Interpretation of maternal and fetal biomarkers in a population with coexisting nutrient deficiencies and infections

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Abstract

Background: Evaluation of pregnancy in remote and impoverished settings, where multiple infections and nutrient deficiencies co-occur, is a clinical challenge. The objective of this thesis was to identify women at risk for adverse pregnancy outcomes with biomarkers of maternal and fetal health in a remote field setting. Thus, in four manuscripts, associations of biomarkers C-reactive protein (CRP), anemia/iron status indicators, blood pressure measurements and symphysis-fundal height (SFH) with multiple infections (caries, scabies, uro-genital infections and intestinal nematodes), nutrient deficiencies (folic acid, vitamins A, B_{12}, D and protein) and inflammation (white blood cells and cytokines) were explored.

Methods: A cohort of 213 indigenous women in rural Panama was recruited during their routine pregnancy follow-up. Information about diet, intake of supplemental iron and multiple-micronutrients (MMN), and exposure to wood smoke was gathered. A physical exam evaluated anthropometrics, blood pressure (systolic (SBP) and diastolic (DBP)), from which mean arterial pressure (MAP) and pulse pressure were calculated. Also, in the absence of sonography, fetal size was evaluated using symphysis-fundal height (SFH) Z-scores, according to new international standards for the detection of small for gestational age. Infections were evaluated both clinically and using laboratory methods available in the field, and a subsample of 120 women provided stool samples for parasitic analyses. Caries, scabies, urinary tract infection, vaginal bacterial, fungal and parasitic infections, as well as intestinal nematodes were detected. Blood samples provided information on complete blood cell count, and serum was processed for iron status indicators (ferritin, serum iron, serum transferrin receptor –sTfR), inflammation biomarkers (C-reactive protein –CRP and cytokines), and hepcidin, and several nutritional status indicators including protein status (retinol-binding protein) and micronutrients (folic acid, vitamin B_{12}, vitamin A and D). Multiple logistic and linear regression models for biomarkers of maternal and fetal health were utilized to evaluate possible associations with infections, nutrients and inflammation indicators.

Results: In *Paper 1 (CRP)*, inflammation indicated through elevated CRP was found in 20.8%, whereas mild-moderate infections were associated both positively and negatively with CRP. Higher CRP was associated with the presence of caries (P= 0.012) and the intestinal parasite hookworm (P= 0.014), but normal vaginal bacteria (P= 0.02) and *Ascaris* (P= 0.002) were associated with lower CRP. Folic acid, and vitamins A, B_{12}, and D did not show significant associations with CRP, but wood smoke exposure was associated with increased odds of elevated CRP (P= 0.034). Manuscript 1 set the context to characterize the range of adverse factors present in this population of pregnant women having **M**ultiple **I**nfections, **N**utrient **D**eficiencies and **I**nflammation (MINDI).

In *Paper 2 (ANEMIA/IRON)*, it was found that anemia (38%) was not only associated with iron deficiency (P= 0.002), but also with lower folic acid (P= 0.044), lower vitamin A (P= 0.045) and with hepcidin >6.1μg/L (P= 0.023); lower hemoglobin was also associated with higher CRP (P= 0.018). All iron status indicators (ferritin, serum iron and sTfR) had positive and negative associations with MINDI, particularly sTfR which showed that erythropoiesis was negatively associated with IL17 (P= 0.015) but positively associated with IL13 (P= 0.037) and therefore, sTfR was not useful for detecting iron deficiency in this context of MINDI. By combining low ferritin and low serum iron, iron deficiency was found in 77.9%. High hepcidin concentrations confirmed the presence of iron restriction due to inflammation in 71.4%. Multi-causal anemia and associations of iron status indicators with MINDI helped to explain the lack of association between the time that mothers took iron supplements and Hb concentrations.

In *Paper 3 (BLOOD PRESSURE)*, no hypertension was found using conventional SBP ≥140 mmHg or DBP ≥90 mmHg, but MAP, a known predictor of hypertension in pregnancy, was elevated in 11.3%. Bi-directional associations of MAP with MINDI were found. Higher MAP was associated with protein-deficiency (RBP<30 mg/L, P= 0.015), higher MMN intake (P= 0.020), higher TNFα (P= 0.033) and hookworm infection (P= 0.016), but lower MAP was associated with *Ascaris* (P= 0.028) and *Trichomonas vaginalis* (P= 0.009) infections. On the other hand, hypotension (SBP/DBP <100/60 mmHg), which also leads to adverse outcomes, was found in 24.4%. The

odds of low blood pressure was increased by the presence of *Ascaris* (P= 0.040), but decreased by higher IL17 (P= 0.016), higher intake of MMN/d (P= 0.001) and more animal-source foods/wk (P= 0.012). Among blood pressure measurements, by creating factor variables, only pulse pressure <30 mmHg was associated with lower SFH-Z scores but predicted only 6% of the variability of SFH, which lead us to investigate other determinants.

In *Paper 4 (FETAL GROWTH)*, using INTERGROWTH standards in women ≥16 wk gestation (n= 174), half of the fetuses were below the 10th centile. Logistic regression analyses allowed to differentiate two distinct subgroups: small for gestational age –SGA (SFH between the 3rd and the 10th centiles, 12.7%), and very small for gestational age –VSGA (below the 3rd centile, 38%). SGA was associated with higher serum iron (P= 0.016) and a pro-inflammatory response [higher lymphocytes (P= 0.013) and IL17 (P= 0.001) but lower IL10 (P= 0.023)], whereas VSGA was associated with low pulse pressure (P= 0.014), low protein status (P= 0.039), higher hepcidin (P= 0.008) and the presence of *Trichuris* (P= 0.025). Higher TNFα (P= 0.006) (which was associated with higher MAP), and higher eosinophil count (P= 0.044) decreased the odds of VSGA.

Conclusions: Results show that multiple chronic mild-moderate infections are able to modulate inflammation in pregnancy. In general, a pro-inflammatory Th1 environment was associated with adverse conditions in pregnancy (lower iron status/iron restriction due to inflammation, higher MAP, low SFH), whereas a Th2 response was associated with a better iron status, lower MAP and higher SFH for gestational age. Several nutrient deficiencies contributed to anemia (iron, folic acid and vitamin A) and protein deficiency was associated with increased MAP, lower pulse pressure, and with poor fetal growth. These findings highlight the need for adjusting current public health policies, especially universal iron/MMN supplementation and deworming. A comprehensive evaluation and therapeutic approach for assessing maternal nutritional status (including iron status and protein deficiency) and controlling infections is necessary in order to minimize inflammatory responses during pregnancy in an effort to improve maternal and fetal outcomes in the MINDI context.

Résumé

Introduction: L'évaluation de la grossesse dans des régions éloignées et pauvres, où des infections et des carences nutritionnelles coexistent, est un défi clinique. L'objectif de cette thèse était d'identifier, dans des régions éloignées, les femmes à risque d'aboutir à une grossesse défavorable, à l'aide d'indicateurs de santé maternelle et fœtale. Quatre études ont alors été réalisées pour explorer les associations de la protéine C-réactive (CRP), des marqueurs du bilan martial, de la pression artérielle et de la hauteur utérine, avec des infections (la caries, la gale, des infections uro-génitaux et des parasites intestinaux), les carences nutritionnelles (acide folique, vitamines A, B_{12}, D et protéine) et l'inflammation (globules blancs et cytokines).

Méthodes: Une cohorte de 213 femmes autochtones, vivante dans les zones rurales du Panama, a été recrutée au cours de leur suivi de grossesse. Ces femmes ont fait l'objet d'un questionnaire sur leur régime alimentaire, la prise de suppléments en fer et de micronutriments, et sur les heures d'exposition à la fumée de bois. Il a été également relevé les indicateurs anthropométriques, la pression artérielle systolique (PAS) et diastolique (PAD), à partir desquelles la pression artérielle moyenne (PAM) et la pression différentielle ont été calculées. De plus, et due à l'absence d'échographie, la taille du fœtus a été évaluée à l'aide de la hauteur utérine qui a été comparée avec des valeurs de Z conformément aux nouvelles valeurs centrées réduites internationales pour la détection des retards de croissance. Des infections ont été évaluées cliniquement et biologiquement à l'aide de méthodes de laboratoire disponibles sur le terrain, et une sous-population de 120 femmes a fourni des échantillons de selles pour l'étude des parasitoses. Des caries, de la gale, des infections urinaires, des infections vaginales bactériennes, fongiques et parasitaires, et aussi des nématodes intestinaux ont été identifiés. Les échantillons de sang ont fourni des informations sur la formule sanguine complète et le sérum a été utilisé pour le bilan martial (ferritine, fer sérique, récepteur soluble de la transferrine –sTfR), des biomarqueurs de l'inflammation (la CRP et les cytokines), l'hepcidine, le statut protéique (protéine de liaison du rétinol) et des micronutriments (acide folique, vitamine B_{12}, vitamines A et D). Des modèles de régression logistique et linéaire multiples pour les biomarqueurs de la santé maternelle et fœtale ont été utilisés pour évaluer

des possibles associations avec les infections, les nutriments et les indicateurs de l'inflammation.

Résultats: *Dans l'étude 1 (CRP)*, l'inflammation indiquée par une CRP élevée était retrouvée chez 20,8% des femmes, alors que les infections légères à modérées étaient liées positivement et négativement à la CRP. Une CRP plus élevée était associée à la présence de caries (P= 0.012) et à un parasite intestinal, l'*Ankylostome* (P= 0.014), mais les bactéries vaginales commensales (P= 0.02) et l'*Ascaris* (P= 0.002) étaient au contraire associées à une CRP plus basse. L'acide folique et les vitamines A, B_{12} et D n'ont pas montré d'association significatives avec la CRP, mais l'exposition à la fumée de bois était associée à un rapport de vraisemblance accrue de CRP élevée (P= 0.034). L'étude 1 a défini un contexte pour caractériser l'ensemble de facteurs adverses qui sont présents dans cette population de femmes enceintes avec des infections/carences en nutriment multiples et inflammation (MINDI).

Dans l'étude 2 (ANÉMIE / FER), il a été établi que l'anémie (38%) était liée non seulement à une carence en fer (P= 0.002), mais également à des concentrations plus basses en acide folique (P= 0.044), en vitamine A (P= 0.045) et en hepcidine >6.1µg/L (P= 0.023). Des niveaux d'hémoglobine plus basses ont été associés à une CRP plus élevée (P= 0.018). Tous les éléments du bilan martial présentaient une association positive et négative avec MINDI, en particulier le sTfR qui a montré que l'érythropoïèse était négativement associé avec IL17 (P= 0.015), mais positivement associé avec IL13 (P= 0.037) et que, par conséquent, le sTfR n'a pas été utile pour détecter une carence en fer. La combinaison d'une ferritine et du fer sérique bas, a mieux détecté une carence en fer dans 77.9% des cas. Des concentrations élevées en hepcidine ont confirmé la présence d'une restriction en fer due à l'inflammation dans 71.4% des cas. L'anémie multi-causale et les associations d'indicateurs du statut en fer avec des MINDI ont permis d'expliquer l'absence d'une association entre le temps du traitement de la supplémentation en fer chez les mères et les concentrations d'hémoglobine retrouvées.

Dans l'étude 3 (PRESSION SANGUINE), aucune hypertension artérielle n'a pas été détectée à l'aide de la PAS ≥140 mmHg ou de la PAD ≥90 mmHg conventionnelles, mais la PAM, facteur prédictif connu des troubles hypertensifs de la grossesse, était élevée chez 11,3%. Des associations bidirectionnelles de PAM avec MINDI ont été observées. Une PAM plus élevée a été associée avec un déficit en protéine (RBP <30 mg/L, P= 0.015) et une quantité plus élevée de micronutriments consommée par jour (P= 0.020), avec un TNFα plus élevé (P= 0.033) et avec une infection par l'*Ankylostome* (P= 0.016). Par contre, une PAM plus basse a été associée avec une infection par *Ascaris* (P= 0.028) ou par *Trichomonas vaginalis* (P= 0.009). Par ailleurs, une hypotension (PAS / PAD <100/60 mmHg), entraînant également des effets indésirables, a été retrouvée chez 24,4% des cas. Le rapport de vraisemblance d'hypotension artérielle était augmenté par la présence d'*Ascaris* (P= 0.040), mais diminuée par une IL17 plus élevée (P= 0.016), par une consommation plus élevée de micronutriments journalière (P= 0.001) et d'aliments d'origine animale par semaine (P= 0.012). Parmi les mesures de pression artérielle, seule la pression différentielle <30 mmHg était associée à la hauteur utérine pour l'âge gestationnel plus bas, mais ne prédisait que 6% de la variabilité de la hauteur utérine, ce qui nous a amenés à étudier d'autres déterminants.

Dans l'étude 4 (CROISSANCE FOETALE), en comparant la hauteur utérine de la population avec les données INTERGROWTH-21st chez les femmes avec une âge gestationnel ≥16 sem (n= 174), la moitié des fœtus se situaient sous le 10ème centile. Des analyses de régression logistique ont alors permis de distinguer deux sous-groupes distincts : petit pour l'âge gestationnel (PAG) (Hauteur utérine entre le 3ème et le 10ème centile, 12,7%) et très petit pour l'âge gestationnel (TPAG) (inférieur au 3ème centile, 38%). Le PAG était associé à un fer sérique plus élevé (P= 0.016) et à une réponse pro-inflammatoire [lymphocytes (P= 0.013) et IL17 (P= 0.001) plus élevée, mais IL10 plus faible (P= 0.023)], tandis que le TPAG était associé à une pression artérielle différentielle basse (P= 0.014), à un statut protéique faible (P= 0.039), à une concentration plus élevée en hepcidine (P= 0.008) et à la présence de *Trichuris* (P= 0.025).

Un TNFα plus élevé (P= 0.006) (qui avait été associé à une MAP plus élevée), et un nombre d'éosinophiles plus élevé (P= 0.044) ont été associés à un rapport de vraisemblance diminué de TPAG.

Conclusions: Les résultats suggèrent que plusieurs infections chroniques légères à modérées sont capables de moduler l'inflammation pendant la grossesse. En général, un environnement pro-inflammatoire (Th1) était associé à des conditions défavorables pendant la grossesse (statut inférieur en fer / restriction en fer due à l'inflammation, une pression artérielle moyenne (PAM) élevée, une hauteur utérine diminuée), alors qu'une réponse Th2 était associée à un meilleur statut en fer, à une PAM inférieure et à une hauteur utérine supérieure pour l'âge gestationnel. Plusieurs carences en éléments nutritifs ont contribué à l'anémie (fer, acide folique et vitamine A), tandis qu'une carence en protéine était associée à une PAM accrue, mais aussi à une diminution de la pression différentielle et à une faible croissance fœtale. L'ensemble de ces éléments soulignent la nécessité d'ajuster les politiques de santé publique actuelles, notamment la supplémentation universelle en fer et en micronutriments, et le déparasitage systématique. Une évaluation complète et une approche thérapeutique pour définir l'état nutritionnel de la mère (y compris le statut en fer et la carence en protéines) et le contrôle des infections sont nécessaires afin de minimiser les réponses inflammatoires durant la grossesse et améliorer les résultats pour la mère et le fœtus dans le contexte des MINDI.

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I feel honored and grateful for having had the opportunity to work with two of the best in their respective fields, Dr Kristine Koski and Marilyn Scott. I specially thank them for being able to read my skills beyond my capacity to express them, for their understanding of my sometimes different way to see things, which has opened already some new opportunities to work with international collaborators. Throughout the time of my Master's and PhD, I learned from them the essential values held by McGill tradition. Clean and honest research, put into practice not only with hard work but also with kindness. Their influence has made a deep impression in the professional I have become.

During my time in Panama, Emerita Pons accompanied me in all the process of getting government permits, ethical approval, laboratory materials, and was a key person all the way through the field work, transportation and analyses of samples. Recruitment and collaboration of nurses at the Health Centers would not have been possible without Delfina Rueda, who kindly put all her energy on the project. Administrative and Laboratory staff at San Felix Hospital, and at the Ngäbe Region Health Department, as well as collaborators from the Ministry of Health (Odalis Sinisterra), and the University of Panama (Dr. Enrique Murillo) were important for the development of the fieldwork. 'Teacher Elsa' and her family, Dr. Cesar Gantes, Lachlan Crawford and Antonio Mendoza, loyal friends and companions provided warmth support along the field part of the research. I thank my lab mates in Montreal: Carli, Marie-Pierre, Maurice, Lisa, Brock, Zsofia, Karine, Hillary, Anne-Marie, Chen, Revathi and Manjurul, for their encouragement and support.

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I also want to thank all women who participated in this research, with the hope that the content of this thesis may serve as support for making public health changes in benefit of their communities.

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Thanks to my brother Cesar, his wife Ruth and her Canadian family for opening their home to me during the time of statistical analyses and writing. To my mother Sara and my brother Silvio who from Colombia have followed me in every step of my career; they are my strength and motivation. I finally want to dedicate this thesis in loving memory of my father Cesar Alberto Gonzalez Medina.

Contribution to original knowledge

This thesis studied a population of indigenous pregnant women living in extreme poverty in rural Panama, in Central America. The research developed in a rural context, in health centers with limited water and electricity services, and with a reference hospital with basic laboratory technology. Pregnant women had difficult access to health care, and had multiple socio-demographic risks for adverse pregnancy outcomes. Novel findings from the four manuscripts in this thesis are highlighted below.

Paper 1. **C-reactive protein is differentially modulated by co-existing infections, vitamin deficiencies and maternal factors in pregnant and lactating indigenous Panamanian women**

Multiple infections that are prevalent in our population, oral, skin, uro-genital infections and intestinal nematodes, are known to elicit different types of immune responses, by stimulating the production of cytokines categorized as Th-1, Th-2 or Th-17. To determine inflammation, we used peer-reviewed cut-offs by trimester, finding that 20.8% of pregnant women had elevated CRP, which contrasts with 95% of the women having at least two infections. Our findings indicate first, that women at gestational age 25 ± 12 wk with elevated CRP were at risk of complications later in pregnancy, and second, that despite multiple infections, there are factors modulating the immune response, so pregnancy can be conserved.

There were basically three infections associated with higher CRP or increasing the risk of elevated CRP: presence of dental caries, hookworm and diplococcal infection. Caries has been described before as a risk factor for adverse pregnancy outcomes related with inflammation, but our study first describes hookworm and *Diplococcus* as associated with systemic low-grade inflammation in pregnancy. On the other hand, it was surprising to find that *Ascaris* and vaginal commensal or pathogenic bacteria were associated with lower CRP, explaining in part the relative low prevalence of inflammation given the amount of combined infections, and proposing some benefit in having those Th2 infections in the presence of pro-inflammatory pathogens. Folic acid, vitamins A, B_{12}, and D did not enter our final models for CRP, but

exposure to wood smoke emerged as an important and modifiable factor associated with higher CRP.

Paper 2. **State of anemia and iron status and their determinants, in pregnant women with multiple infections and nutrient deficiencies**

The high prevalence of anemia in indigenous areas is a main concern of the Panamanian Ministry of Health, given that substantial health resources have been invested in supplementation campaigns, trying without much success, to improve maternal anemia.

We observed 38% prevalence of anemia (hemoglobin <110 g/L) that was associated not only with ferritin <20 µg/L, but also with lower concentrations of folic acid, lower vitamin A and with hepcidin >6.1µg/L, highlighting the importance of other nutrients and of iron restriction due to inflammation as contributors to anemia. Moreover, we found lower concentrations of protein associated with higher hemoglobin, which together with normal or high hematocrit in 96% and urinary gravity >1020 in 26.9%, indicated that hemoconcentration may be underestimating of the prevalence of anemia. To overcome limitations regarding the cut-off for hemoglobin when analyzing the continuous variable, we found that, although iron deficiency contributed importantly to lower hemoglobin, infection by *Trichuris,* a bleeding-producing intestinal parasite, and higher CRP together were contributing to most of the variability captured by the hemoglobin model. Therefore, the lack of association between hemoglobin and the intake of iron supplements may be explained by factors other than iron deficiency, including blood loss due to infection and inflammation.

Using hepcidin measurements, we were able to uncover that 71% of our women had iron restriction due to inflammation, that is to say, most women were sequestering iron in response to infection, a situation where providing iron supplements is contra-indicated.

In contrast to WHO recommendations which advocate for the use of serum transferrin receptor (sTfR) as the best iron indicator in populations with infections on the assumption that it was unaffected by inflammation, we found that sTfR was a less-useful indicator of maternal iron status in a MINDI population because: (1) it detected only 16% of the prevalence of iron deficiency compared with 68% found with ferritin, and (2) it was associated with nutrients (vitamin A and D), with inflammation (IL17 and IL13), and with infections (urinary tract infections, caries and hookworm).

On the other hand, both ferritin and serum iron detected a prevalence of 78% iron deficiency. Both biomarkers shared similar associated determinants and they also were affected by nutrient deficiencies and infections in similar directions, despite both being associated with inflammation in opposite directions. A pro-inflammatory environment (higher monocytes), and/or bleeding (hookworm infection), were associated with lower iron status, whereas an anti-inflammatory environment (eosinophils / *Ascaris*), was associated with higher ferritin and serum iron. Higher vitamin B_{12} was also associated higher iron status indicators.

This paper highlights the need to differentiate anemia and iron deficiency as different entities, since hemoglobin, although a good indicator of maternal health, was a poor indicator of iron status in our MINDI population. It also highlights the need to use alternate measures of iron status (serum iron plus ferritin), and/or of inflammation (hepcidin) in populations with multiple infections and nutrient deficiencies.

Paper 3. **Determinants and utility of blood pressure measurements for the detection of high-risk pregnancies in a rural environment with high prevalence of infections and nutrient deficiencies**

A second main concern of health authorities in Panama is the high prevalence of hypertensive disorders of pregnancy (HDPs), which are a leading cause of maternal mortality, particularly among their indigenous population. It is known that inflammation and severe anemia are

associated with the development of HDPs. Therefore we hypothesized that multiple infections, nutrient deficiencies and inflammation were associated with blood pressure measurements.

We did not find pathological elevations of systolic or diastolic blood pressure (≥160/90 mmHg) in our cohort of pregnant women attending normal pregnancy follow up. Instead, 24.4% had low blood pressure (<100/60 mmHg) which has also been associated with adverse pregnancy outcomes. *Ascaris* infection increased the odds of hypotension, whereas higher intake of animal-source foods and of multiple micronutrients (MMN) were protective, together with higher IL17 concentrations. Those findings add to our previous observation in paper 2, showing that poor protein nutrition may be contributing to an hypovolemic status, manifested as low blood pressure, and that in response to hypovolemia or as a consequence of infection, women had higher IL17.

In contrast, when calculating mean arterial pressure (MAP), a predictor of HDPs, it was elevated in 11.3% women. Elevated MAP was associated with the presence of *Trichuris* infection and with folic acid deficiency. Interestingly, MAP was higher in women with higher TNFα (a known cytokine associated with HDPs), higher intake of MMN and low protein status, but lower in women infected by *Ascaris* and *Trichomonas vaginalis*.

Another alternative blood pressure measurement, pulse pressure (PP, indicator of peripheral perfusion), was positively associated with TNFα and negatively associated with IL17, highlighting the dual role of cytokines where TNFα improved perfusion by increasing blood pressure, and where IL17 increased in response to either infection and/or low placental perfusion. Interestingly, PP was the only blood pressure measurement associated with lower fetal size determined using symphysis-fundal height (SFH). Using those two indicators together, PP and SFH might help to detect pregnancies complicated with poor fetal growth in areas where there is no access to sonography.

Paper 4. **Classification of small- and very-small- for gestational age fetuses using symphysis-fundal height INTERGROWTH-21 standards in an indigenous population identifies multiple infections, nutrient deficiencies and inflammation as risk factors for impaired in-utero growth: The MINDI Cohort**

The application of the new INTERGROWTH standards for the use of symphysis-fundal height (SFH) in the detection of small-for-gestational age (SGA) fetuses allowed us to observe an impressive prevalence of 37.9% of SFH <3rd centile (very small for gestational age –VSGA) in this vulnerable population. Mothers with higher hepcidin, higher number of lymphocytes and higher IL17 but lower IL10 (more inflammation) had increased odds of having VSGA fetuses. On the other hand, mothers with higher hepcidin but lower TNFα, lower protein status and having lower PP (protein malnutrition, low perfusion plus iron restriction due to inflammation) had higher odds of severe-SGA fetuses. Finally, mothers exposed to wood smoke had smaller fetuses.

Multiple infections, nutrient deficiencies and inflammation were associated both positively and negatively with the most used biomarker of inflammation, CRP, with iron status indicators and blood pressure measurements. In general, infections eliciting a Th-1 type immune response were associated with higher CRP, lower iron status and higher blood pressure, whereas infections eliciting a Th-2 type immune response were associated with lower CRP, higher iron status and lower blood pressure. However, the lower quartile of pulse pressure was associated with the smallest fetuses. Low perfusion, indicated by lower pulse pressure and inflammation indicated by higher hepcidin, lymphocyte count and IL17, was associated with retarded fetal growth. Our findings together imply caution in interpreting common biomarkers in impoverished settings.

Contribution of Authors

This thesis is the continuation of my Master's degree in Parasitology, for which a study designed in collaboration with *Dr Marilyn Scott* and *Dr Kristine Koski*, collected a comprehensive range of clinical and laboratory information from indigenous pregnant women belonging to the Ngäbe-Buglé community in Panama.

Initially intending to evaluate interactions between infections and malnutrition during pregnancy, the Master's project involved inter-institutional collaborations with the University of Panama, where *Dr. Enrique Murillo* was the link with Panamanian institutions for accessing funding and processed samples for vitamin A in INDICASAT (*Instituto de Investigaciones Científicas y Servicios de Alta Tecnología*); with the Panamanian Ministry of Health (MINSA), where *MSc Odalis Sinisterra* allowed to work in collaboration with the team of the Nutritional Department, and where *Emerita Pons* coordinated all the laboratory work for detection of infections in the field and for processing and transportation of blood samples. The regional-indigenous section of MINSA, in particular the Maternal-Infant department in head of *RN Delfina Rueda*, facilitated the recruitment of participants and the collection of clinical data and laboratory samples. The Master's work uncovered an entire health problematic in this community and lead to further research questions regarding biomarkers of maternal and fetal health, that are addressed in this PhD thesis in four manuscripts.

Dr Enrique Murillo (Principal Investigator), *Dr Marilyn Scott* (co-PI) and *Dr Kristine Koski* (co-PI) obtained funding from SENACYT (*Secretaría Nacional de Ciencia, Tecnología e Innovación*) for fieldwork and vitamin A analyses and Drs Scott and Koski received additional funding from the McGill Vitamin Fund, and NSERC (*Natural Sciences and Engineering Research Council* of Canada) for biochemical analyses. The PhD thesis was written by *Doris Gonzalez-Fernandez*. I was responsible coordinating the fieldwork, performed physical exams of participants, took and analyzed vaginal smears, ran and interpreted statistical analyses, and wrote the manuscripts in co-authorship with *Dr. Kristine Koski* and *Dr. Marilyn Scott*.

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List of abbreviations

AGP	α-1-acid-glycoprotein
BOND	Biomarkers of Nutrition for Development
BP	Blood pressure
BRINDA	Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia
CRP	C-reactive protein
HDPs	Hypertensive disorders of pregnancy
HIV	Human immunodeficiency virus
IL	Interleukin
INDICASAT	Institute of Scientific Research and High Technology Services (Panama)
INF	Interferon
INSPIRE	Inflammation and Nutritional Science for Programs/ Policies and Interpretation of Research Evidence
IUGR	Intra-uterine growth retardation
LBW	Low birth weight
LMIC	Low- and middle- income countries
MAP	Mean arterial pressure
MINDI	Multiple Infections, Nutrient Deficiencies and Inflammation
MINSA	Panamanian Ministry of Health
MMN	Multiple micronutrients
MTHF	Methyltetrahydrofolate
NLR	Neutrophil-lymphocyte ratio
PP	Pulse pressure
RCT	Randomized control trial
SFH	Symphysis-fundal height
SGA	Small-for-gestational-age
sTfR	Serum transferrin receptor
Th1	T-lymphocyte helper 1
Th2	T-lymphocyte helper 2

TNF	Tumor necrosis factor
UTI	Urinary tract infection
VSGA	Very-small-for-gestational-age

INTRODUCTION

Most Latin-American countries have improved maternal and infant health since release of the Millennium Development Goals in 2015 [1], however some populations living in remote areas, marginalized communities and indigenous peoples have been left behind [2]. For example, indigenous pregnant women in Latin America are less likely to benefit from health-care services and present higher rates of adverse pregnancy outcomes than the majority of the population [3]. Moreover, remote/vulnerable populations in low- and middle-income countries (LMIC) experience multiple infections, nutrient deficiencies and inflammation that may be leading to adverse pregnancy outcomes.

Several adverse pregnancy outcomes are of high relevance at the global level and highly prevalent in LMIC. These include: (1) anemia, which affects 42.7% of pregnant women in LMIC and increases the risk of low birth weight, preterm birth, perinatal mortality, and neonatal mortality [4]; (2) hypertensive disorders of pregnancy (HDPs), with a global prevalence of 3-10%, which are a leading cause of maternal mortality [5] and also a risk factor for perinatal death, preterm birth and low birthweight [6], and (3) small for gestational age (SGA) that affects 19.3% of live-births in LMIC, and that increases the risk of delayed neurodevelopment, poor linear growth, morbidity and mortality in the perinatal period and beyond [7]. For early identification of women at risk of those adverse pregnancy outcomes, health care providers are limited by disparities in the access to conventional technology, which limits assessment of maternal and infant health and contributes to unaccomplished improvement in global maternal and infant health [1].

Those inequities include limited access to biomarkers, defined as a characteristic (physiological measurements, chemical analyses of biological samples or measurements from images) that are objectively measured and evaluated as indicators of normal or pathological biological processes [8]. Biomarkers can be used as surrogate endpoints, meaning that they are able to predict health outcomes, but few fulfill the characteristics of being statistically associated with, and believed to be pathophysiologically related to a clinical outcome [9], in order to be completely

reliable, as is the case of blood pressure as a surrogate for cardiovascular disease [10]. Moreover, validation of biomarkers as surrogate endpoints during pregnancy is challenging, due to variations in the biomarker depending on race, gestational age, and to the difficulty in standardize cut points [11]. For example, the diagnosis of anemia uses hematological cutoffs adjusted for the physiologic hemodilution of pregnancy, and assumes iron deficiency as the main cause of anemia [12], whereas biomarkers proposed for early detection of HDPs and SGA require an important laboratory/sonography infrastructure [13], usually not available in remote settings of LMIC.

Moreover, interpretation of biomarkers and further public health implications might not be so forthright in LMIC, where it has been recognized that multiple micronutrient deficiencies are present, leading to the need for supplementation programs [14, 15], and where malnutrition may interact with prevalent infections [16]. In fact, the increased risk of malaria complications among children who received iron supplementation [17, 18], led to the creation of an international working group (the Iron and Malaria project) to evaluate adverse effects associated with the administration of iron supplements under conditions of malaria and high infection exposure [19]. Therefore, possible adverse effects of supplementation in presence of infections during pregnancy have been studied, but evidence has shown that iron supplementation did not increase malaria [20] or lower genital tract infections [21], further supporting the continuity of the current recommendation of supplementing iron to pregnant women, even in malaria-endemic areas. Still, no studies address the issue of supplementation during pregnancy in malaria-free contexts.

Following the Iron and Malaria project in 2007, awareness has been raised regarding the impact of malnutrition and inflammation on nutritional biomarkers, which led to international initiatives such as “BOND” (Biomarkers of Nutrition for Development) in 2010, with the aim of creating consensus on the biomarkers that could more accurately assess nutritional status under specific conditions; these studies have focused on folate, iodine, iron, vitamin A, vitamin

B_{12}, and zinc [22]. This group concluded that it is crucial to address the impact of life stage, as well as inflammation and infection on nutritional assessment, particularly iron [23].

In 2012, the Inflammation and Nutritional Science for Programs/Policies and Interpretation of Research Evidence, "INSPIRE" gathered expert groups to summarize evidence on multilateral interactions between nutrition, the immune function, and inflammation, and to provide approaches to interpret biomarkers in the presence of inflammation [24]. The extended and detailed review of findings highlights interesting points, such as protein-energy malnutrition as the deficiency with larger effects on innate immunity and on biomarkers of inflammation, followed by other nutrients (iron, vitamin A, zinc). The report also emphasized the need to take into account multiple infections as source of inflammation and possible structural organ damage impacting bioavailability of nutrients. The report concludes that there is a the need for reliable biomarkers of inflammation in the field and suggests that CRP as probably the best indicator to measure inflammation [24].

More recently, the "BRINDA" (Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia) studies addressed questions about factors associated with inflammation biomarkers in children and women of reproductive age; they also studied iron/vitamin A status indicators and their adjustment for inflammation and the presence of malaria in population studies [25]. Importantly, BRINDA studies have questioned the use of CRP as a sole indicator of inflammation [26], and highlighted the need for controlling for inflammation when using serum transferrin receptor as indicator of iron status [27].

Another challenge in developing settings lacking technology and/or trained health practitioners is the evaluation of fetal health. New international standards for symphysis-fundal height (SFH) as measurement of fetal growth, based on serial measurements from the Fetal Growth Longitudinal Study of the INTERGROWTH-21st Project, was conducted between 2009 and 2014 and in eight countries, Brazil, China, India, Italy, Kenya, Oman, UK, and USA, and included data from healthy, well-nourished mothers at low risk of adverse outcomes [28]. This project

addressed issues around lack of uniformity in the detection of SGA, which before was based on different charts from different populations, that were not always adaptable to LMIC [29]. However, studies in vulnerable populations applying the new INTERGROWTH-21st standards for the detection of SGA are still missing.

To further explore these issues, the present study recruited 213 pregnant Ngäbe-Buglé women from 14 communities in rural Panama. Questionnaires on obstetric history, indoor wood smoke exposure and fieldwork, and a clinical exam including anthropometry were conducted. Urine, vaginal and fecal samples were obtained for the assessment of infections. Venous blood was analyzed for complete blood cell counts, and serum was used for measuring protein and iron status indicators, vitamins A, B_{12}, D, and folic acid, and inflammation markers (CRP, neutrophil/lymphocyte ratio (NLR), plateletcrit and cytokines). This thesis addresses in part this gap in the scientific literature and focuses on factors associated with commonly used biomarkers in pregnant women from a remote setting, where multiple infections, nutrient deficiencies and inflammation are present.

Setting and conceptual framework

All experts groups agree on the need for studies on the interpretation of biomarkers during pregnancy in settings where infections and inflammation are prevalent, yet population studies in pregnant women continue using biomarkers with traditional standards, that have been established in developed settings with lower prevalence of infection or inflammation.

