variable	OR ± SE	P	95% CI	Model P
Log of CRP, mg/L	2.33 ± 0.80	0.015	1.2, 4.6	<0.0001
Coffee, cups/day	1.80 ± 0.48	0.030	1.0, 3.0	
No iron or micronutrient supplements[2]	7.31 ± 4.7	0.002	2.1, 25.8	
Severity of clinical vaginitis[3]	0.52 ± 0.2	0.094	0.2, 1.1	
b. PRESENCE OF SCABIES[1]				
Independent variable	OR ± SE	P	95% CI	Model P
Portions of protein/w	0.8 ± 0.1	0.042	0.7, 1.0	<0.0001
Severity of impetigo[3]	11.1 ± 10.0	0.008	1.9, 65.2	
Severity of clinical vaginitis[3]	2.1 ± 0.5	0.004	1.2, 3.4	
Maternal weight category[5]	0.4 ± 0.2	0.017	0.2, 0.8	
Vitamin A, log μg/dL	4.9 ± 3.4	0.021	1.2, 19.1	
Lymphocytes, %	1.1 ± 0.4	0.003	1.0, 1.2	
c. PRESENCE OF CARIES[1]				
Independent variable	OR ± SE	P	95% CI	Model P
sTfRs, Log μg/mL	2.5 ± 0.9	0.014	1.2, 5.1	0.0007
CRP, Log mg/L	1.5 ± 0.3	0.031	1.0, 2.1	
Severity of BV (diagnosed by clue cells)[4]	1.4 ± 0.2	0.026	1.0, 1.9	
Severity of vaginal candidiasis[4]	1.6 ± 0.4	0.048	1.0, 2.5	
Vitamin D >50 mg/L[1]	1.8 ± 0.7	0.13	0.8, 3.7	
d. PRESENCE OF AB/UTI[1]				
Independent variable	OR ± SE	P	95% CI	Model P
Severity of vaginitis[3]	1.4 ± 0.2	0.046	1.0, 2.0	0.0001
Presence of pica[1]	0.1 ± 0.1	0.005	0.02, 0.5	
Both Vit B12 and Vit D deficiencies[1]	0.5 ± 0.2	0.042	0.3, 1.0	
sTfR < 9 μg/mL (iron sufficiency)[1]	2.8 ± 1.3	0.036	1.0, 7.1	

[1] Coded as 0 = no; 1 = yes

[2] Coded as 0= Taking supplements, 1= No taking supplements

[3] Coded as 0= absent; 1= mild; 2= moderate; 3= severe

[4] Coded as 0= absent; 1= few; 2= moderate amount; 3= abundant (per hpf)

[5] Underweight=1, normal weight=2, overweight=3

Table 3. *Multiple ordered logistic regression models for severity of cervico-vaginal infections of 213 pregnant Ngäbe women from Western Panama, pooled across trimesters.*

a. SEVERITY OF BACTERIAL VAGINOSIS (Amount of clue cells in wet-mount)		OR ± SE	P	95% CI	Model P
Model 1	Presence of folic acid deficiency anemia[1]	2.50 ± 1.0	0.022	0.1, 5.4	<0.0001
	No iron or micronutrient supplementation[1]	2.22 ± 0.73	0.015	1.2, 4.2	
	Vitamin D <25 nmol/L[1]	2.90 ± 1.46	0.035	1.1, 7.8	
	Severity of vaginal trichomianosis[2]	0.44 ± 0.10	0.001	0.3, 0.7	
Model 2	MCHC, g/dL	0.61 ± 0.06	<0.0001	0.5, 0.8	<0.0001
	No iron or micronutrient supplementation[1]	2.36 ± 0.80	0.011	1.2, 4.5	
	Vitamin A, Log μg/dL	2.23 ± 0.90	0.043	1.02, 4.8	
	Severity of vaginal trichomiasis[2]	0.49 ± 0.12	0.004	0.3, 0.8	
b. SEVERITY OF TRICHOMONIASIS		OR ± SE	P	95% CI	Model P
Model 1	Vitamin D above 75 nmol/L[1]	7.30 ± 6.13	0.018	1.4, 38.0	<0.0001
	Vitamin B12, Log pmol/L	3.11 ± 1.60	0.026	1.1, 8.5	
	Severity of BV (amount of clue-cells)[2]	0.69 ± 0.11	0.016	0.5, 0.9	
	Severity of vaginal candidiasis[2]	1.93 ± 0.42	0.002	1.2, 3.0	
Model 2	Urinary leukocyte esterase[3]	2.27 ± 0.5	<0.0001	1.5, 3.5	<0.0001
	MCHC, g/dL	1.5 ± 0.25	0.019	1.1, 2,1	
	Combined deficiency of FA and vitamin B12[1]	2.1 ± 0.9	0.09	0.9, 5	
c. SEVERITY OF VAGINAL CANDIDIASIS		OR ± SE	P	95% CI	Model P
Model 1	Vitamin B12, log pmol/L	0.36 ± 0.18	0.041	0.1, 0.9	0.026
	Ferritin > 15 ng/mL[1]	2.02 ± 0.70	0.041	1.0, 4.0	
	Folic acid deficiency anemia[1]	2.41 ± 1.08	0.051	1.0, 5.8	
Model 2	Vitamin D >75 nmol/L[1]	8.6 ± 7.2	0.010	1.6, 44.1	0.0011
	First pregnancy[1]	0.39 ± 0.16	0.029	0.2, 0.9	
	Urinary pH	1.51 ± 0.28	0.027	1.0, 2.2	
d. SEVERITY OF GONOCOCCAL INFECTION		OR ± SE	P	95% CI	Model P
Independent variables	Presence of gingivitis[1]	30.8 ± 28.6	<0.0001	5, 190	<0.0001
	Severity of scabies[1]	3.10 ± 1.83	0.056	1, 10	
	Severity of BV (Nugent score)	0.50 ± 0.10	<0.0001	0.3,0.7	
	Combined FA and vitamin B12 deficiencies[1]	5.88 ± 3.70	0.005	1.7, 20.1	
	Presence of vaginal trichomoniasis[1]	6.21 ± 4.03	0.005	1.7, 22.2	
	Vitamin D <25 nmol/L[1]	4.20 ± 3.85	0.12	0.7, 25.4	
	Ferritin ≥30 ng/mL[1]	2.73 ± 1.82	0.13	0.7, 10.1	

[1] Coded as 0 = no; 1 = yes. [2] Coded as 0= absent; 1= few; 2= moderate amount; 3= abundant (per hpf). [3] Coded as 0= no reaction; 1= (+); 2= (++); 3= (+++). MCHC= Mean corpuscular hemoglobin concentation. BV= Bacterial vaginosis. FA= Folic acid.

Table 4. *Multiple regression models on Trichuris, hookworm and Ascaris eggs per gram feces in data pooled across all trimesters in pregnant Ngäbe women from Western Panama.*

	a. *Trichuris*, epg		
Independent Variables	β coefficient ± SE	P	Overall Model
Hookworm, epg	0.38 ± 0.03	<0.0001	
Vitamin D >75 nmol/L[1]	779.4 ± 330.3	0.021	$F_{4, 69}$ = 51.82
Age > 35 y[1]	-566.3 ± 244.7	0.024	P < 0.0001
Number of pregnancies	66.7 ± 35.7	0.066	Adjusted R^2 = 0.73
Constant	-344 ± 134	0.012	
	b. HOOKWORM, epg		
Independent Variables	β coefficient ± SE	P	Overall Model
Trichuris, epg	1.67 ± 0.13	<0.0001	$F_{2, 71}$ = 97.64
Folic acid, log nmol/L	-1002 ± 369	0.008	P <0.0001
Constant	3209 ± 989	0.002	Adjusted R^2 = 0.72
	c. *Ascaris*, epg		
Independent Variables	β coefficient ± SE	P	Overall Model
Presence of gingivitis[1]	3185 ± 1206	0.010	
Presence of dermatomycosis[1]	7601 ± 3098	0.017	
Presence of vaginal candidiasis[1]	1831 ± 808	0.020	$F_{5, 67}$ = 5.81
Presence of AB/UTI[1]	1796 ± 755	0.020	P = 0.0002
Vitamin D <50 nmol/L[1]	1162 ± 788	0.14	Adjusted R^2 = 0.25
Constant	-1591 ± 873	0.073	

[1] Coded as 0 = no; 1 = yes

AB/UTI= Asymptomatic bacteriuria/Urinary tract infection

Table 5. *Comparison of C Reactive Protein (CRP) (mean± SE) between pregnant Ngäbe women from Western Panama, with and without single or paired micronutrient deficiencies or infections.*

Micronutrient Deficiency (cut-off)	**Condition Present**		**Condition Absent**		**P>|t|[1]**
	n	**CRP (mg/L)**	**n**	**CRP (mg/L)**	
Iron (ferritin <30 ng/mL)	176	4.9 ± 0.3	37	6.6 ± 1.1	0.96
Folic acid (<10 μg/mL)	51	5.7 ± 0.7	162	5.0 ± 0.4	0.22
Vit B12 (<150 pg/mL)	181	5.0 ± 0.3	32	6.8 ± 1.1	0.20
Vit D (<50 nmol/L)	138	5.2 ± 0.4	75	5.1 ± 0.5	0.24
Vit A (<20 μg/dL)	**12**	**10.3 ± 2.0**	**201**	**4.9 ± 0.3**	**0.004**
Both Vit A and Vit B12 deficiencies	**10**	**9.1 ± 2.2**	**203**	**5.0 ± 0.3**	**0.02**
Both Vit A deficiency and Vit D <50 nmol/L	**8**	**10.6 ± 2.7**	**205**	**5.0 ± 0.3**	**0.02**
Infection	**Condition Present**		**Condition Absent**		**P>|t|**
	n	**CRP (mg/L)**	**n**	**CRP (mg/L)**	
Respiratory	**13**	**11.5 ± 2.1**	**200**	**4.8 ± 0.3**	**0.005**
Oral	**49**	**6.7 ± 0.8**	**164**	**4.7 ± 0.3**	**0.02**
Skin	43	5.7 ± 0.8	170	5.1 ± 0.4	0.36
Cervico-vaginal	196	5.2 ± 0.4	17	5.1 ± 1.2	0.60
Bacterial Vaginosis (BV)	194	5.2 ± 0.3	19	5.4 ± 1.6	0.92
AB/UTI	117	5.1 ± 0.5	91	5.0 ± 0.5	0.50
Intestinal parasites	87	5.6 ± 0.6	33	4.8 ± 0.6	0.78
Ascaris	**39**	**4.6 ± 0.8**	**81**	**5.8 ± 0.6**	**0.02**
Hookworm	68	5.8 ± 0.7	52	4.8 ± 0.7	0.30
Trichuris	15	3.5 ± 1.1	105	5.7 ± 0.5	0.17
Respiratory + oral	**4**	**13.7 ± 3.4**	**209**	**5.0 ± 0.3**	**0.02**
Respiratory + skin	**3**	**16.9 ± 2.3**	**210**	**5.0 ± 0.3**	**0.01**
Respiratory + cervico-vaginal	**11**	**11.1 ± 2.4**	**202**	**4.8 ± 0.3**	**0.02**
Respiratory + BV	**11**	**11.8 ± 2.2**	**202**	**4.8 ± 0.3**	**0.006**
Respiratory + hookworm	**3**	**20.3 ± 3.1**	**117**	**5.0 ± 0.4**	**0.007**
Oral + cervico-vaginal	**48**	**6.5 ± 0.8**	**165**	**4.8 ± 0.4**	**0.04**
Oral + BV	**47**	**6.9 ± 0.8**	**166**	**4.7 ± 0.4**	**0.01**

[1] Analysis done on log transformed data

Table 6. *Multiple ordered logistic regression models for presence of high CRP (a) defined as CRP >3.0 mg/L in the first trimester, >20.3 mg/L in the second trimester and >8.1 mg/L in the third trimester.*

Independent variables	**OR ± SE**	**P**	**95% CI**	**Model P**
Both respiratory and oral infections[1]	11.81 ± 15.2	0.055	0.95, 147.0	P < 0.0001
Vitamin D <50 mg/L[1]	3.27 ± 1.6	0.020	1.20, 8.90	
MCHC, g/dL	1.47 ± 0.27	0.034	1.03, 2.11	
Folic acid, log nmol/L	0.28 ± 0.15	0.017	0.10, 0.80	
Both iron and vitamin B12 deficiency[1]	0.18 ± 0.10	<0.0001	0.07, 0.46	
Wood smoke exposure, h/d	1.35 ± 0.19	0.031	1.02, 1.77	

[1] Coded as 0 = no; 1 = yes

MCHC= Mean corpuscular hemoglobin concentration

Fig.1. *Venn diagram of single or multiple micronutrient deficiencies (a) and single and multiple infections (b) in pregnant Ngäbe women in western Panama.*

(a) n = 213; iron (ferritin < 30 ng/mL), folic acid (<10 nmol/L), vitamin B12 (<150 pmol/L), vitamin D (<50 nmol/L). (b) n = 208; skin (scabies, dermatomycosis or impetigo), oral cavity (caries or gingivitis), urinary tract infections or asymptomatic bacteriuria, or vaginal tract infection (bacterial vaginitis, vaginal trichomoniasis, vaginal candidiasis, gonococcal infection). Note that data on intestinal parasites were not available for many of the women, and therefore intestinal parasites have not been included in the figure. Neither the lowest prevalent infection (respiratory) nor the lowest prevalent deficiency (vitamin A) is included.

(a)Prevalence of micronutrient deficiencies *(b)Prevalence of infections*

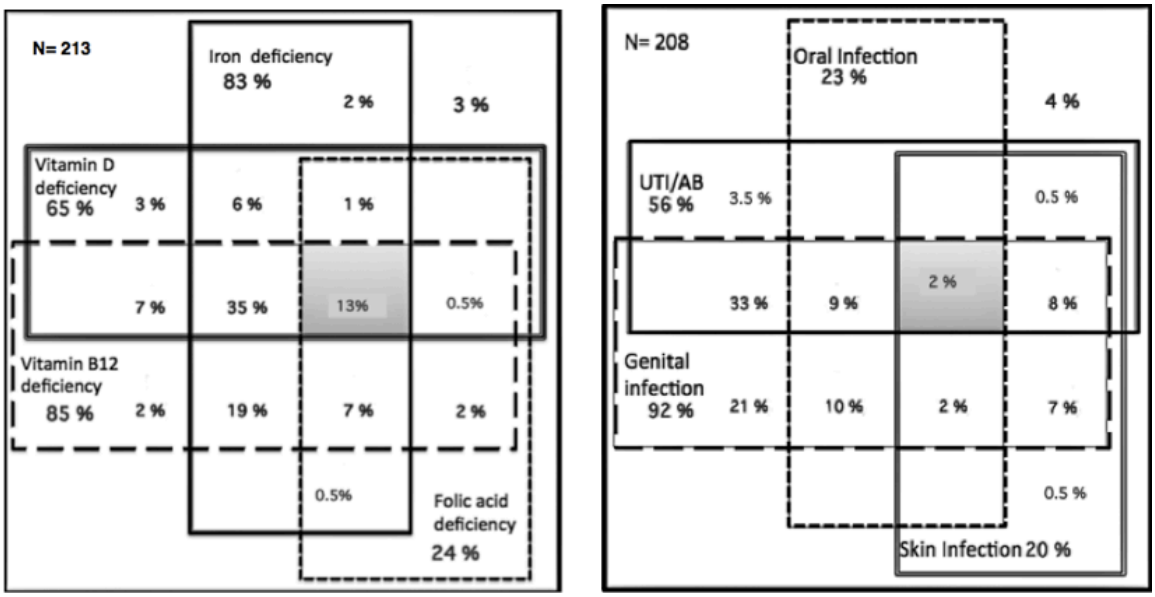
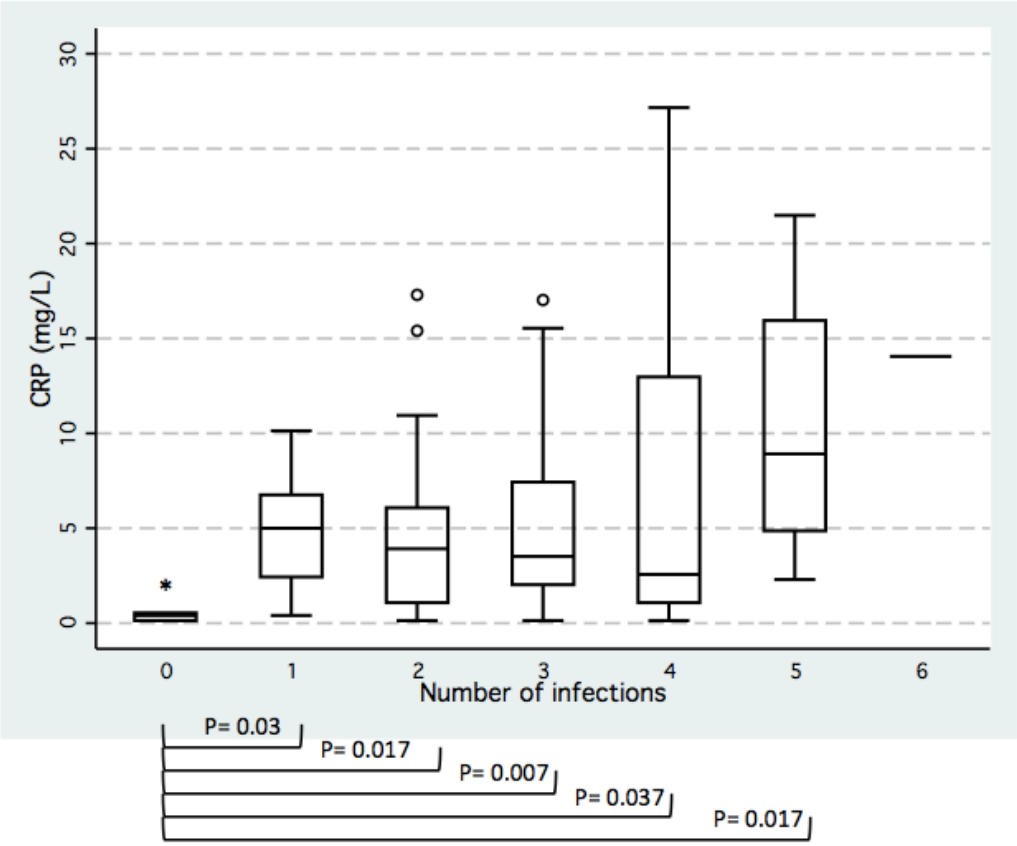


***Fig. 2**. Boxplot of CRP levels (mg/L) of 118 pregnant women by number of co-occurrent infections.*

T-test of log transformed CRP showed that women with no infection (*) had significantly lower levels of CRP compared with women with one or more infections.



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CONNECTING STATEMENT

The first paper ("The Impact of Maternal, Environmental and Nutrition Factors on Infections and Inflammation During Pregnancy, in a Rural Community in Panama") provided an overview of the wide range of factors that may affect the health of pregnant women in this indigenous community in Panama, suggesting grave consequences in terms of pregnancy outcomes.

Fetuses in our population were surrounded first by a maternal environment that was deficient in micronutrients that are important for fetal development, and by extra-uterine infections whose potential influence on the fetus is poorly understood.

Given the cross sectional nature of our rural study, we used simple tools available in the field to the effects of these micronutrient deficiencies and infections on fetal development. Using the measurement of fundal height, fetal cardiac rate and fetal movements as perceived by the mothers, the next paper ("The impact of environmental factors, maternal infections and maternal nutrition on basic antenatal fetal measurements as indicators of fetal wellbeing in a rural indigenous community in Panama") explores interactions between infections and micronutrient deficiencies and fetal development.

Chapter IV - ENVIRONMENTAL FACTORS, MATERNAL INFECTIONS AND MATERNAL NUTRITION MODIFY FETAL MEASUREMENTS ASSOCIATED WITH FETAL WELLBEING IN A RURAL INDIGENOUS COMMUNITY IN PANAMA

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ABSTRACT

We enrolled 213 pregnant women from the Ngäbe-Buglé indigenous community of rural Panama living in extreme poverty and lacking a developed health infrastructure to assess maternal health and fetal well-being. In this cross-sectional study, 3 field appropriate methods - fundal height (FH), fetal cardiac rate (FCR) and maternal counts of fetal movements (FM) in the previous hour were measured in the second (n= 80) and third trimesters (n= 109) as were several maternal factors associated with these outcomes. Results showed that maternal BMI was positively associated with weekly servings of protein whereas coffee intake, pica, presence of skin infections, anemia and vitamin A and D deficiencies were negative predictors of maternal BMI. While controlling for gestational age, FH in the 2nd trimester was negatively associated with hours of wood smoke exposure and two markers of infection (monocytes, urinary pH) and positively with iron plus multivitamin usage and mean corpuscular hemoglobin concentration (MCHC); in the 3rd trimester, maternal BMI was positive and vitamin D and scabies were negative predictors of FH. These models explained 73% and 50% of FH respectively. FCR in the 2nd trimester was positively associated with several infection indicators, higher iron and lower platelet concentrations; in the 3rd trimester FCR was positively associated with gingivitis, MCHC, and negatively with the intake of iron plus multiple micronutrient supplements. Self reported FM were positively associated with the presence of vaginal infections and negatively with scabies and folic acid in the 2nd trimester; in the 3rd trimester, the presence of cervicitis and wood smoke exposure were negative predictors whereas higher hematocrit was positively associated with FM. Our findings validate the usefulness of simple fetal measurements as indicators of fetal wellbeing in developing countries.

INTRODUCTION

Accurate evaluation of fetal wellbeing in rural settings is limited due to lack of access to facilities such as ultrasound and more advanced obstetric and

laboratory techniques. Thus, pregnancy follow up in rural areas is done using only simple clinical measurements including fundal height, fetal cardiac rate and fetal movements.

Fundal height (FH) is measured with a tape from the pubic symphysis up to uterine fundus. FH is applied to WHO tables of fetal growth percentiles for gestational age to determine if fetuses have normal growth, intra-uterine growth retardation (IUGR) or are large for gestational age (LGA). IUGR leads to low birth weight (LBW) that is associated with early newborn morbidity and mortality (WHO, 2002). On the other hand, LGA fetuses have increased metabolic and mechanical obstetric risks at birth. Both conditions have been implicated on long-term adverse outcomes later in life (Ng et al., 2010). Accuracy of FH in detecting fetal growth abnormalities is controversial, since amniotic fluid and maternal tissues surround the fetus, but its usefulness has been demonstrated particularly as a tool to detect IUGR during the second trimester (Fournie et al., 2007). Furthermore, FH correlates with birth weight when measured during the third trimester (Indraccolo et al., 2008).