A collaboration of McGill University with the University of Panama, the Panamanian research-funding body SENACYT, and the Panamanian Ministry of Health, allowed the collection of cross-sectional data in 213 pregnant indigenous women from the Ngäbe-Buglé comarca in Western Panama, where population lives in extreme poverty and maternal mortality (283/100 000 live births) and low-birth-weight (14%) rates are way above the country average [30, 31]. Our meetings with local institutions previous to the project, allowed us to know that the Ministry of Health was interested in looking closer to the anemia problem, persisting despite iron

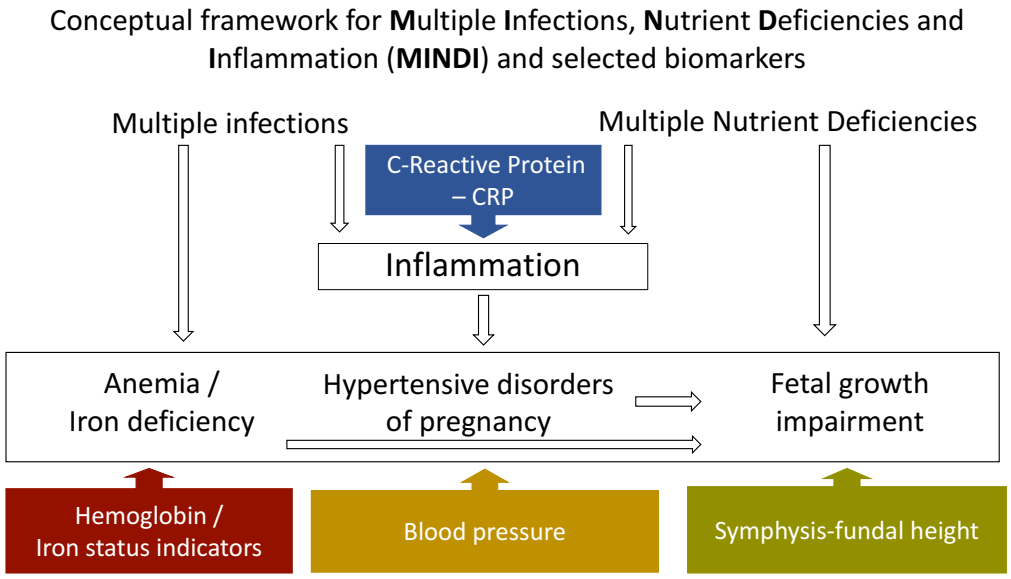
supplementation, and to factors that might be contributing to the high rates of maternal mortality and SGA.

We were able to gather information on maternal-fetal health parameters, infection and nutrient status. My first study, which was part of my Master's Thesis in Parasitology, showed that Ngäbe indigenous women experienced a series of chronic mild-moderate infections: caries, scabies, urinary tract infection (UTI), vaginal infections (bacterial vaginosis – BV, vaginal yeast infections) including sexually-transmitted diseases (vaginal trichomoniasis and diplococcal infections), as well as intestinal nematode infections (*Ascaris*, hookworm and *Trichuris*) [32]. The population had also a high prevalence of nutrient deficiencies, mainly vitamins B_{12} <150 pmol/L (85%), D <50 nmol/L (64.8%), low vitamin A (<1.05 nmol/L) in 41.3% and low folate (<10 nmol/L) in 24% [33].

Given the high prevalence of multiple infections, nutrient deficiencies and reported adverse pregnancy outcomes in a non-endemic area for malaria and where HIV has been ruled out, this population provides a privileged opportunity to study maternal-fetal health using biomarkers available to clinicians in the field.

Therefore, the following conceptual framework is based on the hypothesis that the combination of **M**ultiple **I**nfections, **N**utrient **D**eficiencies and **I**nflammation – **MINDI** affect surrogate endpoints for adverse pregnancy outcomes, using as outcome variables biomarkers available to the Panamanian Ministry of Health for assessing anemia (hemoglobin) and the iron status of their population (ferritin, serum transferrin-receptor), blood pressure for the detection of HDPs, and SFH for the detection of SGA:

Figure A Conceptual framework for experimental design



It is known that not only infections contribute to inflammation leading to adverse pregnancy outcomes [34, 35], but also that nutrient deficiencies are associated with inflammation [36], and are risks factors during pregnancy on their own [37]. Therefore, in our first paper, we wanted to observe the state of inflammation of the population measured through C-reactive protein (CRP), considered an early indicator of adverse pregnancy outcomes [38]. In a second paper, under assumption of the presence of inflammation, we wanted to assess how MINDI were associated with anemia and with iron status indicators, and also to test CRP as indicator of inflammation in the study of iron status under MINDI conditions. Given the high prevalence of HDPs in the population, we wanted to observe in a third paper, if there were women with hypertension in our population, using both systolic and diastolic blood pressure, in addition to the alternative measurements of mean arterial pressure and pulse pressure. We hypothesized that blood pressure measurements would be associated with MINDI, and that these associations could help to define public health strategies for the prevention or early detection of HDPs. Finally, a fourth paper explores associations of all maternal MINDI and fetal size for gestational age measured through SFH using the new international Intergrowth standards. We hypothesized that, as high rates of low birth weight are present in the population, SFH will be able to detect women with SGA fetuses. Moreover, if we would find meaningful associations of

MINDI with SFH, this can help validating the usefulness of SFH in remote areas as indicator of fetal growth.

This thesis will explore a holistic approach to routinely used biomarkers in a population of pregnant indigenous women with MINDI.

Literature review

Pregnancy complications have in common a disbalanced pro-inflammatory immune response [39], that is still subject of intense research. The understanding of physiologic immune response during pregnancy in order to favor fetal development has evolved during last decades, first acknowledging that immune-modulation rather than immune-suppression happens during gestation [40], and that a hormonal/placental-induced shift from a T-helper (Th) 1 towards a Th2 immune response is needed for appropriate development of pregnancy [41]. However, studies with normal pregnant women have shown that a counter-regulatory pro-inflammatory immune response physiologically happens with more advanced gestational age. Higher concentrations of IL12, INFγ [42], IL1β and IL6 have been found in the third trimester [43], indicating that both Th1 and Th2 responses are needed at particular points of time for the good development of pregnancy. In general, it is accepted that naïve T-cells differentiate into regulatory T cells (Treg) in normal pregnancy [44], creating the shift towards a Th2 biased state. However, depending on the cytokine milieu, this physiologic response could turn into the production of pro-inflammatory Th17 cells [45] and Th1 cells, leading to adverse pregnancy outcomes [46].

Whereas acute severe inflammation is known to follow infections during pregnancy and to produce pregnancy loss [39], little is known about the effect of chronic/low-grade inflammation during pregnancy. There is experimental evidence that chronic low-grade inflammation in pregnancy can lead to intra-uterine growth retardation (IUGR) [47]. Also, pro-inflammatory cytokines such as IL1β, IL6, TNFα and INFγ have been associated with adverse pregnancy outcomes such as HDPs and preterm birth [46]. It has also been suggested that low-grade inflammation elicited by common infections such as periodontal diseases and urogenital infections during pregnancy can predispose to HDPs [48], but the impact of multiple mild-moderate infections on adverse pregnancy outcomes is far from being understood as there are significant gaps in the literature.

Overview of infection interactions during pregnancy in LMIC

There is growing interest for the study of combined infections during pregnancy in the context of soil-transmitted infections co-existing with malaria [49], and their associations with human immunodeficiency virus (HIV) [50] and anemia [51-53]. There is also a focus on the co-occurrence of sexually transmitted diseases [54] and their association with HIV [55] during pregnancy is of current concern. Surprisingly, other common infections such as oral, skin and urogenital infections prevalent in non-malaria, non-HIV contexts have not been studied.

Mild-moderate Infections have shown to stimulate the production of pro-inflammatory cytokines, such as IL1β, TNFα and INFγ in caries [56], IL6 and IL17 in urinary tract infection (UTI) [57], IL1β and IL6 in bacterial vaginosis [58], IL1β, IL6, IL17 and TNFα in *Trichomonas vaginalis* [59, 60], IL17 in vaginal yeast [61] and in diplococcal [62] infections. On the other hand, other infections common in LMIC elicit Th2 cytokines such as IL10 in the case of scabies [63], IL10 and IL13 in *Ascaris* [64], whereas INFγ or IL10/IL13 depending on parasitic stage, load and co-infection with other helminths [65] are elicited during hookworm infections. Chronic *Trichuris* infection leads to increased INFγ, TNFα and IL17, whereas a Th2 response through IL10 and IL13 is necessary for parasitic expulsion [66].

Evidence of periodontitis [67-69] and increased vaginal pH [70] (which is in turn associated with bacterial vaginosis [71]) have demonstrated to trigger systemic inflammation and adverse pregnancy outcomes. Although the way multiple co-infections interact in pregnant women has not been described, given current knowledge on immunity during pregnancy, we could infer that infection-specific immune responses may have an impact on the development of adverse pregnancy outcomes.

Beyond the impact of infections on immune balance, they have an impact on the nutritional state of individuals. Infections may produce or aggravate nutrient deficiencies by increased losses, intake and interfering with utilization of nutrients [72]. Anorexia produced by infections can lead to nutritional deficiencies and infections can cause malabsorption and loss of nutrient

as product of diarrhea or vomiting; there is also during infection and inflammation, a redistribution of energy towards the immune system [73]. One of the most studied infection-related deficiencies in pregnancy is anemia. Malaria and HIV have been found independently associated with moderate to severe anemia and low birth weight (LBW) in pregnant women in Sub-Saharan Africa [74], and hookworm infection was found associated with lower hemoglobin concentrations in pregnant women from LMIC [75], in whom this parasite may also aggravate iron and protein-energy deficiencies secondary to blood loos, malabsorption and appetite inhibition [74].

Although, it is commonly accepted that intestinal nematodes contribute to the burden of malnutrition in LMIC, given the geographical overlap between malnutrition and soil-transmitted helminth infections, the evidence about this topic is still weak [76]. The negative impact of respiratory or enteric infections, *Ascaris* in particular, on vitamin A absorption, has been described in children [77], but a recent meta-analysis showed that mass deworming for soil-transmitted helminths *Ascaris*, hookworm and *Trichuris* in children did not improve weight, height, cognition, school attendance or mortality [78]. There is less information about the effect of deworming in pregnant women. Although some studies have identified benefits for perinatal mortality, maternal anemia, hemoglobin, iron deficiency, and birthweight [79], a recent meta-analysis did not find significant benefit of deworming for mentioned outcomes [80].

Overview of nutrition interactions in LMIC

Although energy and protein intakes of pregnant women in developing countries have improved during the past several decades [81], pregnant women from indigenous communities in Australia have been reported to present higher undernutrition rates compared with National statistics [82]. Data among Latin American countries is limited due to weak surveillance nutritional systems [83], and data from indigenous communities in Latin America is missing, but the latest review on the topic showed deficiencies in iron in 36.7% pregnant women in Argentina, B_{12} in 18.6% pregnant women in Colombia, and in women in reproductive age, a prevalence of 4.4% of vitamin A deficiency in Mexico and 3.8% folate deficiency in Costa Rica

were found [83]. In fact, in LMIC micronutrient deficiencies are more common than in developed settings [84], and concurrent micronutrient deficiencies have been documented to be more frequent in pregnant than in non-pregnant non-lactating women [85]. This happens as a result of increased physiologic needs, and also because of short inter-pregnancy periods, multiparity, aggravated with poor diets, adolescent pregnancy, food-related cultural practices and difficult access to health care [14].

There is evidence that diet quality and healthy food patterns that include whole grains, fish, fruit, and green vegetables, and that are low in red meat and saturated fat, have been associated with lower levels of inflammation [86], whereas high red meat, high-fat dairy products and refined grains intake have been associated with inflammation indicated CRP and IL-6 [87]. It is also known that excessive energy intake and adiposity can produce systemic inflammation, but calorie restriction without malnutrition has anti-inflammatory effects [88]. However, the impact of malnutrition secondary to food insecurity in LMIC on inflammation has received less attention.

Experimental work in laboratory settings and population studies with children in LMIC have shown increased susceptibility to a range of infections in the presence of malnutrition, mainly gastrointestinal, respiratory and systemic infections by virus, bacteria and protozoa [89]. Protein-energy malnutrition has been associated with decreased number and function of T-lymphocytes, decreased phagocytic activity of neutrophils and macrophages, impaired skin and mucosal integrity, but antibody response seems not to be affected [90]. Other studies in malnourished children showed an impaired acute-phase protein response to infection, which was more pronounced in severe protein malnutrition than in protein-caloric malnutrition [91] and in African prepubertal boys, a higher prevalence of elevated serum markers of inflammation was associated with reduced bone formation during seasons of food scarceness [92]. There is also evidence that undernutrition is associated with higher re-infection rates of intestinal nematode infections when compared with individuals with normal nutrition status [76] and with Vitamin A deficiency in stunted children in Panama [93].

Infection increases energy requirements of the immune system; activated immune responses involve the production acute-phase reactants, cytokines, enzymes and immune cells, all depending on proteins [94] and micronutrients [73]. Protein malnutrition also reduces most amino acid concentrations, which in turn are needed for the regulation of T-cells, B-cells, natural killer (NK) cells and macrophages; they are also needed for lymphocyte proliferation, antibody and cytokine production [95]. However, and mainly due to ethical constraints, research describing the effect of malnutrition on infection and immunity during pregnancy is extremely limited. It has been experimentally demonstrated that protein deficiency when combined with intestinal nematode infection was able to decrease fetal mass and length [96], and pregnant mice fed a low protein diet had early mortality and inability to carry pregnancy to term when infected with malaria [97].

The association between low protein intake in pregnancy and LBW has been well established, as well as the need for a high percentage of energy from protein (not from carbohydrates) and particularly that from dairy protein, have been demonstrated to have a positive impact on newborn nutritional status [98]. Amino acids, such as methionine, serine, and glycine, besides contributing to protein mass, have a role in one carbon metabolism, therefore in the regulation of cellular proliferation and may impact fetal growth [99].

Notably methionine, an essential amino acid, is required for the synthesis of purines and pyrimidines [100]. Once methionine provides its terminal methyl group for those reactions, it originates homocysteine, which is known to be associated with cardiovascular diseases, pregnancy complications and neurological disorders [101]. Homocysteine can be converted back to methionine using methionine synthase, which depends on vitamin B_{12} as a co-factor; here, folate provides the methyl group of methyltetrahydrofolate (MTHF) to convert homocysteine into methionine and tetrahydrofolate [100, 102]. Another pathway for homocysteine metabolism is via transulfuration, which transfers of sulfur of homocysteine to serine to form cysteine and alpha ketobutyrate [99]. In fact, it has been proposed that protein

malnutrition blocks the hepatic transulfuration pathway, contributing to increased homocysteine concentrations, independently of folate and B_{12} deficiencies, in an attempt of the protein-depleted body to preserve methionine [103], but leading to the development of adverse pregnancy outcomes [99].

Folic acid and vitamin B_{12} are hydro-soluble vitamins that share metabolic pathways and functions, both nutrients participating in one-carbon metabolism [100], which generates precursors for nucleotide biosynthesis and methyl groups for methylation reactions [104]. Increased needs like the ones occurring in pregnancy, poor nutrition and malabsorption (including impairment of parietal cell secretions in the case of B_{12}), contribute to deficiencies of both vitamins in LMIC [105].

The deficiency of folic acid or B_{12} can produce impaired DNA synthesis, with decreased cellular and humoral immune responses leading to decreased resistance to infections [106, 107]. Moreover, the impairment of DNA and protein synthesis in folic acid deficiency can produce maturation arrest of the cell cycle and retardation of DNA replication, which manifest in all blood cell lines but red blood cells show the most marked changes with large oval macrocytes and large variability in size and shape [108]. Folic acid deficiency is considered the main cause of macrocytosis in pregnancy, but despite the known possible contribution of B_{12} deficiency to anemia during pregnancy in LMIC [109], recent studies on this issue come from high income countries, where B_{12} deficiency is not a problem [110]. A study with data from Argentina, Chile, Colombia, Costa Rica, Mexico and Venezuela found that folic acid deficiency was not a public health problem following folic acid supplementation policies, however, low or marginal B_{12} was found in most locations and across population groups [111]. The authors raised the question, based on epidemiological studies in elderly populations, of a possible role of high folic acid status with more pronounced vitamin B_{12} deficiency [111]. Although this has not been definitively proven, it is known that the conversion of folic acid from supplements into tetrahydrofolate does not require B_{12} as a cofactor, therefore, folic acid supplements would correct hematological changes, masking B_{12} deficiency [112].

On the other hand, if vitamin B_{12} deficiency occurs, the conversion of homocysteine to methionine is blocked. Therefore 5-MTHF is trapped in this form, given that its synthesis is a product of an irreversible reaction [104], leading to secondary folate deficiency [113]. However, this secondary folate deficiency may not be captured by serum folic acid concentrations, since it measures both functional folic acid and 5-MTHF [114]. Under such circumstances, folate concentrations return to baseline only after B_{12} replacement [115]. Also, a falsely elevated folate concentrations occur in hemolysis like that observed in malaria [115]. This is important given that serum folate is measured to determine the status of populations, but its usefulness in LMIC has been questioned given the frequent co-occurrence of folate deficiency with B_{12} deficiency and/or infections that induce hemolysis [115].

Other consequences of inadequate maternal folate status have been widely studied, and include abruptio placentae, preeclampsia, spontaneous abortion, stillbirth, preterm delivery, LBW, as well as neural tube defects such as spina bifida and anencephaly [109, 116], this is the reason why in many Latin-American countries, supplementation strategies providing folic acid as part of the prenatal follow up are in place [116, 117]. On the other hand, despite the close relationship of folate and B_{12}, the latter effects of deficiency during pregnancy have received less attention. B_{12} deficiency is an independent risk factor for neural tube defects [118], and a recent meta-analysis found increased risk of SGA and LBW in B_{12} deficient women in India, but failed to show significant associations when analyzing pooled data with developed countries [100].

Hypertensive disorders of pregnancy (HDPs) have been associated with lower concentrations of folate and B_{12}, and higher homocysteine concentrations in Iranian women with HDPs compared with controls [119]. Similarly, in a recent large case-control study in Colombia, low folic acid concentrations and higher homocysteine concentrations were associated with increased risk of hypertensive disorders of pregnancy, but B_{12} concentrations lost protective significance after adjusting for confounders [120]. Another study in Turkey showed a positive association with

homocysteine concentrations whereas folate and B_{12} were not significantly associated with and hypertensive disorders of pregnancy [121]. These studies support the current concept of homocysteine being the link of the association between HDPs and deficiencies in B vitamins [101], through a cascade of events that include increased oxidative stress, vascular fibrosis and a pro-coagulant state leading to endothelial damage [101].

Folate and vitamin B_{12} deficiencies are linked, and the combined imbalance between the two micronutrients can lead to adverse pregnancy outcomes itself, such as decreased anthropometric measurement in newborns [122] and gestational diabetes [123], which has been of raising concern in pregnant women who widely receive folic acid supplementation, without having in account the important interactions with vitamin B_{12} deficiency [104, 124] .

Vitamin A, a fat soluble vitamin, has roles in cell growth and communication, immune function, vision and reproduction [125]. Homeostasis of vitamin A is tightly regulated, since both excess and deficiency have been associated with increased risk of mortality with infectious diseases, blindness [126], anemia and congenital malformations [125]. Moreover, appropriate absorption of vitamin A depends on the integrity of intestinal mucosa, and on the existence of a gradient that allows its passive diffusion or protein-mediated trans-epithelial transport; vitamin A is esterified and storage in the liver, from where it is transported by retinol-binding protein (RBP) to tissues [126]. RBP concentrations are affected in conditions of urinary protein loss or severe malnutrition [127].

Vitamin A is necessary for transport and secretion of immunoglobulin A, therefore its deficiency has been associated with increased susceptibility to infections, especially those affecting the integrity of the gastrointestinal mucosal barrier [128]. Vitamin A is also a co-factor for the development of Treg cells while it inhibits Th17 cell differentiation, as well as INFγ production, directly modulating the immune response [129]; on the other hand, under conditions of inflammation or auto-immunity, vitamin A promotes effectors T-cells, increasing inflammation [130]. A clinical trial with African pregnant women exposed to malaria, found that vitamin A

supplementation increased INFγ and the INFγ/IL10 ratio [131], and the authors suggested a possible protective role of vitamin A supplementation in decreasing placental malarial infection [132].

Vitamin A also interacts with iron and with vitamin D in the process of erythropoiesis since vitamin A has a regulatory role in the expression of genes involved in iron metabolism [128], and transcriptional activity of the nuclear retinoic acid receptors, which can form heterodimers with the vitamin D receptor and participate in the modulation of hematopoiesis [125]. Those associations support findings by a systematic review and meta-analysis, showing that vitamin A supplementation reduced the risk of anemia by 19% in pregnant women [133].

Although vitamin A deficiency has been associated with increased incidence of preterm birth (OR 1.99, 95% CI 1.12–3.53) (Radhika *et al.* 2002), vitamin A supplementation has not shown improvement in adverse perinatal outcomes such as SGA or LBW [133]. Current guidance is to avoid excessive vitamin A intake during pregnancy from either supplement or dietary forms due to potential teratogenic effects [134]. Supplementation is only recommended for populations where vitamin A deficiency is a severe public health problem, at daily doses of up to 10,000 IU (equivalent to 3000 mcg retinol) or 25,000 IU weekly after day 60 of pregnancy, since increased dosages have been associated with miscarriage and fetal malformations [127]. In the general population, serum retinol concentrations of 1.05, 0.70 and 0.35 µmol/L indicate inadequate, deficient and severely deficient vitamin A status [127]. During pregnancy, serum concentrations of <1.05 µmol/L are considered low [128], but the latest Cochrane review on the topic does not support a antenatal vitamin A supplementation to reduce maternal or perinatal mortality [127].

Vitamin D is synthesised in the skin following exposure to sunlight or it can be ingested orally from oily fish, liver, egg yolks or fortified foods [84]. Vitamin D is transported in serum by the vitamin D binding protein or albumin, undergoes 25-hydroxylation in the liver leading to the main circulating form, 25(OH)D [135]. A further activation of vitamin D occurs in the kidney to produce the active metabolite of vitamin D, $1,25(OH)D_3$ [136]. In pregnancy, vitamin D

concentrations are elevated in serum and in the placenta, facilitating fetal bone development and tolerance, acting as immune modulator by decreasing cytokine synthesis by NK cells and increasing the expression of the antimicrobial peptide cathelicidin [136]. Ex-vivo studies have demonstrated that vitamin D blocks the production of IL6, TNFα, INFγ and also of IL10 in placental cell cultures [137]. Currently $25(OH)D_3$ is considered as the best biomarker for vitamin D, and deficiency in pregnancy is considered at concentrations <50 nmol/L [135].

Vitamin D deficiency has been associated with the development of adverse pregnancy outcomes including hypertensive disorders of pregnancy [138]. A recent review has proposed that vitamin D, through its immune modulating properties may prevent placental vasoconstriction, regulate endothelial and vascular smooth muscle cell proliferation, regulate angiogenesis by stimulation the expression of vascular endothelial growth factor in vascular smooth muscle cells [139]. Despite this evidence, population data on decreased risk of HDPs with higher maternal vitamin D concentrations has been inconsistent [140], and vitamin D supplementation has not yet been recommended in part due to gaps in research studies, that include safety issues, minimal effective dosage, timing of initiation of supplementation and its combination with other nutrients [141].

Maternal vitamin D deficiency has also been associated with SGA infants in most studies included in the review of Agarwal et al [139], however a study with 1491 newborns in China showed that cord blood vitamin D had an inverted U-shaped relationship with fetal weight at birth and with the risk of SGA [142]. A similar pattern was observed in babies from white pregnant women in the US, who showed a U-shaped relation between vitamin D and risk of SGA [143]. Also, smaller newborn head circumference with higher vitamin D concentrations was found in a study from Spain [144]. The latest systematic review and meta-analysis of randomized controlled trials (RCTs) found that maternal vitamin D supplementation had a positive effect on birth weight, length and head circumference, and reduced the risk of LBW and SGA [145]. Of note, the inclusion criteria accepted studies of pregnant women of any gestational age without pregnancy complications, and the analysis of LBW and SGA included 3

RCTs from the UK. The effect of vitamin D supplementation on fetal outcomes in the context of multiple infections and nutrient deficiencies or inflammation has not been studied.

In conclusion, there is growing awareness that Multiple Infections, Nutrient Deficiencies and Inflammation (MINDI), common in LIMC, may affect pregnancy outcomes. Given their widespread coexistence in developing countries, the importance of studying their interactions may be in understanding the mechanisms leading to adverse pregnancy outcomes. However, limitations in biomarkers utility under such circumstances have been noticed, particularly the dual role of pro- and anti-inflammatory balance during pregnancy and selection of the appropriate biomarkers of inflammation needed to fully interpret iron status, as well as the combined influence of MINDI on measurements of blood pressure and fetal size. This thesis tries to add information to the literature studying the MINDI Cohort, a population of indigenous pregnant women in Panama with multiple infections, nutrient deficiencies and inflammation.

PAPER 1. C-Reactive Protein is differentially modulated by co-existing infections, vitamin deficiencies and maternal factors in pregnant and lactating indigenous Panamanian women

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List of abbreviations

AB/UTI: Asymptomatic bacteriuria/urinary tract infection; ANOVA: Analysis of variance; BMI: Body mass index; BV: Bacterial vaginosis; CRP: C-reactive protein; HIV: Human immunodeficiency virus; IL: Interleukin; IQR: Interquartile range; INDICASAT: Institute of Scientific Research and High Technology Services (Panama); INF: Interferon; MPV: Mean platelet volume; NLR: Neutrophil/Lymphocyte ratio; PDW: Platelet distribution width: SENACYT: Secretaría Nacional de Ciencia, Tecnología e Innovación; Th2: Lymphocyte T helpers 2; TNF: Tumor necrosis factor; VIF: Variance inflation factor; WBC: White blood cells; WHO: World Health Organization

Abstract

Background: The usefulness of C-reactive protein (CRP) as a non-specific marker of inflammation during pregnancy and lactation is unclear in impoverished populations where co-existing infections and vitamin deficiencies are common.

Methods: This cross-sectional study in Panama recruited 120 pregnant and 99 lactating Ngäbe-Buglé women from 14 communities in rural Panama. Obstetric history, indoor wood smoke exposure, fieldwork, BMI, vitamins A, B_{12}, D, and folic acid, and inflammation markers (CRP, neutrophil/lymphocyte ratio (NLR), plateletcrit and cytokines) were measured. Multiple regressions explored both associations of CRP with other inflammatory markers and associations of CRP and elevated CRP based on trimester-specific cut-offs with maternal factors, infections and vitamin deficiencies.

Results: CRP was higher in pregnancy (51.4 ± 4.7 nmol/L) than lactation (27.8 ± 3.5 nmol/L) and was elevated above trimester specific cut-offs in 21% of pregnant and 30% of lactating women. Vitamin deficiencies were common (vitamin A 29.6%; vitamin D 68.5%; vitamin B_{12} 68%; folic acid 25.5%) and over 50% of women had two or more concurrent deficiencies as well as multiple infections. Multiple regression models highlighted differences in variables associated with CRP between pregnancy and lactation. In pregnancy, CRP was positively associated with greater indoor wood smoke exposure, caries and hookworm and negatively associated with *Ascaris* and vaginal *Lactobacillus* and *Bacteroides/Gardnerella* scores. Consistent with this, greater wood smoke exposure, caries as well as higher diplococcal infection score increased the odds of trimester-elevated CRP concentrations, whereas longer gestational age lowered the likelihood of a trimester-elevated CRP. During lactation, folic acid deficiency was associated with higher CRP whereas parity, number of eosinophils and *Mobiluncus* score were associated with lower CRP. Also, a higher BMI and *Trichomonas vaginalis* score increased the likelihood of an elevated CRP whereas higher parity and number of eosinophils were associated with lower likelihood of an elevated CRP.

Conclusions: Infections both raise and lower CRP concentrations in pregnant and lactating mothers. Only folic acid deficiency during lactation was associated with higher CRP

concentrations. Caution is required when interpreting CRP concentrations in pregnant and lactating women who have co-existing nutrient deficiencies and multiple infections.

Key Words: CRP, intestinal parasites, caries, vaginal infections, folic acid deficiency, wood smoke, neutrophil/lymphocyte ratio, plateletcrit, pregnancy, lactation

Background

C-reactive protein (CRP) is widely used in clinical practice as a non-specific acute-phase indicator of inflammation [1], given its rapid production by the liver and release into circulation [2] and its stimulation by several cytokines including IL6, IL1β and TNFα [3]. CRP also helps the body recognize the presence and severity of infections [4-6] and is used as an indicator of low-grade inflammation in chronic infections [7, 8] and chronic diseases [9]. In addition, CRP responds to a variety of maternal factors. Multiparity [10, 11] and exposure to wood smoke [12, 13] have been associated with elevated CRP in pregnancy and/or lactation whereas recreational exercise has been shown to decrease CRP [14, 15]. CRP normally increases during gestation [16], returning to basal concentrations shortly after delivery [17]. A recent study in a population of indigenous Australian women showed CRP concentrations differed by trimester [18]. Previously, some authors have recommended use of trimester-specific cut-offs to define elevated concentrations of CRP during pregnancy [19, 20] whereas other authors have tried to identify cut-offs for predicting adverse pregnancy outcomes such as gestational diabetes and choriamnionitis [21].

CRP is modulated by nutritional status and deficiencies of vitamins A [22], D [23] and folic acid [24] but several studies have reported no association of CRP with vitamin B_{12} [25, 26]. CRP reportedly is higher in obese pregnant women [27], but this association was lost by the end of pregnancy [28]. Folic acid concentration has been negatively correlated with CRP in pregnant women from Korea [24], and in non-pregnant non-lactating population in the US [25]. Also, serum vitamin A concentrations were negatively correlated with CRP in pregnant women from Guinea-Bissau [29] and Ethiopia [22], and in lactating women from Mali [30]. Vitamin D supplementation was reported to decrease CRP concentrations in healthy Iranian pregnant women [31], but not in African-American women in their second trimester [32]. However, a large study with pregnant women in Nepal showed no effect of multiple micronutrient supplementation on indicators of inflammation including CRP [33].

CRP is a well-known soluble pattern recognition molecule that responds to infections [34]. The higher elevation of CRP in the presence of combined viral and bacterial pneumonia [35], combined human immunodeficiency virus (HIV) and tuberculosis [36], dengue and malaria [37] than in the presence of single infections, highlights the likelihood that co-occurring chronic infections are likely to cumulatively increase concentrations of CRP. On the other hand, evidence suggests that helminth infections can lower inflammation [38].

In developing countries where biomarkers of inflammation are not always available in remote settings, CRP is a widely available and cost-effective marker of inflammation [6] that can be used alongside the neutrophil/lymphocyte ratio (NLR) and platelet indices as predictors of adverse pregnancy outcomes [39-41]. Our previous work in an indigenous population living in conditions of extreme poverty in Panama revealed not only a range of bacterial, fungal, protozoan, helminth, and ectoparasite infections among pregnant and lactating women but also synergistic and antagonistic interactions among the various pathogens [42]. Furthermore, informal conversations with physicians highlighted the challenge they faced in relying on CRP as a marker of inflammation under these conditions. This led us to investigate whether CRP may respond differently depending on the set of infections and/or micronutrient deficiencies experienced by the pregnant or lactating women.

Our specific objectives were (1) to record CRP concentrations in a population of indigenous pregnant and lactating women; and (2) to assess which among a range of other inflammatory markers (platelets, neutrophils, cytokines), environmental factors (wood smoke, field work), infections (caries, urogenital, intestinal, skin), and micronutrient deficiencies (folate, vitamin A, B_{12}, D) were associated with CRP concentrations during pregnancy and lactation.

Methods

Design

A detailed description of the study design and methodology and inclusion/exclusion criteria was previously published [42]. Briefly, this cross sectional study included 120 pregnant women (11

first, 43 second and 66 third trimester) and 99 lactating women belonging to the Ngäbe-Buglé community in Western rural region of Panama and was conducted between August and December 2010. Questionnaires and clinical exams were conducted, and venous blood, urine, vaginal and fecal samples were obtained.

Anthropometry, obstetric history, environmental factors

Weight and height were recorded. Body mass index (BMI) [weight/(height)^2] was calculated and was corrected in pregnant women by subtracting estimated fetal weight using Johnson's formula [43]. Pregnant women were classified individually as underweight, normal weight or overweight/obese according to the Pan-American Health Organization weight-for-height chart [44]. The BMI for lactating women was classified as for the general population: underweight (BMI <18.5 kg/m^2), normal weight (BMI between 18.5 and 24.9 kg/m^2), overweight (BMI ≥ 25 kg/m^2), and obese (BMI ≥ 30 kg/m^2) [45]. Participants answered questions on obstetric history, hours of indoor wood smoke exposure and fieldwork hours per day.

C Reactive Protein

CRP was processed at the Central Reference Laboratory in Public Health of the Gorgas Memorial Institute for Health Studies in Panama City in duplicate using solid phase enzyme-linked immunosorbent assay (MP Biomedicals, Orangeburg, NY), with a minimum detectable concentration of 0.9 nmol/L. The cut-offs for elevated CRP were set at 193.3 nmol/L and 77.1 nmol/L in the second and third trimesters, respectively [20]. During the first trimester and during lactation, the cut-off for non-pregnant women of 28.5 nmol/L was used [20].

Infections and co-morbidities

Oral, skin and urogenital infections were assessed clinically. Urine (118 pregnant and 94 lactating women), vaginal (119 pregnant and 79 lactating women) and fecal samples (120 pregnant and 23 lactating women) were analyzed for infections as previously described [42]. Presence or absence of caries, scabies, asymptomatic bacteriuria/urinary tract infection (AB/UTI), *Ascaris*, hookworm and *Trichuris* were recorded and semi-quantitative scores (0 to 4)

of vaginal *Lactobacillus*, *Bacteroides/Gardnerella*, *Mobiluncus*, *Trichomonas vaginalis*, yeast and diplococcal infections were recorded. Bacterial vaginosis (BV) was diagnosed using the Nugent score, calculated as: *Bacteroides/Gardnerella* score + (4 – *Lactobacillus* score) + (*Mobiluncus* score/2) [46]. HIV and gestational diabetes were ruled out and the study area was non-endemic for malaria. Mothers reported no allergic conditions or history or symptoms of chronic cardiovascular, renal or autoimmune diseases.

Micronutrient concentrations

Serum samples were analyzed for folic acid and vitamin B_{12} concentrations using immunoelectro-chemiluminescence on the analyzer MODULAR E170 (Roche Diagnostics GmBH, Mannheim, Germany) at the Canadian Diagnostics Laboratory, a national reference laboratory in Montreal. Folic acid deficiency was defined as <10 nmol/L and vitamin B_{12} deficiency as <150 pmol/L [47].

Serum vitamin A was detected using high-performance liquid chromatography at the Institute of Scientific Research and High Technology Services-INDICASAT in Panama City [48]. The cut-off for vitamin A deficiency was set at <1.05 µmol/L during pregnancy and lactation [49], and the cut-off for higher than normal vitamin A was set at >1.5 µmol/L for both pregnancy [20] and lactation [50].

Serum OH vitamin D was assayed using the LIAISON, DiaSorin direct competitive chemiluminescence immunoassay (lot 125756, kit 72947). The intra-assay coefficients of variation were 1.2% and 3.3% for pregnant women and 2.2% and 3.1% for lactating women. Respective inter-assay variabilities were 3.8% and 17.1%. We used a cut-off for vitamin D deficiency of <50 nmol/L [51].

White blood cells (WBC) and platelets

Blood samples were analyzed for complete blood cell count using a BC-5500 Mindray Auto Hematology Analyzer. Data were recorded on total white blood cells, number of neutrophils,

lymphocytes, eosinophils and basophils, NLR, and number of platelets (120 pregnant and 99 lactating women). Plateletcrit, mean platelet volume (MPV) and platelet distribution width (PDW) were also recorded.