Fetal cardiac rate (FCR), besides indicating viability of the fetus, is also an indicator of physiologic or pathologic states of fetal oxygenation. FCR regulation depends among other factors on autonomous nervous system. Sympathetic tonus is present early in fetal life, whereas parasympathetic tonus progressively establishes later in pregnancy. During the second trimester it is possible to observe higher basal FCR, due to predominance of sympathetic tonus, whereas in the third trimester, when the vagal tonus is more developed, there is a reduction of the basal FCR, higher FCR variability (fluctuations of FCR in more than 2 cycles per minute), and more frequent accelerations. Therefore, the reaction of the fetus facing hypoxia differs according to the gestational age (GA), and is more evident during the third trimester (Nomura et al., 2010). For example, experimental studies have shown that pathogens are able to activate the parasympathetic system causing pronounced bradyarrhythmias, followed by

rapid and prolonged desensitization (Fairchild et al., 2011), contributing to the common finding of decreased FCR variability and transient heart rate decelerations observed in human neonatal sepsis (Fairchild and O'Shea, 2010). Less is known about FCR response to infection in earlier stages of pregnancy. A study done in extremely premature infants (25 weeks or less) showed that infants with fetal inflammation had significantly higher FCR (Furukawa et al., 2008). Fetal responses under mild-moderate maternal infection-inflammation have not been studied.

Maternal counting of fetal movements (FM) is a very useful tool in remote areas to assess fetal viability since it is available to all women and does not require any technology. Decreased FMs help to identify pregnancies at risk of IUGR and stillbirth (Tveit et al., 2010). Mothers, in general, are able to feel up to 90% of fetal movements after week 24 of gestation. They tend to be better felt at evening time, and in supine position. Liston and collaborators found that FM do not relate to maternal physical activity, glucose levels or stress, but that smoking and depressant drugs are able to temporarily decrease FM (Liston et al., 2007). Another study showed that maternal glucose levels were significantly correlated with maternal perception of fetal movements (Mirghani et al., 2005). It is considered that 3 or less FM per hour can be considered as abnormal, according to Sadovsky's scale, and this is one of the most used methods in population studies (Sadovsky et al., 1981).

The current study examines associations between of maternal, environmental, nutritional and infectious measurements on fetal wellbeing evaluated through FH, FCR and FM in a group of mothers belonging to the Ngäbe-Buglé indigenous community in rural Panama.

METHODS AND PROCEDURES

The details of the study approval, design and sampling are described in the previous manuscript. In brief, ethical approval was obtained from McGill

University and Panamanian and indigenous authorities. Traditional midwives and community health workers were trained to explain the research study in their communities and encouraged pregnant women to come to the nearest health center. We recruited these women along with those who arrived either for pregnancy follow up or to consult about missing menses where a pregnancy test was positive.

After obtaining informed consent, pregnant women received a complete medical consultation that included obstetric history, a questionnaire on factors affecting maternal/fetal health such as iron or micronutrient supplementation, coffee consumption, wood smoke exposure, hours of field work per day; and review of systems looking for infection. The physical examination included anthropometry, vitals, and obstetric and genital examination. Clinically diagnosed infections in the 213 women included caries, gingivitis, respiratory infection, skin infections (scabies, impetigo and dermatomycosis) and cervicitis or vaginitis were classified into 1 = mild, 2 = moderate, and 3 = severe.

A single blood (n= 213), urine (n= 208) and stool (n= 120) sample and a vaginal smear (n= 213) were obtained from mothers. Serum was used for complete blood count (n=213, processed with an auto hematology analyzer), HIV (n=213, through ELISA antibody test), VDRL (n=172, latex agglutination test indicator of syphilis infection) and glucose (n=178, processed with spectrophotometry) in the local hospital. Ferritin enzyme immunoassay, Ramco's soluble transferrin receptor (sTfR) enzyme immunoassay and high sensitivity enzyme immunoassay for C-reactive protein (hsCRP ELISA) were assayed at the Gorgas Memorial Institute (GMI) in Panama City; vitamin A concentrations in plasma were determined using HPLC at the Institute of Scientific Research and High Technology Services (NDICASAT) in Panama City. Remaining serum (n= 213) was stored at minus 20°C for analysis of folate, vitamin B12 and vitamin D (25OHD) concentrations, which were measured at McGill University using immuno electro-chemiluminescence and LIAISON 25 OH Vitamin D assay respectively.

Several measures of infection were assessed. Urine was obtained from 208 participants and was assayed using microscopic analysis for asymptomatic bacteriuria/urinary track infection (AB/UTI) that was ruled out in samples with high amounts of mucus and epithelial cells; a dipstick test allowed detection of urinary pH, gravity, semi-quantitative amount of occult blood, proteins, nitrites, leukocyte esterase and glucose. Presence of Giardia, Entamoeba coli, fecal yeasts and leukocytes and intensity (eggs per gram of feces) of intestinal nematodes (Ascaris, Trichuris and hookworm) and occult blood were assayed in 120 stool samples, using a direct smear. Kato-Katz and Flotac were processed when amount and characteristics of the stool sample allowed (n= 106 and 73 respectively). Vaginal smears (n=213) were examined using direct microscopic exam (wet mount) to obtain semi-quantitative data on leukocytes, bacteria, clue-cells, Trichomona, Candida and Diplococcus. Measurements using a Gram stained slide to obtain semi-quantitative information on Lactobacillus, Mobiluncus and Bacteroides-Gardnerella were used to calculate the Nugent score for bacterial vaginitis (Nugent, 1991). A score of 7 or higher was considered diagnostic; a score 4-6 was considered as intermediate vaginal microflora, and a score of 0-3 was considered as normal vaginal microflora. Details regarding all methods are found in Gonzalez-Fernandez et al. (Chapter III).

As ultrasound was not available, fetal health was monitored through three clinical measurements. FH was used as a measure of fetal growth. FCR through direct auscultation or Doppler (when available in the health centers) and mother-reported FM in the previous hour were used as signs of fetal viability and wellbeing. The viability of all fetuses (>20 weeks GA or earlier when possible) was confirmed at the time of the clinical exam of the women.

Fetal wellbeing was determined using Panamanian guidelines (Panamanian Ministry of Health, 2007), based on WHO's charts for 10^{th} and 90^{th} percentiles for FH for GA by determining the presence of IUGR, normal growth or LGA infants; fetal tachycardia was defined according to WHO guidelines when FCR was above 160/min and fetal bradycardia when FCR was below 120/min (WHO, 1996). Fewer than 4 FM in the previous hour was considered abnormal as evidence shows that low FMs at this level are associated with IUGR, low Apgar score and perinatal mortality (Sadovsky et al., 1981).

STATISTICS

The majority of statistical analyses were done only on mothers in the second and third trimester because of under-representation of women in the first trimester and because of our inability to obtain FH, FCR and FM from many women during the first trimester. In addition, whenever data permitted, analyses were done separately for the second and third trimester because of the known physiological differences between trimesters, particularly with respect to vital signs and hematological values (Torgersen and Curran, 2006). The complete list of maternal, environmental and hematological independent variables are shown in table 1a.

Detailed list of infections that were studied are shown in table 1b. Some variables had very few observations, particularly fungal skin infections and impetigo, and variables derived from urine analysis - urinary nitrates, proteins, occult blood and crystals (oxalate, phosphate and urate). For these, absence/presence was used in separate analyses for pooled data for second and third trimesters.

All data were analyzed using STATA 10. Correlation matrices were used for WBC and RBC measurements, ferritin, serum folic acid (FA) levels and C-Reactive protein (CRP) to determine which combination of variables could be included together within the same regression models. For each dependent variable (FH,

FCR, FM), we used a series of stepwise multiple regression models to identify the set of factors that best explained variability in the dependent variable (highest R^2). First we constructed sets of models based on known maternal risk factors, on maternal infections, and on micronutrient concentrations. We then ran composite models that incorporated those factors that entered the above models at $P < 0.15$.

RESULTS

We were able to recruit 213 pregnant women for this study of whom 11.3%, 37.5% and 51.2% were in the first, second and third trimester respectively. Women ranged in age from 13 to 45 years old, with an average of 24.9 (SD 7.2; Fig. 2a). Mean maternal systolic blood pressure (SBP) was 103 ± 11 mmHg, and mean diastolic blood pressure (DBP) was 62 ± 9 mmHg.

FH could not be measured in 11 mothers in the first trimester or in 2 mothers in the second trimester. IUGR was found in 7% of the fetuses and 27.2% were LGA. A scatter plot of FH for GA compared with WHO 10^{th} and 90^{th} percentiles is presented in Fig1a. FCR was undetectable during the first trimester; we detected FCR in 41% (n= 33) of the mothers during the second trimester and in 99% (n= 108) of the mothers in the third trimester. Only 1 mother (4%) reported FM during the first trimester, 48 (60%) in the second trimester and 93 (85%) in the third trimester. FCR significantly differed between the second and third trimesters (143.3 ± 1.8 and 139 ± 1.13 respectively, P= 0.049) but FM did not (7.9 ± 0.6 and 8.6 ± 0.3, respectively). Both FCR and FM were within physiological ranges. FCR was borderline correlated with GA (P= 0.053; adjusted R^2= 0.02) but FM were not significantly related with GA (P= 0.53) (Fig. 1b and 1c).

Most mothers in this study had at least one obstetric risk factor (Fig.2): 30% were adolescents and 13% were older than 35 years (Fig 2a), 28% were primarous and 32% were in their fifth or more gestation (Fig 2b), 92% were exposed to indoor wood smoke associated with cooking (Fig 2c), 10% were

underweight for GA and 23% were overweight for GA according to Panamanian guidelines which are based on WHO percentiles of weight for GA (Panamanian Ministry of Health, 2007) (Fig. 2d).

Nearly one third (31.5%) of the pregnant women were receiving only iron supplements, 6% received multiple micronutrient (MMN) supplementation, 45% received both iron tablets plus MMN supplementation, and 17.4% took no supplements[1]. Sixty eight percent began iron/MMN in the third trimester.

As described in Gonzalez-Fernandez (Chapter III), 38% of the mothers were anemic (hemoglobin <11 gr/dL), with 48% having mean corpuscular hemoglobin concentration (MCHC) below normal values (32 – 35 g/dL). Overlapping causes of anemia in this population were iron deficiency (35%), followed by vitamin B12 and folate deficiencies (34 and 12.7% respectively). For vitamin D, only 3% of the mothers achieved recommended levels above 75 nmol/L, and 65% had levels below 50 nmol/L, considered by most as the cut-off for deficiency. In contrast, vitamin A had a lower prevalence of deficiency (5.7%, mean = 33.9 ± 0.7 μg/dL) with 49% of the mothers classified as having vitamin A levels above recommended concentrations for pregnancy, according to a recent review (Duerbeck and Dowling, 2012).

Most women in our study had a combination of two or more infections in conjunction with two or more micronutrient deficiencies. Cervico-vaginal infections were the most frequent (96.7%), followed by intestinal parasitism (61.6%), AB/UTI (56.5%), oral infection (23%), cutaneous infection (20.2%) and respiratory infection (6.1%). The mean urinary pH in the study was 6.4 ± 0.8. As part of the urine analysis, dipstick semi-quantitative measurement of urinary

1 Iron tablets contain 60 mg of elemental iron. Ministry of Health provides a Multiple Micronutrient mix, containing for each 100 gr of powder: 11.1 mg of iron; 36 μg of folic acid; 0.5 μg of vitamin B12; 222 μg of vitamin A. The mix also contains vitamin E, B1, B2, B6, calcium, phosphate, iodine and zinc.

proteins showed mild proteinuria in 5.3% of the samples, always accompanying AB/UTI. No signs or symptoms of pregnancy-induced hypertension were found in this population. Urinary occult blood was found in 5% of the samples, erythrocytes were eumorphic and their presence was not associated with erythrocyte casts, proteinuria or crystaluria; besides, mothers did not report urinary system symptoms, making the diagnosis of other nephropathies unlikely.

Maternal BMI (controlled for GA) was related not only with dietary factors (particularly the availability of protein intake) or micronutrient deficiencies (such as hypovitaminosis D and A), but also with environmental factors such as the habits of pica and coffee consumption and with infections like scabies, which was a negative predictor, and bacterial skin infection that was a positive predictor of maternal BMI (Table 2).

Regression Models (Stepwise and Multiple Linear Regressions) for Fundal Height (FH)

Anthropometry and environmental factors: In order to determine whether the following known determinants of IUGR - maternal weight, height, BMI, gestational age, parity and wood smoke exposure - were predictors of FH in our population, we included them in stepwise regression models. GA always entered as significant, whereas wood smoke exposure and maternal BMI were negatively associated with FH in the second and third trimesters respectively (Table 3a). Neither maternal weight, height, parity nor maternal exercise[2] was significantly related with FH in either trimester. Therefore, in all subsequent analyses of FH, we controlled for GA and wood smoke exposure in the second trimester, and for GA and maternal BMI in the third trimester.

Following this analysis, a series of stepwise multiple regressions were performed in 78 mothers in the second trimester and in 109 mothers in their third trimester

[2] Exercise was assessed as the number of hours per day of fieldwork

unless otherwise specified, first for vital signs and micronutrient supplementation, next for infection parameters and finally for biochemical indices. These were then used to form a composite multiple linear regression model that included the significant independent variables from each of these progressive stepwise regressions.

Vital signs and micronutrient supplementation: During the second trimester, the stepwise multiple regression model of FH that considered maternal vital signs, iron and multi-micronutrient supplementation while controlling for GA and wood smoke exposure revealed that the combination of iron plus MMN supplementation was positively related with FH (P=0.003; adjusted R^2= 0.67) (stepwise model not shown).

In the third trimester, coffee consumption was positively associated with FH (P= 0.001) and maternal cardiac rate entered as a non-significant negative factor (P=0.09) while controlling for GA and maternal BMI (adjusted R^2= 0.45). Neither iron nor MMN supplementation were significant in this analysis (stepwise model not shown).

Maternal infections: Given the wide range of infections, we investigated a variety of models of FH based on either presence/ absence of clinically detected respiratory, skin, oral or genital infections or severity by system (oral, skin, genital, urinary, intestinal) using clinical or laboratory data. In the second trimester (n=77 urine samples), FH was positively associated with urinary pH (P= 0.029) and number of leukocytes in urine/hpf (P=0.009), and negatively associated with the number of bacteria in urine/hpf (P=0.022) and the percent, while controlling for GA and wood smoke exposure. A negative association of FH with the presence of urate crystals (P= 0.043), phosphate crystals (P= 0.027) and of oxalate crystals in urine (P= 0.056) was found when running second and third trimesters together (n= 182; adjusted R^2= 0.81, n= 182; stepwise model not shown).

In the third trimester, FH was negatively associated with severity of scabies (n= 109; P=0.011; adjusted R^2= 0.45; stepwise model not shown) when controlling for GA and BMI. Another model showed that FH was positively associated with Ascaris epg (n= 59; P=0.025; adjusted R^2=0.52; stepwise model not shown) while controlling for GA, maternal BMI and wood smoke exposure.

Blood count and serum micronutrients: When controlling for GA, maternal height and wood smoke exposure, FH was negatively associated with MCHC (P=0.005) in the second trimester (adjusted R^2= 0.69; stepwise model not shown). In a separate model, FH was positively associated with sTfRs (P=0.037; adjusted R^2=0.65), but only if vitamin B12 was included in the model where it entered as a borderline negative factor (P=0.13) (stepwise model not shown).

In the third trimester, FH was negatively associated with vitamin D (P=0.004) when controlling for maternal BMI (adjusted R^2=0.45; stepwise model not shown).

Composite Multiple Regression Models for FH

In entering borderline and significant variables from our stepwise models into a single multiple regression where we controlled for gestational age, we found that FH in the second trimester was positively associated with, iron plus MMN supplementation and urinary pH and negatively associated with wood smoke exposure, MCHC and percent of monocytes (Table 3b). In the third trimester, FH was negatively associated with maternal vitamin D concentration and severity of scabies and positively associated with coffee consumption and maternal BMI (Table 3c).

Regression Models (Stepwise and Multiple) of Fetal Cardiac Rate (FCR)

In the second trimester, FCR was positively associated with maternal age and negatively associated with systolic blood pressure (SBP) (Table 4a). During the third trimester, FCR was positively associated with maternal cardiac rate (MCR)

(Table 4a) though the model only explained 3% of the variation in FCR. The adjusted R^2 was increased to 0.07 by inclusion of iron plus MMN supplementation (P=0.050) and hours of fieldwork (P=0.11) both of which were positively associated with FCR. For all subsequent regression models, we controlled for maternal age and SBP in the second trimester, and for MCR in the third trimester. The following describes a series of stepwise regressions by variable class, in a population of 33 mothers in the second trimester and 108 in the third trimester.

Infections: FCR was negatively associated with presence of skin infection in the second trimester (P=0.025; adjusted R^2=0.28; stepwise model not shown). We also found a negative association between FCR and presence of scabies in the second trimester (P= 0.10; adjusted R^2= 0.3; stepwise model not shown).

In the third trimester, weak but significant models showed a positive association with presence of gingivitis (P=0.08, adjusted R^2=0.02, model not shown), and with severity of cervicitis (P=0.14, adjusted R^2=0.05, stepwise model not shown).

Complete blood count and serum micronutrient levels: All three blood cell lines (red blood cells (RBC), white blood cells (WBC) and platelets) were related to FCR in the second trimester. The simplest model revealed elevated FCR in association with higher WBC count (P= 0.003), lower platelet deviation width (PDW) (P=0.007) and RBC count (borderline significance: P= 0.097) (model R^2= 0.51; stepwise model not shown). Particularly interesting was the relationship among the different WBC lines with respect to FCR when controlling for PDW. FCR was positively associated with number of monocytes (P= 0.024) and showed a borderline positive association with percent of neutrophils (P= 0.08) (adjusted R^2= 0.46; stepwise model not shown), whereas a separate model revealed that percent of lymphocytes (P= 0.017) was negatively related with FCR and mean corpuscular volume (MCV) was positively related (P= 0.025) (adjusted R^2= 0.48; stepwise model not shown). Another significant model included vitamin D as

negatively related with FCR (P=0.039). In this model, number of neutrophils entered as a positive variable (P=0.005) while controlling for PDW (adjusted R^2= 0.52; stepwise model not shown).

In the third trimester, hemoglobin indicators MCHC (P= 0.022; adjusted R^2= 0.07; stepwise model not shown) and mean corpuscular hemoglobin (MCH) (P= 0.041; adjusted R^2= 0.06; stepwise model not shown) appeared to be positively related with FCR in separate models. Interestingly, vitamin A levels entered both models with better overall significance when combined with MCHC (vitamin A, log μg/dL P= 0.004, adjusted R^2= 0.14, stepwise model not shown).

Composite Models for FCR

The model with the best R^2 (0.53) in the second trimester showed a positive relationship between FCR and infections as indicated by number of monocytes and percent of neutrophils whereas platelets deviation width and systolic blood pressure were negative predictors; on the other hand, concentrations of ferritin, an indicator of iron storage were positively related with FCR while controlling for maternal age (Table 4b).

In the third trimester, FCR, when controlling for maternal cardiac rate, was positively associated with maternal height, presence of gingivitis, MCHC levels and vitamin A levels, but negatively associated with iron/MMN supplementation (Table 4c), although this model only explained 22% of the variability in FCR.

Regression Models (Stepwise and Multiple) of Fetal Movements (FM)

To establish factors that needed to be controlled for in regression models for FM, we explored several factors that might be related. Our first models suggested that FM might be associated with hours of fieldwork and maternal glucose concentrations in the second trimester (P=0.078 and 0.064 respectively; adjusted R^2= 0.17; stepwise model not shown). In the third trimester, FM were positively associated with maternal cardiac rate (P=0.018). More hours per day of wood

smoke exposure was associated with fewer FM (P=0.053) (adjusted R^2=0.08; stepwise model not shown). In subsequent regression models (n= 48 for the second trimester; n= 93 for the third trimester), we controlled for MCR in the third trimester.

Infections: FM were negatively associated with the presence of cervicitis in the second trimester (P= 0.034; adjusted R^2= 0.07; model not shown), and with the presence of both vaginitis (P= 0.03) and cervicitis (P=0.04) in the third trimester (adjusted R^2= 0.13) (stepwise models not shown). In separate analyses using the smaller number of stool samples, FM was negatively associated with Ascaris epg (n= 24; P=0.017; adjusted R^2=0.20) and positively associated with hookworm epg (n= 17; P=0.003; adjusted R^2= 0.41) in the second trimester (stepwise models not shown).

Complete blood count and serum micronutrient levels: In the second trimester, FM were positively associated with percent of monocytes (P=0.015) and negatively associated with folic acid concentrations (P=0.019; adjusted R^2= 0.18). Hemoglobin was negatively related with FM in a separate model (P= 0.045; adjusted R^2= 0.06), but lost significance when combined with other variables. Two other variables entered a model in the second trimester: percent of eosinophils (P= 0.071) was borderline positively related, and vitamin D levels (P= 0.13; adjusted R^2= 0.08) were borderline negatively related with FM (stepwise models not shown).

In the third trimester, FM was positively associated with percent of basophils (P= 0.024), although basophil counts were among normal ranges (mean= 0.3 % ± 0.01; normal= 0 to 2%), and hematocrit was a borderline positive factor (P= 0.14; adjusted R^2= 0.10).

Composite Models for FM

The variables that significantly entered the composite model for the second trimester were presence of vaginal trichomoniasis, BV and percent of monocytes (positive associations) and presence of scabies and folic acid levels (negative associations) (Table 5a). Ascaris, not included in the model because smaller sample size, was also positively related with FM (model not shown).

In the third trimester, FM controlled for MCR was positively associated with number of basophils and hematocrit, and negatively associated with hours of wood smoke exposure and presence of cervicitis (Table 5b).