Concentrations of cytokines (Interleukin (IL) 1β, IL4, IL6, IL10, IL12, IL13, IL17, TNFα and INFγ) were analyzed via Luminex (Luminex Corp., U.S.A.) as part the Human 10-plex Cytokine/Chemokine Magnetic Bead Panel (Cat. HCYTOMAG-60K; Millipore Corporation Canada) according to manufacturer instructions at the School of Dietetics and Human Nutrition, McGill University. Minimum detection limits (pg/mL) were: IL1β = 0.8, IL4= 4.5, IL6= 0.9, IL10= 1.1, IL12= 0.6, IL13= 1.3, IL17= 0.7, TNFα=0.7, INFγ= 0.8. For each essay, standards and quality controls were analyzed in duplicate and quality controls were within accepted ranges.

Statistical analyses

Statistics were performed using STATA 14 (StataCorp LP, Texas, USA). CRP was not normally distributed and was log transformed unless otherwise indicated. Kolmogorov-Smirnov test compared those non-normally distributed variables (age, parity, wood smoke, BMI and interleukins). Student's t-tests compared normally distributed vitamin D, MPV, PDW and plateletcrit, and log transformed folic acid, vitamin B_{12}, vitamin A, WBC counts and total platelets, between pregnancy and lactation. Chi^2 analysis compared the frequency of vitamin deficiencies between pregnancy and lactation. T-tests were also used to compare log CRP between binary classifications of exposure (yes/no) to wood smoke and fieldwork, presence/absence of specific infectious and micronutrient deficiencies in both pregnant and lactating women. One-way ANOVA compared log CRP by trimesters, by age (<19 yrs, 19-35 yrs and >35 yrs), by parity (1, 1-4 and ≥5 pregnancies) and by BMI (underweight, normal weight, overweight) classifications. Spearman correlations were used to identify correlations of non-transformed CRP with WBC, platelet indices and cytokines and Kruskal Wallis test to compare the number of women with 0, 1, 2, or 3 concurrent micronutrient deficiencies between pregnancy and lactation. P-values < 0.05 were considered significant. A multiple linear

regression analysis associated inflammatory markers (WBC, platelet and cytokines) with log-transformed CRP in pregnancy and in lactation.

Separate stepwise multiple linear regression models for log CRP and multiple logistic regression models for elevated CRP were done for both pregnant and lactating women, controlling for parity and BMI as well as gestational age of pregnant women and weeks post-partum for lactating women. In the final linear regression model for log CRP and logistic regression model for elevated CRP, we included environmental factors, infections with a prevalence > 10%, and vitamin deficiencies. The following variables were entered: hours of wood smoke exposure and fieldwork; presence of caries, semi-quantitative scores of vaginal *Lactobacillus*, *Bacteroides/Gardnerella*, *Mobiluncus*, *T. vaginalis*, yeast and diplococcal infections; and deficiencies of folic acid, vitamins B_{12}, A and D. In pregnancy, presences of *Ascaris*, hookworm and *Trichuris* were also included. In lactation, eosinophil count was included.

All models were tested for collinearity using variance inflation factors (VIF) and the stability of the regression coefficients using the condition number. Models were considered suitable if VIF< 10 and condition number <30. AB/UTI and scabies were excluded from models of lactating women due to collinearity.

Results

Maternal characteristics and infections

The population was characterized by high rates of adolescent pregnancy (26.9%), grand-multiparity (27.4%), and indoor wood smoke exposure (94.5%). The majority of women (62.1%) were of normal weight, 6.8% were underweight and 31% of women were overweight. About half of the women (46.1%) worked in the field. Almost all women (97%) were infected with at least one vaginal pathogen. Other common infections included hookworm (55.2%), AB/UTI (45.7%), *Ascaris* (30%), caries (19.6%), scabies (15.5%) and *Trichuris* (11.9%), as reported previously [42].

Vitamins

Deficiencies of vitamin B_{12} (85.8% in pregnancy; 46% in lactation, χ^2 test $P < 0.0001$) and vitamin A (38.6% in pregnancy; 18.5% in lactation, χ^2 test $P = 0.001$) were more common in pregnancy. Neither deficiency of vitamin D (68.3% in pregnancy; 68.7% in lactation, χ^2 test $P = 0.95$) nor folic acid (31.3% in pregnancy; 20.8% in lactation, $p = 0.07$) differed between pregnancy and lactation. Concurrent micronutrient deficiencies were more often found in pregnant women (Kruskal-Wallis test: $P = 0.0003$) (Fig. 1). Two concurrent deficiencies were found in 48% of pregnant and 43.3% of lactating women, 3 concurrent deficiencies occurred in 23.5% of pregnant and 11.3% lactating women, and 4 concurrent deficiencies were found in 7.5% of pregnant and 3.1% of lactating women. T-test comparisons of vitamin concentrations revealed that pregnant women had higher folic acid concentrations but lactating women had higher concentrations of vitamins A and B_{12} (Table 1).

Inflammation Markers

The numbers of WBC and neutrophils, the NLR, and PDW were higher in pregnant women, but numbers of lymphocytes, eosinophils, basophils, platelets and plateletcrit were higher in lactating women (Table 1). Concentrations of all measured cytokines (IL1β, IL4, IL6, IL10, IL12, IL13, IL17, INFγ, TNFα) were higher in pregnant than in lactating women (Table 1).

CRP

Mean CRP concentration was higher in pregnancy than lactation (Table 1), but did not differ between first (35.1 ± 8.4 nmol/L; median 31.4; interquartile range (IQR) 51.4), second (58.4 ± 9.7 nmol/L; median 32.4; IQR 57.1) and third (50.0 ± 5.7 nmol/L; median 37.6; IQR 62.8) trimesters. Based on trimester-specific cut-offs, elevated CRP occurred in 63.6%, 7.0% and 22.7% of first, second and third trimester women, respectively, and in 30% of lactating mothers (Fig.2).

CRP did not differ among women according to age, parity or BMI classifications. CRP concentrations were compared between binary categories for a variety of maternal variables (Table 2). In pregnancy, CRP concentrations were higher in women with caries (Table 2) and

lower in those with *Ascaris* (Table 2). During lactation, CRP concentrations were higher in women with above normal vitamin A (>1.5 μmol/L) and lower in grand-multiparous women and those with caries (Table 2).

In pregnancy, CRP was positively correlated with NLR (r_s = 0.24, P = 0.008), platelet count (r_s = 0.20, P = 0.032), plateletcrit (r_s = 0.23, P = 0.012), IL6 (r_s = 0.23, P= 0.011), IL10 (r_s= 0.25, P = 0.005), IL12 (r_s = 0.28, P = 0.002), IL13 (r_s = 0.25, P = 0.005), and TNFα (r_s = 0.22, P = 0.015). In lactation, CRP was positively correlated with numbers of neutrophils (r_s = 0.30, P = 0.002), NLR (r_s = 0.29, P = 0.003), platelet count (r_s= 0.40, P < 0.0001), plateletcrit (r_s = 0.34, P = 0.0006), and IL4 (r_s = 0.20, P = 0.042) and negatively correlated with number of eosinophils (r_s = -0.25, P = 0.010) and MPV (r_s = -0.27, P = 0.006).

To determine whether CRP was associated with other markers of inflammation and to confirm significant correlations, multiple regression analyses controlling for gestational age, parity and BMI during pregnancy revealed a positive association of log CRP with NLR and plateletcrit and accounted for 14.5% of the variability in CRP (Table 3). During lactation, log CRP was also positively associated with plateletcrit and negatively associated with number of eosinophils when controlling for weeks postpartum, parity and BMI and the model explained 27.5% of variability in CRP. No cytokines emerged in either model (Table 3).

Role of Maternal Factors, Infections and Vitamin Deficiencies as Determinants of CRP

During pregnancy, our final multiple linear regression model explained 18.9% of variation in log CRP (Table 4). Wood smoke, presence of caries and hookworm were positively associated with CRP, and presence of *Ascaris*, and scores for vaginal *Lactobacillus* and *Bacteroides/Gardnerella* were negatively associated with CRP. Using trimester specific cut-offs for elevated CRP (Table 4), our multiple logistic regression model showed that presence of caries, higher diplococcal infection score and greater indoor wood smoke exposure were associated with higher likelihood of elevated CRP, and higher gestational age was associated with a lower likelihood of elevated CRP. The Pseudo R^2 was 0.243.

During lactation, our composite multiple linear regression model captured 24.4% of the variability in log CRP. Folic acid deficiency was positively associated with CRP; the number of eosinophils, the *Mobiluncus* score and parity were negatively associated with CRP (Table 5). Our multiple logistic regression model for elevated CRP in lactation had a Pseudo R^2 of 0.243 (Table 5). Higher *T. vaginalis* score and BMI were associated with a higher likelihood of elevated CRP, and a higher number of eosinophils and parity were associated with lower likelihood of elevated CRP.

Discussion

Several key findings emerged from this study. First, our results differentiated between those infections that were associated with higher CRP and those associated with lower CRP. During pregnancy, dental caries, hookworm and vaginal diplococcal infection were associated with higher CRP or increased the odds of trimester-specific elevated CRP whereas *Ascaris*, *Lactobacillus* and *Bacteroides/Gardnerella* were negatively associated with an elevated CRP. During lactation, vaginal trichomoniasis was associated with an elevated CRP whereas vaginal *Mobiluncus* and eosinophil counts were associated with lower CRP and/or lower odds of an elevated CRP. Second, although deficiencies of vitamins A, B_{12}, D and folic acid were common, only folic acid deficiency was associated with higher CRP in lactating women. Third, higher daily indoor wood smoke exposure was positively associated with CRP and increased the odds of elevated CRP in pregnancy. Together, these results show that, after correcting for the normal elevation of CRP during pregnancy, the systemic CRP concentration is influenced by the mix of diverse infections, folic acid deficiency and maternal wood smoke exposure in this vulnerable population.

Our finding that CRP was positively associated with NLR and plateletcrit is consistent with their usefulness as markers of inflammation [2, 52]. NLR is thought to be an early marker of bacteremia given its positive association with CRP [53], and NLR has also been associated with placental inflammation when NLR ≥ 6.48 and CRP ≥ 71 nmol/L [54]. Activation of platelets

occurs in response to inflammatory stimuli [55], and activated platelets are enlarged resulting in higher plateletcrit [56]. Our negative association of eosinophils with CRP during lactation is consistent with the production of Th2 cytokines, particularly IL4, by eosinophils [57]; both Th2 and IL4 are required for protection against intestinal nematodes and for suppression of a Th1 pro-inflammatory cytokine response [58].

Although CRP was positively correlated with four cytokines during pregnancy and three during lactation, cytokines did not emerge in the multiple regression model. We interpret this to mean that CRP was a more specific marker of inflammation than cytokines in this population. Thus, we suggest that plateletcrit and NLR that have been useful for the diagnosis of obstetric pathologies in tertiary care [39, 59] would be alternatives to evaluate inflammation in pregnant and lactating women in settings where a complete blood count, but not CRP measurement, is possible.

Among the four vitamin deficiencies detected in this population, only folic acid deficiency was associated with higher CRP and only in lactating women. This is consistent with the observation that folate intake was protective against elevation of CRP above 28.5 nmol/L in lactating women from Kenya [60] and that young Vietnamese women with higher folic acid intake had lower CRP [61]. The absence of a relationship between folic acid deficiency and CRP during pregnancy is most likely because women in our study were receiving folic acid supplementation during pregnancy. These findings suggest that there may be a need to extend folate supplementation beyond the 3 months post-partum established in the national protocol.

Among the many infections detected [42], several were associated with higher CRP and/or a higher likelihood of an elevated CRP. In pregnancy, the finding that caries was associated with higher CRP is consistent with reports showing that periodontal disease is associated with elevated CRP in pregnancy [62] and correlated with CRP [63]. Furthermore, aggressive vaginal microorganisms such as *Diplococcus* and *T. vaginalis* were found to induce a systemic inflammatory response. To our knowledge this is the first report of diplococcal infection being

associated with higher CRP in pregnancy. Also, the positive association detected between trichomoniasis and CRP in lactation extends the findings of a systemic inflammatory response measured through CRP and granulocyte-macrophage colony-stimulating factor found in pregnant women with vaginal trichomoniasis [64]. It is also consistent with a mouse study that demonstrated signs of systemic inflammation associated with trichomoniasis [65].

One striking observation was that several vaginal bacteria were associated with lower, not higher CRP. The negative relationship between *Lactobacillus* and CRP in pregnancy is consistent with the observation that *Lactobacillus* dominates the vaginal flora in healthy women [66] and that many species of *Lactobacillus* are considered to be protective [67]. It is also consistent with the lower amount of *Lactobacillus* but higher CRP in pregnant compared with non-pregnant women in the US [68]. In addition, however, the typical pathogenic bacteria associated with BV, *Bacterioides/Gardnerella* and *Mobiluncus* [69] were also associated with lower CRP in pregnancy and lactation, respectively. As BV has been linked with inflammation during pregnancy [70, 71], we would have expected both *Bacteriodes/Gardnerella* and *Mobiluncus* to be associated with higher CRP but they weren't. Together, these contrasting findings show that the vaginal tract has organisms that both increase and decrease CRP and is supported by a recent study showing women with BV and adverse pregnancy outcomes had different vaginal microbiota profiles compared to women with BV and no adverse pregnancy outcomes [71] indicating different host-parasite adaptations depending the type of vaginal microorganisms. This requires further investigation.

We were also intrigued that *Ascaris* was negatively associated with CRP whereas hookworm was positively associated with CRP in pregnancy. There is considerable evidence of the anti-inflammatory effect of *Ascaris* based on the *in vitro* suppressive effects of *Ascaris suum*-derived protein (PAS-1) on pro-inflammatory cytokine production [72], the down-regulation by low intensity *Ascaris* infection of the IL6 response to intestinal giardiasis [73], the anti-inflammatory effect of *Ascaris* antigen on cytokine production by peripheral mononuclear cells from children when co-incubated with allergens [74], and the consistent protective effect of *Ascaris* against

allergic sensitization found in a meta-analysis [75]. However, to our knowledge, this is the first report of a negative association of CRP with *Ascaris* during pregnancy. Also, the possibility that this anti-inflammatory influence would reduce CRP in the presence of multiple infections and vitamin deficiencies has not been previously considered. This may have important public health implications during pregnancy given the health risks of pathologies often associated with inflammation.

There are two reasons why we would have also expected a negative association of hookworm with CRP. First, secretory products released by adult hookworms during feeding can down-regulate the inflammatory response in humans by suppressing TNFα secretion [76]. Second, although experimental hookworm infection in a human volunteer showed a transient increase in CRP as well as pro-inflammatory (IFNγ) and T helper 2 (Th2) cytokines (IL5, IL13) soon after infection, this response was dampened once adult worms had reached the intestine and started shedding eggs [77]. It is unlikely that hookworm was a transient infection in women in our study, as the prevalence based on egg counts was over 50% [42], indicating ongoing transmission. The observed positive association between CRP and presence of hookworm in pregnancy may be explained by different degrees of tissue damage caused by the adult worms. In contrast to the non-invasive manner by which adult *Ascaris* worms feed [78], hookworm adults invade the mucosa and submucosa of the small intestine where the release of hydrolases and mechanical injury associated with feeding results in considerable tissue damage [79]. An additional justification for the different response of CRP between hookworm and *Ascaris* in pregnancy is explained by the capacity of the adult hookworm *Necator Americanus* to produce proteases that specifically cleave the eosinophil chemoattractant eotaxin [80] whereas *Ascaris* induces higher transcription levels of eotaxin at least in pigs [81].

In this population where wood is used as fuel for cooking, increased hours of wood smoke exposure during pregnancy increased the odds of elevated CRP. Studies from India have reported that wood fuel use is associated with low birth weight [82], more frequent stillbirth and increased risk of preterm delivery [83], outcomes that are in turn associated with elevated

CRP [84, 85]. To our knowledge, this is the first report of an association between biofuel exposure and inflammation during pregnancy, although the finding is consistent with a study of women living in rural India that found increased serum CRP concentration in those exposed to wood smoke [12].

Strengths and Limitations

This cross sectional screening of indigenous pregnant and lactating women in a remote outpatient setting allowed us to collect data on biomarkers of inflammation, infection and nutritional status and to associate these with CRP concentration during the three trimesters of pregnancy and 6 months postpartum. Nevertheless, we acknowledge the following limitations. The cross-sectional design precluded us from monitoring CRP over time and from relating CRP to pregnancy outcomes. The results of our multiple logistic regression models for trimester-specific elevations in CRP in pregnancy may need to be viewed with caution because of the lack of a consensus on trimester-specific cut-offs for CRP [85]. We were unable to explicitly consider intestinal nematodes in our regression models during lactation due to the high proportion of women who did not provide a fecal sample.

Conclusion

Both within the intestine and within the vaginal tract, some organisms raised and others lowered systemic CRP. This highlights the complexity of the associations of pathogens and CRP, and raises questions about how to interpret CRP in settings with multiple infections. It is possible that treatment of ascariasis may inadvertently increase CRP and that the treatment of BV may lead to the opportunistic growth of concurrent/more aggressive microorganisms and to an elevation in CRP, which could adversely affect pregnancy outcomes. Further, folic acid deficiency may play an under recognized role in leading to increased CRP concentrations in lactating women, in which case it might be recommended that folic acid supplementation be extended beyond 3 mo postpartum. Finally, consideration should be given to reducing exposure to indoor wood smoke particularly during pregnancy, given its role in increasing CRP.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from Institutional Review Board of McGill University in Canada (IRB Study Number A03-M25-08B), the Gorgas Memorial Institute Ethics Board in Panama, the Panamanian Ministry of Health, provincial and local health authorities, and indigenous authorities. Pregnant and lactating women who agreed to participate were recruited from among those seeking routine follow-up at rural Health Centers that covered the indigenous Ngäbe population, in Chiriquí province, Panama. Fully informed consent was obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are not publicly available due to participant confidentially.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

DGF, EM, MES and KGK were involved in the study conception, design, analysis and interpretation of data, drafting and critical revision of the manuscript. DGF, ECP, DR and OTS coordinated fieldwork and data collection. EM coordinated official relationships with SENACYT, the Panamanian funding agency, and INDICASAT for the processing of samples for vitamin A determination. KGK coordinated other vitamin processing with National reference laboratories in Quebec. All authors read and approved the final manuscript.

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Tables and figures

Table 1 Comparisons between impoverished pregnant and lactating women in rural Panama

	Pregnancy Mean ± SE	Lactation Mean ± SE	Pregnancy Median (IQR)[2]	Lactation Median (IQR)[2]
MATERNAL CHARACTERISTICS				
Age (y)	24.8 ± 0.6	25 ± 0.7	24 (11)	23 (10)
Parity (#)	3.4 ± 0.2	3.5 ± 0.2	3 (4)	3 (3)
Wood smoke, h/d	2.5 ± 0.1	2.3 ± 0.1	2 (1)	2 (1)
Fieldwork, h/d	4.5 ± 0.3	4.7 ± 0.3	4 (3.5)	4 (4)
BMI (kg/m^2)	25.1 ± 0.3	25.5 ± 0.3	24.7 (4.6)	25.1 (4.9)
NUTRITIONAL STATUS INDICATORS				
Folic acid, nmol/L	16.0 ± 0.7^{a*}	13.5 ± 0.6^{b}	14 (8.1)	11.9 (7.3)
Vitamin B_{12}, pmol/L	111.1 ± 4.3^{b}	170.2 ± 7.2^{a*}	100 (48)	153 (62)
Vitamin A, µmol/L	1.20 ± 0.03^{b}	1.47 ± 0.04^{a**}	1.17 (0.46)	1.42 (0.66)
Vitamin D, nmol/L	43.1 ± 1.4	43.0 ± 1.4	39.7 (22.0)	42.2 (19.8)
WHITE BLOOD CELLS (WBC)				
Total WBC x 10^3/dL	8.92 ± 0.21^{a*}	8.21 ± 0.18^{b}	8.6 (2.8)	7.8 (2.4)
Neutrophils x10^9/L	6.07 ± 0.20^{a**}	4.55 ± 0.15^{b}	5.9 (2.3)	4.1 (1.7)
Lymphocytes x10^9/L	2.01 ± 0.04^{b}	2.45 ± 0.06^{a**}	1.9 (0.6)	2.3 (0.8)
Monocytes x10^9/L	0.39 ± 0.01	0.38 ± 0.01	0.37 (0.14)	0.36 (0.12)
Eosinophils x10^9/L	0.39 ± 0.02^{b}	0.78 ± 0.06^{a**}	0.36 (0.29)	0.61 (0.57)
Basophils x10^9/ L	0.03 ± 0.00^{b}	0.05 ± 0.00^{a**}	0.03 (0.02)	0.04 (0.03)
Neutrophil/lymphocyte ratio (NLR)	3.15 ± 0.14^{a**}	1.96 ± 0.08^{b}	2.9 (1.1)	1.7 (0.9)
PLATELETS				
Total platelets x10^9/L	262.8 ± 5.6^{b}	323.4 ± 1.0^{a**}	262 (82.5)	306 (113)
MPV, fL	8.9 ± 0.1	8.8 ± 0.10	8.8 (1.2)	8.7 (1.4)
PDW	15.9 ± 0.03^{a**}	15.6 ± 0.04^{b}	15.9 (0.6)	15.6 (0.5)
Plateletcrit, %	23.5 ± 0.4^{b}	28.3 ± 0.71^{a**}	23 (7)	26.9 (8)
CYTOKINES,pg/mL				
IL1-B	4.9 ± 0.5^{a**}	1.5 ± 0.4^{b}	1.72 (7.7)	0.02 (1.3)
IL4	19.7 ± 2.5^{a**}	3.9 ± 0.6^{b}	9.1 (23.4)	2.1 (4.0)
IL6	8.8 ± 1.1^{a**}	4.9 ± 1.2^{b}	1.6 (11.8)	1.6 (0)
IL10	3.9 ± 0.6^{a**}	1.3 ± 0.3^{b}	1.2 (4.8)	0.3 (1.2)
IL12	15.6 ± 3.1^{a*}	4.6 ± 0.8^{b}	1.5 (21.5)	1.3 (5.7)
IL13	4.4 ± 0.6^{a**}	1.2 ± 0.2^{b}	1.6 (7.4)	0.9 (0.9)
IL17	6.5 ± 0.7^{a**}	3.5 ± 1.2^{b}	2.3 (10.9)	0.8 (1.2)
INF-γ	9.0 ± 1.0^{a**}	7.6 ± 2.6^{b}	3.6 (13.2)	1.6 (3.4)
TNF-α	7.5 ± 0.7^{a*}	6.0 ± 0.5^{b}	6.5 (12.4)	4.4 (6.9)
CRP (nmol/L)	51.6 ± 4.7^{a**}	27.6 ± 3.5^{b}	32.8 (58.1)	12.3 (30.4)

[1]Values are means ± SD or IQR, n = 120 for pregnancy with the exception of wood smoke (114), fieldwork (60), vitamin A and cytokines (119), MPV, PDW and plateletcrit (115). For lactation, n= 99 with the exception of wood smoke (93), fieldwork (41), vitamin A and MPV, PDW and plateletcrit (97). Means with different superscripts are significantly different at *P <0.05 and ** P <0.0001

[2]IQR= Q3 – Q1 value

Table 2 Mean CRP concentrations (nmol/L) in the presence and absence of the specified maternal condition in pregnancy and lactation

	PREGNANCY		LACTATION	
Condition	**Yes**	**No**	**Yes**	**No**
Environmental hazards				
Wood smoke	52.2 ± 4.9 (n= 114)	41.7 ± 19.8 (n= 6)	28.0 ± 3.7 (n= 93)	24.4 ± 8.4 (n= 6)
Fieldwork	52.3 ± 7.5 (n= 60)	51.0 ± 5.9 (n= 60)	24.0 ± 4.7 (n= 41)	30.4 ± 5.0 (n= 58)
Micronutrient status				
Folic acid <10 nmol/L	61.1 ± 11.6 (n= 25)	49.2 ± 5.1 (n= 95)	30.1 ± 6.7 (n= 31)	26.7 ± 4.2 (n= 68)
Vitamin B_{12} <150 pmol/L	48.8 ± 4.9 (n= 103)	68.7 ± 15.5 (n= 17)	24.6 ± 4.5 (n= 46)	30.5 ± 5.4 (n= 53)
Vitamin A <1.05 µmol/L	59.5 ± 8.9 (n= 46)	47.1 ± 5.3 (n= 73)	30.2 ± 11.7 (n= 18)	27.3 ± 3.6 (n= 79)
Vitamin A >1.5 µmol/L	35.6 ± 6.5 (n= 22)	55.2 ± 5.5 (n= 98)	30.6 ± 5.5[a] (n= 39)	26.0 ± 4.8[b] (n= 58)
Vitamin D <50 nmol/L	54.2 ± 5.8 (n= 82)	46.1 ± 8.2 (n= 38)	25.7 ± 3.9 (n= 68)	32.3 ± 7.5 (n= 31)
Clinically detected Infections				
Caries	74.0 ± 12.2[a] (n= 25)	45.8 ± 4.9[b] (n= 95)	21.4 ± 8.3[b] (n= 18)	29.2 ± 3.9[a] (n= 81)
Scabies	53.1 ± 11.8 (n= 26)	51.2 ± 5.1 (n= 94)	25.8 ± 9.5 (n= 8)	27.9 ± 3.8 (n= 91)
Laboratory detected infections				
AB/UTI	53.5 ± 7.1 (n= 63)	47.1 ± 6.1 (n= 55)	26.0 ± 6.4 (n= 34)	30.0 ± 4.5 (n= 60)
Bacterial vaginosis	52.5 ± 6.0 (n= 73)	50.8 + 7.9 (n= 46)	21.7 ± 4.3 (n= 50)	22.8 ± 5.6 (n= 29)
Lactobacillus	48.8 ± 6.7 (n= 59)	54.8 ± 6.8 (n= 60)	22.2 ± 6.9 (n= 21)	22.0 ± 3.9 (n= 58)
Bacteroides/ Gardnerella	51.5 ± 4.8 (n= 114)	59.4 ± 29.6 (n= 5)	22.5 ± 3.5 (n= 77)	5.2 ± 4.3 (n= 2)
Mobiluncus	48.8 ± 4.9 (n= 95)	63.9 ± 13.7 (n= 24)	20.6 ± 3.4 (n= 69)	31.4 ± 13.4 (n= 10)
Vaginal Trichomoniasis	52.8 ± 5.8	49.1 ± 8.0	23.0 ± 3.7	12.0 ± 3.7

	(n= 88)	(n= 31)	(n= 72)	(n= 7)
Vaginal yeast infection	47.2 ± 10.3 (n= 33)	53.3 ± 5.2 (n= 87)	16.0 ± 5.7 (n= 9)	22.9 ± 3.8 (n= 70)
Vaginal Diplococcal infection	64.4 ± 14.3 (n= 24)	48.6 ± 4.7 (n= 95)	18.1 ± 4.5 (n= 25)	23.9 ± 4.5 (n= 54)
Ascaris	44.0 ± 8.1[b] (n= 39)	55.3 ± 5.8[a] (n= 81)	35.0 ± 11.9 (n= 4)	35.9 ± 10.4 (n= 19)
Hookworm	55.9 ± 6.5 (n= 68)	46.1 ± 6.8 (n= 52)	34.8 ± 14.7 (n= 11)	36.5 ± 10.6 (n= 12)
Trichuris	33.3 ± 10.9 (n= 15)	54.3 ± 5.1 (n= 105)	49.4 ± 27.3 (n= 2)	34.4 ± 9.4 (n= 21)

[1]Values are means ± SE. Means with different superscripts are significantly different at P <0.05

Table 3 Multiple regression of Log CRP with indicators of inflammation in pregnant and lactating women

CRP (nmol/L) in pregnant women[1]	**Coefficient ± SE**	**P**	**Overall Model**
BMI, kg/m^2	-0.06 ± 0.04	0.039	n= 114
Neutrophil/lymphocyte ratio	0.14 ± 0.07	0.041	$F_{5, 108}$ = 4.85
Plateletcrit, %	0.06 ± 0.02	0.024	P = 0.0005
IL6, pg/mL	0.01 ± 0.009	0.122	Adj. R^2 = 0.145
IL13, pg/mL	0.02 ± 0.01	0.142	VIF= 1.18
Constant	2.83 ± 0.94	0.003	
CRP (nmol/L) in lactating women[2]	**Coefficient ± SE**	**P**	**Overall Model**
Parity	-0.12 ± 0.05	0.026	n= 97
BMI, kg/m^2	0.06 ± 0.04	0.96	$F_{6, 90}$= 7.09
Neutrophil/lymphocyte ratio	0.29 ± 0.16	0.072	P < 0.0001
Eosinophils x10^3/mm$^{\#}$	-0.61 ± 0.19	0.002	Adj.R^2= 0.275
Plateletcrit, %	0.04 ± 0.02	0.016	VIF = 1.12
IL4	0.03 ± 0.02	0.144	
Constant	-0.29 ± 1.23	0.813	

[1]Variables that were explored but did not enter the pregnancy model (P> 0.15): Gestational age, parity, IL4, IL10, IL12, IL17, TNFα

[2]Variables that were explored but did not enter the lactation model (P> 0.15): Weeks postpartum, IL17

Table 4 Multiple linear and logistic regression models for Log CRP and elevated CRP in pregnant women

CRP (nmol/L) in pregnant women[1]	**Coefficient ± SE**	**P**	**95% CI**	**Overall Model**
Wood smoke, h/d	0.15 ± 0.07	0.032	0.01, 0.30	
Caries, presence	0.66 ± 0.26	0.012	0.15, 1.12	
Lactobacillus, score	-0.27 ± 0.11	0.020	-0.5, -0.04	n= 116
Bacteroides/Gardnerella, score	-0.35 ± 0.14	0.019	-0.64, -0.06	$F_{9, 106}$= 3.99
Mobiluncus, score	0.20 ± 0.10	0.059	-0.008, 0.42	P= 0.0002
Ascaris, presence	-0.73 ± 0.23	0.002	-1.20, -0.27	Adj.R^2= 0.189
Hookworm, presence	0.56 ± 0.22	0.014	0.11, 1.02	VIF= 1.52
Trichuris, presence	-0.57 ± 0.33	0.092	-1.24, 0.09	
Vitamin A <1.05 µmol/L	0.34 ± 0.22	0.122	-0.09, 0.78	
Constant	1.55 ± 0.06	0.019	0.26, 2.85	
Elevated CRP in pregnant women[2]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Gestational age	0.92 ± 0.03	0.022	0.87, 0.98	
Wood smoke, h/d	1.50 ± 0.29	0.034	1.03, 2.19	n= 116
Caries, presence	3.86 ± 2.46	0.034	1.10, 13.4	P=0.0006
Vaginal yeast, severity	0.36 ± 0.18	0.050	0.13, 0.99	Pseudo R^2=
Diplococcal infection, severity	2.04 ± 0.53	0.007	1.22, 3.42	0.243
Folic acid <10 nmol/L	3.53 ± 2.34	0.057	0.96, 12.9	VIF= 1.09
Vitamin A <1.05 µmol/L	2.45 ± 1.43	0.124	0.78, 7.72	
Vitamin D <50 nmol/L	2.74 ± 1.91	0.148	0.69, 10.7	

[1]Variables that were explored but did not enter the linear regression model for CRP (nmol/L) in pregnancy (P> 0.15): Gestational age, parity, BMI (kg/m^2), fieldwork (h/d), presence of scabies and AB/UTI, score of trichomoniasis, vaginal yeast and diplococcal infection, folic acid <10 nmol/L, vitamin B_{12} <150 pmol/L, vitamin A >1.5 µmol/L. Vitamin D <50 nmol/L

[2]Variables that were explored but did not enter the logistic regression model for elevated CRP (>28.5 nmol/L, >193.34 nmol/L and >77.14 nmol/L in the first, second and third trimesters, respectively [20]) in pregnancy (P> 0.15): Parity, BMI (kg/m^2), fieldwork (h/d), presence of scabies, AB/UTI, *Ascaris*, hookworm, *Trichuris*, score of *Lactobacillus*, *Bacteroides/Gardnerella*, *Mobiluncus*, trichomoniasis, vitamin B_{12} <150 pmol/L, vitamin A >44.3 µmol/L

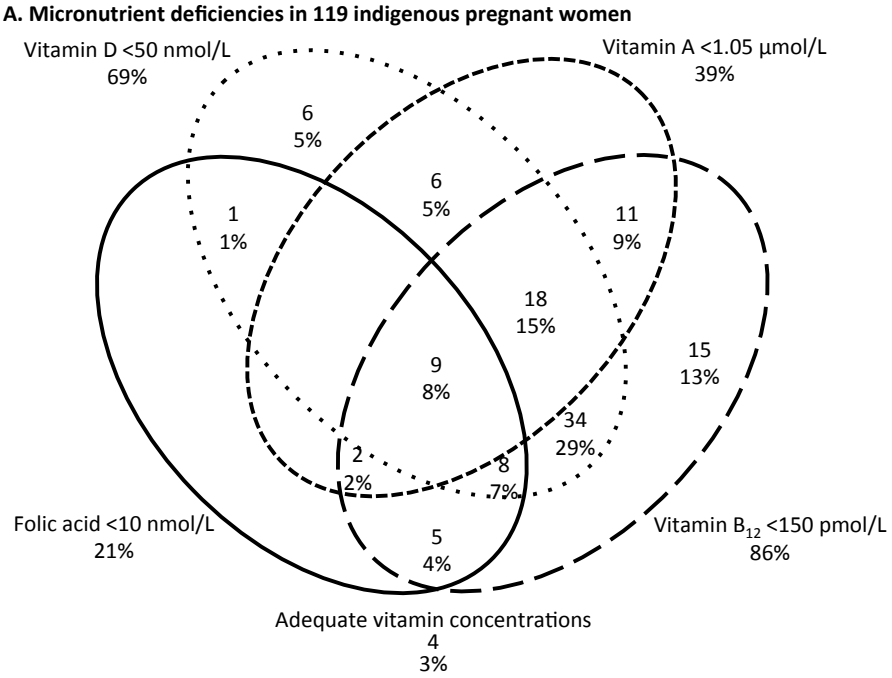
Table 5 Multiple linear and logistic regression models for Log CRP and Elevated CRP in lactating women

CRP (nmol/L) in lactating women[1]	**Coefficient ± SE**	**P**	**95% CI**	**Overall Model**
Parity	-0.17 ± 0.06	0.006	-0.29, -0.05	
Fieldwork, h/d	0.08 ± 0.05	0.119	-0.02, 0.19	n= 78
Eosinophils, number	-0.48 ± 0.20	0.019	-0.88, -0.08	$F_{8, 69} = 4.11$
Caries, presence	-0.61 ± 0.34	0.080	-1.30, 0.07	P= 0.0005
Mobiluncus, score	-0.31 ± 0.13	0.018	-0.57, -0.05	Adj. R^2= 0.244
Folic acid <10 nmol/L	0.64 ± 0.29	0.033	0.05, 1.23	VIF= 1.18
Vitamin A <1.05 µmol/L	-0.73 ± 0.38	0.061	-1.50, 0.03	
Vitamin A >1.5 µmol/L	0.54 ± 0.31	0.085	-0.07, 1.16	
Constant	3.57 ± 0.50	<0.0001	2.56, 4.57	
Elevated CRP in lactating women[2]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Parity	0.53 ± 0.12	0.007	0.34, 0.84	n= 78
BMI	1.31 ± 0.16	0.029	1.02, 1.66	P= 0.0010
Mobiluncus, score	0.51 ± 0.18	0.066	0.25, 1.04	Pseudo R^2= 0.243
Eosinophils, number	0.24 ± 0.16	0.031	0.06, 0.88	VIF= 1.08
Trichomoniasis, score	2.52 ± 0.97	0.016	1.18, 5.36	

[1]Variables that were explored but did not enter the linear regression model for CRP (nmol/L) in lactation (P> 0.15): Weeks post-partum, BMI (kg/m^2), wood smoke (h/d), presence of caries, score of vaginal *Lactobacillus*, *Bactaroides/Gardnerella*, *T.vaginalis*, yeast and diplococcal infection, vitamin B_{12} <150 pmol/L, vitamin D <50 nmol/L.