DISCUSSION

The simple clinical parameters of fundal height, and fetal movements were used to evaluate fetal wellbeing in a setting of food insecurity and multiple infections among indigenous pregnant women attending rural health facilities as part of normal pregnancy follow up. Although few studies have examined the range of factors that may affect fetal growth in such a context, Buscicchio and collaborators described how fetuses were affected by maternal conditions in a study of complicated pregnancies in Italy at week 35-36 of gestation; the baseline FCR was significantly higher in women suffering gestational diabetes, gestational hypertension, IUGR and PROM than in controls; and number of FM per hour were significantly increased among women with pregnancy induced hypertension, but significantly decreased in IUGR and PROM when compared with their controls (Buscicchio et al., 2010). Despite the wide range of infections and the multiple micronutrient deficiencies in our study population, along with known physiological and environmental risk factors during pregnancy, none of the complications were detected in our population. We were extremely interested, however, to find that several mild-moderate disease conditions were significantly associated with FH, FCR and FM even though these parameters of fetal wellbeing fell within normal ranges for the majority of fetuses. Thus, the simple clinical parameters that can be monitored in rural settings are sufficiently

sensitive to pick up effects of infection and micronutrient deficiencies when controlling for known risk factors during pregnancy.

Fetal Growth Measured Through FH

Our findings confirm previous studies on the benefit of iron and MMN supplementation in fetal development (Yakoob and Bhutta, 2011, Christian, 2010). Although there is no international consensus on the use of MMN supplementation during pregnancy, MMN supplementation has been shown to be more effective than iron and folate supplementation in reducing the risk of LBW and small for gestational age (SGA) (Kawai et al., 2011), even though it is not more beneficial than iron-folate supplementation on maternal anemia in the third trimester (Haider et al., 2011). Our findings support that MMN supplements positively influence fetal growth as measured by FH in the second trimester. In contrast, we were unable to detect an association between the combined use of iron and MMN supplements and FH in the third trimester.

The impact of organic fuel exposure during pregnancy in rural areas in Latin America and other parts of the world has been neglected, even though its negative effect on fetal growth is similar to that of tobacco smoke (Perez-Padilla et al., 2010), an observation strongly supported by our data from the second trimester.

It is known that although coffee consumption in Ngäbe-Buglé indigenous community is high, caffeine concentration is low due to the diluted traditional way of preparation but it is extremely sweet (Lardeau et al., 2012). We confirmed this by testing 28 randomly collected samples of coffee among participants of the study; caffeine content was of 6.4 ± 6.5 mg/100 ml and women reported that they drank 1.6 ± 1 cup of coffee per day (González-Fernández et al., Chapter III). According to latest WHO review on the effect of caffeine on fetal growth, evidence is insufficient to draw conclusions; however, caffeine consumption has been associated with increased risk of IUGR when it

exceeds 100 mg/day (CARE, 2008). We might infer then, that the positive relationship between coffee consumption and larger fetal size in the third trimester was not due to caffeine but to other coffee components such as chlorogenic acid and caffeic acid that have antioxidant and anti-inflammatory properties (Butt and Sultan, 2011). Alternatively, the larger fetal size may be linked with the high caloric content of this sweetened beverage.

In the process of building exploratory models, we observed that more infectious-related variables entered as significantly related with FH in the second than in the third trimester. Among them, even if not entered the final model, UTI seemed to impair fetal growth in the second trimester. AB/UTI has been documented as responsible for preterm delivery and low birth weight (Sheiner et al., 2009), but its role in fetal growth is controversial. Mann and collaborators did not find associations between maternal UTI and SGA infants (Mann et al., 2009). We found that more bacteria in urine, more acidic urinary pH and the presence of crystals in urine were associated with poor FH, from which phosphate and urate crystals had a more significant relationship. Urease producing bacteria are able to cause the formation of some kinds of phosphate stones (Hesse and Heimbach, 1999), and with an urinary pH <5.5 urate would precipitate and form crystals that also serve as nucleus for the formation of calcium oxalate crystals (Grases et al., 2011). The relationship between urinary crystals/stones and fetal growth has not been studied, but our findings suggest that factors including AB/UTI might be affecting urinary pH, leading to the formation of crystals and that this level of infection is affecting fetal growth.

Anemia and iron deficiency anemia are known factors affecting birth weight (Akhter et al., 2010, Gonzales et al., 2011), and we found that iron plus MMN were positively associated with fetal growth only in the second trimester, but on the other hand, we found that MCHC had a negative relationship with FH in the second trimester both when considered on its own ($P= 0.017$; adjusted $R^2= 0.66$; $n= 78$) and when included in the composite model. The strength of the

association of MCHC with FH increased when percent of monocytes was included in the composite model, whereas monocytes alone entered only as a borderline negative factor (P= 0.10). As described in Chapter III, CRP was significantly lower in women with iron deficiency than in non-deficient mothers. Those findings suggests that the positive influence of anemia on fetal growth might be related to a protective response to chronic inflammation and/or infection and support observations of other studies; high levels of hemoglobin in the first two trimesters of pregnancy have been associated with increased risk of SGA (Scanlon et al., 2000); failure of hemoglobin to fall below 10.5 g/dL was found to increase the risk of poor pregnancy outcomes (Scholl, 2005) and high concentrations of serum ferritin have been found to have a role in increasing the risk of preterm delivery and gestational diabetes (Soubasi et al., 2010).

We were intrigued by the importance of vitamin D concentration as a negative factor affecting FH in the third trimester and suggest that this is linked with the negative relationship between vitamin D and CRP as reported by Gonzalez-Fernandez et al. (Chapter III). CRP has been found elevated in cord blood of SGA newborns compared with adequate for GA newborns (Amarilyo et al., 2011), and we found in data described in Chapter III, that mothers with higher levels of vitamin D had significantly lower levels of CRP. Whereas both vitamin D levels and CRP concentrations were high throughout the second trimester, and both dramatically decreased with GA in the third trimester, at about week 38, CRP and vitamin D moved in opposite directions. It is at this time when fetuses are at term and the Th2 state starts shifting towards a Th1 response in preparation for delivery. Perhaps vitamin D is produced during pregnancy in higher amounts by the placenta and other organs/cells not only for maternal and fetal growth requirements, but also in response to infection, and that its immune-modulator role during pregnancy might be responsible for the lower than expected impacts of multiple infections on fetal growth in this population.

In fact, we expected to find more infections as negative predictors of FH in our composite models. Instead, only severity of scabies was negatively associated with FH and only in the third trimester. To our knowledge this has never been reported before. Scabies' severity is negatively related with maternal BMI (González-Fernández et al. Chapter III), indicating the close association between fetal growth and maternal nutritional status. Not included in the composite model because of the limited number of stool samples, Ascaris epg was positively associated with FH in the third trimester. Ascariasis is characterized by a strong Th2 response, high levels of IgE synthesis, eosinophilia and mucus hyper secretion (Acevedo and Caraballo, 2011). A similar pattern of high parasite epg at the end of pregnancy and immediately after parturition has been described in livestock and is accompanied by a "relaxation" of immune responses during that period of time, which included decreased eosinophilia, lower levels of plasma total antibody and lower indicators of intestinal mucosa immunity (Beasley et al., 2010). The positive relationship between FH and Ascaris epg in the third trimester might be an indirect consequence of the shift to a Th1 immune response near parturition as well as to hormonal changes at the end of the pregnancy. A high correlation between prolactin levels and fecal nematode egg counts was found in goats (Chartier et al., 1998), and experimental studies show that progesterone is able to inhibit mast cell secretions (Vasiadi et al., 2006), which are responsible for local immune responses such as intestinal muscle hypercontractility and mucus production that lead to expulsion of nematode parasites.

Fetal Cardiac Rate

FCR has different characteristics in the second and in the third trimester. The cross-sectional design of our study precluded us from confirming if FCR declined throughout a mother's pregnancy, as expected (DiPietro et al., 2007), but at the population level, we detected an association between FCR and GA. It is known that in the second trimester the mean basal FCR is higher, but mean of

accelerations over 10 and 15 bpm, and mean short-term variation are lower than in the third trimester, which reflects the immaturity of autonomous system and predominant sympathetic tonus in the second trimester (Nomura et al., 2010). In the third trimester, FCR is a clinical indicator of the autonomic response to stress, where the parasympathetic element tries to conserve energy through vagal stimulus leading to fetal bradycardia, while the sympathetic element prepares the fetus for stressful situations (like the delivery) through vasoconstriction and fetal tachycardia (Buhimschi et al., 2008). Higher FCR has been also found in IUGR fetuses in a longitudinal study in UK (Nijhuis et al., 2000), indicating that increased FCR might be an indicator of poor pregnancy outcomes. But the FCR of our population of fetuses with growth below the 10^{th} percentile (140 ± 8) was not significantly different from the FCR of the normal or large for GA groups in the third trimester (141 ± 13 and 137 ± 10 respectively; P= 0. 15).

Furthermore, FCR variability through fetal monitoring provides a more precise measurement of fetal wellbeing at a point in time (Nijhuis et al., 2000), but this was not available in our rural setting. Nevertheless, a high percentage of variability in basal FCR was explained in regression models for both the second and third trimesters, suggesting not only that our simple measure of FCR was responsive to maternal condition but also that it was sufficiently sensitive to detect associations that within the normal range of FCR observed in the majority of women in this study.

Among maternal vital signs, we found that FCR increased with maternal age but decreased with higher maternal systolic blood pressure in the second trimester. We did not find previous reports on those particular maternal factors, but, in the third trimester, we detected a positive relationship between maternal and fetal cardiac rates, which has been described before. Riedl and collaborators identified a consistent synchronization of the maternal heartbeats preceding the fetal beats in relation with maternal respiratory cycles (Riedl et al., 2009).

After controlling for maternal vital signs, our composite model of FCR in the second trimester identified significant associations: a negative relationship with vitamin D together with a negative relationship with severity of scabies, both which were also negatively associated with FH in the third trimester. As mentioned above, vitamin D seems to have a immune-modulatory effect and was negatively associated with CRP in our population (González-Fernández et al., Chapter III). Thus, mothers with lower serum vitamin D had both elevated CRP and higher FCR. Lower FCR variation has been described as product of hypocalcemia, decreased parathyroid hormone and decreased vitamin D levels (Hagay et al., 1986, Memmi et al., 1993), but more specialized tests would be needed to confirm the link between vitamin D levels and cardiac function in our population. The fact that scabies and vitamin D entered together in models for both FH and FCR suggested that these two variables were positively associated; further research should be conducted to see if vitamin D deficiency might be protective against scabies.

PDW is an indicator of platelet deficiency that constantly entered our models in the second trimester. Platelets have been extensively studied in the context of pregnancy-induced hypertension and its complications, where a decreased number has been associated with higher incidence of IUGR (Ertan et al., 2002). We found that PDW, which indicates platelet deficiency, was negatively related with FCR in the second trimester, indicating that a higher number of platelets would promote lower FCR under normal maternal blood pressure conditions.

Our final model stresses the role of acute and chronic infection (indicated by increased neutrophils and monocytes respectively) in combination with higher iron levels as positively related with FCR during the second trimester, which is a novel relationship requiring further investigation, given the important role of iron during this period of time for neurodevelopment (Lozoff and Georgieff, 2006).

Exploratory models for FCR in the third trimester included the presence of two infections, gingivitis and cervicitis. We know from our parallel study on infections (González-Fernández et al., Chapter III), that the mothers with oral and oral plus genital infections had significantly higher CRP. Kim and collaborators described a negative relationship between CRP and GA at delivery (Kim et al., 2011). Our results suggest that further work is required on the links between these mucosal infections, CRP and fetal cardiac physiology.