[2]Variables that were explored but did not enter the logistic regression model for elevated CRP in lactation (P >0.15): Weeks post-partum, wood smoke exposure (h/d), fieldwork (h/d) presence of caries, score of vaginal *Lactobacillus*, *Bacteroides/Gardnerella*, *Mobiluncus*, yeast and diplcoccal infection, folic acid <10 nmol/L, vitamin B_{12} <150 pmol/L, vitamin A <1.05 µmol/L, vitamin A >1.5 µmol/L, vitamin D <50 nmol/L.

Figure 1 Venn diagram of micronutrient deficiencies in (A) pregnant women, and (B) lactating women



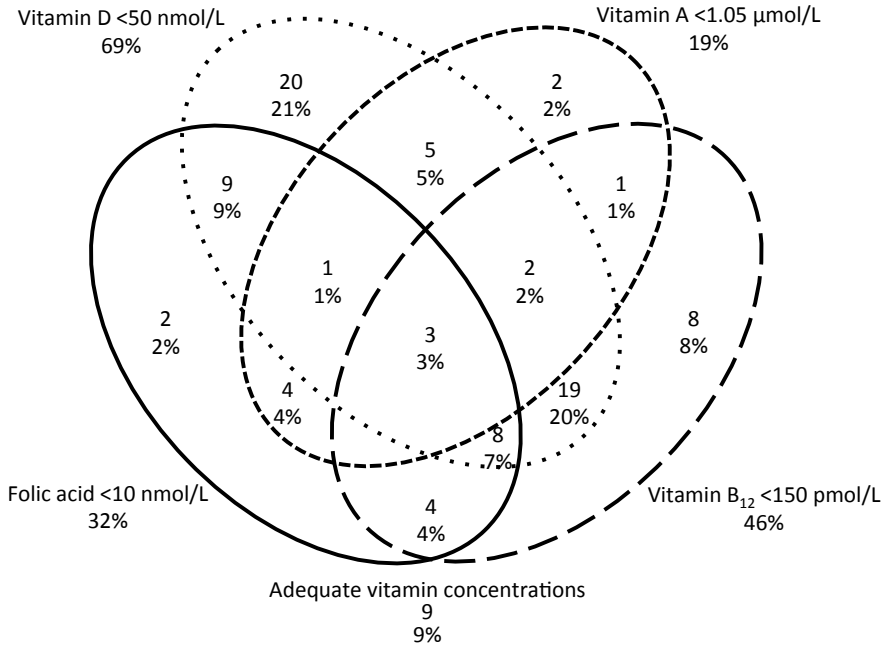
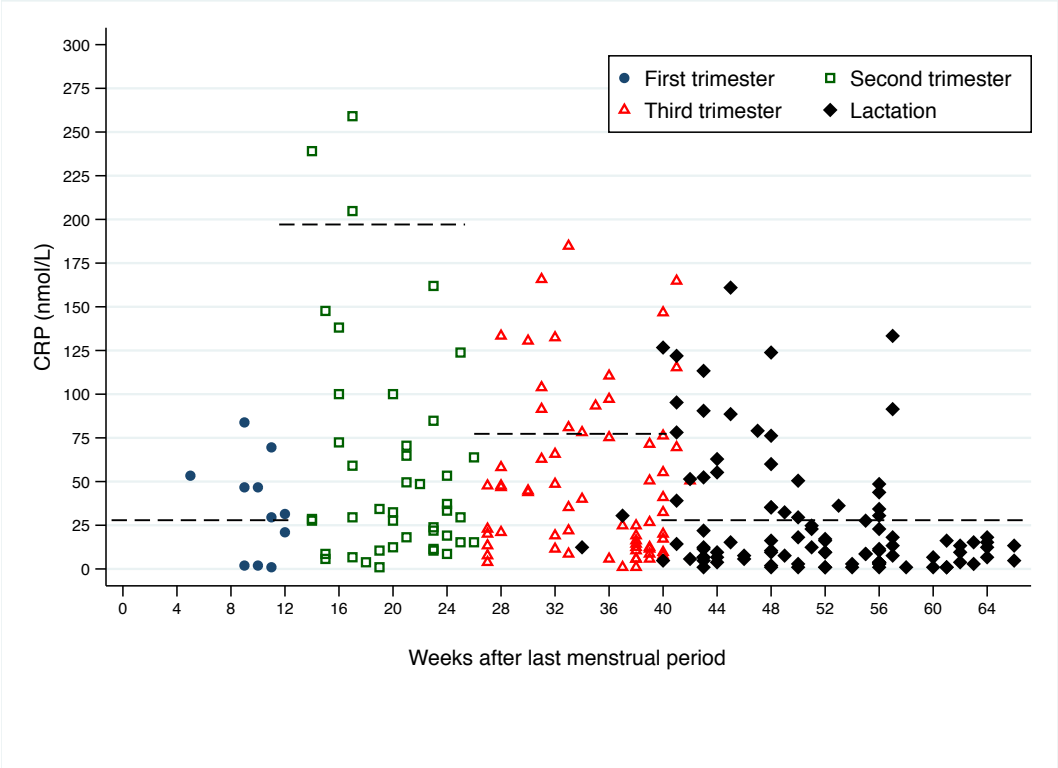


Figure 2 Mean CRP (nmol/L) by weeks after last menstrual period in indigenous pregnant and lactating women.



Circles, squares and triangles indicate pregnant women in their first, second and third trimesters, respectively. Diamond markers indicate lactating women. Dashed horizontal lines denote cutoffs for elevated CRP (first trimester and lactation= 28.5 nmol/L; second trimester= 193.3 nmol/L; third trimester= 77.1 nmol/L) [20].

Connecting statement 1

The paper on C-reactive protein (CRP) raised questions regarding the impact of a high prevalence of inflammation on maternal and fetal outcomes and set the context to term the cohort as having Multiple Infections, Nutrient Deficiencies and Inflammation (MINDI).

We have described for the first time bimodal associations of CRP with vaginal commensal bacteria and infections. Higher CRP was found in women with infections eliciting a Th1 response (caries, *Diplococcus*, hookworm), whereas lower CRP was found in women with higher score of *Lactobacillus* and infections (*Bacteroides/Gardnerella, Ascaris*) known to elicit a Th2 response. Given that CRP is the biomarker most frequently used for the assessment of inflammation in population studies that evaluate anemia and iron deficiency, which are chief priorities for Panamanian health authorities, we aimed to observe how CRP behaved as biomarker of inflammation in a MINDI environment, as well as to understand associations of biomarkers of anemia and iron deficiency with MINDI.

It is suspected that a combination of nutritional anemia and anemia of inflammation might be present in the population, and that CRP might have limitations for evaluating iron status given its possible down-modulation by Th2 eliciting infections.

Paper 2. Anemia and biomarkers of iron deficiency are associated with Multiple Infections, Nutrient Deficiencies and Inflammation in indigenous pregnant women from Panama: The MINDI Cohort

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List of abbreviations

BRINDA: Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia; CRP: C-reactive protein; epg: Eggs per gram; Hb: Hemoglobin; Hct: Hematocrit; IL: HIV: Human immunodeficiency virus; Interleukin; INF: Interferon; MCV: Mean corpuscular volume; MMN: Multiple micronutrients; MINDI: Multiple infections, nutrient deficiencies and inflammation; RBC: Red blood cell; RBP: Retinol-binding protein; RDW-CV: Red cell distribution width–coefficient of variation; RDW-SD: Red cell distribution width–standard deviation; sTfR: Serum transferrin receptor; TNF: Tumor necrosis factor; ULE: Urinary leukocyte esterase; USG: Urinary specific gravity; UTI: Urinary tract infection; VIF: Variance inflation factor; WBC: White blood cells; WHO: World Health Organization.

Abstract

Background: The difficulty of assessing anemia and iron deficiency during pregnancy in the presence of co-existing infections, nutrient deficiencies and inflammation presents challenges regarding development of appropriate public health policies for the treatment of anemia.

Objectives: We aimed to identify determinants of anemia and hemoglobin in a cohort of pregnant women with Multiple Infections, Nutrient Deficiencies and Inflammation (MINDI), and to identify associations of MINDI with iron status indicators: ferritin, serum iron and serum transferrin receptor (sTfR).

Methods: In a cohort of 213 pregnant women attending normal pregnancy follow-up in the Ngäbe-Buglé, indigenous community, we gathered information on maternal exposure to wood smoke; intake of iron and multiple-micronutrient (MMN) supplements; oral, skin, urogenital and intestinal nematode infections; complete blood count; iron indicators (ferritin, serum iron, sTfR) and hepcidin; serum nutrients (retinol-binding protein as indicator of protein status, folic acid, vitamins A, B_{12} and D); and inflammation indicators (C-reactive protein -CRP, cytokines). Multiple logistic and linear regressions identified MINDI variables associated with anemia and iron status indicators.

Results: The prevalence of anemia was 38%. Anemia was associated with ferritin <20 µg/L (P= 0.002), with lower folic acid (P= 0.044) and vitamin A status (P= 0.045) and with hepcidin >6.1 µg/L (P= 0.023). On the other hand, higher hemoglobin was associated with lower CRP (P= 0.018), protein deficiency (P= 0.018) and with higher serum concentrations of folic acid (P= 0.020). Interestingly, iron supplementation was not associated with hemoglobin, serum iron or sTfR, and only entered the ferritin model when intestinal nematodes were included (P= 0.006). In addition, folic acid was positively associated with ferritin (P= 0.001) and serum iron (P= 0.001) whereas lower vitamins A (P= 0.032) and D (P= 0.025) were associated with higher sTfR. With regards to inflammation indicators, a higher monocyte count was associated with both lower ferritin (P= 0.016) and serum iron (P <0.0001) but higher eosinophil count was positively associated with both indicators (P= 0.027 for ferritin and P= 0.009 for serum iron). Cytokines entered the sTfR model, with IL17 associated with lower sTfR (P= 0.015), but IL13 with higher sTfR (P= 0.037), however, these associations lost significance when including intestinal

nematodes in the models. With regards to specific infections, higher *Trichuris* burden was associated with lower hemoglobin (P <0.0001), higher hookworm burden was associated with lower ferritin (P= 0.025) and serum iron (P <0.0001) but with higher sTfR (P= 0.001), whereas higher *Ascaris* load was associated with higher ferritin (P= 0.030) and serum iron (P= 0.008). Multiple deficiencies and iron restriction due to inflammation supported by high hepcidin concentrations helped explaining lack of response of anemia to iron supplementation in this population.

Conclusions: The complexity of associations between iron status indicators and multiple infections, nutrient deficiencies and inflammation in vulnerable pregnant women highlight the need for a more comprehensive public health strategy for treating anemia, which includes nutritional supplementation strategies, and considers the contribution of infections and inflammation to its ethiology.

Introduction

Anemia is indirectly responsible for 50% of severe adverse maternal outcomes, according to a multi-country survey of women from Africa, Asia, Latin America, and the Middle East [1]. The most recent review on anemia in developing countries found associations of anemia in the two first trimesters with low birth weight, preterm birth, and perinatal, neonatal and maternal mortality [2]. Pregnant women are particularly vulnerable to the harmful effect of anemia given the higher nutritional physiological demands, especially in developing countries where populations are usually deficient in more than one micronutrient [3]. In such settings, poor nutrition and infectious diseases remain main causes of anemia despite global efforts to improve maternal health [2].

Most often iron deficiency is considered the main cause of anemia globally [4] and during pregnancy [5], but other causes of anemia like micronutrient deficiencies [6], including vitamin A [7, 8], vitamin D [9-11], and vitamin B_{12} [12, 13], are of public health concern; in contrast folic acid deficiency as cause of anemia in Latin America is now considered unlikely, following implementation of National fortification policies [14, 15].

Anemia has also been associated with parasitic infections [16] and inflammation that usually co-exist in developing settings [17, 18]. Infection by malaria [19], hookworm [20], and the interaction of malaria with soil-transmitted helminth infections [21] are recognized causes of anemia. Indeed, the prevalence of anemia is higher in countries with high infection burden (40%), compared with countries with moderate (12%) and low (7%) infection burdens [22]. On the other hand, the prevalence of inflammation was similarly associated with anemia in different infections settings, indicating that inflammation causing anemia is independent of infections [22]. However, little is known on the range of multiple infections that pregnant women experience in low-resource settings.

In a MINDI context, the selection of biomarker for assessing anemia and iron status has been just partially investigated. In general, for most clinicians, the terms anemia and iron-deficiency

anemia are used interchangeably, and anemia is considered an indicator of iron deficiency [3], where both hemoglobin (Hb) [23] and hematocrit (Hct) [24, 25] are used to interpret the severity of iron deficiency. Red blood cell (RBC) indices are also used to differentiate iron deficiency anemia from other types of anemia by using mean corpuscular volume (MCV) and the heterogeneity of RBC defined by the deviation in red cell width (RDW) [26], given that impaired erythropoiesis or abnormal red blood cell survival increase RDW [27, 28].

Currently, the World Health Organization (WHO) considers the measurement of both serum ferritin and serum transferrin receptors (sTfR) as optimal to predict a change in hemoglobin concentration in response to iron intervention [23]. Serum iron is used in clinical and research practices, but given that it has diurnal fluctuations and decreases in the presence of inflammation [23, 29, 30], its use as the sole indicator of iron deficiency in pregnancy has been discouraged [31]. Serum ferritin reflects iron stores, and is routinely used in both clinical and public health settings for detecting iron deficiency [32], but WHO discourages the use of ferritin as an iron indicator in populations where inflammation is prevalent as it is a positive acute phase reactant [23, 32]. However, the amount of intracellular iron that modulates the amount of surface transferrin receptors in erythroid cells is reflected in serum transferrin receptors (sTfR) [33]. It is believed that sTfR does not react to inflammation and WHO had proposed the prevalence of iron deficiency be based on sTfR, together with a measurement of inflammation, usually C-reactive protein (CRP) [23, 32].

Traditionally CRP has been used to control for inflammation in the presence of anemia in populations with high prevalence of infection [34] including pregnant women [35] despite the known physiological rise of CRP during pregnancy [36]. The emergence of hepcidin as biomarker of iron restriction due to inflammation [37] has allowed researchers to distinguish anemia of inflammation in pregnant women infected with *Schistosoma japonicum* in The Philippines [38], and has been proposed as alternative measurement of iron status following studies performed in Ghana [39]. However, those studies have proposed different cut-offs for recognizing iron restriction due to inflammation [38, 40].

Our study population of women from the Ngäbe-Buglé indigenous communities in West-Panama live in conditions of extreme poverty in a non-endemic zone for malaria, and has high prevalence of anemia (>40% in indigenous communities [41]), despite efforts from the Panamanian Ministry of Health through programs of iron and multiple micronutrient (MMN) supplementation for pregnant women. We had previously reported that 95% of pregnant women from these communities had at least two concurrent chronic mild-moderate infections (caries, scabies, urinary tract infection, vaginal infections and intestinal parasites) and where neither malaria or human immunodeficiency virus (HIV) were present [42]. These women also had deficiencies in vitamins B_{12} (85%), D (64%), A (41%), and folic acid (24%), and inflammation measured through elevated CRP in 17% [43].

In this population with multiple infections, nutrient deficiencies and inflammation (MINDI), our objectives were to explore the contribution of MINDI to the problem of anemia, and to detect associations of Hb and iron status indicators (ferritin, serum iron and sTfR) with MINDI variables. Understanding associations of infections, inflammation and nutrient deficiencies may help to identify the most useful biomarker(s) to assess anemia and iron deficiency in this MINDI cohort, given their importance as risk factors for adverse pregnancy outcomes [2].

Methods

Maternal characteristics and methods for data collection have been fully described previously [42]. Briefly, a total of 213 pregnant women were recruited while attending their routine follow-up in local health centers in the Ngäbe-Buglé indigenous territory located in West-Panama. Of these, 24 women were in their first, 80 in their second and 109 in their third trimester. Data on obstetric history, diet, and hours/d of wood smoke exposure and fieldwork were collected. Mothers also provided information duration and daily intake of iron supplements and tbsp./d of multiple micronutrient supplement powder containing in the recommended 90 g daily portion: 315 Kcal, 10.8 gr of protein, 225 µg of vitamin A, 9 mg of vitamin E, 126 mg of vitamin C, 0.4 mg of thiamine, 0.4 mg of riboflavin, 5.4 mg of niacin, 0.4

mg of vitamin B_6, 0.8 mg of B_{12}, 76.5 µg of folic acid, 10 mg of iron, 7.2 mg of zinc, 225 mg of calcium, 90 mg of phosphorus and 360 mg of cupper [44]. Folic acid distribution was not widely available at the time of the study, and women did not recognize folic acid tablets from other medications, therefore it was not possible to assess folic acid supplementation intake.

Maternal weight by height by gestational age was classified using Pan American Health Organization charts [45]. Presence of caries and scabies were clinically assessed, and urine (n= 208) and vaginal samples (n=211) were used to detect presence and severity of uro-genital infections. Urinary tract infection (UTI) severity was assessed by score (0+ to 3+) of urinary leukocyte esterase (ULE). Urine analysis also detected the presence of hypo-hydration if urinary specific gravity (USG)>1020 [46]. Intensity of intestinal parasites (*Ascaris*, hookworm and *Trichuris*) using Kato-Katz technique [47] were quantified as eggs per gram (epg) in 106 women as previously described [42].

Whole blood was processed for RBC indices for all women as well as total and differential white blood cells (WBC) and platelet indices (BC-5500 Mindray Auto Hematology Analyzer) at the local laboratory of San Félix Hospital, Panama.

Anemia was defined as Hb <110 g/L [23]. Cut-offs by trimester (first, second and third respectively), were used to define low hematocrit (31%, <30% and <28%), mean corpuscular volume for microcytosis (MCV <85, <85.8 fL and <82.4 fL), and macrocytosis (MCV >97.8 fL, >99.4 fL and >100 fL) and high RDW-CV (≥14.9% >14.7% and >16.6%) [48]. An RDW-SD cutoff at >46 fL [49] was used as for the general population, as not defined specifically for pregnancy.

Serum samples were analyzed for serum iron (spectrophotometry, FERENE®-ENDPOINT), ferritin (ELISA, MP Biomedicals), sTfR (RAMCO immunoassay) and CRP (ELISA, MP Biomedicals) in the National Reference Gorgas Institute Laboratory, and vitamin A (HPLC) in INDICASAT in Panama City. Folic acid and vitamin B_{12} (chemiluminescence, MODULAR E170, Roche Diagnostics) were processed at the Canadian Diagnostic Laboratories in Montreal. Interleukin

(IL)1β, IL4, IL6, IL10, IL12, IL13, IL17, interferon (INF)Ɣ, tumor necrosis factor (TNF)α (Luminex Magnetic Bead Panel, Millipore), 1-25 OH vitamin D (LIAISON, DiaSorin), and retinol-binding protein (RBP) (Human RBP4-ELISA, MP Biomedicals) at the School of Human Nutrition (McGill University), and hepcidin (Intrinsic Hepcidin IDx™ ELISA Kit, Intrinsic LifeSciences) at the Center for Iron Disorders, University of California.

Nutrient deficiencies were set as serum iron <8.9 µmol/L, ferritin <20 µg/L [23], high sTfR >8.3 mg/L (laboratory kit RAMCO®), folic acid <10 nmol/L [50], vitamins B_{12} <150 pmol/L [50], A <1.05 µmol/L [51, 52], and D <50 nmol/L [53]. RBP <30 mg/L was considered as low protein status [54, 55], and elevated CRP as >3 mg/L, >20.3 mg/L and >8.1 mg/L in the first, second and third trimester respectively [48].

Statistical analyses:

Statistical analyses were run using STATA 14®. Scatter plots with regression lines were drawn for associations between hemoglobin and months on iron supplements and between hepcidin and the iron status indicators. Student's t-test, Kruskall-Wallis test, Chi^2 or Fisher exact were used to identify differences in independent variables between anemic and non-anemic women. Student's t-tests were also used to assess differences in Hct by elevated USG and low protein status. Venn-diagrams described co-occurrence of anemia with micronutrient deficiencies and with high hepcidin defined as >6.1 µg/L [38]. Also, the co-occurrence of abnormal iron status indicators and of low ferritin, low serum iron and high hepcidin were graphed. Univariate regression models were used to observe the association between our outcome variables anemia (logistic regressions) and hemoglobin (linear regressions) with iron deficiency, indicated by low ferritin, low serum iron and high sTfR. Models were run separately given that iron status indicators were correlated with each other.

A series of initial stepwise backwards multiple regressions were conducted for outcome variables (linear analyses for Hb, log-serum iron, log-ferritin and log-sTfR and logistic analysis for anemia) using several distinct groups of independent variables: 1) maternal characteristics;

2) iron and MMN supplementation; 3) RBC indices; 4) biomarkers of inflammation (CRP, WBC counts, platelets, and cytokines); 5) infections (caries, scabies, UTI, bacterial vaginosis, *Trichomonas*, yeast, *Diplococcus* scores); and 6) nutrients (folic acid, vitamins B_{12}, A and D, RBP). From each model, variables with P<0.15 were included in the final multiple logistic/linear regression models. Both initial and final models were adjusted for trimester. Collinearity was assessed using a variance inflation factor <10 and a condition number <30. The assumption of linearity of entering variables was tested using augmented component-plus-residual plots in linear regression models, and the link test for model specification and Box-Tidwell regression models for logistic regression models. Breusch-Pagan/Cook-Weisberg test and White test were used to assess heteroscedasticity [56]. When running models using the subsample of women with data on intestinal nematode epgs, Little's test for randomness of missing data was used [57].

In order to explore if iron restriction due to inflammation was associated with anemia, the independent continuous variable hepcidin or two cutoffs, one at >6.1 μg/L [38] and a second >2.5 μg/L [40] were separately added to the final model. Also, univariate linear regression models for hepcidin and CRP, ferritin, serum iron and sTfR were run. Significant associations were drawn in scatter plots.

Results

Maternal characteristics of anemic compared with non-anemic women are shown in Table 1. No correlation was found between hemoglobin concentrations and months on iron supplementation, and despite iron supplementation, 38% of women were anemic (Fig 1). Anemic mothers had a history of more pregnancies, lower BMI, and as expected, compared with non-anemic mothers, RDW was higher. Although a low Hct was more frequent in mothers with anemia, its prevalence was only 4.2%. Hct was higher in women with RBP<30 mg/L (35.4 ± 0.4%) compared with women with RBP≥30 mg/L (34.6 ± 0.2, P= 0.043), but did not differ between women with USG >1020, indicative of hypo-hydration, and USG ≤1020 (P=0.43).

Anemia was mostly normocytic, with a low frequency of micro- and macrocytosis (Table 1). Mothers had a combination of deficiencies known to produce micro- or macrocytosis. One or more iron indicators (ferritin, serum iron or sTfR) showed iron deficiency in 78.4% of the women, whereas only 6.5% had microcytosis. Interestingly, most cases of anemia (95.5%) co-occurred with either iron deficiency (ferritin <20 μg/L) or with iron restriction due to inflammation (hepcidin >6.1 μg/L); importantly, 61.7% of women with anemia had evidence of both conditions (Fig 2A). Also most women (86.8%) had either low B_{12} or low folic acid (Fig 2B), but macrocytosis was found in only 12.2%. On the other hand, high RDW-SD captured an important proportion of women (34.8%) with RBC size heterogeneity that better reflected the combination of nutrient deficiencies in both anemic and non-anemic women. Comparisons between mothers with and without anemia also showed that vitamin A concentrations were lower in anemia cases, but distribution of inflammation indicators was not different between women with and without anemia, except for IL10, which showed lower concentrations in anemic mothers. Among infections, only the frequency of UTI was lower in anemic women, but other infections, including intestinal nematodes, did not differ.

Regression models for anemia (Table 2)

Univariate logistic regression models for anemia showed that iron deficiency was associated with higher odds of anemia, but both pseudo R^2 indicated that iron deficiency captured <7% of the variability in the proportion of anemia (Table 2A). Multiple logistic regression models confirmed that low ferritin was associated with increased odds of anemia (Table 2B). Moreover, as expected, higher weight by height classification based on Pan-American Health Organization standards, which was present in 23%, was associated with lower odds of anemia, whereas higher parity was associated with increased odds of anemia. Two other nutrients entered the model. Higher folic acid and vitamin A were associated with lower odds of anemia. When testing the hypothesis that iron restriction due to inflammation may be associated with anemia, the model with the continuous variable hepcidin or with hepcidin >2.5 μg/L, showed no significant associations. However, when adding hepcidin >6.1 μg/L into the model, high

hepcidin was associated with increased odds of anemia and captured 14% of variability and increased the odds (OR 2.39, p< 0.02) of anemia (Table 2C).

Regression models for Hemoglobin (Table 3):

Univariate linear regression models for Hb showed lower Hb concentrations were associated with iron deficiency. However, adjusted R^2s showed that iron deficiency predicted only between 4% and 11.4% of the variability of Hb (Table 3A). Our multiple linear regression model predicted 26.6% of the variability of Hb, and showed that, besides ferritin, higher parity was associated with lower Hb, whereas higher folic acid was associated with higher Hb (Table 3B). Interestingly, low protein status was associated with higher Hb, suggesting the presence of hemoconcentration related to hypoproteinemia. In the model with intestinal nematodes, higher *Trichuris* epg together with higher concentrations of CRP and low ferritin were associated with lower Hb concentrations but neither folic acid nor low protein emerged (Table 3C). Of note, despite a lower sample size, the nematode model captured 32.4% of the variability in Hb.

Multiple linear regression models for iron status indicators:

Log-Ferritin (Table 4):

As expected, higher ferritin was associated with higher CRP. Lower ferritin was associated with more advanced pregnancy and with higher monocyte number, whereas higher ferritin was associated higher concentrations of vitamin B_{12} and folic acid (Table 4A). Higher ferritin was also associated with higher BMI and with more severe UTI. When including intestinal nematodes in the model, higher ferritin was associated with higher *Ascaris* epg, whereas lower ferritin was associated with higher hookworm epg. Vitamin B_{12} and monocyte number remained as positively and negatively associated with ferritin respectively, and eosinophils became positively associated. Of note, folic acid and CRP did not enter the model.

Log-Serum iron (Table 5):

As expected, lower serum iron was associated with higher CRP. Similarly to ferritin, lower serum iron was associated with higher monocyte number, whereas higher serum iron was associated higher concentrations of vitamin B_{12} and folic acid (Table 5A). Higher serum iron was also associated with higher lymphocytes, eosinophils, IL10 and more severe vaginal yeast infection, but lower serum iron was associated with the presence of caries and more severe diplococcal infection (Table 5A). Similarly to ferritin, when including intestinal nematodes in the model, serum iron was associated with higher *Ascaris* epg whereas lower concentrations with higher hookworm epg. Monocyte number remained as negatively associated, as well as eosinophils and vitamin B_{12} remained as positively associated with serum iron. Of note, although folic acid did not enter the model, CRP remained negatively associated with serum iron.

Serum transferrin receptor (Table 6):

Multiple regression models for log sTfR with a larger sample size captured associations with infections, nutrients and inflammation. The presence of caries was associated with higher sTfR, whereas more severe UTI (higher ULE score), with lower sTfR. Vitamin A<1.05 µmol/L and low vitamin D concentrations were associated with higher sTfR. Finally, pro-inflammatory cytokine IL17 was associated with lower sTfR, whereas IL13, known Th2 cytokine, was associated with higher sTfR (Table 6A). Interestingly, when adding intestinal nematode loads (Table 6B), the model better explained the variability of sTfR despite a lower sample size. Whereas inflammation biomarkers lost significance and micronutrients did not enter the model, higher sTfR was associated with higher hookworm epgs, and infections (caries and more severe UTI) remained as significantly associated with sTfR in a similar fashion than the larger n model (Table 6B).

Presence of combined low iron status and iron restriction due to inflammation

More than 78% of women had low iron status characterized by low ferritin or low serum iron or high sTfR (Fig 3A). Ferritin identified the largest proportion of iron deficiency, followed by

serum iron, whereas sTfR alone identified only one woman with increased sTfR but normal ferritin and serum iron (Fig 3A). The Venn diagram of low ferritin, low serum iron and hepcidin >6.1 μg/L (Fig 3B) showed that either low iron status (low ferritin or low serum iron) or iron restriction due to inflammation (high hepcidin) were present in 96% of the population. Finally, univariate linear regression models for log-hepcidin showed that it was positively associated with ferritin (Fig 4A), but was not significantly associated with serum iron (CI: -0.001, 0.03, P= 0.078, R^2= 0.01). Univariate linear regressions also showed that hepcidin was positively associated with CRP (Fig 4B) and negatively associated with sTfR (Fig 4C), our indicator of erythropoiesis.

Discussion

The wide range of available information of indigenous pregnant women in a remote community made possible a comprehensive analysis of anemia and iron status indicators, where our findings clearly indicate that several paradigms regarding the use of biomarkers are not applicable to our population with multiple infections, nutrient deficiencies and inflammation. First, anemia was multi-causal. Anemia was associated with both iron deficiency and iron restriction due to inflammation, and with folic acid and vitamin A deficiencies. Importantly, the combination of multiple factors able to influence RBC indices (Hct, MCV and RDW-CV), made them appear normal, making even more difficult the identification of women with of anemia and/or nutritional deficiencies. Second, the explanatory power of our multiple linear regression models for Hb, serum iron, and sTfR was improved by adding nematode epgs, despite the lower sample size, revealing the importance of intestinal nematodes in the overall iron status of our population. Third, under our MINDI conditions, biomarkers other than CRP such as WBC counts and cytokines were associated with particular iron status indicators and are potential candidates to control for inflammation. In particular, Hb, ferritin and serum iron were bi-directionally associated with markers of inflammation such that a Th1 type of response was associated with lower Hb (CRP) and iron status indicators (monocytes and IL17) whereas a Th2 response was associated with higher Hb (IL10) and iron status (eosinophils, IL13). Fourth, we also provide evidence that inflammation was blunting erythropoiesis, and as a consequence

sTfR was not a good indicator of iron status in this population. Fifth, Hb and iron status were also bi-directionally associated with infections where a severe UTI, vaginal yeast and *Ascaris* were associated with higher iron status, but where higher loads of *Trichuris* were associated with lower Hb and hookworm with low ferritin and serum iron. Finally, elevated hepcidin was present in most women, indicating widespread iron restriction due to inflammation simultaneously with low iron status. Together these results provide the supporting evidence that multiple infections, nutrient deficiencies and inflammation may be behind the lack of response to iron supplementation in this population.

Use of RBC indices in MINDI populations

Diagnosis of anemia is based on Hb concentrations, but also Hct is used in developing settings to diagnose anemia, especially where automated hematology analyzers are not available [58], but Hct is more sensitive to plasma volume changes (e.g. dehydration), making it less reliable for the diagnosis of anemia [25]. In our study we observed that most women had normal Hct despite the presence of anemia or iron deficiency as indicated by low Hb and low ferritin or low serum iron or high sTfR. However, low Hct was present in all anemic women, and no women with normal Hb had low Hct. These facts, further supported by Hct being higher in protein deficient women, show that women may be experiencing hypovolemic hemoconcentration as low protein is a known cause of hypovolemia [59]. Therefore Hct may not be sufficiently sensitive to diagnose anemia in our population.

MCV, which morphologically classifies erythrocytes as microcytic, normocytic or macrocytic [60], is also used as first line evaluation of anemias, but is not always accurate in the diagnosis of deficiencies [61]. Microcytosis generally indicates the presence of iron deficiency [62], but macrocytosis appears when folic acid or vitamin B_{12} deficiencies are present [63]. We found that MCV was normal in 82.2% despite the high prevalence of low ferritin (68%), vitamin B_{12} (85%) and folic acid (24%) deficiencies. We found that 17.3% of anemic women were also microcytic, whereas a low proportion of anemic women (4.2%) had macrocytosis. Therefore, normocytosis reflected a mixed effect of deficiencies that took RBC size in both directions, and

therefore MCV did not help in detecting the presence of iron or folate/B_{12} deficiencies. On the other hand, RDW-CV can be used to detect iron deficiency [62] and has been effective to do so in an Indian population [24], but in our study, RDW-CV was not elevated. However, RDW can be reported either as coefficient of variation (% calculated from MCV) or as standard deviation (RDW-SD in fL); this latter calculation is not influenced by RBC size [49]. In our population, RDW-SD showed RBC size heterogeneity in mothers with and without anemia, better providing insight on the widespread severity of iron, folic acid and B_{12} deficiencies. Although RBC indices are first line tool for most clinicians identifying anemia and nutritional deficiencies associated with micro- and macrocytic anemia, our findings urge for the use of more sensitive diagnostic methods in the field, given that normal hematocrit, MCV and RDW-CV are not detecting women at risk in our MINDI context.

The problem of anemia

Anemia in our population was multi-causal. We observed that despite our final logistic regression models for anemia showed that iron deficiency indicated by low ferritin, low serum iron or high sTfR was associated with anemia. Those biomarkers predicted only 1-6% of the variability in the frequency of anemia, which contrasts with the proportion of anemic women who also had low ferritin in our population (81.5%), and with the reported 53-63% of anemia associated with iron deficiency in Latin America and the Caribbean [64]. Moreover, the ensemble of variables entering the model which included lower BMI, higher parity and lower concentrations of folic acid and vitamin A, predicted only 12.8% of the variability of the proportion of anemia, and although highly prevalent, neither infections nor inflammation indicators entered our final regression models for anemia using the cut-off of Hb<110 g/L. Interestingly, the cut-off of hepcidin >6.1 µg/L indicating iron restriction due to inflammation was associated with 2.3 fold increased odds of anemia, improving the variability captured by the model to 14%. Few other studies have investigated at hepcidin during pregnancy for the diagnosis of anemia of inflammation in developing settings [38, 39, 65, 66], but only Abioye et al found an association of anemia with hepcidin. His study also included women with similar hepcidin concentrations to our study and measured parasitic loads of *Ascaris*, hookworm,

Trichuris and *Schistosoma japonicum* [38]. Thus, we can infer that anemia of inflammation was present in our population. Therefore, anemia reflected the general poor nutritional status of women but was not specific for detecting the magnitude of the impact of nutrient deficiencies, infection or inflammation. Given the multi-causality of anemia, a therapeutic approach with iron supplementation alone may not be enough to decrease the prevalence of anemia.

The multi-causality of anemia drove us to examine associations of Hb as continuous variable with the MINDI variables. Higher Hb was associated with higher BMI, higher folic acid concentrations, higher IL10 and, and also with protein deficiency, whereas, lower Hb was associated with higher CRP, low ferritin and higher parity. Divergent associations of Hb with CRP and IL10 suggest that Hb may respond to pro- and anti- inflammatory environments. This model for Hb explained only 26.6% of the variability of Hb. Although often measured as covariate, Hb has been seldom investigated as dependent variable to observe its associations with cytokines. A study from Russia reported higher concentration of pro-inflammatory cytokines IL2 and IL8 but lower IL10 in pregnant women with anemia [67]. Interestingly, when intestinal nematodes were included in our model, more variability (32.4%) in the Hb concentration was captured. Moreover, despite a high prevalence of hookworm and *Ascaris*, only higher loads of *Trichuris* were associated with lower Hb. Few studies have investigated associations of intestinal nematodes with Hb concentrations in pregnancy. A higher relative risk of anemia has been reported in pregnant women from Venezuela infected by *Ascaris*, hookworm and *Trichuris* [68], whereas no association between anemia and intestinal nematodes infection was found in pregnant women from Papua New Guinea [69]. The fact that higher loads of *Trichuris*, and not the presence/absence of the parasite entered the model, highlights the importance of measuring intensity of parasitic infections for the study of anemia in pregnant women populations, as previously observed [70].

Intestinal nematodes and iron status indicators

One important contribution of this study was the observation of a high prevalence of intestinal nematodes and their association not only with Hb but also with iron status indicators,

suggesting that influence of intestinal nematodes beyond the known local effect on nutrient absorption and intestinal bleeding [71]. Of note, in our study it was not the prevalence of intestinal nematodes but the intensity of infections that entered our models, which was also observed in a study of anemia in pregnant women from Peru [72]. Others have found a negative [73] or no association between *Ascaris* presence [74] or parasitic load [75] and ferritin concentrations during pregnancy. In contrast, we found that higher ferritin and serum iron were associated with higher intensity of *Ascaris*. A possible role of *Ascaris* infection in decreasing inflammation could explain its positive association with iron status, supported by our previous finding of lower CRP associated with the presence of *Ascaris* in this population [43]. Associations of *Trichuris* and hookworm with lower ferritin concentrations in pregnant women have been documented before [75, 76] and are explained, in the case of hookworm, by the direct attachment to duodenal mucosa, where adult-stage parasites consume host blood to obtain nutrition [77], whereas *Trichuris* burrows the cecal mucosa [78] and high parasitic loads can produce bleeding due to mucosal erosions and ulcerations [79]. Beyond local tissue bleeding, we had previously reported that hookworm was associated with elevated CRP in our population of pregnant women [43], and it is known that infections decrease availability of iron via hepcidin's effect on iron retention inside macrophages [80] and decreasing of iron absorption as a host defense mechanisms [81].

Folic acid and vitamin B_{12} deficiencies

Contrary to the assumption that folic acid fortification has eradicated folic acid deficiency in Latin America [15], our study provided evidence that folic acid deficiency is still a problem in this marginalized community. Interestingly, folic acid deficiency had relatively low frequency (23.9%) compared with B_{12} deficiency (85%), but 92.1% of folic acid deficient women were also B_{12} deficient in our study population. It is known that low serum folate levels can be masked by an intracellular metabolic block in folate utilization during vitamin B_{12} deficiency, in which cases folate returns to basal concentrations after vitamin B_{12} replacement [82, 83]. Given that in our population vitamin B_{12} had the highest prevalence (85%), folic acid values may have been under-estimated by B_{12} deficiency. Thus, there is likely a need to correct not only folic acid but

also B_{12} deficiency in this marginalized community that is outside the range of food fortification programs.

Interestingly folic acid concentration was independently and positively associated not only with Hb, but also with ferritin and serum iron. Folate and B_{12} deficiencies can alter RBC nucleic acid synthesis, by impairing DNA and RNA production and leading to ineffective erythropoiesis, with nuclear/cytoplasmic size discrepancy and macrocytosis [84]. On the other hand, although the association of higher folic acid with higher ferritin and serum iron is less understood, there is evidence in the literature for interactions between iron and folic acid/B_{12} metabolism. Reviewed by Bailey et al, the protein that mediates folic acid intestinal absorption (heme-carrier protein 1) is also a heme transporter [82] and there is also, evidence that erythropoietin enhances both iron and folic acid absorption [85], suggesting an interaction between folic acid and iron metabolism. Therefore, our associations between lower folic acid concentrations and increased the odds of anemia and lower folic acid and B_{12} associated with lower ferritin and serum iron concentrations suggest a double adverse effect of those micronutrient deficiencies not only affecting erythropoiesis but also interfering with iron metabolism.

Interpreting sTfR as erythropoiesis indicator

Despite WHO's recommendation for the use of high sTfR as an indicator of iron deficiency in settings where infection/inflammation are prevalent, our data on sTfR in this MINDI cohort was difficult to interpret. Normally, higher sTfR reflects the erythropoietic response to iron deficiency [86] and erythropoiesis is considered as the most important determinant of sTfR concentrations [87]. Given the lack of association of sTfR with anemia and that prevalence iron deficiency indicated by low ferritin (67.6%) or low serum iron (52.1%) was higher than that found from elevated sTfR in only 16.4%, we hypothesized that a normal physiological increase in erythropoiesis in response to iron deficiency anemia was not occurring. Our population had several factors that can lead to decreased erythropoiesis. First, it is known that severe protein malnutrition can impair erythropoiesis [88], however undernutrition was not significantly associated with sTfR in African pregnant women with mild undernutrition [89], and RBP

although entered our preliminary models, lost significance after controlling for other nutrients and inflammation indicators. Second, both vitamins A and D have been associated with erythropoiesis. It is known that vitamin A stimulates later stages of red cell development [90], through contributing to the expression of erythropoietin [91]. However, the down-regulation of erythropoietin by vitamin A deficiency is lost in the presence of iron deficiency [92], which is possibly what we observed in our pregnant women, in whom higher sTfR was associated with low vitamin A. Similarly, it has been proposed that vitamin D may favor erythropoiesis by decreasing pro-inflammatory cytokines and by having a synergistic effect with erythropoietin [93], but we observed lower sTfR associated with higher vitamin D concentrations. In agreement with our findings, Thomas et al found that vitamin D was negatively associated with erythropoietin, but positively associated with Hb in adolescent pregnant women [9]. Interestingly, in our study, associations of vitamin A and D with sTfR were lost after including intestinal nematodes in the model, whereas the association of sTfR with cytokines persisted, indicating that inflammation might dominate over nutrient deficiencies on erythropoiesis.

In support of this, Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia - "BRINDA" studies have reported a consistent pattern of increased sTfR in response to inflammation across studies and have suggested controlling for inflammation using α-1 glycoprotein and not CRP, because the magnitude of the difference in iron-deficient erythropoiesis was minimally captured when adjusting for CRP [34]. In our study, CRP was not associated with sTfR, and among inflammation indicators measured, only two cytokines, IL17 and IL13 entered our final models. It is known that inflammatory cytokines including INFγ and IL6 may impair erythropoiesis by inhibiting the production of erythropoietin and the differentiation and proliferation of erythroid progenitor cells [87, 94, 95]. Our data provide evidence to show that this may be occurring. Higher IL17 was associated with lower sTfR, which contrasts with experimental studies in mice where IL17 was shown to increase erythropoiesis [96, 97], but to our knowledge, ours is the first study reporting a negative association of IL17 with sTfR in pregnant women. Similarly, we also describe for first time a negative association of IL13 with sTfR in pregnancy.

Recent evidence has highlighted that the process of erythropoiesis might overcome iron restriction due to inflammation, given that the higher erythropoietic demand down-regulates the expression of the iron hormone hepcidin and, as a consequence, increases iron absorption [98]. Although international consensus on a cut-off for elevated hepcidin is not available, two studies with pregnant women in developing countries have proposed that hepcidin >6.1 µg/L [38] or ≥2.5 µg/L [40] could identify women with iron restriction due to inflammation. Understanding the limitation that essays used in those studies are different from ours, by using those cut-offs, 71.4% had hepcidin >6.1 µg/L and 95.8% had hepcidin >2.5 µg/L. Given that hepcidin is known to progressively decrease during pregnancy from the first trimester to undetectable concentrations (≤ 1.39 µg/L) during the second and third trimesters [99], we can suspect the presence of iron restriction due to inflammation in most of our population. Moreover, higher hepcidin was associated with higher ferritin and higher CRP but with lower sTfR, further supporting that low concentrations of sTfR despite iron deficiency may be the result of lacking erythropoietic response probably due to inflammation.

In agreement with inflammation blunting erythropoiesis, more severe UTI, known to promote a shift to a pro-inflammatory state during pregnancy [100], was associated with lower sTfR. Interestingly hookworm, associated with higher CRP in our population [43] was associated with higher sTfR. It has been recently shown that *Trichuris muris* is able to induce erythropoiesis by inducing INFγ [101], and also hookworm has been associated with increased production of INFγ [102]. Therefore it would be plausible that some types of intestinal nematodes may be increasing erythropoiesis during pregnancy by modulating the immune response. Taking our findings together, and given sTfR associations with multiple infections, nutrients and inflammation, our findings show that sTfR was not a good indicator of iron deficiency our population with MINDI.

Iron status

Most women in our population (78.5%) had iron deficiency evidenced by either low ferritin or low serum iron or elevated sTfR. However, a high prevalence of elevated hepcidin challenges the therapeutic approach of low iron status in these vulnerable pregnant women, who are fighting infection through withholding iron, but at the same time, have depleted iron stores to maintain a healthy pregnancy. Our mean (range) hepcidin concentration (11.9 (0.3–80.1)µg/L) was closer to values reported in the Philippian study (12.9 (8.2–15.9) µg/L) in women with non-iron deficiency anemia [38], and higher that those reported by the Gambian study (1.59 (0.03–49.79) µg/L) [39] By using the most conservative cut-off of hepcidin (>6.1 µg/L)[38], iron restriction due to inflammation was co-occurring with iron deficiency in 53% of the women, meaning that only 24% of women with nutritional iron deficiency would benefit from iron supplementation. Our data also indicates that most iron deficient women were detected using low ferritin as indicator, with a 10% under-estimation that was captured by low serum iron. Therefore, our results indicate that ferritin was appropriate for detecting iron deficiency, and highlights the need of controlling for iron restriction due to inflammation, which in our population, was not possible to do through CRP or sTfR.

Both ferritin and serum iron are known to be increased and decreased by inflammation respectively [23, 30, 32], however, cytokines did not enter our final models for ferritin nor serum iron, and higher CRP was associated with lower serum iron but did not enter the final ferritin model with intestinal nematodes, indicating that the use of CRP for controlling ferritin for the presence of inflammation would not be accurate in our population.

Interestingly, cells from the differential WBC count consistently entered our models for both ferritin and serum iron. Higher monocytes counts were associated with lower ferritin and lower serum iron, but higher eosinophils were associated with higher ferritin and higher serum iron. It has been recognized that the major cellular system being responsible for the supplying of iron for erythropoiesis is the monocyte/macrophage system [103]. In agreement with our findings, Gomo et al found negative associations of monocyte counts with serum ferritin in pregnant

women from Zimbabwe [104]. Experimental studies have also demonstrated that stimulation of monocytes with intra- and extracellular bacteria, as well as with IL6 infusion, induces the production of hepcidin [105], which helps to explain the consistent association of higher monocytes with lower ferritin and serum iron. It is also known that excess of tissue iron is associated with the eosinophilic response in allergic conditions, and iron chelators have been experimentally investigated to decrease allergic inflammation [106, 107]. However, ours is the first study showing an association of higher eosinophils with higher iron status in pregnant women with multiple infections. Therefore, WBC counts particularly monocytes, would be alternative inflammation biomarkers when assessing iron status of populations with MINDI.

Limitations

We acknowledge several limitations mainly driven by the remoteness location and lack of technology facilities in our field study. The novel finding of sub-clinical hypoproteinemia and hemoconcentration, rather than the normal hemodilution observed during pregnancy may have underscored deficiencies based on cutoffs taking in account the hemodilution of pregnancy (Hb and other RBC indices). Due to field conditions, and that recruitment and sample collection were done on the same day, many mothers were not able to provide stool samples. Thus our sample size for women with nematode epg data was reduced by 44%. However, since data was missing at random and R^2s of our models with nematode epgs were higher than those of models with the total population, we concluded that our sample size was enough to detect meaningful associations between nematode epgs and iron status indicators.

Conclusion

Our study provides evidence that in a population of pregnant women with MINDI, the presence of sub-clinical protein malnutrition may interfere with the diagnosis and evaluation of anemia, and that co-occurrence of deficiencies may result on normal RBC indices. Therefore, RBC indices, which are usually the only locally available biomarker for the evaluation of maternal health, may be insufficient to suspect nutrient deficiencies. As it is frequently assumed that iron deficiency is the main cause of anemia, public health policies usually focus on iron

supplementation, disregarding the impact of other nutrient deficiencies and importantly, of inflammation and infections, on the ethiology of anemia. Importantly, we observed the presence of iron restriction due to inflammation in most women co-occurring with low ferritin and low serum iron, and questioning about the systematic use of iron supplements that could favor certain infections and promote further inflammation. Therefore, the results of this study indicate a need to re-orient public health policies in MINDI populations towards an integrated management of anemia and iron deficiency, by improving protein-source foods, minimizing micronutrient deficiencies and appropriately treating infections in order to reduce anemia in the MINDI context.

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Tables and figures

Table 1 Maternal characteristics by anemia status. Mean ± SD or median (min – max) or frequency (%) according to anemia status are reported

	Hemoglobin ≥110 g/L	Hemoglobin <110 g/L	P[1]		Hemoglobin ≥110 g/L	Hemoglobin <110 g/L	P[1]
Maternal characteristics				**Nutrient biomarkers[8]**			
Age (yr)	23 (13 – 45)	25 (13 – 41)	0.106	Ferritin, µg/L	17.4 (2.3 – 115.8)	7.9 (1.0 – 136.6)	**0.0001**
Parity (n)	2 (1 – 12)	4 (1 – 10)	**0.007**	Ferritin <20 µg/L	78 (36.6%)	66 (31.0%)	**0.001**
Gestational age (wk)	27.5 (4.9 – 42)	25.7 (5 – 40.6)	0.817	Serum iron, µmol/L	10.3 (2.7 – 37.2)	6.5 (0.5 – 54.8)	**0.0001**
Weight by height classification[2]				Serum iron <8.9 µmol/L	54 (25.3%)	57 (26.8%)	**<0.0001**
Underweight	11 (5.6%)	10 (4.7%)	**0.014**	sTfR, mg/L	5.0 (1.6 – 22.6)	5.7 (0.7 - 25.7)	0.27
Normal weight	82 (38.5%)	61 (28.6%)		sTfR >8.3 mg/L	16 (7.5%)	19 (8.9%)	**0.030**
Overweight	39 (18.3%)	10 (4.7%)		Folic acid, nmol/L	15.0 (6.6 – 45.4)	11.7 (6.3 - 45.4)	**0.0012**
Supplements				Folic acid <10 nmol/L	24 (11.3%)	27 (12.7%)	**0.012**
iron	100 (46.9%)	63 (29.6%)	0.736	Vitamin B_{12}, pmol/L	102 (53 – 376)	95 (57 – 289)	0.188
MMN[3]	64 (30.0%)	81 (38.0%)	0.408	Vitamin B_{12} <150 pmol/L	109 (51.2%)	72 (33.8%)	0.211
RBC indices[4]				Vitamin D, nmol/L	45.1 ± 15.6	43.6 ± 14.4	0.251
Hb (g/L)	117 (110 – 143)	104 (57 – 109)	**0.0001**	Vitamin D <50 nmol/L	82 (38.5%)	56 (26.3%)	0.300
Hct, %	36.3 (32.0 – 44.4)	23.6 (23.0 –36.3)	**0.0001**	Vitamin A, µmol/L	1.2 (0.42 – 2.9)	1.1 (0.4 – 2.1)	**0.047**
Low Hct	0	9 (4.2%)	**<0.0001**	Vitamin A <1.05 µmol/L	48 (22.9%)	39 (18.6%)	0.091
MCV, fL	94.6 (86.9 – 107.3)	93.3 (73.4 – 112.3)	**0.013**	RBP, mg/L	49.0 (8.6 – 412.2)	46.5 (2.9 – 302.4)	0.663
Microcytosis	0	14 (6.5%)	**<0.0001**	RBP <30 mg/L	36 (17.0%)	21 (9.9%)	0.804
Normocytosis	115 (54.0%)	58 (27.2%)		Hepcidin (µg/L)	8.4 (0.3 – 75-9)	8.0 (0.8 – 80.1)	0.539
Macrocytosis	17 (8.0%)	9 (4.2%)		**Inflammation indicators[9]**			
RDW, CV (%)[2]	13.3 (12.1 – 16.4)	13.7 (12.4 – 17.6)	**0.0001**	CRP, mg/L	3.3 (0.1 – 27.2)	4.1 (0.1 – 25.1)	0.354
Elevated RDW-CV	0	5 (2.5%)	**0.005**	Elevated CRP	23 (10.8%)	13 (6.1%)	0.795
RDW, SD (fL)	45.9 (40.4 – 61.1)	45.9 (39.3 – 56.7)	0.705	**WBC, number [10]**			
RDW (SD) >46 fL	42 (21.2%)	27 (13.6%)	0.355	Neutrophils	5.9 (1.8 – 10.8)	5.2 (2.5 – 21.9)	**0.023**
Urine analysis[5]				Lymphocytes	2.0 (1.1 – 3.8)	1.9 (0.9 – 3.2)	0.231
Bacteriuria	41 (19.7%)	13 (6.2%)	**0.008**	Monocytes	0.4 (0.1 – 1.4)	0.3 (0.2 – 1.4)	0.138
USG >1020	57 (27.4%)	23 (11.1%)	0.639	Eosinophils	0.4 (0.02 – 2.4)	0.3 (0.03 – 2.5)	0.548
Other infections				Basophils	0.03 (0 – 0.10)	0.03 (0.01 – 0.08)	0.898
Caries	23 (10.8%)	19 (8.9%)	0.283	**Cytokines**			

				(pg/mL) [11]			
Scabies	25 (11.7%)	12 (5.6%)	0.441	IL1β	2.6 (0.07 – 32.5)	1.8 (0.07 – 95.7)	0.908
Vaginal[6]				IL4	6.3 (0 – 134.2)	9.6 (0.03 – 119)	0.655
Yeast	31 (14.5%)	22 (38.0%)	0.547	IL6	1.6 (0.2 – 58.2)	1.6 (0.4 – 122.8)	0.301
Bacterial vaginosis	82 (38.9%)	46 (21.8%)	0.575	IL10	1.6 (0 – 45.7)	1.0 (0 – 43.8)	**0.027**
Trichomonas	94 (44.5%)	65 (30.8%)	0.071	IL12	3.5 (0 – 203.4)	0.9 (0 – 332.9)	0.155
Diplococcus	23 (10.9%)	20 (9.5%)	0.168	IL13	1.6 (0.03 – 50.2)	1.6 (0.03 – 26.3)	0.100
Intestinal nematodes[7]				IL17	3.0 (0.01 – 66.6)	1.7 (0.02 – 48.5)	0.329
Ascaris *Ascaris epg*	24 (20%) 196 (6-1078)	15 (12.5%) 100 (4-980)	0.408 0.477	INFγ	5.5 (0.05 – 47.9)	2.5 (0.05 – 73.9)	0.132
Hookworm Hookworm epg	47 (39.2%) 11 (1-245)	21 (17.5%) 13 (3-735)	0.515 0.891	TNFα	7.2 (0 – 36.9)	5.1 (0 – 27.1)	0.446
Trichuris *Trichuris epg*	11 (9.2%) 5 (2-12)	4 (3.3%) 294 (49-539)	0.394 0.892				

Significant values at P<0.05 are highlighted

n= 132 (no anemia), 81 (anemia), unless otherwise specified

[1] P values were calculated using T-test or Kruskal-Wallis for independent continuous variables, or Chi^2 test or Firsher's exact tests for binary comparisons of frequencies

[2] Weight by height classification: Prevalence of under- normal- and over-weight according to PAHO are shown [45].

[3] MMN: Multiple micronutrients contained in the recommended 90 g daily portion: 315 Kcal, 10.8 gr of protein, 225 µg of vitamin A, 9 mg of vitamin E, 126 mg of vitamin C, 0.4 mg of thiamine, 0.4 mg of riboflavin, 5.4 mg of niacin, 0.4 mg of vitamin B_6, 0.8 mg of B_{12}, 76.5 µg of folic acid, 10 mg of iron, 7.2 mg of Zinc, 225 mg of calcium, 90 mg of phosphorus and 360 mg of cupper {de Caballero, 2003 #3017}.

[4] RBC indices: Red blood cell indices. Cutoffs are provided for the first, second and third trimester respectively [48]:

Hb: Hemoglobin.

Low Hct: Low Hematocrit. Defined as <31%, <30% and <28%

MCV: Mean corpuscular volume

Low MCV (microcytosis): Defined as <85 fL, <85.8 fL and <82.4 fL

High MCV (macrocytosis): Defined as >97.8 fL, >99.4 fL and >100.4 fL

RDW-CV: Red blood cell distribution width-coefficient of variation. n= 129 (no anemia), 69 (anemia)

High RDW-CV: Defined as >14.9%, >14.7% and >16.6%

RDW-SD: Red blood cell distribution width-standard deviation

High RDW-SD: Defined as >46 fL [49].

[5] Urine analysis: n=128 (no anemia), 80 (anemia)

USG: urinary specific gravity

[6] Vaginal smears for detection of bacterial vaginosis, *Trichomonas* and *Diplococcus*. n= 132 (no anemia), 79 (anemia)

[7] Frequencies are based on positive results from direct exam, Kato-Katz or Flotac: n= 80 (no anemia), 40 (anemia) Stool samples processed for Kato-Katz: n=72 (anemia), 34 (no anemia).

Values of median (min–max) of positive cases are reported

[8] RBP: Retinol-binding protein, n=131 (no anemia), 81 (anemia)

[9] CRP: C-reactive protein

Elevated CRP defined as >3 mg/L, >20.3 mg/L and >8.1 mg/L in the first, second and third trimester respectively [48].

[10] WBC: White blood cell counts /mm^3

[11] Cytokines: n= 131 (no anemia), 81 (anemia)

IL: Interleukin

INF: Interferon

TNF: Tumor necrosis factor

Table 2 Multiple logistic regression models for anemia. A. Simple logistic regression models for anemia and low iron status using low ferritin, low serum iron and high sTfR, separately. B. Composite logistic regression model for anemia adjusting for infections, nutrition and inflammation indicators. C. Including hepcidin to the model.

A. Anemia	**OR ± SE**	**P**	**95% CI**	**Overall model (n= 213)**
Ferritin <20 µg/L	3.05 ± 1.02	0.001	1.57, 5.89	Pseudo R^2=0.043
Serum iron <8.9 µmol/L	3.43 ± 1.03	<0.0001	1.90, 6.19	Pseudo R^2= 0.063
sTfR >8.3 mg/L	2.22 ± 0.83	0.033	1.07, 4.62	Pseudo R^2= 0.016
B. Anemia[1]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Trimester	0.70 ± 0.18	0.168	0.42, 1.16	n= 203 P <0.0001 Pseudo R^2= 0.128 VIF= 1.08 Condition number= 18.68 Goodness-of-fit test= 0.231
Weight by height classification	0.48 ± 0.14	0.012	0.27, 0.85	
Parity	1.14 ± 0.07	0.034	1.01, 1.29	
Folic acid, nmol/L	0.95 ± 0.02	0.027	0.90, 0.99	
Vitamin A, µmol/L	0.36 ± 0.17	0.028	0.15, 0.90	
Ferritin <20 µg/L	2.93 ± 1.13	0.005	1.37, 6.25	
Constant	14.11 ± 16.5	0.024	1.42, 140.4	
C. Anemia[2]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Trimester	0.75 ± 0.19	0.275	0.45,, 1.25	n= 203 P <0.0001 Pseudo R^2= 0.148 VIF= 1.09 Condition number= 20.93 Goodness-of-fit test= 0.198
Weight by height classification	0.44 ± 0.13	0.007	0.24, 0.80	
Parity	1.14 ± 0.07	0.040	1.006, 1.29	
Folic acid, nmol/L	0.95 ± 0.02	0.044	0.91, 0.99	
Hepcidin >6.1 µg/L	2.39 ± 0.91	0.023	1.13, 5.06	
Ferritin <20 µg/L	3.57 ± 1.43	0.002	1.62, 7.83	
Vitamin A, µmol/L	0.40 ± 0.18	0.045	0.16, 0.98	
Constant	5.52 ± 6.84	0.168	0.48, 62.7	

Variables included in the stepwise process, but had P>0.15:
[1]Model B. P> 0.15: IL10 (pg/mL), IL1β (pg/mL), IL6 (pg/mL), WBC count, TNFα (pg/mL), CRP (mg/L), score of *Diplococcus*, ULE (+), score of *Mobiluncus*, *Trichomona*s and *Bacteroides/Gardnerella*
[2]Model C. P> 0.15: : IL10, WBC count, score of *Diplococcus*, ULE score, IL6 (pg/mL), IL1β (pg/mL), TNFα (pg/mL), CRP (mg/L), scores of *Bacteroides/Gardnerella*, *Trichomonas* and *Mobiluncus*.

Table 3 Multiple linear regression model for hemoglobin (g/L). A. Simple linear regression models for Hb (g/L) and iron deficiency indicated by low ferritin, low serum iron and high sTfR separately. B. Composite linear regression model for Hb (g/L), adjusting for infections, nutrition and inflammation indicators. C. Multiple linear regression model in the subsample of women with intestinal nematode infection data

A. Hb (g/L)	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model (n=213)**
Ferritin <20 µg/L	-7.53 ± 1.57	<0.0001	-10.62, -4.43	-0.313	Adjusted R^2= 0.094
Serum iron <8.9 µmol/L	-7.73 ± 1.45	<0.0001	-10.60, -4.86	-0.344	Adjusted R^2= 0.114
sTfR >8.3 mg/L	-6.40 ± 2.04	0.002	-10.4, -2.37	-0.211	Adjusted R^2=0.040
B. Hb (g/L)[1]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	0.61 ± 1.10	0.578	-1.55, 2.77	0.037	n= 207 $F_{10, 196}$= 7.70 P <0.0001 Adj. R^2= 0.266 VIF= 1.11 Condition number= 21.18 Breusch-Pagan <0.0001 White test= 0.455
Parity	-0.54 ± 0.26	0.042	-1.07, -0.02	-0.125	
Weight by height classification	4.34 ± 1.21	<0.0001	1.94, 6.73	0.218	
Wood smoke (yes/no)	-4.19 ± 2.48	0.093	-9.09, 0.71	-0.105	
Folic acid, nmol/L	0.21 ± 0.09	0.020	0.03, 0.38	0.147	
RBP <30 mg/	3.72 ± 1.55	0.018	0.65, 6.79	0.149	
CRP, mg/L	-0.30 ± 0.13	0.025	-0.57, -0.04	-0.139	
Diplococcus, score	-1.09 ± 0.61	0.076	-2.30, 0.11	-0.108	
Ferritin <20 µg/L	-7.10 ± 1.58	<0.0001	-10.21, -3.98	-0.299	
IL10, pg/mL	0.31 ± 0.10	0.002	0.11, 0.50	0.197	
Constant	107.82 ± 4.62	<0.0001	98.71, 116.92		
C. Hb (g/L)[2]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	1.88 ± 1.47	0.206	-1.05, 4.81	0.109	n= 103 $F_{5,97}$= 10.62 P <0.0001 Adj. R^2= 0.324 VIF= 1.07 Condition number= 10.683 Breusch-Pagan = 0.223 White test= 0.661 MCAR test= 0.782
IL10, pg/mL	0.25 ± 0.15	0.092	-0.04, 0.55	0.146	
CRP, mg/L	-0.40 ± 0.16	0.018	-0.72, -0.07	-0.204	
Ferritin <20 µg/L	-6.11 ± 1.95	0.002	-9.98, -2.23	-0.262	
Trichuris, epg (Kato-Katz)	-0.10 ± 0.02	<0.0001	-0.13, -0.07	-0.490	
Constant	108.64 ± 4.86	<0.0001	98.99, 118.28		

Variables included in the stepwise process, but had P>0.15:

[1]P>0.15: Presence of caries, portions of animal-source foods/wk, IL1β pg/mL, IL17 pg/mL, lymphocytes (classification), vitamin A<1.05 µmol/L, IL6 pg/mL

[2] P>0.15: weight by height classification, IL17 (pg/mL), portions of animal-source foods/wk, RBP<30 mg/L, parity, hookworm (epg), *Diplococcus* (score), folic acid (nmol/L), IL6 (pg/mL), lymphocytes (category), IL1β (pg/mL), wood smoke (yes/no), *Ascaris* (epg), vitamin A<1.05 µmol/L, presence of caries

Table 4 Multiple linear regression models for ferritin (µg/L). A. In the larger sample size, B. in the subsample of women with intestinal nematodes data

A. Log Ferritin (µg/L)[1]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	-0.53 ± 0.11	<0.0001	-0.74, -0.32	-0.37	n= 207 $F_{11, 195}$= 9.51 P <0.0001 Adj. R^2= 0.312 VIF= 1.24 Condition number= 23.98 Breusch-Pagan test= 0376 White test= 0.684
Weight by height classification	0.26 ± 0.10	0.013	0.06, 0.47	0.147	
Months on iron	0.05 ± 0.03	0.119	-0.01, 0.11	0.112	
Monocytes, number	-1.08 ± 0.05	0.016	-1.96, -0.20	-0.167	
Eosinophils, number	0.42 ± 0.22	0.058	-0.01, 0.85	0.134	
CRP, mg/L	0.03 ± 0.01	0.006	0.009, 0.05	0.167	
IL1β, pg/mL	-0.01 ± 0.006	0.109	-0.02, 0.002	-0.096	
Caries, presence	-0.25 ± 0.15	0.082	-0.54, 0.03	-0.103	
ULE (0-3+)	0.17 ± 0.06	0.009	0.043, 0.30	0.156	
Folic acid, nmol/L	0.02 ± 0.008	0.001	0.01, 0.04	0.202	
Vitamin B_{12}, pmol/L	0.002 ± 0.001	0.041	0.0001, 0.005	0.127	
Constant	2.44 ± 0.42	<0.0001	1.61, 3.27		
B. Model with intestinal nematodes	**Coeff. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	-0.44 ± 0.14	0.002	-0.72, -0.17	-0.339	n= 102 $F_{8\ 93}$= 6.77 P <0.0001 Adj. R^2= 0.313 VIF= 1.39 Condition number= 17.90 Breusch-Pagan test= 0.344 Whithe test= 0.945 Little's MCAR test= 0.120
Months on iron suppl.	0.11 ± 0.04	0.006	0.03, 0.19	0.293	
Monocytes, number	-1.51 ± 0.61	0.016	-2.73, -0.28	-0.260	
Eosinophils, number	0.71 ± 0.31	0.027	0.08, 1.33	0.244	
ULE (+)	0.36 ± 0.08	<0.0001	0.29, 0.53	0.372	
Ascaris, epg (Kato)	0.0009 ± 0.0004	0.030	0.0001, 0.002	0.217	
Hookworm, epg (Kato-Katz)[3]	-0.003 ± 0.001	0.025	-0.006, -0.0004	-0.227	
Vitamin B_{12}, pmol/L	0.004 ± 0.002	0.032	0.0003, 0.007	0.197	
Constant	2.97 ± 0.43	<0.0001	2.11, 3.82		

Variables included in the stepwise process, but had P>0.15:

[1] P>0.15: NLR

[2] P> 0.15: IL1B, NLR, weight by height classification, CRP mg/L, presence of caries, *Trichuris* epg (Kato-Katz), folic acid nmol/L

[3] One influential outlier was excluded from analyses in order to achieve linearity

Table 5 Multiple linear regression models for serum iron (µmol/L). A. In the larger sample size, B. in the subsample of women with intestinal nematodes data

A. Serum iron, µmol/L[1]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	0.07 ± 0.06	0.285	-0.06, 0.20	0.071	n= 206 $F_{11, 194}$= 7.99 P <0.0001 Adj. R^2= 0.273 VIF= 1.24 Condition number= 23.50 Breushc-Pagan test= 0.026 White test= 0.258
Monocytes, number	-1.81 ± 0.34	<0.0001	-2.50, -1.13	-0.405	
Eosinophils, number	0.53 ± 0.15	0.001	0.22, 0.83	0.245	
Lymphocytes, number	0.23 ± 0.10	0.022	0.03, 0.43	0.161	
CRP, mg/L	-0.03 ± 0.008	0.001	-0.04, -0.01	-0.217	
IL10, pg/mL	0.01 ± 0.006	0.018	0.002, 0.02	0.148	
Caries, presence	-0.29 ± 0.10	0.007	-0.49, -0.08	-0.169	
Diplococcus, score	-0.10 ± 0.04	0.011	-0.17, -0.02	-0.160	
Yeast, score	0.12 ± 0.06	0.037	0.007, 0.24	0.131	
Folic acid, nmol/L	0.02 ± 0.005	0.001	0.007, 0.03	0.206	
Vitamin B_{12}, pmol/L	0.002, 0.0009	0.012	0.0005, 0.004	0.169	
Constant	1.61 ± 0.30	<0.0001	1.01, 2.21		
B. Model with intestinal nematodes[2]	**Coeff. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	0.07 ± 0.10	0.450	-0.12, 0.27	0.070	n= 102 $F_{8, 93}$= 7.42 P <0.0001 Adj. R^2= 0.337 VIF= 1.28 Condition number= 18.04 Breushc-Pagan test= 0.439 White test= 0.478 Little's MCAR test= 0.356
Monocytes, number	-1.46 ± 0.47	0.003	-2.39, -0.52	-0.318	
Eosinophils, number	0.63 ± 0.24	0.009	0.16, 1.11	0.279	
CRP, mg/L	-0.04 ± 0.01	<0.0001	-0.06, -0.02	-0.350	
Vaginal yeast score	0.19 ± 0.08	0.028	0.02, 0.35	0.191	
Ascaris, epg (Kato)	0.0008 ± 0.0003	0.008	0.0002, 0.001	0.236	
Hookworm, epg (Kato)[3]	-0.003 ± 0.0006	<0.0001	-0.004, -0.001	-0.351	
B_{12}, pmol/L	0.004 ± 0.001	0.008	0.001, 0.006	0.245	
Constant	2.10 ± 0.36	<0.0001	1.38, 2.83		

Variables included in the stepwise process, but had P>0.15:

[1] P>0.15: ULE (+), wood smoke exposure (y/n), parity, score of *Lactobacillus*, iron supplementation (yes/no), platelets number

[2] P> 0.15: Presence of caries, iron supplementation (y/n), score of *Lactobacillus* and *Diplococcus*, parity, folic acid (nmol/L), ULE (+), IL10 (pg/mL), platelets number, wood smoke exposure (y/n), lymphocytes number, *Trichuris* epg (Kato-Katz).