Vitamin A has an important role during pregnancy (Strobel et al., 2007) particularly in the physiopathology of the fetal heart (Zile, 2001), with both, deficiency and excess as identified causes of congenital malformations (Pan and Baker, 2007), but its relationship with FCR or its patterns has not been described. We found higher vitamin A levels associated with higher FCR in the third trimester, whereas iron and MMN supplements were negatively associated with FCR. To our knowledge, there is only one study from describing how other micronutrients influence FCR. Merialdi and collaborators (Merialdi et al., 2004) reported that supplementation with the combination of zinc, iron and folate decreased FCR and improved FCR variability compared with fetuses of mothers supplemented only with iron and folate. This result is consistent with the pattern detected in our study. The association between FCR and MCHC in the composite model was positive. Together, it appears that the third trimester fetus has a higher FCR with higher maternal hemoglobin levels.

Fetal Movements (As Perceived by Mothers)

Decreasing FM with gestational age is a pattern that has been described using four-dimensional ultrasound (Kurjak et al., 2006), and is what we observed, especially during the third trimester. Also using four-dimensional ultrasound, Basgul and collaborators elegantly showed the patterns of different fetal behavior, demonstrating a tendency on increasing movements in the first and

second trimesters, and a decreasing tendency for others in the third trimester as head retroflection and rotation (Yigiter and Kavak, 2006).

The frequency of fetal movements is known to be influenced by IUGR, where movements are slow with small amplitude preceding the decrease in FCR and hypoxemia; by caffeine intake, where movements are considerably increased accompanied by increased FCR; and by maternal stress that has a positive correlation with FM and with infant activity after birth (Visser et al., 2010). We did not find significant relationships between coffee consumption and FM, maybe due to the low caffeine levels in coffee. We did not evaluate maternal psychological stress, but we measured MCR (influenced among other factors by maternal stress) and it emerged as the stronger maternal factor associated with higher FM in the third trimester.

From the nutritional side, folic acid concentration was negatively associated with FMs, indicating a calmer state of the fetus in mothers with higher folate levels during the second trimester. Interestingly, Schlotz and collaborators recently found that low maternal red cell folate in early pregnancy was associated with higher scores on hyperactivity and peer problems in the offspring, and positively associated with head circumference at birth (Schlotz et al., 2010). Our findings may indicate that FM may be a useful field indicator of folate deficiency in the second trimester.

During the second trimester, FM responded differently to those infections that stimulate a Th2-response (ascariasis and scabies) that were associated negatively with FM compared with those that induce a Th-1 response (trichomoniasis, bacterial vaginosis) which were positively associated with FM. Furthermore, the percent of monocytes was also positively associated with FM, suggesting that maternal inflammation may increase FM during the second trimester. To our knowledge, none of these responses have been previously observed. Whereas FM were lower under conditions of a Th2 immune response in the second

trimester, the opposite was observed in the third trimester. In the third trimester, percent of basophils consistently emerged as positively associated with FM. Basophils are critical for the induction of Th2 immunity, they have a role in anti-parasite immune responses through their activation by immune serum and production of cytokines and they're also involved in mediating immunity against ecto-parasites such as ticks (Min et al., 2011). Basophils are also known mediators of allergic responses (Voehringer, 2011), but none on the mothers reported allergies during the interviews. The relationship between basophils and fetal movements has not been described before. FM also showed the opposite relationship between trimesters with vaginal infections, as FM were lower in mothers with cervicitis in the third trimester. Taken together, these results suggest that a pro-inflammatory state induces FM during the second trimester but reduces FM in the third trimester.

In the third trimester, the combination of a positive association between hematocrit and FM but a negative association between wood smoke exposure and FM suggest that better oxygenation may lead to better fetal behavior. No information is available regarding organic fuel exposure and fetal behavior, and evidence on the acute and chronic effects of maternal smoking on fetal behavior is inconclusive. A Canadian study compared smoking and non-smoking women and found no differences in spontaneous fetal behaviors but a delayed onset of response to the maternal voice in fetuses <37 weeks of smoking women (Cowperthwaite et al., 2007); another older study showed that fetal activity was significantly decreased in smoking mothers compared with non-smoking mothers, and that this difference was independent of FCR variation (Coppens et al., 2001).

CONCLUSIONS

Despite the occurrence of oral, urogenital, intestinal and skin infections, of folate, iron and vitamins A, D and B12 deficiencies and a state of chronic

inflammation in pregnant women in rural Panama, the majority of fetuses were developing normally as indicated by FH, FCR and FM. Our simple field indicators were sufficiently sensitive to detect maternal factors known to influence fetal wellbeing differentially between the second and third trimester. They were also sensitive enough to uncover additional factors that are associated with fetal wellbeing, factors that when included in composite models increased their explanatory power by 10-20%. Our results suggest that the negative influence of some infections and deficiencies on fetal outcomes may be counterbalanced by positive influences of other infections and deficiencies leading to relatively normal growth, highlighting the importance of a holistic approach to maternal-fetal health. Additionally, our study provides valuable information about issues that are not well documented including the effect of wood smoke and scabies on fetal health and gives an insight of those factors that would be interesting targets for future research.

TABLES AND FIGURES

Table 1.a. *List of maternal, environmental and nutritional indicators used as independent variables in sets of regression, logistic regression and ordered logistic regression models*

Maternal variables	**Environmental variables**	**WBC and Platelets**	**RBC indices**	**Serum micronutrient levels**
Age[c] *Adolescent pregnancy*[d] *More than 35 years old*[d] Number of pregnancies[c] *First pregnancy*[d] *5 or more pregnancies*[d] Maternal BMI[c] (Kg/m^2) Systolic and diastolic blood pressure (mmHg)	Iron and/or Multiple micronutrient supplementation[d] (0= no; 1= yes) Coffee consumption[c] (cups/day) Fieldwork[c] (hours/day) Wood smoke exposure[c] (hours/day)	WBC count[c] Number and percent of: *Neutrophils*[c] *Lymphocytes*[c] *Eosinophils*[c] *Basophils*[c] Platelets count[c] PDW[c] (platelets deviation width) Platelecritc (volume of packed platelets)	RBC count Hemoglobin[c] (gr/dL) Hematocrit[c] (%) MCV[c] (fL) Volume of packed cells/ RBC count MCH[c] (pg/cell) Hemoglobin/ RBC count MCHC[c] (gr/dL) Hemoglobin/ Volume of packed cells RDW-SD[c] (fL) Red cell distribution width	Serum Transferrin Receptors[c/log] (sTfRs, µg/mL) Ferritin[c/log] (ng/mL) CRP[c/log] (mg/L) Folic acid[c/log] (nmol/L) Vitamin B12[c/log] (pmol/L) Vitamin D[c] (nmol/L) Vitamin A[c/log] (µg/dL)
c = Continuous; d= Dichotomous; o= Ordinal; log= Logistically transformed				

Table 1b. *List of infections used as independent variables in sets of regression, logistic regression and ordered logistic regression models*

INFECTIONS		
Type of varible	**Dicotomous (d)**	**Ordinal (o) or continuous (c)**
Clinically detected	Respiratory[d]	
	Oral[d] Caries[d] Gingivitis[d]	
	Genital[d] Vaginitis[d] Cervicitis[d]	
Laboratory detected	Uro-Genital VDRL[d] Nugent score (BV)[c] UTI/ AB[d]	Amount of Clue cells (BV)[o] *Trichomona*[o] *Candida*[o] *Gonococci*[o]
	Intestinal *Giardia*[d] *E. coli*[d]	Epg/hpf of *Ascaris*[c] Hookworm[c] *Trichuris*[c]

c = Continuous; d= Dichotomous; o= Ordinal.
Dichotomous variables were categorized: 0= Absent and 1= Present.
For VDRL (Syphilis): 0= Non-reactive; 1= Reactive (any dilution).
Ordinal variables: 0= Absent; 1= Few; 2= Moderate amount; 3= Abundant/high power field (hpf).
BV= Bacterial vaginosis; AB/UTI= Urinary tract infection/asymptomatic bacteriuria; Epg= eggs per gram.

Table 2. *Multiple regression model of maternal BMI controlled for gestational age for environmental factors (weekly portions of protein, coffee consumption and pica), presence of infections (scabies and impetigo) and nutritional factors (anemia and combined deficiency of vitamin D and vitamin A) of 213 pregnant Ngäbe women from Western Panama, pooled across trimesters.*

	MATERNAL BMI		
Independent Variables	β coefficient ± SE	P	Overall Model
Gestational age	0.14 ± 0.02	<0.0001	$F_{8, 204}$ = 13.93 P = <0.0001 Adjusted R^2 = 0.33
Portions of protein/w	0.21 ± 0.06	<0.0001	
Coffee consumption[1]	-1.16 ± 0.51	0.024	
Presence of pica[1]	-2.03 ± 0.71	0.005	
Presence of scabies[1]	-0.63 ± 0.27	0.018	
Presence of impetigo[1]	3.0 ± 1.30	0.022	
Presence of anemia[1]	-0.90 ± 0.36	0.016	
Vitamin D plus vitamin A deficiencies[2]	-2.17 ± 0.92	0.02	
Constant	23.0 ± 0.66	<0.0001	

[1] Codes as 0 = no; 1 = yes

[2] Coded as 0= no; 1= yes, vitamin D deficiency was defined as <50 nmol/L and vitamin A deficiency as levels <20 μg/dL

Table 3a. *Stepwise multiple regression of Fundal Heightin 2nd and 3rd third trimesters*

FUNDAL HEIGHT, cm	**Second Trimester**		**Third Trimester**	
	β Coef ± SE	P	β Coef. ± SE	P
Gestational age, wks	1.14 ± 0.10	<0.0001	0.52 ± 0.070	<0.0001
Wood smoke exposure	-5.33 ± 1.45	<0.0001	–	
Maternal BMI, kg/m^2	–		0.38 ± 0.10	<0.0001
Constant	0.60 ± 2.65	0.82	2.63 ± 3.43	0.44
Overall model fit	$F_{2, 75}$ = 68.45 P < 0.0001 Adjusted R^2= 0.63		$F_{2, 106}$ = 39.89 P < 0.0001 Adjusted R^2 = 0.41	
– indicates that the variable that did not enter the final model				

Table 3b. *Composite multiple regression model of Fundal Height in the 2nd trimester*

FUNDAL HEIGHT, cm	**Second Trimester**		**Overall model fit**
	β Coef. ± SE	P	
Gestational age, weeks	1.03 ± 0.10	<0.0001	$F_{6, 70}$ = 34.91 P < 0.0001 Adjusted R^2 = 0.73
Wood smoke exposure	-6.40 ± 1.28	<0.0001	
Iron plus MMN supplementation	2.14 ± 0.71	0.004	
MCHC, gr/dL	-0.94 ± 0.28	0.001	
Monocytes, %	-0.92 ± 0.45	0.047	
Urinary pH	0.92 ± 0.41	0.029	
Constant	30.8 ± 10.2	0.003	

Table 3c. *Composite multiple regression model of Fundal Height in the 3rd trimester*