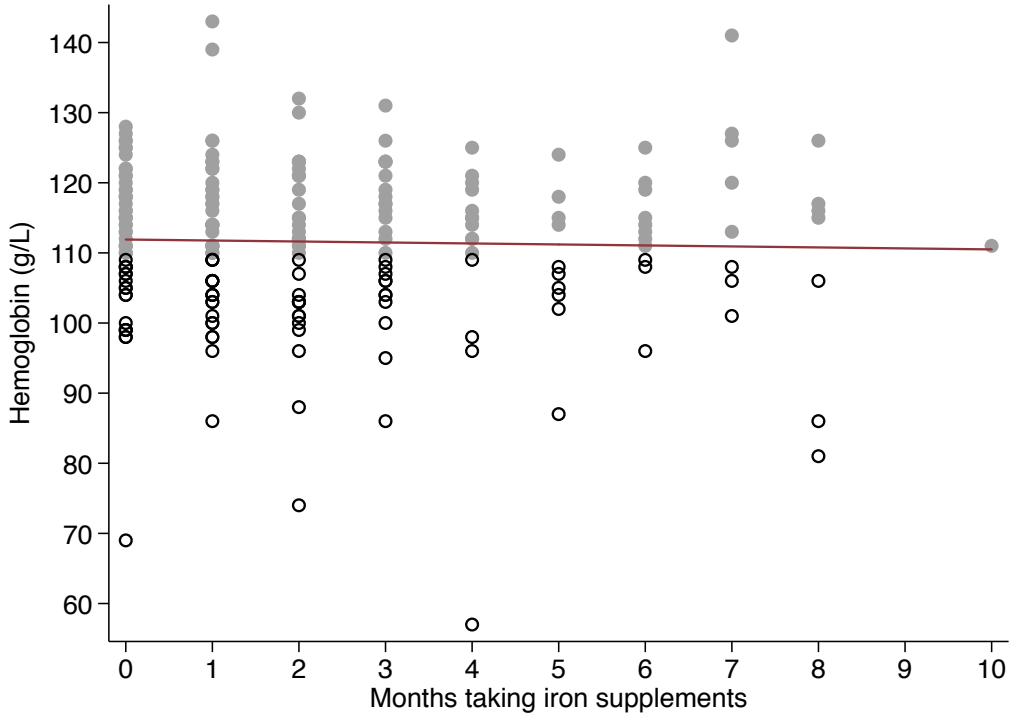
[3]One influential outlier was excluded from analyses in order to achieve linearity

Table 6 Multiple linear regression models for serum transferrin receptors (sTfR). A. In the larger sample size, B. in the subsample of women with intestinal nematodes data

A. Log sTfR[1]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	0.17 ± 0.05	<0.0001	0.08,0.27	0.238	n= 203 $F_{9, 193}$= 6.72 P <0.0001 Adj. R^2= 0.203 VIF= 1.50 Condition number= 16.42 Breusch-Pagan test= 0.461 White test= 0.596
Parity	-0.04 ± 0.01	0.002	-0.07, 0.01	-0.209	
Eosinophils, number	-0.18 ± 0.10	0.088	-0.38, 0.03	-0.111	
IL13, pg/mL	0.01 ± 0.007	0.037	0.0009, 0.03	0.229	
IL17, pg/mL	-0.01 ± 0.005	0.015	-0.02, -0.002	-0.270	
Caries, presence	0.16 ± 0.08	0.041	0.006,0.32	0.130	
ULE (+)	-0.11 ± 0.04	0.002	-0.19, -0.04	-0.208	
Vit. A<1.03 µmol/L	0.14 ± 0.06	0.032	0.02, 0.27	0.139	
Vitamin D, nmol/L	-0.005 ± 0.002	0.025	-0.009, -0.0006	-0.143	
Constant	1.69 ± 0.18	<0.0001	1.33, 2.05		
B. Model with intestinal nematodes [2]	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall model**
Trimester	0.14 ± 0.07	0.043	0.004, 0.27	0.182	n= 101 $F_{8, 92}$= 5.68 P <0.0001 Adj. R^2= 0.273 VIF= 1.73 Condition number= 13.71 Breusch-Pagan test= 0.838 White test= 0.924 Little's MCAR test= 0.506
Parity	-0.04 ± 0.02	0.048	-0.07, -0.0004	-0.187	
Hookworm, epg (Kato-Katz)	0.002 ± 0.0004	0.001	0.0006, 0.002	0.293	
ULE (+)	-0.18 ± 0.05	0.001	-0.28, -0.07	-0.315	
CRP, mg/L	0.01 ± 0.008	0.056	-0.0004, 0.03	0.178	
IL13, pg/mL	0.02 ± 0.01	0.093	-0.003, 0.04	0.271	
IL17, pg/mL	-0.02 ± 0.009	0.074	-0.03, 0.001	-0.289	
Caries, presence	0.22 ± 0.10	0.046	0.004, 0.43	0.180	
Constant	1.46± 0.21	<0.0001	1.04, 1.88		

Variables included in the stepwise process, but had P>0.15:
[1]P> 0.15: folic acid (nmol/L), score of *Diplococcus* CRP (mg/L), RBP (mg/L), iron supplementation (y/n)
[2]P> 0.15: Ascaris (epg, Kato), iron supplements (y/n), vitamin D (nmol/L), eosinophils number, folic acid (nmol/L), RBP (mg/L), *Trichuris* (epg, Kato), Diplococcus score, vitamin A <1.05 µmol/L

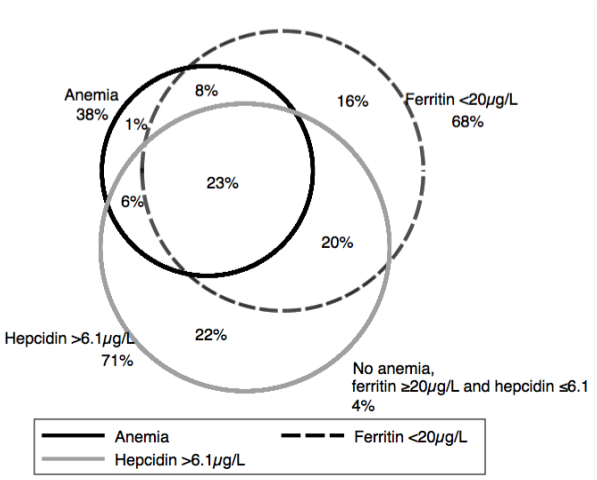
Figure 1 Scatter plot of hemoglobin (g/L) and reported months taking iron supplementation



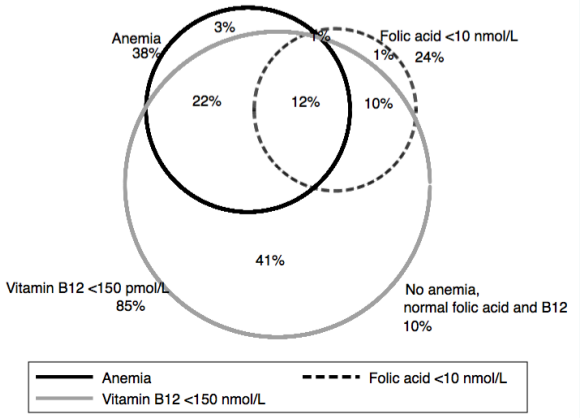
The regression line shows no association between hemoglobin concentrations and reported time (mo) taking iron supplements (Adjusted R^2= -0.004, P=0.680). Hollow circles correspond to women with hemoglobin <110 g/L, and gray circles correspond to women with hemoglobin ≥110 g/L.

Figure 2 Venn-diagrams showing overlapping frequencies of anemia and A. iron deficiency and elevated hepcidin, B. folic acid and vitamin B_{12} deficiencies, C. folic acid and low vitamin A concentrations

A.



B.



C.

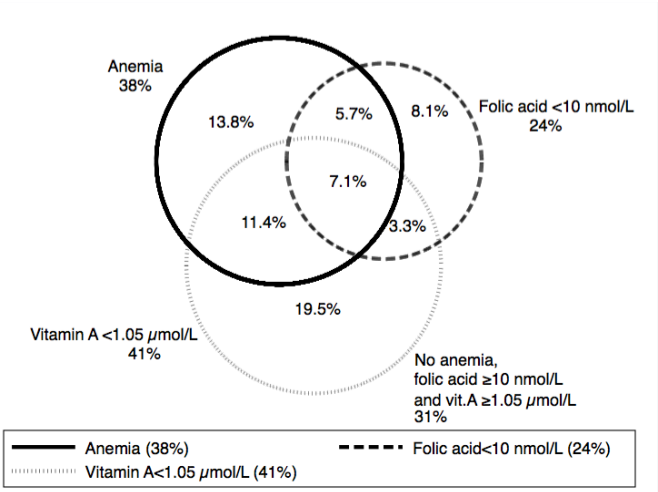
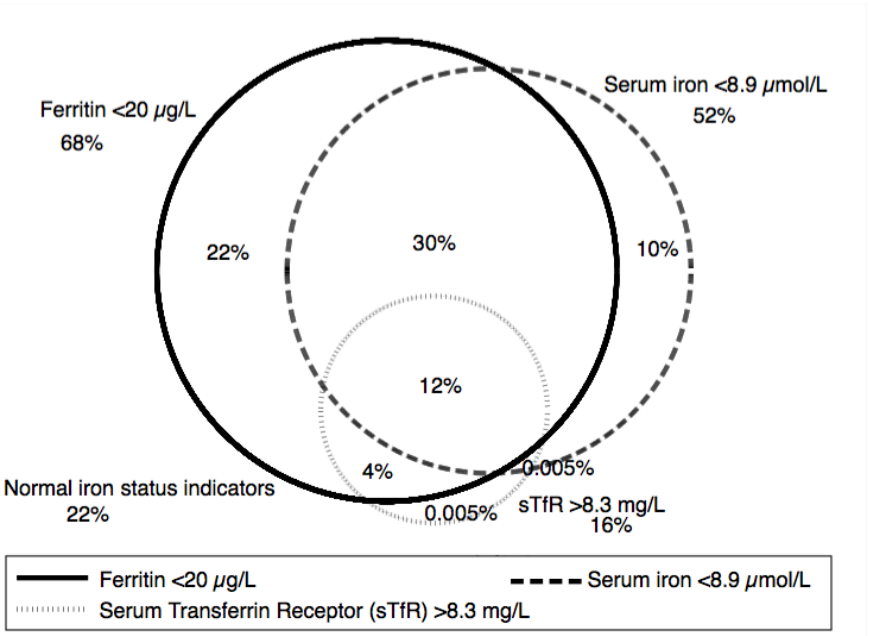


Figure 3 Venn-diagrams showing overlapping frequencies of A. low ferritin, low serum iron and high serum transferrin receptor (sTfR), B. low ferritin, low serum iron and hepcidin >6.1 µg/L

A.



B.

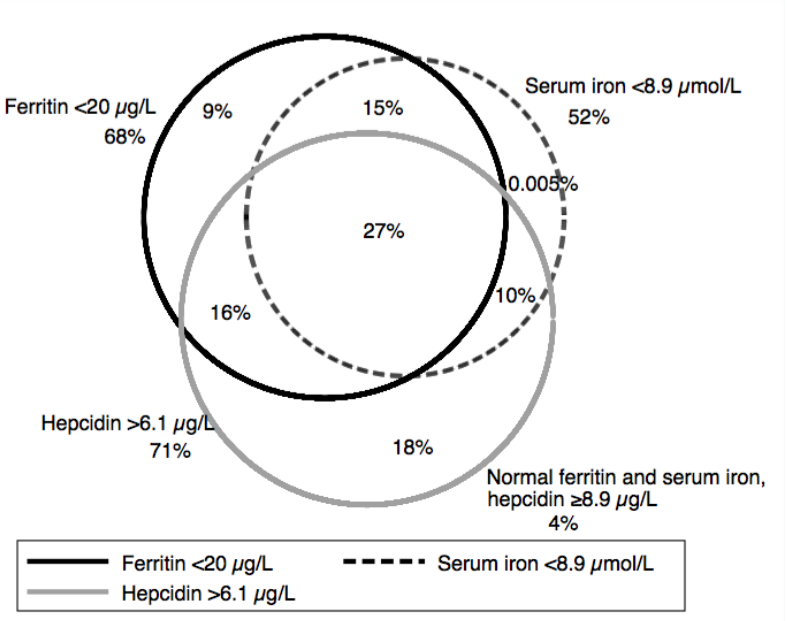
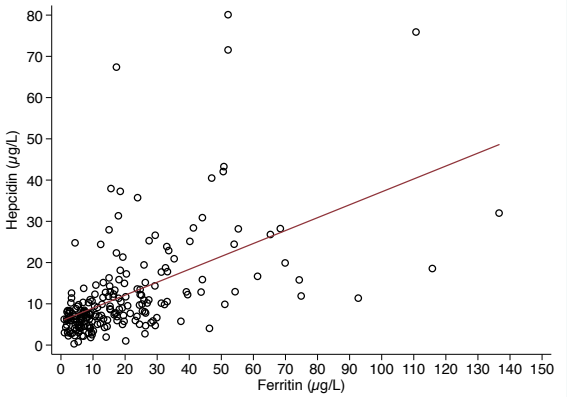
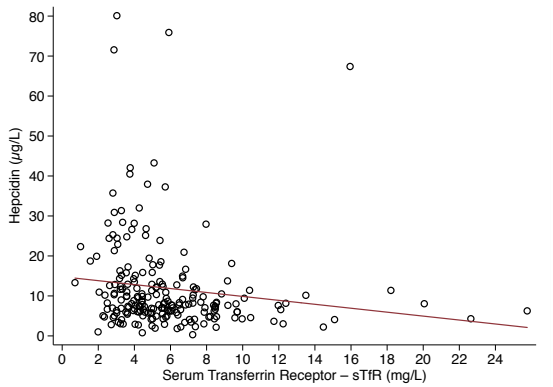


Figure 4 Scatter plot of hepcidin (µg/L) and A. ferritin (µg/L), B. serum transferrin receptor (mg/L), C. C-reactive protein (mg/L)

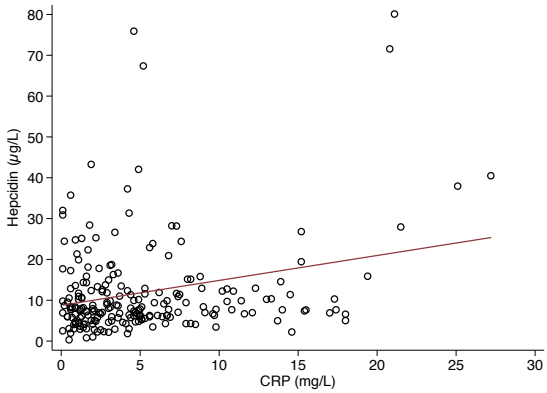
A.



B.



C.



Regression lines show associations of hepcidin with A. ferritin (Adj. R^2= 0.28, P<0.0001), B. C-reactive protein (Adj. R^2= 0.051, P= 0.0005), and C. Serum transferrin receptor (Adj. R^2= 0.031, P= 0.006). Log hepcidin was used in regression calculations.

Connecting statement 2

Together with anemia and iron deficiency, hypertensive disorders of pregnancy (HDPs) are main causes of morbidity and indirect/direct mortality in Panamanian pregnant women, particularly in indigenous communities. Anemia is a known risk factor for HDPs and also for low fetal growth. In the next paper we explored alternative maternal blood pressure measurements (mean arterial pressure and pulse pressure) for evaluating the risk for HDPs, as well as associations of systolic and diastolic blood pressure, mean arterial pressure and pulse pressure with MINDI (including anemia/iron status), and assessed if any/all of them were associated with fetal growth evaluated through symphysis-fundal height Z-scores.

Given findings on papers 1 and 2, it is possible that inflammation and iron status might be associated with blood pressure measurements, and that a trend to high blood pressure might negatively affect fetal growth.

PAPER 3. Determinants of ambulatory blood pressure measurements and their association with low symphysis fundal height in indigenous Panamanian pregnant women: The MINDI Cohort

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List of abbreviations

BP: Blood pressure; BMI: Body mass index; CRP: C-Reactive protein, DBP: Diastolic blood pressure; eMAP: Elevated mean arterial pressure; GA: Gestational age; HDPs: Hypertensive disorders of pregnancy; HIV: Human immunodeficiency virus; INF: Interferon; IL: Interleukin; IUGR: Intra-uterine growth retardation; LBW: Low-birth weight; MAP: Mean arterial pressure; MMN: Multiple micro-nutrients; MINDI: Multiple infections, nutrient deficiencies and inflammation; NLR: Neutrophil/lymphocyte ratio; PP: Pulse pressure; PTB: Preterm birth; RBC: Red blood cells; RBP: Retinol-binding protein; SENACYT: "Secretaría Nacional de Ciencia, Tecnología e Innovación"; SBP: Systolic blood pressure; SFH: Symphysis-fundal height; SGA: Small for gestational age; TNF: Tumor necrosis factor; USG: Urinary specific gravity; WBC: White blood cells.

Abstract

Objective: The objectives of this cross-sectional study were to determine whether maternal infections, nutrient deficiencies and inflammation (MINDI) were associated with four measures of maternal blood pressure (BP) and to determine their association with fetal size assessed using symphysis fundal height (SFH)

Design: Cross-sectional

Setting: Rural Republic of Panama

Population: 213 pregnant women

Methods: BP, environmental and dietary factors, intake of iron and multiple-micronutrients (MMN), markers of inflammation, protein, anemia, folate, vitamins B_{12}, A and D status, and urogenital, skin, oral and intestinal nematode infections were measured. Adjusted multiple linear and logistic regression models were employed.

Main outcome measures: Systolic and diastolic blood pressure (SBP, DBP), hypotension (SBP<100 and DBP<60), mean arterial pressure (MAP), elevated MAP (eMAP), pulse pressure (PP), SFH.

Results: Despite absence of elevated SBP or DBP, 11.2% of woman had eMAP. Furthermore, 24.1% had hypotension. MINDI were bi-directionally associated with blood pressure indicators. Infections known to elicit an anti-inflammatory response (*Ascaris, Trichomonas,* scabies) were associated with lower BP whereas those eliciting a pro-inflammatory response (hookworm, *Trichuris*, urinary tract infection), with higher BP. Higher intakes of MMN (OR 0.35, 95%CI: 0.18 – 0.64) and animal source foods (OR 0.70, CI: 0.54 – 0.92) reduced the likelihood of low blood pressure; moreover, low serum protein (RBP< 30 mg/L) was associated with higher MAP (β 0.20, CI: 0.81 – 7.19) and folic acid deficiency with an increased likelihood of eMAP (OR 6.98, 95% CI: 1.44 – 33.79). Higher TNFα was associated with higher MAP (β 0.18, CI: 0.01 – 0.33), but higher IL17 was associated with lower odds of hypotension (OR 0.87, CI: 0.78 – 0.97) and was also associated with lower PP (β -0.29, CI: -0.39 – -0.09). Low PP was associated with the smaller fetuses detected using SFH Z scores (β-0.26, CI: -4.37 – -1.32).

Conclusion: In this MINDI cohort, multiple infections, nutrients and inflammation emerged as novel factors associated with maternal BP. The presence of eMAP rather than SBP/DBP

identified women at risk of hypertension during pregnancy. Low pulse pressure emerged as a risk factor for poor fetal growth. Infections both raised and lowered BP indices, as did several cytokines. On the other hand, deficiencies of folic acid and protein were associated with higher MAP. Therefore, MAP and PP may help in detecting women at risk of adverse pregnancy outcomes in settings with limited access to technology.

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Introduction

Appropriate pregnancy follow-up is a major challenge in settings where access to health care is limited. Fortunately, maternal blood pressure (BP) is widely available even in remote settings. It is considered as an indicator of quality antenatal care [1] and useful in predicting hypertensive disorders of pregnancy (HDPs), preterm-birth (PTB) and small for gestational age (SGA) infants [2]. In addition to systolic and diastolic blood pressure (SBP and DBP), both mean arterial pressure (MAP = DBP + 0.33 [SBP-DBP]) and pulse pressure (PP = SBP - DBP) [3] also have clinical value. SBP, DBP [4], MAP [5] and PP [6, 7] have been found to be elevated in early pregnancy before the development of HDPs whereas low DBP and MAP have been associated with poor fetal outcomes [8, 9]. However, clinical interpretation may be misleading if co-existing conditions such as multiple infections, nutritional deficiencies and inflammation (MINDI) modulate the relationship between blood pressure and pregnancy outcomes, including poor intrauterine growth.

Maternal nutrient deficiencies have been associated with both abnormal maternal blood pressure and decreased fetal growth. Maternal protein-energy malnutrition can lead to intra-uterine growth retardation (IUGR)[10], and low protein intake (<65 g/d) more than tripled the risk of HDPs [11]. Although severe anemia is one of the main factors associated with HDPs [12], elevated hemoglobin (≥132 g/L) was positively associated with SBP and DBP [13], and hematocrit was positively correlated with MAP and with peripheral vascular resistance [14]. With respect to vitamins, improvement of Vitamin D status by ≥ 30 nmol/L from the first to third trimester lowered the odds of preeclampsia [15]. Homocysteine, which increases in response to low folate or vitamin B_{12} [16], was positively associated with MAP during pregnancy [17], and has been implicated in the physiopathology of HDPs and IUGR [16]. Studies on vitamin A are contradictory. Women with retinol concentration >1.08 µmol/L had decreased risk of HDPs in Peru [18] whereas retinol concentrations > 1.05 µmol/L increased the risk of HDPs in Zimbabwe [19]. Together, these studies highlight the potential associations of nutrients blood pressure, but no studies have explored the combined impact of co-existing nutrient deficiencies on maternal/fetal health.

The limited evidence to date shows that common infections in impoverished rural populations affect blood pressure and pregnancy outcomes in a pathogen-specific manner. A prospective-cohort study showed that pregnant women with acute malaria had lower SBP, DBP and MAP that non-infected controls but no difference was found between the PP of infected and non-infected mothers [20] whereas both urinary infections [12] and the protozoan tissue parasite, *Toxoplasma gondii* [21] increased the odds of hypertension in pregnancy. The nature of the relationship between infection and blood pressure may depend on whether the infections induce a pro- or anti-inflammatory response. Inflammation modulates blood pressure in pregnancy [22] as evidenced by the association of HDPs with inflammatory markers including C-reactive protein (CRP) [23, 24] and with two pro-inflammatory cytokines, interleukin (IL)6 and tumor necrosis factor (TNF)α [25]. In contrast, down-regulation of the pro-inflammatory response by IL10 and T-regulatory cells has been shown to reduce the pathology of HDPs [26].

To date, study of the direct association between maternal blood pressure and fetal growth has been difficult in areas where infections and nutrient deficiencies are common because of the absence of standards for assessing fetal growth in the absence of ultrasound [27]. Fortunately, new standards for symphysis-fundal height (SFH) according to gestational age (GA) have been recently developed by the INTERGROWTH project based on a large database of pregnant women from Brazil, China, India, Italy, Kenya, Oman, United Kingdom, and United States [28]. The INTERGROWTH Project now allows SFH to be used as a first level screening tool for detection of IUGR where ultrasound is not available.

The objectives of this cross-sectional study using our MINDI cohort were to determine if multiple infections, nutrient deficiencies and inflammation were associated with measures of maternal blood pressure (SBP, DBP, MAP, PP, elevated MAP (eMAP), and low blood pressure) and to determine which of these were associated with SFH using the new INTERGROWTH standards.

Methods

Study Population

This cross-sectional study was conducted between August and December 2010 in the extremely impoverished Ngäbe-Buglé indigenous population in western Panama, where approximately 40% of deliveries occur at home. In Panama, 13% of maternal mortality has been attributed to HDPs [29]. Based on government statistics, 6.7% of institutional deliveries are low-birth weight (LBW) infants at the country level [30], whereas LBW rates are as high as 14% in the Ngäbe population [31]. We have previously reported that pregnant women in our study, although from a zone non-endemic for malaria, had a high prevalence (>50%) of several vaginal and intestinal infections as well as oral, skin and urinary tract infections, human immunodeficiency virus (HIV) was ruled-out, as well as gestational diabetes [32]. Despite distribution of iron and micronutrient supplements by the Ministry of Health, >40% of these women had deficiencies of vitamins A, D and B_{12}, and CRP was elevated in 16.4% of these women [33].

Ethics and Recruitment

Ethics approval was obtained from the Gorgas Memorial Institute in Panama and from McGill University. Pregnant women living within 2 hours walking-distance from community health centers belonging to the Ngäbe-Buglé region in Chiriquí, Panama were invited to participate as part of their normal pregnancy follow-up [32]. Based on 2010 data reporting that 2127 live-births occurred in the Ngäbe-Buglé community [30], a prevalence of 4.5% for HDPs in a population of women ≤20 y from Panama [34], and on successful use of ambulatory blood pressure to detect 4.69% HDPs in a large cohort study with women from Saudi Arabia [35], we estimated that a sample size of 67 pregnant women would allow us detect women at risk of HDPs. Based on 6.7% prevalence of IUGR using LBW as a proxy [30], we estimated that 92 pregnant women would be sufficient to detect IUGR with a level of confidence of 95%. We were able to recruit 213 pregnant women, 174 of whom were beyond 16 wk of pregnancy, the minimal GA for SFH to be compared with the INTERGROWTH standards [28].

Questionnaire and Physical examination

Pregnant women answered questions about reproductive history, intake of iron supplements (60 mg tablets), multiple micronutrient (MMN) supplements (tbsp/d), and wood smoke exposure (h/d). Anthropometry was measured and the body mass index (BMI) was classified as underweight, normal or overweight using Pan American Health Organization standards for GA in pregnant women [36]. When used as a continuous variable, maternal BMI was calculated as maternal weight/height^2.

Maternal blood pressure (Omron HEM-790IT® automatic BP monitor) was measured in a sitting position and re-measured if elevated (SBP ≥ 140 or DBP ≥ 90 mmHg). URISCAN® dip-stick strips on a Miditron-M semi-automated reflectance photometer were used for semi-quantitative measurements of urinary specific gravity (USG) and protein. Hypertension was defined as a combination of elevated SBP and DBP. HDPs were considered if hypertension and dipstick-proteinuria ≥ 1+ or symptoms of preeclampsia were present after week 20 of pregnancy [37]. Low blood pressure was defined as SBP <100 and BP <60 mmHg [3]. MAP was calculated as DBP + 1/3 (SBP-DBP) [38]. Trimester-specific cutoffs for elevated MAP (eMAP) in pregnancy were >87 mmHg (10-18 wks), >84 mmHg (18-34 wks), and >86 mmHg (after 34 wks) [39], and low MAP was define as <70 mmHg [40]. Pulse pressure was calculated as the difference between SDP and DBP [3]. Cut-offs for elevated (>68 mmHg) and low PP (<42 mmHg) were based on a large population study (n= 235) of normotensive white pregnant women from Spain [41].

Symphysis-fundal height (SFH) was measured after the mother had recently emptied her bladder and while she was in a supine position. A non-elastic tape was placed at the upper border of the symphysis pubic bone, and straightened over the uterus until reaching the fundus. The cubital edge of the hand was used to hold the tape at the point of the fundus while it was turned to see the numbers; the value was recorded to the nearest complete half centimeter. The INTERGROWTH standards for SFH were used to calculate SFH Z-scores and centiles in women with GA ≥16 weeks (n= 177). Fetuses below the 10th centile were classified as

small for gestational age (SGA) and those above the 90th centile were classified as large for gestational age [28].

Infections

Maternal infections were evaluated both clinically and using laboratory measurements as previously described [32]. Briefly, oral caries and skin lesions compatible with scabies were detected during the clinical exam (yes/no), and bacteriuria based on microscopic analysis of centrifuged urine was scored 0 (absent), 1+ (few), 2+ (moderate) and 3+ (abundant). Vaginal Gram smears were assigned semi-quantitative scores (0 – 4) for *Lactobacillus, Bacteroides/Gardnerella* and *Mobiluncus*, and these scores were used to diagnose bacterial vaginosis (BV) based on a Nugent score ≥7, calculated as *Bacteroides/Gardnerella* score + (4 – *Lactobacillus* score) + (*Mobiluncus* score/2) [42]. Semi-quantitative scores were similarly assigned for Gram-detected vaginal trichomoniasis and diplococcal infection, and for vaginal yeast detected by direct smears. Presence of intestinal nematodes (*Ascaris*, hookworm and *Trichuris*) was identified using direct microscopic fecal examination, Kato-Katz and Flotac® from the 120 women who provided stool samples, as previously described [32].

Laboratory analyses

Hematological status and inflammation: Complete red (RBC) and white blood cell (WBC) counts (BC-5500 Mindray Auto Hematology Analyzer) were performed. Anemia was defined as hemoglobin <110 g/L [43]. Hematocrit (%) was compared against normal ranges for the first (31-41), second (30-39) and third (28-40) trimesters [44]. The degree of hemo-concentration was classified for the <25th centile and >75th centiles (33.2% and 36.9%, respectively). Hypohydration was assessed as USG >1020 [45]. Serum was analyzed for CRP using solid phase ELISA (MP Biomedicals, Orangeburg, NY) with a minimum detectable concentration of 0.95 nmol/L. Serum cytokines IL-1β, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, interferon (INF)-γ and TNF-α were quantified by Luminex using a Human Cytokine/Chemokine Magnetic Bead Panel (Millipore Corporation Canada).

Maternal nutritional status: Serum samples were processed for folic acid and vitamin B_{12} concentrations using immunoelectro-chemiluminescence (MODULAR E170, Roche Diagnostics GmBH, Mannheim, Germany); for 25-OH vitamin D using the LIAISON, DiaSorin direct competitive chemiluminescence immunoassay; for vitamin A using HPLC [46]; and for retinol binding protein (RBP) using Human RBP4 ELISA (MP Biomedicals) with a standard curve range between 0.14 – 100 ng/mL. Folic acid deficiency was defined as <10 nmol/L and vitamin B_{12} deficiency as <150 pmol/L [47]. We used a cutoff for vitamin D deficiency of <50 nmol/L [48]. The cutoff for low vitamin A was set at <1.05 µmol/L [49, 50]. Low protein status was defined as RBP <30 mg/L [51].

Statistical analysis

All statistical analyses were performed using STATA 14 (StataCorp, TX, USA). Given that a few women did not provide urine (n=5) or vaginal samples (n=2), that volume of serum samples was insufficient to process vitamin A analysis (n=3) and cytokine assays (n=1), and that only 120 women provided stool samples for intestinal nematode screening, multiple linear regression models included the STATA complete-case analysis function [52] which allowed us to both maximize the sample size of each final model and confirm the randomness of missing data using Little's chi-squared test [53]. The stepwise process filtered entering variables at P<0.15. If this yielded more variables than permitted given the sample size of the regression model, the filter was lowered to P<0.10 or P<0.05 until the number of variables was appropriate for the sample size. Depending on the sample size for each final model, inclusion of 6-10 independent variables allowed us to have power of 0.80 and a medium effect size [54]. We controlled for gestational age in all models. We also confirmed the absence of collinearity based on a variance inflation factor (VIF) <10 and the stability of regression coefficients by a condition number <30. Significance of variables in the final models was set at P<0.05. Only the final models are presented.

Initial exploratory models SBP, DBP, MAP and PP were developed using stepwise multiple linear regressions on seven distinct clusters of variables: 1) maternal characteristics (age, parity, BMI

category, hypohydration); 2) environmental/supplementation variables (fieldwork, wood smoke, intake of iron and MMN supplements); 3) RBC indices including hematocrit quantile and anemia; 4) inflammation indicators (WBC count and differential, neutrophil-lymphocyte ratio (NLR), CRP and nine cytokines); 5) nutritional deficiencies (low protein and deficiencies of folic acid, vitamin B_{12}, A and D); 6) intestinal nematode infection with *Ascaris*, hookworm and *Trichuris*; and 7) other infections with prevalence ≥10% (presence of caries, scabies, and BV; semi-quantitative scores for urinary bacteria, and vaginal *Lactobacillus, Bacteroides/Gardnerella*, *Mobiluncus*, trichomoniasis, diplococci and yeast). Spearman correlations were calculated among independent variables within each set to avoid inclusion of significantly correlated variables in the same regression model. The independent occurrence of physiologically-related variables (low RBP with vitamin A deficiency and elevated CRP; anemia with low hematocrit) was tested using Chi^2 analysis. Choice of independent variables to be included was based on the P value (maximum $P < 0.10$). Then, final stepwise multiple linear regression models were developed including all independent variables from among the seven initial exploratory models with $P < 0.05$. Given that at least one of the intestinal nematodes was significant in the final SBP, DBP and MAP models despite availability of data for only 120 of the 213 women, we compared explanatory power for final stepwise regression models that included or excluded women who had provided fecal samples.

Variables associated with eMAP and low blood pressure were explored using multiple logistic regression analysis, following the same sequence of steps as used for the multiple linear regressions.

In order to determine which of the blood pressure indicators were asscociated with SFH Z-score, an independent factor linear regression analysis was conducted. For each blood pressure indicator, the data were separated into three "factors" (factor 1: <10^{th} centile; factor 2: 10^{th} – 90^{th} centile; factor 3: >90^{th} centile) as determined by 10^{th} and 90^{th} centile values from our data (SBP, 90 and 117.6 mmHg; DBP, 51.4 and 72 mmHg; MAP, 65.6 and 86.6 mmHg; and PP, 30 and 51.6 mmHg). Regression models for SFH-Z-score were constructed using factor 2 as the

reference factor. Reported coefficients compared women in factor 1 and in factor 3 with those in factor 2. Significance was set at $P<0.05$. Associations were further tested after adjusting for two known risks for IUGR, low BMI and wood smoke exposure.

Results

Population characteristics

Descriptive data are reported in Table 1. The population had a series of risk factors for adverse pregnancy outcomes, including pregnancy in adolescents and those over 35 y, and primiparous and grand-multiparous (≥5) pregnancies. Most women used wood as fuel for cooking. Both low weight for GA and overweight/obesity were present. Over 50% of women had vitamin B_{12} or D deficiency, and over 20% had low RBP or vitamin A or folic acid deficiency. RBP concentration was not correlated with concentration of vitamin A (r_s= 0.04, P= 0.55) or CRP (r_s= -0.10, P= 0.12) which supports use of RBP as a protein status indicator in this population. Of the two supplementation programs in the communities, iron tablets reached over 75% of women, and half the women took MMN supplements, although at a median intake well below the recommended 6 tbsp/d. Despite this, over one-third of the women were anemic. Infections were extremely common and 96.7% of women had at least two infections. The most prevalent were vaginal infections, followed by intestinal nematodes, urinary, oral and skin infections (Table 1).

In our population 27% of the women had USG>1020 indicating hypo-hydration and 27% had RBP<30 mg/L indicating low protein status and suggesting low oncotic pressure. Moreover, despite the high prevalence of anemia (38%), all women had normal RBC count and most had normal (93.4%) or high (2.3%) hematocrit percentage, further supporting the presence of hypovolemia in our population and suggesting that the normal hemo-dilution of pregnancy is not appropriately happening.

Trimester-specific blood pressure measurements are summarized in Table 1. None of the pregnant women had high SBP or DBP and only one woman had DBP = 90 mmHg (Figure 1A).

Furthermore, none of the women presented with clinical manifestations of HDPs, and although urinary protein =1+ was found (2.9%), those women had urinary tract infection. However, using eMAP, risk of HDPs was detected in 11.3% women (Fig 1B).

With regard to PP, none of the women had elevated PP but 52.6% had low PP (Figure 1C) and the 10th centile corresponded to a PP of 30 mmHg. Based on the INTERGROWTH standards for SFH, 50.6% (n=88) of women had SFH measurements below the 10th centile and 9.2% (n= 16) had measurements above the 90th centile (Fig 2).

Multiple regression models for blood pressure measurements

Multiple infections entered our regression models for SBP, DBP and MAP, and were associated with both higher and lower BP measurements. Presence of scabies was associated with lower SBP, and presence of trichomoniasis with lower SBP and lower MAP. It is important to highlight that hookworm or *Trichuris* were consistently associated with higher blood pressure (SBP, DBP, MAP, eMAP models) whereas *Ascaris* was associated with lower blood pressure measures (DPB, MAP, and low blood pressure) (Tables 2, 3, 4). Furthermore, despite the much lower sample size, inclusion of these intestinal nematodes increased the adjusted R^2 of the final SBP, DBP and MAP models by more than 75% compared with models where data on intestinal nematodes were not included (see comparison of Tables 2 and 3 with Table S1). Similarly, inclusion of intestinal nematodes increased the Pseudo R^2 of the eMAP (0.272 vs 0.198) and low blood pressure (0.418 vs 0.262) models, compared with models not including intestinal nematodes (Table S2).

Regarding diet and nutrition, higher reported intake of MMN was associated with higher SBP, DBP, MAP and PP, but was not associated with eMAP (Tables 2-4) whereas higher intake of animal-source foods was associated with decreased odds of hypotension (Table 4). Also, two nutrient biomarkers were associated with BP, folic acid deficiency was associated with increased DBP and with increased odds of eMAP, and low RBP with higher DBP and MAP (Tables 2-4).

Among inflammation indicators, two cytokines prevailed in our models. TNFα was associated with higher SBP (Table 2), higher MAP and PP (Table 3); however, higher IL17 was associated with lower peripheral perfusion indicated by lower PP (Table 3). Interestingly, IL17 was not associated with SBP or DPB, but was associated with decreased likelihood of maternal hypotension (Table 4).

Factor linear regressions of SFH Z-scores

In order to determine which of the blood pressure measurements (SBP, DBP, MAP, PP) was associated with SFH, all four were included separately in a three-level factor model for SFH Z scores. Only PP emerged as significantly associated with SFH Z-score (Table 5). Compared with women whose PP was between the 10^{th} and 90^{th} centile, SFH-Z score was lower in women in the $<10^{th}$ centile but similar to those in the $>90^{th}$ centile (Fig. 3).

Discussion

The Ngäbe-Bugle indigenous community has one of the highest rates of adverse pregnancy outcomes in Panama, due in part to its remoteness and difficult access to health care, making it imperative from the public health perspective, to be able to detect women at high risk. Compared with traditional reliance on SBP and DBP, we have observed that MAP and PP provided important information about the risk of both high and low blood pressure in our study population. High MAP has been validated as a risk factor for HDPs [55], and in fact, eMAP was the only BP measurement that identified the risk of HDPs in 11.3% of the women. However, few studies have investigated the prevalence of low BP in marginalized communities. We identified a high prevalence of low BP, and particularly low PP was associated with the lowest SFH for gestational age. These findings emphasize importance of routine blood pressure measurements in remote areas to identify women at risk of poor pregnancy outcomes. Consistent with our hypothesis, we also provide evidence that individual infections, nutrient deficiencies and cytokines, our markers of inflammation, differentially modified each blood pressure measurement. With regard to the presence of multiple infections, those that were associated

with an anti-inflammatory (Th2) response (*Ascaris, Trichomonas,* scabies) were associated with lower blood pressure (SBP, DBP, MAP, and SBP/DBP <100/60 mmHg) whereas those associated with a pro-inflammatory (Th1) response (hookworm, *Trichuris*, UTI) were associated with higher blood pressure measurements (SBP, DBP and MAP). With regard to inflammation, we found that TNFα, which is a hallmark cytokine for HDPs, was associated not only with higher SBP but also with higher MAP, supporting the use of MAP for early detection of women at risk of HDPs. In contrast, IL17, known to increase with placental hypoperfusion, was negatively associated with PP. With regards to specific nutritional deficiencies, low protein and folic acid deficiency were associated with higher MAP and eMAP respectively. Thus against the backdrop of low blood pressure in nearly 25% of the population, we report for the first time that higher intakes of MMN and animal source foods were associated with reduced likelihood of low blood pressure.

Nutrition and blood pressure

There is growing evidence that blood pressure is modulated by multiple nutrients and not just sodium [56]. Recently the INTERMAP Study [57] identified protein, insoluble fibre, phosphorus, calcium, magnesium and non-heme iron as having inverse relationships with SBP and DBP. In our study, pregnant mothers consumed a limited diet but were provided with a dietary supplement by the Ministry of Health [58] containing in addition to energy (400 kcal), protein (12.0 g), lipids (12 -14 g), several micronutrients (vitamins A , E, B_1, B_2, B_3 , B_6 ,B_{12}, and folic acid and calcium , phosphorus, iron, zinc and copper). Noteworthy among our findings was the observation that even though women in our study consumed less of the MMN than recommended, its intake was associated with a modest increase in SBP, DBP, MAP and PP, which aligns with a previous MMN supplementation study with protein and folic acid that was also positively associated with SBP, DBP, MAP and PP and not to the development of HDPs [59]. In our study, the MMN supplement was also associated with decreasing the odds of hypotension, suggesting that adequate intake of one or more of its nutrients might normalize the low blood pressure in our population of pregnant women.

Several studies have examined the consequences of maternal dietary protein intakes on pregnancy outcomes, particularly SGA. Although a Cochrane review concluded that there is no justification for prescribing high-protein nutritional supplements to pregnant women [60], a more recent meta-analysis of studies from low-medium income countries found that balanced protein-energy supplementation of undernourished women significantly improved birthweight [61]. And although the link between maternal protein malnutrition in further hypertension in the offspring has been established [62], only one study with Dutch women showed that protein intake-related acid load was not associated with HDPs; however a higher vegetable protein/potassium ratio was associated with lower DBP [63], which is consistent with a meta-analysis in non-pregnant populations from developed countries that showed that increased intake of dietary protein relative to carbohydrate was associated with lower blood pressure [64]. In our marginalized community, there was clinical evidence of dietary protein deficiency with 26% of mothers having low RBP and 85% having low B_{12}, but our finding that higher intakes of animal source foods reduced the odds of low blood pressure agrees with observations from developing settings where higher protein intakes in carbohydrate-rich diets could decrease blood pressure [64, 65]. Our study is the first to observe an association of higher protein intake with the reduced odds of hypotension not previously reported in marginalized communities.

Another nutrient deficiency that emerged as increasing the odds for elevated MAP was that of folic acid. We had previously shown that folic acid deficiency was positively associated with elevated CRP in lactating women in our population [33], which aligns with evidence that folic acid deficiency may promote increased BP probably as consequence of increased inflammation [66]. Folic acid supplementation for the prevention of neural tube defects and LBW has been studied [67], but its use for the prevention of HDPs is still controversial. Although folic acid supplementation did not change SBP or DBP in Iranian [68] or Netherlander [59] women, and folic acid levels were similar between normal and hypertensive pregnancies in India [69], it has been reported that folate supplementation decreased the risk of HDPs in populations from China [70] and Korea [71]. The association of folic acid deficiency with increased odds of eMAP

points towards a favorable effect of folic acid supplementation for the prevention of HDPs and aligns with the significant positive correlations among serum homocysteine and high MAP in women with pregnancy complicated by HDP [17].

BP and infections

A particularly intriguing and novel observation was the bi-directional nature of associations between infections and blood pressure indicators. Hookworm was associated with higher SBP, DBP and MAP and also was associated with lower odds of low blood pressure, and *Trichuris* with increased odds of eMAP. In contrast, a third intestinal nematode, *Ascaris*, as well as the scabies mite and the vaginal protozoan, *Trichomonas* were associated with lower blood pressure and *Ascaris* also increased the odds of low blood pressure. The only data linking any of these infections with blood pressure comes from *Strongyloides,* an intestinal nematode that penetrates the skin and migrates to the intestine like hookworms. In rats, adult *Strongyloides venezuelensis* increased both SBP and MAP possibly reflecting the impact of nematode-induced inflammation on cardiovascular function [72]. These results are consistent with the higher blood pressure observed in our pregnant women who were infected with hookworm.

The contrast in direction of association for *Ascaris,* scabies and trichomoniasis compared with hookworm and *Trichuris* is also consistent with the distinctiveness of their inflammatory responses. First, in these same pregnant women, C reactive protein (CRP) was shown to be negatively associated with *Ascaris* but positively associated with hookworm [33], suggesting that *Ascaris* was anti-inflammatory whereas hookworm was pro-inflammatory. Second, the immune response to intestinal nematodes [73], parasitic infections including scabies mites [74]and *Trichomonas vaginalis* [75, 76] are able to modulate the host immune response with production of classical Th2 cytokines, IL4, IL5 and IL13, mastocytosis, eosinophilia, IgE and alternatively-activated macrophages known to play a critical role in tissue repair [77]. In contrast, hookworm releases molecules that down-regulate the strong Th2 response through a mixed Th2/Th1 response with elevation of pro-inflammatory cytokines including TNFα and tolerance to allergens [78-80] similar to the pro-inflammatory response observed in response to

chronic low dose infection of mice with *Trichuris muris* [81]. Taken together, it is not surprising that the association with blood pressure differed for *Ascaris,* scabies and trichomoniasis compared with hookworm and *Trichuris,* and it is likely that this was linked to the anti-inflammatory and pro-inflammatory responses, respectively.

Cytokines and inflammation

Two cytokines entered our models in opposite directions. TNFα was positively associated with SBP, MAP and PP, whereas IL17 was negatively associated with PP and was associated with decreased odds of low BP. It is known that TNFα increases under placental hypoperfusion, and that TNFα is able to increase BP by activating humoral and endothelial factors [82]. TNFα has been consistently found to be elevated in women with HDPs, but serum concentrations (pg/mL) reported in pre-eclamptic women varied among studies [83] [84-86]. Importantly, we found that serum values of TNFα found in our population were comparable with pre-eclamptic women from these studies, and its association with MAP support the use of eMAP as early indicator of women at risk for HDPs in MINDI populations.

On the other hand, IL17 was negatively associated with PP and with decreased odds of low BP. Experimental studies have shown that Th17 cells from animals with reduced uterine perfusion are able to elevate BP and to induce IUGR [87]. More recent studies in humans have shown that Th17 cells or IL17 are higher in women with pre-eclampsia [88, 89]. Moreover, PP is a surrogate measurement of arterial stiffness of large arteries [90] which explains the associations found by others between higher PP and lower birth weighs [91]. However, having in mind that PP is also the main indicator of organ's blood perfusion [3] and that the association of PP with later pregnancy-induced hypertension has been inconsistent [4], it is possible that the negative association of PP with IL17 in our study reflects a role of IL17 in decreased maternal perfusion.

BP measurements and symphysis-fundal height

It is known that women who do not achieve the physiologic increase of blood volume are prone to HDPs, fetal growth restriction and SGA [92, 93]. Aside from the context of pre-eclampsia,

which is an important cause of IUGR [94], few studies have evaluated the use of BP measurements as determinant of offspring size. European [95] and Asian studies [96] have shown that elevated SBP and DBP were associated with impaired fetal growth, but few studies have addressed hypotension as potential risk factor for SGA. Warland et al found increased risk of stillbirth in women with low DBP and low MAP [9], whereas Zhang and Klebanoff found that low BP defined as DBP <80 mmHg was associated with PTB and SGA, but the association was lost after controlling for maternal age, BMI, socio-economic status and race [8]. Previously others had used PP to explain associations between higher PP and lower birth weighs [91]. Among our BP measurements, only low pulse pressure was associated with low SFH, demonstrating that hypotension may be an unrecognized factor contributing to impaired fetal growth in our MINDI cohort.

Conclusion

In conclusion our results demonstrate that routine blood pressure measurements, which reflect overall exposure to multiple infections, nutritional deficiencies and inflammation, can be used to assess pregnancies at risk in remote settings. Specifically, the association of higher MAP with indicators of a pro-inflammatory responses support its use in the earlier detection of women at risk of HDPs. On the other hand, the association of low PP with smaller fetuses makes it an interesting indicator to suspect SGA in remote areas where sonography is not available.

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Tables and figures

Table 1 Population characteristics in pregnant Ngäbe-Buglé women from rural Panama (n= 208 to 213).

Blood pressure measurements (Mean ± SD)'		**Nutritional characteristics**	
Systolic blood pressure, mmHg		Body mass index category	
First trimester	104.8 ± 10.6	Underweight, %	9.8
Second trimester	100.6 ± 10.8	Overweight or obese, %	23.0
Third trimester	103.9 ± 9.6	Anemia, %	38.0
Diastolic blood pressure, mmHg		Retinol-binding protein < 30 mg/L, %	26.7
First trimester	64.0 ± 7.4	Vitamin B_{12} < 150 pmol/L, %	84.9
Second trimester	59.5 ± 8.5	Vitamin D < 50 nmol/L, %	64.8
Third trimester	62.5 ± 8.5	Vitamin A < 1.05 µmoll/L, %	40.8
Mean Arterial Pressure, mmHg		Folic acid < 10 nmol/L, %	23.9
First trimester	77.6 ± 7.2	Supplementation with iron, %	76.5
Second trimester	73.2 ± 8.5	Supplementation with MMN[1], %	50.7
Third trimester	76.3 ± 7.9	**Vaginal infections**	
Pulse pressure, mmHg		*Trichomonas vaginalis*, %	75.3
First trimester	40.7 ± 9.8	Bacterial vaginosis, %	60.6
Second trimester	41.0 ± 8.3	*Lactobacillus*, %	53.5
Third trimester	41.3 ± 8.5	*Bacteroides/Gardnerella*, %	93.8
Maternal characteristics		*Mobiluncus*, %	82.4
Age		Yeast, %	24.8
≤ 19 yrs, %	29.1	*Diplococcus spp.*, %	20.3
≥ 35 yrs, %	13.1	**Intestinal parasites** [2]	
Parity		Hookworm, %	56.6
First gestation, %	28.2	*Ascaris*, %	32.5
≥5 gestations, %	32.4	*Trichuris*, %	12.5
Wood smoke, %	91.5	**Other infections**	
Hemoglobin, g/L	111.6 ± 11.3	Bacteriuria ≥ 2+, %	25.9
Hematocrit (%), mean ± SD	34.8 ± 3.2	Caries, %	19.7
Mean corpuscular volume, fL	93.7 ± 6.0	Scabies, %	17.3
Urinary specific gravity >1020	26.9		

[1]MMN: Multiple micronutrients containing in every 100 g, energy (400 kcal), protein (12.0 g), lipids (12 to 14 g), vitamin A (250 µg), vitamin E (10 mg), vitamin B_1 (0.50 mg), vitamin B_2 (0.50 mg), vitamin B_3 (6.0 mg), vitamin B_6 (0.50 mg), vitamin B_{12} (0.90 µg), folic acid (85 µg), iron (4.0 mg iron bisglycinate), zinc (4.5 mg amino chelated), calcium (100 mg), phosphorus (55 mg) and copper (400 µg) [58].

[2] n = 120

Table 2 Multiple stepwise linear regression models for systolic blood pressure (SBP) (Model A) and diastolic blood pressure (DBP) (Model B) in pregnant Ngäbe-Buglé women from rural Panama [1, 2]

Model A. SBP [3]	**Coefficient ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA, wks	0.09 ± 0.08	0.279	-0.07, 0.26	0.091	n= 116 P< 0.0001 $F_{8, 107}$ = 7.54 Adjusted R^2= 0.313 VIF= 1.11 Condition number= 15.294
BMI category	4.08 ± 1.44	0.005	1.23, 6.93	0.239	
MMN, tbsp/d	1.53 ± 0.50	0.003	0.54, 2.52	0.262	
TNFα, pg/mL [4]	0.35 ± 0.09	<0.0001	0.16, 0.54	0.294	
Caries, presence	-3.3 ± 1.89	0.075	-7.14, 0.35	-0.142	
Scabies, presence	-4.28 ± 1.89	0.026	-8.03, -0.53	-0.182	
Trichomonas, presence [4]	-6.99 ± 1.82	<0.0001	-10.62, -3.38	-0.309	
Hookworm, presence [4]	3.21 ± 1.61	0.049	0.02, 6.40	0.162	
Constant	91.20 ± 4.18	<0.0001	82.91, 99.49		
Model B. DBP [5]	**Coefficient ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA, wks	0.12 ± 0.09	0.173	-0.05, 0.29	0.139	n= 116 P< 0.0001 $F_{9, 106}$ = 4.58 Adjusted R2= 0.219 VIF= 1.18 Condition number= 15.23
BMI category	2.45 ± 1.30	0.063	-0.13, 5.04	0.166	
Urinary gravity >1020 [4]	3.34 ± 1.72	0.054	-0.06, 6.75	0.164	
MMN, tbsp/d	0.98 ± 0.46	0.036	0.06, 1.91	0.195	
Iron supplementation (yes/no)	-3.98 ± 2.08	0.059	-8.11, 0.14	-0.183	
RBP < 30 mg/L [4]	4.44 ± 1.79	0.015	1.88, 8.00	0.214	
Folic acid <10 nmol/L	2.85 ± 1.78	0.113	-0.69, 6.38	0.136	
Ascaris, presence [4]	-3.77 ± 1.57	0.018	-6.88, -0.65	-0.207	
Hookworm, presence [4]	3.04 ± 1.50	0.046	0.06, 6.02	0.177	
Constant	51.51 ± 3.58	<0.0001	44.40, 58.62		

[1] Abbreviations: GA, gestational age; BMI, body mass index; MMN, multiple micronutrient supplement; RBP, retinol binding protein

[2] Unstandardized coefficient and standardized β

[3] Additional variables included: wood smoke exposure (yes/no), hematocrit quantile, IL13 (pg/mL), IL17 (pg/mL), bacteriuria score, *Ascaris* (yes/no). Little's Chi-squared test for randomness of missing data, P = 0.698.

[4] Missing data for TNFα (1), RPB (1), *Trichomona*s (2), urinary gravity (5), hookworm (93), *Ascaris* (93)

[5] Additional variables included in final model: hematocrit quantile, hemoglobin (g/L), IL13 (pg/mL), vitamin D<50 nmol/L, *Trichomonas* (yes/no). Little's Chi-squared test for randomness of missing data, P = 0.197.

Table 3 Multiple stepwise linear regression models for mean arterial pressure (MAP) (Model A), and pulse pressure (PP) (Model B) in pregnant Ngäbe-Buglé women from rural Panama [1, 2]

Model A. MAP [3]	**Coefficient ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA, wks	0.07 ± 0.07	0.293	-0.06, 0.21	0.092	n= 116 P< 0.0001 $F_{9, 106}$ = 6.32 Adjusted R^2= 0.294 VIF= 1.14 Condition number= 15.63
BMI category	3.24 ± 1.17	0.007	0.91, 5.56	0.233	
Urinary gravity > 1020 [4]	3.03 ± 1.55	0.053	-0.03, 6.10	0.158	
MMN, tbsp/d	1.00 ± 0.42	0.020	0.16, 1.84	0.210	
TNFα, pg/mL [4]	0.17 ± 0.08	0.033	0.01, 0.33	0.178	
Trichomonas, presence [4]	-4.02 ± 1.52	0.009	-7.03, -1.00	-0.217	
RBP < 30 mg/L [4]	4.00 ± 1.61	0.015	0.81, 7.19	0.205	
Ascaris, presence [4]	-3.13 ± 1.40	0.028	-5.91, -0.35	-0.182	
Hookworm, presence [4]	3.28 ± 1.34	0.016	0.63, 5.94	0.204	
Constant	63.6 ± 3.4	<0.0001	56.9, 70.3		
Model B. PP [5]	**Coefficient ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA, wks	-0.05 ± 0.06	0.355	-0.18, 0.06	-0.066	n= 206 P= 0.0007 F6, 203= 4.08 Adjusted R2= 0.083 VIF= 1.36 Condition number= 13.31
Age, yrs	-0.20 ± 0.08	0.013	-0.36, -0.04	-0.169	
MMN, tbsp/d	0.79 ± 0.38	0.040	0.03, 1.55	0.145	
Basophils, number/mm^3	0.06 ± 0.04	0.110	-0.01, 0.14	0.109	
IL17, pg/mL [4]	-0.24 ± 0.07	0.002	-0.39, -0.09	-0.290	
TNFα, pg/mL [4]	0.27 ± 0.09	0.005	0.08, 0.46	0.261	
Constant	44.58 ± 2.83	<0.0001	39.0, 50.16		

[1] Unstandardized coefficient and standardized β

[2] GA, gestational age; BMI, body mass index; MMN, multiple micronutrient supplement; RBP, retinol binding protein

[3] Additional variables included in final model: folic acid <10 nmol/L, hematocrit quantile, urinary protein score, hemoglobin (g/L), IL13 (pg/mL), vitamin D <50 nmol/L, vaginal yeast score. Little's Chi-squared test for randomness of missing data, P = 0.163.

[4] Missing data for TNFα (1), IL17 (1), RBP (1), *Trichomona*s (2), urinary gravity (5), hookworm (93), *Ascaris* (93)

[5] Additional variables included in final model: neutrophils (#), urinary gravity, *Mobiluncus* and *Bacteroides/Gardnerella* scores. Little's Chi-squared test for randomness of missing data, P = 0.647.

MAP = DBP + 0.33 [SBP-DBP]; PP = SBP-DBP

Table 4 Multiple logistic regression model for elevated mean arterial pressure (eMAP)1 (Model A) and low blood pressure1 (Model B) in pregnant Ngäbe-Buglé women from rural Panama[1]

Model A. eMAP [2]	**Odds Ratio ± SE**	**P**	**95% CI**	**Overall Model**
Age, yrs	1.18 ± 0.07	0.003	1.06, 1.32	n= 117 P= 0.0003 Pseudo R^2= 0.272 VIF= 1.03 Condition number= 7.76
Folic acid <10 nmol/L	6.98 ± 5.62	0.016	1.44, 33.79	
Trichuris, presence [3]	6.72 ± 6.48	0.048	1.01, 44.56	
Constant	0.0003 ± 0.0005	<0.0001	5.6^{-6}, 0.01	
Model B. Low blood pressure [4]	**Odds Ratio ± SE**	**P**	**95% CI**	**Overall model**
GA, wks	0.93 ± 0.03	0.035	0.86, 0.99	n= 119 P< 0.0001 Pseudo R^2= 0.418 VIF= 1.32 Condition number= 27.43
BMI, kg/m^2	0.75 ± 0.07	0.002	0.62, 0.90	
Field work, h/d	0.60 ± 0.09	0.001	0.45, 0.81	
Ascaris, presence [3]	3.63 ± 2.27	0.040	1.06, 12.39	
MMN, tbsp/d	0.35 ± 0.11	0.001	0.18, 0.64	
IL17, pg/mL[3]	0.87 ± 0.05	0.016	0.78, 0.97	
INFγ, pg/mL [3]	1.08 ± 0.05	0.082	0.99, 1.18	
Hookworm, presence [3]	0.33 ± 0.21	0.081	0.09, 1.15	
Animal-source foods/wk	0.70 ± 0.10	0.012	0.54, 0.92	
Constant	66399.6 ± 201973.8	<0.0001	171.0, 2.6^7	

[1] eMAP defined as >87 mmHg between weeks 10-18, >84 mmHg in weeks 18-34, and >86 mmHg after week 34 [39]; low blood pressure defined as SBP <100 and DBP <60 mmHg); GA, gestational age; BMI, body mass index; MMN, multiple micronutrient supplement; RBP, retinol binding protein; NLR, neutrophil-lymphocyte ratio; MCV, mean corpuscular volume

[2] Additional variables included in final model: BMI category, NLR, MCV (fL), IL1β (pg/mL), IL17 (pg/mL), iron supplementation (mo), MMN supplementation (tbsp/d), RBP <30 mg/L, *Ascaris* (yes/no), vaginal yeast (yes/no), bacteriuria(yes/no). Little's Chi-squared test for randomness of missing data, P = 0.948.

[3] Missing data for IFNγ (1), IL1β (1), IL17 (1), RBP (1), vaginal yeast (2), bacteruria (5), *Ascaris* (93)*, Trichuris* (93), hookworm (93). Two outliers of IL17 were removed.

[4] Additional variables included in final model: Hb (g/L), hematocrit quantile, green leafy vegetable intake/wk, iron supplementation (mo), folic acid <10 nmol/L, vitamin D (nmol/L), scabies (yes/no). Little's Chi-squared test for randomness of missing data, P = 0.801.

Table 5 Multiple linear regression models of symphysis fundal height (SFH) Z-scores and pulse pressure (PP) in 177 pregnant Ngäbe-Buglé women from rural Panama with gestational age ≥16 wks.[1]

Adjusted Model B. SFH-Z score [2,3]	Coefficient ± SE	P	95% CI	β	Overall Model
PP < 10^{th} centile	-2.85 ± 0.77	<0.0001	-4.37, -1.32	-0.262	n= 177
PP > 90^{th} centile	-0.06 ± 0.53	0.899	-1.12, 0.98	-0.009	$F_{4, 172}$= 6.87
BMI category	0.57 ± 0.26	0.029	0.06, 1.08	0.158	P< 0.0001
Wood smoke (h/d)	-0.30 ± 0.09	0.002	-0.50, -0.11	-0.222	Adj. R^2= 0.117
Constant	-2.00 ± 0.60	0.001	-3.19, -0.80		VIF = 1.02 Condition Number = 15.45

[1] A three-level factor was used for our independent variable, pulse pressure (< 10^{th} centile, ≥10^{th} - ≤90^{th} centiles, and > 90^{th} centile), and comparisons were made against the base category (≥10^{th} - ≤90^{th} centile). Adjusted for BMI category and wood smoke exposure (h/d).

[2] SFH, symphysis-fundal height; PP, pulse pressure; BMI, body mass index

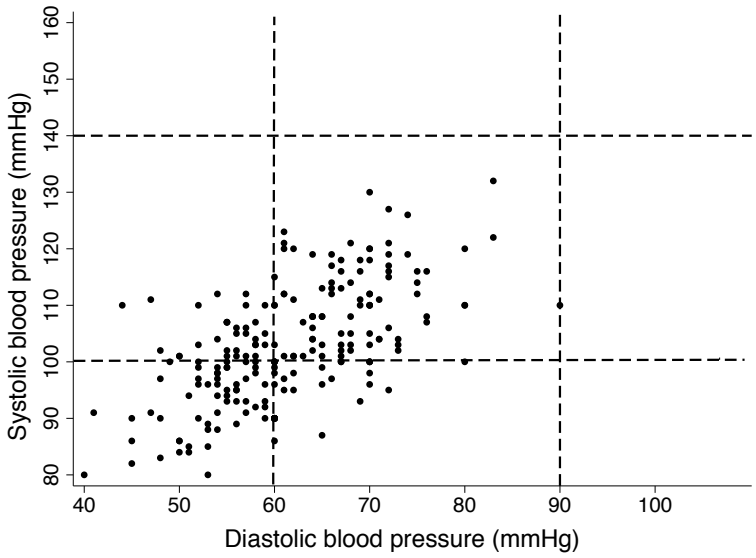
[2] Missing data for SFH (15)

[3] Little's Chi-squared test for randomness of missing data, P = 0.466.

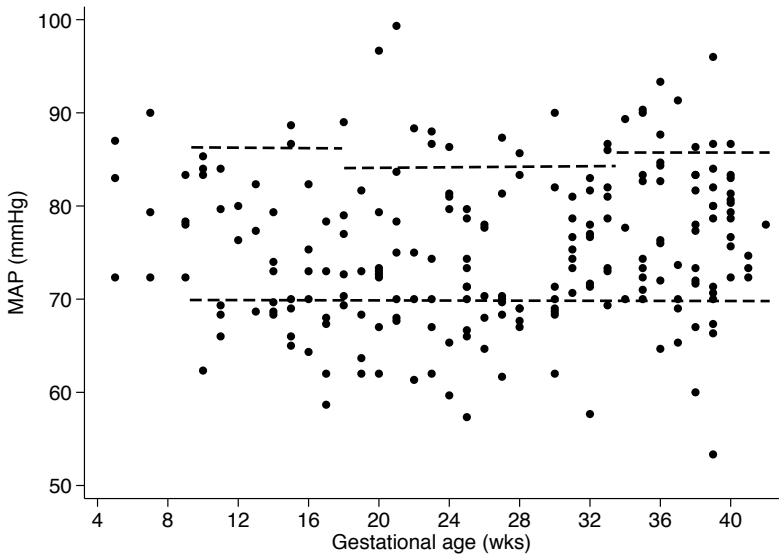
Figure 1 Ambulatory blood pressure measurements in 213 pregnant Ngäbe-Buglé women from rural Panama.

A. Scatter plot of systolic vs diastolic blood pressure. Dashed lines represent blood pressure limits for hypertension during pregnancy (≥140 mmHg for SBP and ≥90 mmHg for DBP) [37], and lower values were defined following the most conservative cut-offs (<100 mmHg for SBP and <60 mmHg) found for a large population of pregnant women [41]. **B**. Scatter plot of mean arterial pressure (MAP) according to gestational age. Dashed lines represent cutoffs for elevated MAP: >87 mmHg between weeks 10-18, >84 mmHg in weeks 18-34, and >86 mmHg after week 34 [39], and low values <70 mmHg according to Henry et al [40]. **C.** Scatter plot of pulse pressure according to gestational age. Points below the dashed line represent low PP (<42 mmHg) [41].

A.



B.


MAP (mmHg)
100
90
80
70
60
50
4
8
12
16
20
24
28
32
36
40
Gestational age (wks)

C.

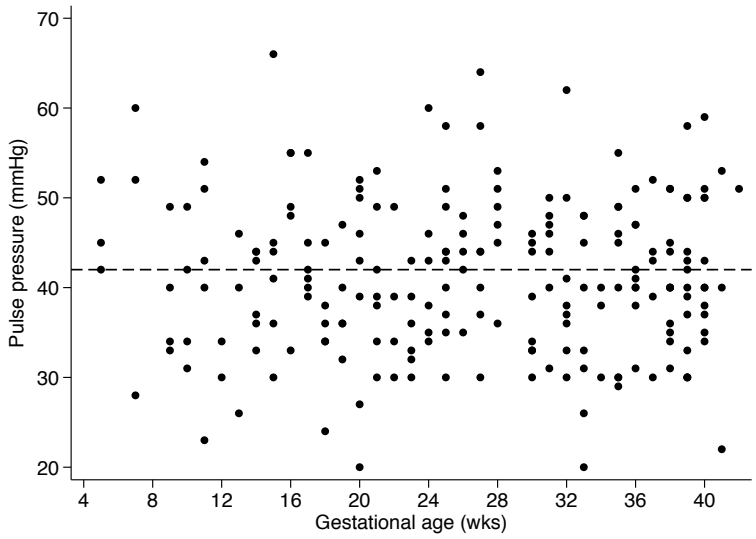

Pulse pressure (mmHg)
70
60
50
40
30
20
4
8
12
16
20
24
28
32
36
40
Gestational age (wks)

Figure 2 Scatter plot of symphysis-fundal height Z-scores based on INTERGROWTH standards for gestational age in 177) pregnant Ngäbe-Buglé women from rural Panama.

Reference lines mark the 10th centile, below which fetuses are considered to be small for gestational age, and the 90th centile, above wich fetuses are considered large for gestational age [28].

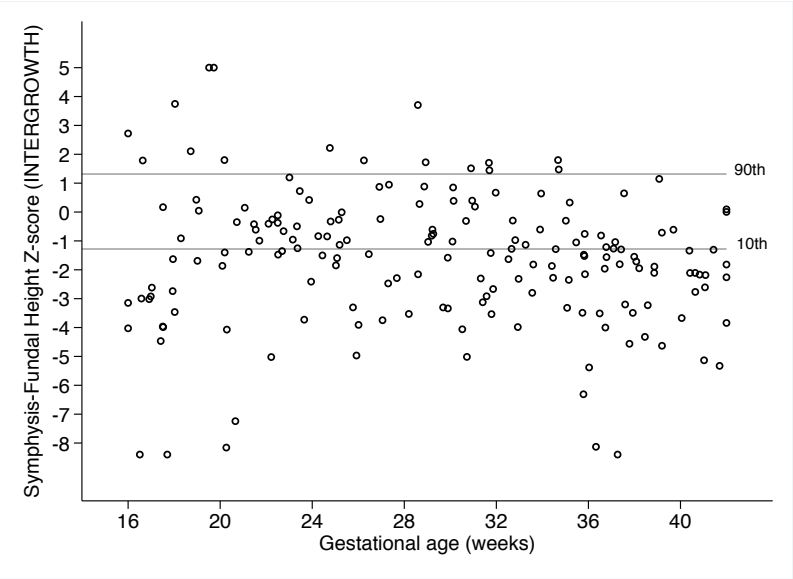
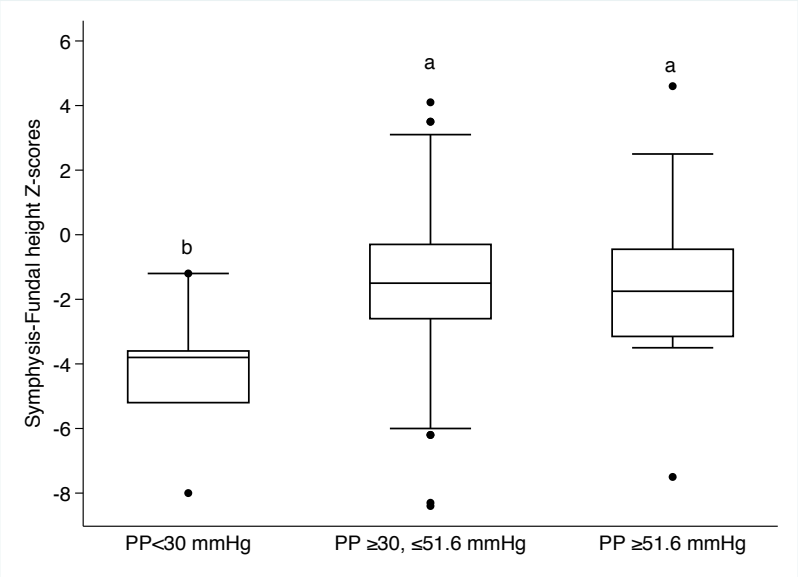


Figure 3 Box and whisker plots of symphysis-fundal height for three pulse pressure (PP) categories (<10th centile, 10th – 90th centile, and ≥90th centile) based on data from 177 pregnant Ngäbe-Buglé women from rural Panama.

The bottom and top of each box represent the 25th and 75th centiles respectively; the horizontal line inside the box represents the median. Whiskers show the minimum and maximum values and dots represent outside values. Different lower case letters denote significant differences at P ≤0.05.



Supplementary tables

Table S1 Multiple linear regression models for A. systolic (SBP), B. diastolic (DBP) blood pressure and C. mean arterial pressure (MAP) in the dataset of indigenous pregnant Ngäbe-Buglé women (n= 206-207) where data on intestinal nematodes were not included.

A. SBP	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA	-0.006 ± 0.07	0.927	-0.14, 0.13	-0.006	n= 206 P< 0.0001 $F_{9, 196}$ = 5.65 Adjusted R^2= 0.170 VIF= 1.29 Condition number= 17.73
BMI category	3.28 ± 1.26	0.010	0.78, 5.77	0.176	
MMN, tbsp./d	1.62 ± 0.45	<0.0001	0.72, 2.52	0.248	
IL17	-0.20 ± 0.08	0.024	-0.37, -0.02	-0.203	
TNFα	0.32 ± 0.11	0.004	0.10, 0.53	0.263	
Hematocrit quantile	1.28 ± 0.60	0.035	0.09, 2.46	0.139	
Scabies, presence	-3.86 ± 1.75	0.028	-7.32, -0.41	-0.143	
Urinary bacteria (+)	-1.94 ± 1.14	0.092	-4.20, 0.32	-0.109	
Trichomonas, presence	-3.81 ± 1.57	0.016	-6.92, -0.70	-0.159	
Constant	95.79 ± 3.81	<0.0001	88.27, 103.3		
B. DBP	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA	0.05 ± 0.06	0.416	-0.07, 0.16	0.057	n= 207 P= 0.0001 $F_{7, 199}$ = 4.75 Adjusted R^2= 0.113 VIF= 1.08 Condition number= 15.72
Urinary gravity >1020	3.32 ± 1.25	0.009	0.85, 5.80	0.175	
BMI category	2.30 ± 1.05	0.029	0.23, 4.37	0.150	
MMN, tbsp./d	0.82 ± 0.38	0.034	0.06, 1.58	0.152	
Hematocrit, quantile	1.30 ± 0.50	0.010	0.31, 2.28	0.172	
Trichomonas, presence	-2.22 ±1.34	0.100	-4.86, 0.42	-0.112	
Vitamin D< 50 nmol/L	-2.03 ± 1.16	0.082	-4.32, 0.26	-0.115	
Constant	53.07 ± 3.09	<0.0001	46.97,59.17		
C. MAP	**Coef. ± SE**	**P**	**95% CI**	**β**	**Overall Model**
GA	0.004 ± 0.05	0.939	-0.11, 0.12	0.005	n= 206 P< 0.0001 $F_{9, 196}$ = 5.60 Adjusted R^2= 0.168 VIF= 1.10 Condition number= 16.35
BMI category	2.87 ± 0.99	0.004	0.92, 4.83	0.194	
Urinary gravity >1020	3.05 ± 1.18	0.011	0.71, 5.39	0.167	
MMN, tbsp./d	1.03 ± 0.36	0.005	0.31, 1.74	0.198	
TNFα	0.10 ± 0.06	0.114	-0.02, 0.22	0.103	
Hematocrit, quantile	1.34 ± 0.48	0.006	0.39, 2.29	0.185	
Trichomonas, presence	-2.26 ± 1.25	0.072	-4.74, 0.20	-0.119	
Folic acid <10 nmol/L	3.82 ± 1.27	0.003	1.31, 6.32	0.200	
RBP <30 mg/L	1.83 ± 1.22	0.136	-0.58, 4.25	0.099	
Constant	63.0 ± 2.9	<0.0001	57.3, 68.7		

Table S2 Multiple logistic regression models for A. elevated MAP (eMAP) and B. low blood pressure (SBP<100 and DBP <60 mmHg) in the dataset of indigenous pregnant Ngäbe-Buglé women (n= 212), where data on intestinal nematodes were not included.