FUNDAL HEIGHT, cm	**Third Trimester**		**Overall model fit**
	β Coef. ± SE	P	
Gestational age, weeks	0.52 ± 0.06	<0.0001	$F_{5, 103}$ = 22.73 P < 0.0001 Adjusted R^2 = 0.50
Maternal BMI, kg/m^2	0.40 ± 0.09	<0.0001	
Vitamin D, nmol/L	-0.04 ± 0.016	0.005	
Severity of scabies	-0.91 ± 0.38	0.020	
Coffee consumption, cups/day	0.55 ± 0.26	0.037	
Constant	3.62 ± 3.35	0.30	

Table 4a. *Stepwise multiple regression of Fetal Cardiac Rate - related variables in the second and third trimesters*

FETAL CARDIAC RATE, beats/min	**Second Trimester**		**Third Trimester**	
	βCoef. ± SE	P	βCoef. ± SE	P
Maternal age, years	0.51 ± 0.19	0.012	–	
Maternal systolic blood pressure, mmHg	-0.35 ± 0.14	0.019	–	
Maternal cardiac rate, beats/min	–		0.18 ± 0.08	0.023
Constant	165.9 ± 16	<0.0001	124.1 ± 6.7	<0.0001
Overall model fit	$F_{2, 30}$ = 7.93 P = 0.0017 Adjusted R^2=0.30		$F_{1, 106}$ = 5.32 P = 0.023 Adjusted R^2= 0.03	
– indicates that the variable that did not enter the final model				

Table 4b. *Composite multiple regression model of Fetal Cardiac Rate in the second trimester*

FETAL CARDIAC RATE, beats/min	**Second Trimester**		**Overall model fit**
	βCoef. ± SE	P	
Age, years	0.84 ± 0.19	<0.0001	$F_{6, 25}$= 6.78 P= 0.0002 Adjusted R^2= 0.53
Systolic blood pressure, mmHg	-0.37 ± 0.11	0.004	
Platelets deviation width	-7.80 ± 4.14	0.071	
Ferritin, log ngr/mL	3.06 ± 1.46	0.047	
Monocytes, number x 10^3	38.91 ± 13.71	0.009	
Neutrophils, %	0.39 ± 0.18	0.039	
Constant	232 ± 67	0.002	

Table 4c. *Composite multiple regression model of fetal cardiac rate in the third trimester*

FETAL CARDIAC RATE, beats/min	**Third Trimester**		**Overall model fit**
	βCoef. ± SE	P	
Maternal cardiac rate, beats/min	0.16 ± 0.07	0.026	$F_{6, 100}$ = 5.93 P < 0.0001 Adjusted R^2= 0.22
Maternal height, cm	0.53 ± 0.22	0.021	
Presence of gingivitis	4.91 ± 2.0	0.015	
MCHC, gr/dL	2.03 ± 0.82	0.016	
Vitamin A, log μg/dL	7.60 ± 0.02	0.025	
Iron plus MMN supplementation	-4.53 ± 2.20	0.042	
Constant	-43.03 ± 44.05	0.33	

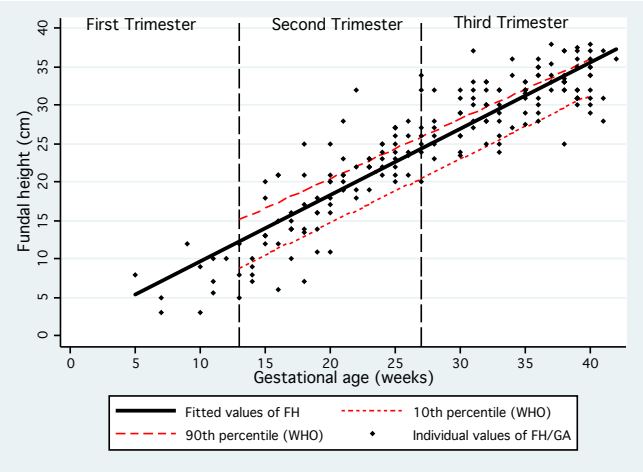
Table 5a. *Composite multiple regression of Fetal Movements in the second trimester*

PERCEIVED FETAL MOVEMENTS/HOUR	**Second Trimester**		**Overall model fit**
	βCoef. ± SE	P	
Presence of vaginal trichomoniasis	3.64 ± 1.39	0.012	$F_{5, 42} = 5.48$ P = 0.0006 Adjusted R^2= 0.32
Presence of bacterial vaginosis	3.00 ± 1.40	0.038	
Monocytes, %	1.72 ± 0.58	0.006	
Presence of scabies	-2.88 ± 1.15	0.017	
Folic acid, nmol/L	-0.12 ± 0.05	0.021	
Constant	0.16 ± 2.98	0.95	

Table 5b. *Composite multiple regression of Fetal Movements in the third trimester*

PERCEIVED FETAL MOVEMENTS/HOUR	**Third Trimester**		**Overall model fit**
	βCoef. ± SE	P	
Maternal cardiac rate, beats/min	0.07 ± 0.02	0.002	$F_{5, 84} = 5.59$ P < 0.0001 Adjusted R^2= 0.21
Basophils, $x10^3/mm^3$	51 ± 21.5	0.020	
Hematocrit, %	0.17 ± 0.08	0.041	
Wood smoke exposure, hours/day	-0.40 ± 0.20	0.048	
Presence of cervicitis	-1.35 ± 0.60	0.028	
Constant	-1.65 ± 3.75	0.66	

Figure 1a. Scatter plot fundal height (FH) for gestational age (GA)



Markers indicate observations of fundal height for gestational age with associated linear regression (solid black line). Dashed vertical lines divide GA into trimesters. Dashed diagonal lines indicate the 10th and 90th percentiles of FH/GA according to WHO.

Figures 1b and 1c. Scatter plot of fetal cardiac rate (FCR) and fetal movements (FM) in the second and third trimesters.

Markers indicate observations of FCR (n= 33, 108) and FM (n= 48, 93) in the second and third trimester, showing the fitted lines. Cutoffs for normality are shown as dashed lines. GA was negatively associated with FCR (P= 0.049), but no significant relationship was found between GA and FM (P= 0.5).

Fig. 1b.

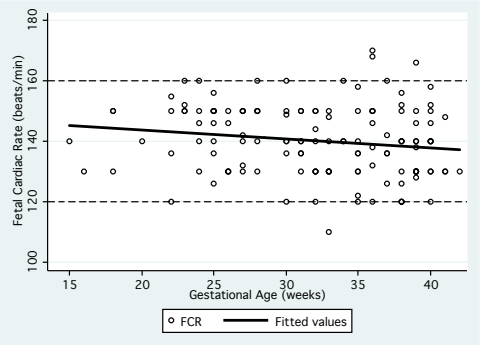


Fig. 1c.

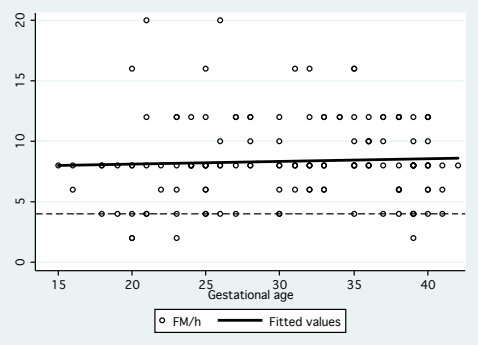


Figure 2. *Frequency of known risk factors during pregnancy.*
Histograms of (a) age; (b) parity; (c) exposure to indoor wood smoke; (d) weight for height; and (e) anemia. Dashed lines indicate cutoffs for risk according to WHO.

Fig. 2a.

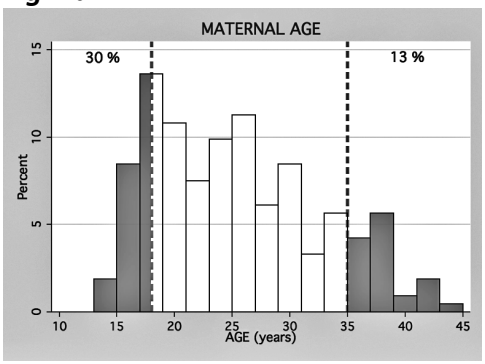


Fig. 2b.

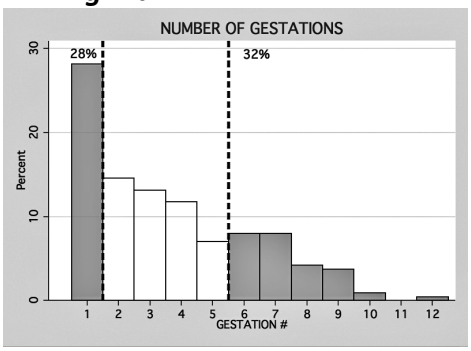


Fig. 2c.

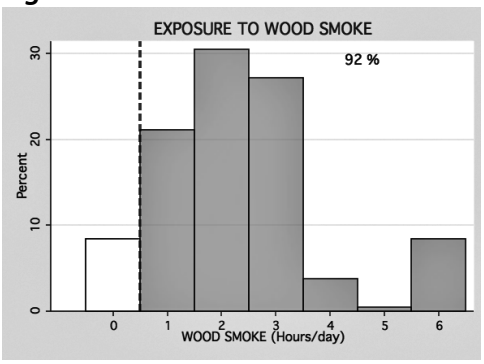


Fig.2d.

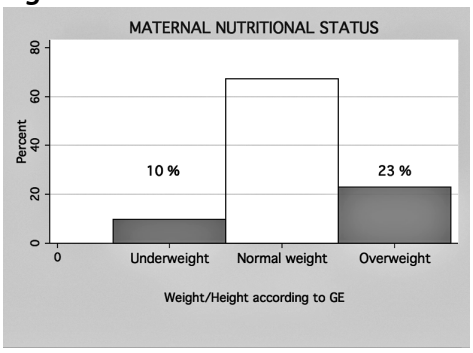
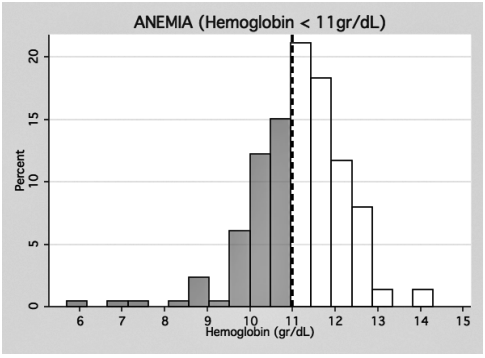


Fig. 2e.



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FINAL CONCLUSIONS

To our knowledge, this is the first study to examine the interrelationship among a large number of mild-moderate infections and micronutrient deficiencies in a non-malaria non-HIV population of pregnant women living in extreme poverty and their combined effect on fetal health. Indigenous pregnant women (n=213) belonging to the Ngäbe-Buglé community in rural Panama were assessed for respiratory, oral, skin, urogenital infections and intestinal parasites, and for iron, folic acid, vitamin B12, vitamin A and vitamin D deficiencies in a cross sectional study. Fetal wellbeing was assessed through simple field measurements. Infections and micronutrient deficiencies were extremely prevalent, with all mothers affected by at least one condition and most affected by several. Inflammation indicated by high levels of CRP was found in 16.5% of the mothers. CRP was significantly higher in women with oral infection and/or respiratory infections alone or in combination with genital infection or hookworm infection. CRP was significantly lower in women with lower levels of iron and vitamin B12, in women with vitamin D levels above 75 nmol/L and vitamin A levels above 20 μg/dL. Intra-uterine growth retardation was found in 7% of the fetuses, 27% were categorized as large for gestational age, but the majority were found as adequate for gestational age (66%). FCR and FM were within normal limits. Indicators of infections, micronutrient deficiencies, and maternal inflammation were associated in complex manner with our simple measures of fetal wellbeing. Indicators of both nutritional status and infection entered regression models for FH, FCR and FM, indicating early fetal responses to mild-moderate maternal infections and early nutritional deficiencies.

The high prevalence of infections and micronutrient deficiencies in our population are the result of multi-factorial social and economical conditions. Factors affecting maternal health in the Ngäbe-Bugle community in Panama are complex and start with the difficult access to health facilities, lack of roads, electricity, and water and sanitation services. Furthermore, traditional beliefs

make home delivery the first option for majority of pregnant women. These deliveries are attended by traditional midwives, who have experience but minimal theoretical knowledge. Also the majority of them are not able to read and write. Government programs encourage monthly professional pregnancy follow up through the implementation of conditional cash transfer in the region that includes delivery of multiple-micronutrient supplements to the mother. The implementation of such kind of programs brings benefits the health of mothers and children (Halpenny et al., 2012), and during pregnancy we observed that iron and micronutrient supplementation contributed to a lower prevalence of respiratory infections, less severity of BV and decreased CRP in the mother, better fetal growth in the second trimester and to modulate the FCR in the third trimester when both, iron and micronutrients are taken together. Despite high levels of infection and deficiencies exist, the majority of fetuses in our study were growing within normal limits.

Of interest was the high prevalence of sexually transmitted infections, with over 90% of pregnant women infected with at least one vaginal pathogen. We suspect that this may be related to the extreme poverty that forces men to migrate to other provinces in the search of job opportunities, usually in agricultural labor, leaving women at home in charge of their children. Such social conditions favor promiscuity of the men and subsequent transmission of infections such as vaginal trichomoniasis and gonococcal infection and syphilis to the women. Health and sexual education could positively impact these problems.

Periodontal disease also emerged in these women. Of all the potential relationships with inflammation, presence of oral infection was associated with high levels of CRP. Moreover, iron deficiency was also associated with severity of caries. These results support the few other studies that have linked oral health with anemia (Pradeep and Anuj, 2011).

The problem of anemia is more complex due to its multiple etiologic factors. Early detection and treatment of infections would help to reduce the anemia related with inflammation, but this would require extra effort from the health system since it involves transportation and analysis of laboratory samples, and the delivery of treatment to remote areas. Local health staff also need to be aware that the provision of multiple micro-nutrients to pregnant women is not enough to improve their nutritional status, and that intestinal parasites are not the only infections of these women. Furthermore, deworming in the presence of co-occurring other parasitic, fungal or bacterial infection could interfere with the "balance" of the Th2 response that intestinal parasites might be providing.

From our findings we suggest that iron supplementation should be given only after a complete assessment of infections and treatment, in order to prevent an increase in infections that seem to be iron-dependent such as vaginal trichomoniasis, UTI and ascariasis. In fact, any kind of supplement should be ideally provided when infections have been ruled out or treated. Such is the case of vitamin B12, the most common micronutrient deficiency and the second more important cause of anemia after iron deficiency. Beyond the improvement of micronutrient status through supplementation, it would be important to identify affordable ways to increase protein ingestion in order to improve vitamin B12 and hemoglobin status, both which emerged as important protector factors against several infections.

Interestingly, the impact of vitamin D on infections differed markedly depending on the pathogen. Low levels of vitamin D increased severity of gonococcal infection, *Ascaris* epg and risk of BV whereas high levels of vitamin D were positively related to the severity of trichomoniasis, candidiasis, hookworm and trichuriasis and higher risk of caries and AB/UTI. Overall vitamin D was negatively associated with CRP. Furthermore, vitamin D deficiency together with scabies were associated with poor fetal growth. These findings on vitamin D raise questions about strategies for supplementation during pregnancy and

suggest an important area for further studies, because of its positive relationship with some infections (UTI, trichuriasis), and its negative relationship with fundal height in the third trimester.

Our study had a few limitations. Our sample size in the first trimester precluded us from making inferences about early fetal development. Research tools for assessing fetal wellbeing are typically more sophisticated than those that were feasible in this study. Had we been able to use such techniques, it is likely that additional impacts of the multiple infections and multiple micronutrient deficiencies on fetal health would have been observed. Oral infections were only assessed through a clinical exam. Involvement of a dentist in future studies is warranted. We were extremely fortunate to work with a wealth of outcome variables. Although it may appear that a large number of regression models were run, we were careful to focus our analysis using a well-thought-through strategy. This allowed us to describe associations among infections of multiple organ systems, several micronutrients, indicators of inflammation and fetal growth in a manner that, to our knowledge, has not been done before. The results provide a number of hypotheses for further investigation.

We add valuable information about the usefulness of simple clinical parameters: FH, FCR and FM in the evaluation of fetal health in extremely impoverished areas lacking of health infrastructure. We were able using these simple techniques identify health risks and benefits to mothers and there fetuses. There could still be enormous benefits from introducing ultrasound together with trained health workers to this impoverished region. We believe that more accurate measurements of fetal health would identify other health risks and benefits results in addition to the ones found.

To date, the vast majority of epidemiological research on infection and malnutrition during pregnancy in vulnerable populations has focused on a very limited number of explanatory variables of fetal growth while controlling for

known risk factors. Through this study, we have first demonstrated that pregnant women in our study are simultaneously infected with a wide range of pathogens and experience deficiencies of many micronutrients. It is our thesis that advances in maternal/infant health require a better understanding not only of this set of health challenges, but also of the complex set of interactions that occur among them, and their impact on maternal and fetal health. The research presented in this thesis provides a key first step in advancing research to this new level.

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APPENDIX 1. QUESTIONNAIRE AND CLINICAL FORM FOR PREGNANT WOMEN

***Code No._____ Age_____ Provenance:_____**

Date of last menstrual period_____Gestational age according to LMP_____

Trimester ☐1st ☐2nd ☐3rd

G_____T_____P_____A_____L_____

History and description of pregnancy complications_____

Current medication (including iron/vitamins)_____

Smoking ☐No ☐Yes. How many cigarettes per day?_____

Alcohol: ☐ No ☐ Yes Which drink and how many cups per day?_____

***In the last week, how many times have you eaten: Animal products____, yellow fruits____, green vegetables____?**

***Pica ☐No ☐Yes Which kind? _____ Since when? _____Frequency_____**

***Coffee consumption: ☐ No ☐ Yes How many cups per day? _____**

***Wood smoke exposure ☐No ☐Yes Hours of exposure per day_____**

***Number of fieldwork hours/day _____**

PHYSICAL EXAMINATION: Weight:_____Height:_____***Skin type_____**

Heart rate_____ Blood pressure_____ Temperature_____

Fundal Height_____Fetal cardiac rate_____Number of fetal movements in 1 hour_____

Fetal lie: ☐ longitudinal ☐ transverse ☐ oblique

Infection: Classified as L=mild (ambulatory treatment); M=moderate (requiring close follow up) or S=severe (near-miss obstetrical event).

Time of onset	Respiratory	Gastro-intestinal	Uro-genital	Cutaneous	Diagnosis

Non-infectious complications:

☐IUGR ☐Fetal distress ☐Pregnancy induced hypertension ☐Second trimester bleeding

Other: _____

Filled by:_____Date:_____

INSTRUCTIVE FORM:

For field assistants: Please provide a code to the participant and fill up **bold (*)** sections previous to consultation. Ask the mother to start counting fetal movements while waiting for consultation. Include the form into the patient's file.

For physicians:

Nägele's Rule for calculating gestational age according to last menstrual period:

LMP= 1st day of LMP + 7 days – 3 months

First trimester: From detection of pregnancy (around week 5) – week 12

Second trimester: Weeks 13-27

Third trimester: 28 weeks or more

G: Gravidity: Total number of pregnancies of any gestation. Includes current pregnancy, abortions, ectopic pregnancies and hydatidiform moles (twins count for one pregnancy)

T: Number of term infant delivered (>37 weeks)

P: Number of premature infants delivered (20-37 weeks)

A: Number of abortions (loss of intrauterine pregnancy prior to viability of fetus, meaning <20 weeks and/or <500 gr fetal weight), either induced (therapeutic) or spontaneous (miscarriage).

L: Number of living children.

In current medication, please include the generic name of the medication, the dosage administrated and the time it has been prescribed for.

Please write patient's skin type I-VI according to FZ classification:

Fitzpatrick (FZ) scale measures of skin phototypes (Roberts, 2009)

Type FZ I white skin	Always burns, never tans
Type FZ II white skin	Always burns, minimal tan
Type FZ III white skin	Burns minimally, tans moderately and gradually
Type FZ IV light brown skin	Burns minimally, tans well
Type FZ V brown skin	Rarely burns, tans deeply
Type FZ VI dark brown/black skin	Never burns, tans deeply

Fetal movements: The presence, decrease or absence of fetal movements will be recorded in mothers during their second and third trimester using Sadovsky's method: A member of the RT will ask mothers to start counting fetal movements while they wait

for consultation. Four movements should be felt within one hour, if not, patients should monitor movement for a second hour. If after two hours four movements have not been felt, further evaluation is needed to assess fetal viability (Boog, 1988; Heazell & Froen, 2008)

Please write diagnosis of infection in the table, signaling if it's mild, moderate or severe. Any others non-infectious diagnoses are written at the end of the form.

Please stick a small mark in the file to recognize that the participant is already included, in order to avoid repetitive inclusion.

Thank you!

Maternal increase of weight and fundal height according to gestational age

Incremento del Peso Materno (Kg) Percentilos		Semanas	Altura Uterina ** (cm) Percentilos	
25	90		10	90
0.4	3.5	13	8.0	12.0
1.2	4.0	14	8.5	14.5
1.3	4.5	15	9.5	15.0
1.8	5.4	16	11.5	18.0
2.4	6.1	17	12.5	18.0
2.6	7.0	18	13.5	19.0
2.9	7.7	19	14.0	19.5
3.2	8.3	20	15.0	21.0
4.1	8.6	21	15.5	21.5
4.5	9.4	22	16.5	22.5
4.8	10.2	23	17.5	23.0
5.1	10.8	24	18.5	24.0
5.6	11.3	25	19.5	25.5
5.9	11.6	26	20.0	25.5
6.1	11.7	27	20.5	26.5
6.4	11.9	28	21.0	27.0
6.6	12.1	29	22.5	28.0
7.0	13.5	30	23.5	29.0
7.1	13.9	31	24.0	29.5
7.6	14.5	32	25.0	30.0
7.7	14.7	33	25.5	31.0
7.9	15.0	34	26.0	32.0
7.9	15.4	35	26.5	33.0
8.0	15.6	36	28.0	33.0
8.0	15.8	37	28.5	34.0
8.0	16.0	38	29.5	34.0
8.0	16.0	39	30.5	34.0
8.0	16.0	40	31.0	34.5

Fundal height measurements are obtained from the superior border of pubis to the bottom of the uterine fundus, displacing the tape measure between the 2nd and 3rd fingers.