A. eMAP	**OR ± SE**	**P**	**95% CI**	**Overall Model**
Age	1.11 ± 0.04	0.003	1.03, 1.18	n= 212 P= 0.0001 Pseudo R^2= 0.198 VIF= 1.16 Condition number= 11.34
Folic acid <10 nmol/L	3.14 ± 1.63	0.027	1.13, 8.69	
MMN, 139bsp../d	1.25 ± 0.17	0.109	0.95, 1.64	
NLR	0.61 ± 0.17	0.084	0.35, 1.07	
IL1β	1.11 ± 0.05	0.017	1.02, 1.21	
IL17	0.93 ± 0.04	0.074	0.85, 1.01	
Constant	0.01 ± 0023	0.001	0.001, 0.18	
B. Low blood pressure	**OR ± SE**	**P**	**95% CI**	**Overall Model**
Gestational age	1.00 ± 0.02	0.895	0.95, 1.05	n= 210 P< 0.0001 Pseudo R^2= 0.263 VIF= 1.34 Condition number= 16.83
Field work, h/d	0.81 ± 0.06	0.008	0.70, 0.95	
Iron supplementation, months	0.76 ± 0.09	0.029	0.59, 0.97	
MMN, tbsp./d	0.52 ± 0.10	0.001	0.35, 0.76	
Animal-source foods, portions/wk	0.86 ± 0.06	0.050	0.74, 0.99	
IL17	0.90 ± 0.03	0.003	0.84, 0.96	
INFγ	1.06 ± 0.03	0.031	1.00, 1.12	
Scabies, presence	2.26 ± 1.07	0.08	0.89, 5.74	
Mobiluncus, score	1.59 ± 0.29	0.013	1.10, 2.29	
Anemia, presence	4.04 ± 1.67	0.001	1.79, 9.11	
Folic acid <10 nmol/L	0.19 ± 0.10	0.002	0.06, 0.55	
Vitamin D, nmol/L	0.97 ± 0.01	0.074	0.095, 1.002	
Constant	2.35 ± 2.38	0.399	0.32, 17.18	

Connecting statement 3

After understanding from paper 2 (anemia/iron deficiency) and 3 (blood pressure) that in our population of pregnant women hypo-perfusion was present, that hypotension was more prevalent than the risk of hypertension, and that hypotension predicted only 5% of the variability of symphysis-fundal height Z-scores, we finally wanted to evaluate associations of all adverse conditions including MINDI with fetal growth.

Since sonography or other technologies were not available in the field, symphysis-fundal height was used to measure fetal growth and was, at the same time, tested as biomarker under conditions of MINDI.

It was hypothesized that small for gestational age might be prevalent and that the association of low blood pressure with smaller symphysis-fundal height might be lost after adjusting for other factors.

Paper 4. Classification of small- and very-small- for gestational age fetuses using symphysis-fundal height INTERGROWTH-21 standards in an indigenous population identifies multiple infections, nutrient deficiencies and inflammation as risk factors for impaired in-utero growth: The MINDI Cohort

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List of abbreviations

AGA: adequate for gestational age; ANOVA: Analysis of variance; BMI: body mass index; CRP: C-reactive protein; DBP: diastolic blood pressure; GA: gestational age; HIV: human immunodeficiency virus; IL: interleukin; INF: interferon; LGA: large for gestational age; MAP: mean arterial pressure; MINDI: Multiple infections, nutrient deficiencies and inflammation; MMN: multiple micronutrients; proMBP: eosinophil major basic protein; NLR: Neutrophil-lymphocyte ratio; RBC: Red blood cell; RBP: Retinol-binding protein; SBP: systolic blood pressure; SGA: small for gestational age; SFH: symphysis-fundal height; sTfR: serum transferrin receptors; TNF: tumor necrosis factor; VSGA: very small for gestational age; WBC: White blood cell; WHO: World Health Organization

Abstract

Background: The detection of fetal size in resource-deprived settings with no access to ultrasound is difficult. The newly developed international standards for symphysis-fundal height (SFH) provide an opportunity to assess the prevalence and to identify the determinants of poor fetal growth in remote settings where the co-occurence of multiple infections and nutrient deficiencies are common.

Methods: For this cross-sectional study, we recruited a representative sample of 174 indigenous women with pregnancies ≥16 weeks from the Ngäbe-Buglé comarca in Panama. Fetal size was assessed–using new international INTERGROWTH SFH standards. Comparisons among fetuses classified as very small (VSGA, SFH<3rd centile), small (SGA, SFH>3rd and less than the 10th centile), adequate (AGA, SFH ≥10th and ≤90th centile) and large (LGA, SFH>90th centile) for gestational age, were performed using one-way ANOVA. Factors associated with of poor fetal growth were explored using a backward stepwise logistic regression modeling framework. Maternal characteristics (age, parity, wood smoke exposure), infections (caries, scabies, urogenital and intestinal nematode infections), markers of inflammation (white blood cell count, C-reactive protein, hepcidin and cytokines) and nutrient deficiencies (vitamins A, D, B_{12}, and folic acid, iron and protein status defined as retinol-binding protein <30 mg/L) were considered as independent variables.

Results: The prevalence of all fetuses with SFH <10% was 50.6%; from those, 12.6% were between the 10th and the 3rd centiles and those with SFH<3rd centile were 37.9%. Pro-inflammatory cytokines were higher in the SGA compared with the VSGA, but protein deficiency was more common in mothers with VSGA. Multiple logistic regression analyses showed that higher odds of SFH between the 3rd–10th centiles was associated with higher lymphocytes, IL17 and serum iron whereas IL-10 was associated with lower odds. For VSGA, higher hepcidin was associated with increased likelihood of SFH<3rd, but higher protein status, higher TNFα and higher pulse pressure were associated with lower likelihood of VSGA. Among nematodes, only the presence of *Trichuris* increased the likelihood of SFH<3rd centile.

Conclusion: In our MINDI cohort, we provide evidence that the presence of intestinal nematode infections, nutrient deficiencies and biomarkers of inflammation were associated with impaired fetal growth. We highlight that inflammation biomarkers and low pulse pressure likely driven by low placental perfusion, as well as iron restriction due to inflammation together with protein malnutrition are overlooked factors associated with fetal growth in marginalized communities. We validate SFH as a useful biomarker capable of identifying determinants of SGA and VSGA in a remote indigenous community.

Introduction

Small for gestational age fetuses (SGA) are at higher risk of morbidity and mortality [1-3] and to growth, hormonal and developmental problems later in life [4]. SGA is been defined as fetuses <10^{th}, 5^{th} or 3^{rd} centile for gestational age according to the World Health Organization (WHO), based on birthweight-for-gestational-age measures compared to a gender-specific reference population [5, 6].

Causal factors associated with nearly 50% of SGA births include maternal factors such as low maternal height, chronic malnutrition, hypo- or hypertension, smoking and infections such as HIV, Zika virus, toxoplasmosis and rubella; placental abnormalities, congenital problems and other infections account for about 5% of SGA cases, but no cause for SGA has been identified in 40% of the cases [7, 8]. Idiopathic SGA has been associated with placental insufficiency [9], mainly in well-nourished populations [10]. However, associations of SGA with co-existing infections, nutrient deficiencies and inflammation, common in developing country settings, have been only partially explored.

Several studies in Africa report associations between intestinal parasites and SGA but only in populations with malaria [11] and human immunodeficiency virus (HIV) [12]. Inflammatory markers are reportedly higher in cord blood from SGA infants compared to adequate for gestational age (AGA) infants in the Netherlands [13], and maternal interleukin (IL)-13 and interferon (IF)-γ were found inversely associated with SGA in Vietnam [14], indicating a role of inflammation in the origins of SGA. On the other hand, the use of multiple micronutrient supplementation reportedly lowers SGA rates in undernourished settings [15]. Folic acid supplementation [16] and higher intake of vitamin B_{12} [17] have been found to protect against SGA. However, vitamin D supplementation studies, although showing decreased risk of SGA, were found to lack robustness in sensitivity and subgroup analyses in a recent systematic review [18]. Likewise, studies on iron supplementation during pregnancy have shown that iron status has a U- shaped curve suggesting an increased risk of adverse pregnancy outcomes including SGA with both low and high intakes [19].

Clinically measured symphysis-fundal height (SFH) is the first level screening tool often used for assessing fetal size in marginalized communities in the absence of ultrasound [20]. Despite controversial evidence concerning its accuracy because different populations with relatively small samples were used to create reference charts [21, 22], the INTERGROWTH-21st project targeted the development of new international SFH standards using a large cohort of healthy pregnant women from Brazil, China, India, Italy, Kenya, Oman, United Kingdom and the United States [23].

Therefore, the objectives of our study were to identify, using the new international INTERGROWTH standards, if maternal risk factors, including infections, nutrient deficiencies and inflammation (MINDI), were associated with SFH using centile classifications in a cross-sectional cohort of pregnant indigenous Ngäbe-Buglé in rural Panama.

Methods

Context, Recruitment and Ethics

Pregnant women from the Ngäbe-Buglé indigenous community in Panama live in extreme poverty , with deficiencies of vitamins A, D and B_{12} being reported in >40% of women [24], they have a high prevalence (>50%) of several vaginal and intestinal infections as well as oral, skin and urinary tract infections, [24]. Only clinical assessment was possible as ultrasound was not available and several local health centers even lacked electricity and a water supply. Malaria is non-endemic, and HIV and gestational diabetes were ruled out in all participants. The majority of pregnant women had home deliveries assisted by traditional midwives.

As part of their normal pregnancy follow-up (August - December 2010), indigenous pregnant women living within 2 hours walking-distance from a community health center were invited to participate. For this cross sectional study, we determined that 92 pregnant women were required to detect SGA with a level of confidence of 95%, based on a prevalence of 6.7% for low birth weight reported by the Panamanian Ministry of Health [25]. To this end, we were able to recruit 213 pregnant women, of whom 174 had ≥ 16 wks of gestation, the minimal gestational

age for SFH to be compared with international INTERGROWTH standards [23], were included in the study. Ethics approval was obtained from the Gorgas Memorial Institute in Panama and from McGill University.

Questionnaire and Physical examination

Participants answered questions about reproductive history, intake of iron (60 mg tablets) or multiple micronutrient (MMN) supplement (tbsp/d) and wood smoke exposure (h/d). Anthropometry was measured, BMI was calculated and corrected for fetal weight, and women were classified as underweight, normal or overweight using Pan American Health Organization standards for gestational age (GA) [26].

Maternal blood pressure (Omron HEM-790IT® automatic BP monitor) was measured in a sitting position and re-measured if elevated systolic (SBP≥140) or diastolic (DBP≥90) blood pressure was found. Mean arterial pressure (MAP) was calculated as DBP + 1/3 (SBP-DBP) [27]. Pulse pressure was calculated as the difference between SDP and DBP [28].

Symphysis-fundal height (SFH) was measured while the mother was in a supine position (after emptying her bladder) using a non-elastic tape from the pubic symphysis to uterine fundus. Initially, the tape was placed at the upper border of the symphysis pubis bone, and straightened over the uterus until reaching the fundus; measurements were recorded to the nearest complete half centimeter. GA was calculated using the days of amenorrhea according to last menstrual period. The INTERGROWTH standards for SFH were used to calculate centiles for GA. Fetal size was classified as very small for gestational age fetuses (VSGA) if SFH <3rd centile, small for GA (SGA) if SFH <10th centile, adequate for GA (AGA) if SFH was ≥10th to ≤90th centiles, and large for GA (LGA) if SFH >90th centile [23].

Infections

Maternal infections were evaluated both clinically and using laboratory measurements as previously described [24]. Briefly, oral caries and skin lesions compatible with scabies were

detected during the clinical exam (yes/no). Urinary infection was assessed using microscopic analysis of centrifuged urine. Gram of vaginal smears was used to identify trichomoniasis and diplococcal infection, as well as *Lactobacillus, Bacteroides/Gardnerella* and *Mobiluncus* required to diagnose bacterial vaginosis using a Nugent score ≥7 calculated as *Bacteroides/Gardnerella* score + (4 – *Lactobacillus* score) + (*Mobiluncus* score/2) [29]. Vaginal yeast was detected by direct vaginal smears. Presence of intestinal nematodes (*Ascaris*, hookworm and *Trichuris*) was identified using direct microscopic fecal examination, Kato-Katz and Flotac® from the 101 women who provided stool samples, as previously described [24].

Laboratory analyses

For the evaluation of hematological status and inflammation, a complete red (RBC) and white blood cell (WBC) counts (BC-5500 Mindray Auto Hematology Analyzer) were performed. Anemia was defined as hemoglobin <110 g/L [30]. Eosinophilia was defined as eosinophils count >0.6 $x10^3/mm^3$ [31].

Serum was analyzed for C-reactive protein (CRP), using solid phase ELISA (MP Biomedicals, Orangeburg, NY), with a minimum detectable concentration of 0.95 nmol/L, for hepcidin (Intrinsic Hepcidin IDx™ ELISA Kit, Intrinsic LifeSciences), and for cytokines IL-1β, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, INF-γ and tumor necrosis factor (TNF)-α, that were quantified via Luminex using a Human Cytokine/Chemokine Magnetic Bead Panel (Millipore Corporation Canada) .

For evaluating maternal nutritional status, serum samples were processed for 1) folic acid and vitamin B_{12} concentrations using immunoelectro-chemiluminescence (MODULAR E170, Roche Diagnostics GmBH, Mannheim, Germany); 2) 25-OH vitamin D using the LIAISON, DiaSorin direct competitive chemiluminescence immunoassay; 3) vitamin A using HPLC (Gundersen *et al.*, 1997); 4) retinol binding protein (RBP) using Human RBP4 ELISA (MP
Biomedical) with a standard curve range between 0.14 – 100 ng/mL; 5) serum iron (spectrophotometry, FERENE®-ENDPOINT), ferritin (ELISA, MP Biomedicals) and serum transferrin receptor (sTfR) (RAMCO immunoassay).

Folic acid deficiency was defined as <10 nmol/L and vitamin B_{12} deficiency as <150 pmol/L [32]. We used a cutoff for vitamin D deficiency of <50 nmol/L [33]. The cutoff for low vitamin A was set at <1.05 µmol/L [34]. Low protein status was defined as RBP <30 mg/L [35]. Cut-offs for iron deficiency were set as serum iron <8.9 µmol/L, ferritin <15 µg/L [30] and high sTfR >8.3 mg/L (laboratory kit RAMCO®).

Statistical analysis

All statistical analyses were performed using STATA 14 (StataCorp, TX, USA). Differences in maternal characteristics, nutritional, inflammation and infection status were assessed among women having VSGA, SGA, AGA or LGA. One-way ANOVA or Kruskal-Wallis analyses were used depending on the nature of the variable. Frequency comparisons of binary variables were made among groups using Chi^2 or Fisher's exact tests.

Multiple logistic regression controlling for exposure to wood smoke and maternal weight by height classification (under- normal- overweight category), were ran to explore determinants of SFH below the 10th centile, between the 3rd and 10th centiles and below the 3rd centile. Stepwise regression models of outcome variables with the following clusters of independent variables were explored: 1) maternal/environmental characteristics (age, parity, weight by height category, exposure to wood smoke and to field work); 2) diet/supplementation variables (intake of animal-source foods and fruits/vegetables per week, intake of iron and MMN supplements); 3) RBC indices and anemia, 4) inflammation indicators (WBC count and differential, neutrophil-lymphocyte ratio (NLR), CRP and cytokines (IL-1β, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-α, INF-γ)); 5), serum concentration of nutrients (folic acid, vitamin B_{12}, A and D, protein and iron status) or their deficiencies; 6) infections with prevalence ≥10%: presence of caries and scabies, microscopically scored urinary bacteria, semi-quantitative scores (0 – 4) for vaginal *Lactobacillus, Bacteroides/Gardnerella* and *Mobiluncus*/bacterial vaginosis, and similarly assigned semi-quantitative scores of vaginal trichomoniasis, diplococcal infection and vaginal yeast. and 7) final models were challenged with presence and parasitic loads of intestinal

nematodes *Ascaris*, hookworm and *Trichuris* in a smaller sample size, while adjusting for number of eosinophils and basophils. Spearman's correlations were run among independent variables, and significantly correlated variables were avoided in the same regression model.

Variables with P < 0.15 within each cluster were combined in final stepwise backwards logistic regression models; CRP was included in the model when entering variables were biomarkers known to be influenced by inflammation (serum ferritin, hepcidin, RBP). Given that only 101 women were able to provide stool samples, missing data were not imputed but a complete-case analysis was performed [36] and to validate models for the randomness of missing data, Little's chi-squared test was used when intestinal parasites were included in the models [37]. Linearity of entering variables were tested using Box-Tidwell models [38]. When non-linear associations were detected, affected independent variables were transformed into their respective ordinal variables with three categories: $<25^{th}$, $\geq 25^{th} - \leq 75^{th}$ and $>75^{th}$ quantiles. Final sample sizes (n=170) allowed the inclusion of 25 independent variables for obtaining models with a power of 0.80 and a medium effect size, but those with n=108 (nematode models) allowed the inclusion of a maximum 7 independent variables [39]. Significance was set at $P<0.05$ and model pseudo-R^2s, indicating a proportion in terms of the log likelihood [38] are reported. Absence of collinearity and instability of regression coefficients were evaluated using a variance inflation factor (VIF) <10 and a condition number <30 respectively. Hosmer and Lemeshow's goodness-of-fit tests were applied to the logistic regression models [38]. Additional logistic regression models including interaction terms were used to test whether the impact of one outcome variable was modified by another variable [40].

Results

Maternal characteristics: Maternal characteristics are described in Table 1. Most women attending clinics as part of their routine pregnancy care, had a normal weights, but 92% were exposed to wood smoke used for cooking. Moreover, SBP and DBP were within normal ranges, but we observed hypotension in 24% of the population, and MAP was elevated in 12.6%. Following Ministry of Health recommendations, 75% took iron supplements and 50% took

MMN, however 38% had anemia. Infections were highly prevalent; 50% had 2-3 co-occurring infections, including bacterial, protozoal, fungal and nematode infections. In general, WBC indices were within normal limits, but we found eosinophilia in 15%. Protein deficiency (29%) as well as multiple micronutrient deficiencies (vitamin B_{12}, iron, D, A and folic acid) were common.

Comparisons among fetal size classification according to INTERGROWTH standards

The prevalence of SFH <3rd centile was 37.9%, with another 12.6% between the 3rd and the 10th centiles; 40.2% women had normal SFH for GA and 9.2% had fetuses with SFH>90th centile (Table 2). This classification was used to compare differences in maternal characteristics by fetal size. Women with fetuses between the 3rd – 10th centiles were characterized by having higher cytokine concentrations (IL4, IL6, IL12 IL17 and TNFα) compared to those with SFH<3rd centile (Table 2). Nutritional status indicators showed that women with SFH below the 3rd centile had lower BMI compared with mothers with AGA fetuses, whereas the prevalence of RBP<30 mg/L, indicative of low protein status, was more frequent in mothers with VSGA. Interestingly, hepcidin concentrations were higher in the VSGA group compared with AGA but not with SGA mothers. There were no differences between AGA and LGA mothers, except for IL4, which was higher in LGA mothers. Of note, no significant differences were found across SFH sizes by iron/MMN supplementation, infectious status, anemia or RBC parameters, iron status or cellular immune response (WBC or differential).

Multiple logistic regression models for SFH

Despite the high prevalence of positive cases for SFH<10th centile (50.9%), only higher hepcidin (Table 3A) was associated with increased likelihood of SFH< 10th centile. In the multiple logistic regression model for the subgroup of women between the 3rd and the 10th centile (Table 3B) hepcidin did not enter, but higher serum iron concentrations were associated with increased likelihood of SFH between the 3rd and the 10th centile. Moreover, inflammation indicators including lymphocytes and IL17 were associated with increased odds of SGA, whereas the anti-inflammatory cytokine IL10 was associated with lower odds of SGA.

For the 37.9% of the population with SFH <3rd centile, the logistic regression model showed that protein deficiency, higher hepcidin, and also higher vitamin B_{12} were associated with increased odds of VSGA, whereas mothers with higher concentrations of TNFα and with higher pulse pressure had decreased odds of VSGA fetuses (Table 4A). Interestingly, for the subset of women with measures of intestinal nematodes, the presence of *Trichuris* increased the odds of VSGA as did hepcidin, but higher eosinophil counts were associated with lower odds of SFH <3rd centile (Table 4B).

Given the apparent contradictory association between higher B_{12} concentrations with increased odds of VSGA, further analyses based on literature evidence for possible elevated B_{12} due to hypoproteinemia or eosinophilia, we ran logistic regression models for VSGA and the interactions between B_{12} quantile-classification and low protein (RBP <30 mg/L) (Table 5A), and between B_{12} quantile-classification and eosinophilia (>0.6 $x10^3/mm^3$) (Table 5B). Our model showed that interactions between medium B_{12} (≥82 and ≤127 pmol/L) or high B_{12} (>127 pmol/L) with low protein, increased the odds for VSGA (95% CI: 1.05 – 9.84 and 1.47 – 58.47 respectively) (Table 5A), whereas no significant associations were found between VSGA and the interaction of high B_{12} and eosinophilia (Table 5B).

Discussion

SFH is widely used in developing countries to screen for fetal growth despite concerns about its sensitivity; however, its specificity is reportedly ≥80% in all studies [20, 41, 42]. Cochrane reviews had concluded that there was insufficient evidence to recommend the use of SFH in the detection of SGA [21, 43], but the only study included in the reviews [44] was conducted in an obstetric facility in Denmark. None of the earlier studies had considered using SFH to investigate early determinants of fetal growth in marginalized communities. Our goal had been to apply the new international INTERGROWTH standards for SFH [23] to our MINDI Cohort in order to identify determinants of poor fetal growth in a marginalized population of pregnant women experiencing multiple infections, nutrient deficiencies and inflammation. Our findings revealed that more than 50% of our study population had a SFH<10th centile and 37.9% had a

SFH< 3rd centile. These high prevalences exceed the highest world estimates (34%) found in South-Asia [3]. Beyond well-known determinants of SGA fetuses that include low maternal height, chronic malnutrition, hypo- or hypertension, smoking and HIV [7, 8] , we confirmed that higher BMI was associated with a lower likelihood of SFH< 3rd centile, only in the model including intestinal nematodes. Also, although wood smoke was associated with SGA and VSGA in exploratory models, it did not remained in final models for either SGA and VSGA despite known associations of smaller fetuses with environmental tobacco smoke [45]. Instead, in our MINDI Cohort, a more complex set of factors that included infections, nutritional deficiencies and markers of inflammation were associated with the variability of SGA and VSGA and helped to differentiate them as distinct entities.

Inflammation and SGA

The classical biomarker of inflammation in population studies, CRP, has been found elevated in cord blood of SGA compared with AGA infants [46], but did not emerge in any of our models for SGA. We had previously reported in this population that CRP was lower with the presence Th2-eliciting infections [47], indicating that CRP might not be appropriate in the screening for inflammation-related SGA in populations with multiple infections, nutrient deficiencies and inflammation. In our MINDI cohort, higher lymphocyte count and higher IL17 were associated with SGA, which have been linked to uterine hypo-perfusion. In experimental animal studies, lymphocytes have been implicated in the development of uterine hypo-perfusion induced by ischemia leading to IUGR [48]. Also, the transfer of Th17 lymphocytes from animals with reduced uterine perfusion pressure were able to decrease fetal weight in normal pregnant animals [49]. Moreover, IL17 has been reported elevated as well in human pregnancies complicated with placental insufficiency [50]. Moreover, the emergence of IL10 into this model also agree with the known protective role of IL10 in adverse pregnancy outcomes [51] and the observed lower IL10 in IUGR with placental insufficiency compared with IUGR without placental insufficiency [52]. Together, these findings point towards the presence of placental hypo-perfusion in our subsample of fetuses between the 3rd and the 10th centiles.

In contrast, in our model for VSGA, different inflammation biomarkers emerged. TNFα, which is known for its role in the physiopathology of hypertensive disorders of pregnancy [53] and which has shown to be positively associated with higher MAP in this population [54], emerged as a biomarker that was associated with decreased odds of VSGA. It is known that inflammatory markers including TNFα are higher in amniotic fluid of obese pregnant women compared with normal or lean women [55]. However, in our models, the association with TNFα was significant after controlling for BMI. Previously, low concentrations of TNFα were found in pregnancies complicated with SGA, but only in idiopathic but not pre-eclampsia- associated SGA [56]. Interestingly, in our model with intestinal nematodes, higher eosinophil count, a biomarker of Th2 immune response [57], decreased the odds of VSGA, supporting the hypothesis that an increased Th2 immune response elicited by nematodes [58] would dampen the effect of Th1 response that leads to pregnancy complications including IUGR [59]. In contrast, the only population study exploring the association of eosinophils with SGA found that a higher eosinophil percentage associated with increased odds of SGA in Chinese women [60]. However, it is known that the precursor of the most abundant protein in eosinophil granules, eosinophil major basic protein (proMBP), is decreased in SGA, and is used as biomarker of adverse pregnancy outcomes in early pregnancy [61]. Although the contribution of parasitic-related increase of eosinophils during pregnancy to proMBP has not been reported, our study suggests that higher eosinophils count might be protective for SGA in MINDI contexts.

Trichuris infection increased the odds of VSGA

Among the many infections present in our population, no infections were directly associated with SFH <10th centile. In contrast, the presence of *Trichuris*, despite its low prevalence (13.8%), was significantly associated with increased odds of VSGA. Other studies have reported high prevalence of *Trichuris* during pregnancy [62], while others have reported the association of *Trichuris* with anemia during pregnancy [63, 64], but to our knowledge, ours is the first report of a direct link of *Trichuris* with VSGA. Moreover, in our study this association persisted even after adjusting for hemoglobin concentrations and the presence of anemia. It has been experimentally demonstrated that chronic *Trichuris* infections elicit a persistent Th1 immune

response that stimulates bone marrow cell production via INFγ [65]. Therefore, chronic inflammation could explain the association of *Trichuris* infection with VSGA fetuses.

Iron and SGA

In our population characterized by high prevalence of anemia, and where most women were receiving iron supplements, higher serum iron increased the odds of SGA . The role of iron deficiency on SGA is controversial [19]. Higher maternal hemoglobin, hematocrit and/or ferritin have been associated with SGA [66] or IUGR [67], and associations between iron and SGA vary depending on iron status indicators used, and the confounding contribution of failure in plasma volume expansion and infection/inflammation on decreased fetal growth [19]. Besides, hepcidin was associated with both SGA and VSGA, indicating the presence of iron restriction due to inflammation. Hepcidin is a protein synthetized in the liver in response to inflammation, it inhibits intestinal iron absorption and the release of iron from reticuloendothelial system cells [68]. Hepcidin is suppressed during normal pregnancy, given the increase of iron requirements to supply augmented erythropoiesis and fetal growth [69]. However, studies looking for associations of hepcidin with pregnancy outcomes are just emerging. Hepcidin was shown to be a better indicator of iron deficiency than hemoglobin in African pregnant women [70]. Higher hepcidin concentrations in early pregnancy were found in women who developed preeclampsia compared to normal pregnancies [71]. To our knowledge, ours is the first report of significantly higher hepcidin associated with SGA. Collectively these findings show that higher iron status and higher iron restriction due to inflammation increased the odds of SGA and VSGA respectively, and suggest that iron supplementation in this population with a high prevalence of infections and inflammation may be counterproductive for fetal growth. Moreover, the use of hepcidin as indicator of iron restriction due to inflammation may be helpful identifying women in whom iron is contra-indicated due to inflammation, as already evidenced in a population of anemic African pregnant women [72].

Nutritional status and SGA

It was very interesting to find hidden, non-symptomatic protein deficiency in the population as defined by low RBP, which has previously been considered to be a very sensitive indicator of protein status even before clinical signs of malnutrition appear [73], and to observe that the lower RBP concentrations were evident among mothers with VSGA fetuses. Despite the high prevalence of malnutrition in developing countries, few studies have directly linked protein malnutrition and SGA [74]. Famine studies have revealed that exposed women delivered babies with lower birthweights, and that this outcome was more evident in mothers during the third trimester [75]. We were able to identify RBP <30 mg/L in nearly 29% of our mothers, that increased the odds of SFH<3rd centile. We had also reported that low protein status was associated with low pulse pressure [54], indicating that besides the known nutritional adverse effects of maternal low protein status on fetal growth, the association between VSGA with low protein may be related to hypoproteinemia contributing to low blood-volume and hypoperfusion.

Pregnant women in our study showed low concentrations of vitamin B_{12}, which was not surprising given that B_{12} is mainly available from animal-source foods [76], which were scarce for our population. However, women in the highest quartile of B_{12} concentrations (127 – 376 pmol/L) had higher odds of SFH<3rd centile. We found two possible explanations for this association. First, high B_{12} concentrations have also been observed in presence of eosinophilia given that eosinophils can produce B_{12} binding proteins [77]. In our population, eosinophilia (eosinophils >0.6 x10^3/mm^3 in 17.4%), was frequent, and B_{12} was positively correlated with the number of eosinophils (r_s =0.13, P= 0.05). On the other hand, higher eosinophil count reduced the odds of VSGA, while vitamin B_{12} was no longer significant after including intestinal nematodes in the model. Therefore, we considered that the association found between B_{12} and VSGA was probably due to eosinophilia. Second, one study has reported low protein status but high serum concentrations of vitamin B_{12} in Indian pregnant women [78], and they also reported a similar relationship in protein-malnourished children with fatty liver infiltration, hypoproteinemia and high B_{12} concentrations [79]. More recent studies using m B_{12} radioassay

techniques have confirmed increased B_{12} concentrations in severe protein malnutrition in children that declined after nutrition therapy [80]. Therefore, protein malnutrition would be a possible explanation for the association between higher B_{12} and VSGA. When testing our two possible explanations for the association between VSGA and higher B_{12} concentrations in logistic regression models for VSGA and interactions between B_{12} categories and low RBP and eosinophilia, only interactions between higher B_{12} and low protein emerged as significantly associated with increased odds of VSGA, highlighting the subclinical impact of low protein status on maternal health and fetal growth in our population.

Pulse pressure and SGA

It is known that elevated blood pressure is an important risk factor for SGA [81, 82]. However, elevated MAP was not associated with any of the classifications of small SFH our study population. On the other hand, decreased pulse pressure, an indicator of peripheral perfusion [28], increased the odds of SHF<3rd centile. The effect of low blood pressure on pregnancy outcomes [83], particularly with stillbirth [84], has been described, and a recent Latin-American study found signs of placental hypoperfusion in SGA [85], but low blood pressure in pregnancy is a neglected subject in current literature. Interestingly, the association between low blood pressure during pregnancy and poor perinatal outcomes has been reported in "younger, shorter, lighter, leaner, poorer, and more often a minority" women in a study from the US [8]. Importantly, we have reported that TNFα increased MAP but decreases the odds of hypotension, and was associated with higher pulse pressure in this population [54]. Since TNFα decreased the odds of VSGA and low pulse pressure increased the odds of SFH < 3rd centile, our findings suggest that a compensatory increase in TNFα is occurring to improve placental perfusion, while increasing maternal risk for HDPs. Our results may indicate that pulse pressure could be used together with low SFH in remote areas for the detection of pregnancies at risk for SGA.

Conclusion

This study shows for the first time determinants of low SFH in the context of multiple infections, nutrient deficiencies and inflammation, in a vulnerable population of pregnant indigenous women. Our regression models helped to distinguish between SGA, which was associated with inflammation indicators, and VSGA which was associated with iron restriction due to inflammation, protein deficiency and low pulse pressure. Public health interventions leading to dietary improvements by providing adequate intakes of protein and addressing the complexity of the inflammatory response may be required to improve maternal and fetal health, then reducing the prevalence of SGA in marginalized and remote settings.

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Tables

Table 1 Maternal characteristics of indigenous Panamanian pregnant women

Maternal characteristics	Median (min – max), Mean ± SD, or %	Maternal characteristics	Median (min – max), Mean ± SD, or %
General characteristics		**Complete blood cell count**	
Age	24 (13 – 45)	RBC $x10^6$	3.68 ± 0.31
Parity	3 (1 – 12)	Hemoglobin, g/L	112 (57 – 141)
Weight by height category		Anemia	37.9
Underweight	12.1	Hematocrit, %	34.9 (23 – 44)
Normal weight	63.8	MCV, fL	94.6 (73.4 – 112.3)
Overweight	24.1	MCH, pg	30.3 (19.1 – 37.3)
Blood pressure measurements		MCHC, g/L	319 (248 – 356)
Systolic blood pressure	102.7 ±10.4	RDW-CV, %	13.4 (12.1 – 17.6)
Diastolic blood pressure	61.5 ± 8.6	RDW-SD, fL	45.9 (39.3 – 61.1)
Mean arterial pressure (MAP)	75.2 ± 8.4	Total WBC x103	8.5 (3.4 – 14.0)
Elevated MAP	12.6	Neutrophils	5.9 (1.8 – 10.8)
Blood pressure <100/60 mmHg	24.1	Lymphocytes	1.9 (0.9 – 3.8)
Pulse pressure	41.2 ± 8.4	Monocytes	0.4 (0.1 – 1.4)
Supplementation		Eosinophils	0.3 (0.02 – 2.47)
Taking iron supplements	76.5	Eosinophils >0.6 $x10^3/mm^3$	14.9
Taking MMN supplements	50.7	Basophils	0.03 (0 – 0.08)
Infections		Platelets $x10^3$	255.5 (73 – 445)
Caries	19.5		
Scabies	19.0	**Nutritional indicators**	
UTI (n= 169)	25.4	Ferritin, µg/L	9.9 (1.0 – 115.8)
Vaginal microorganisms		Ferritin <20 µg/L	73.6
Lactobacillus (n= 173)	53.7	sTfR, mg/L	5.3 (0.7 – 25.7)
Bacteroides/Gardnerella (n= 173)	93.1	sTfR >8.3 mg/L	18.4
Mobiluncus (n= 173)	84.4	Serum iron, µmol/L	8.4 (0.5 – 54.8)
Trichomonas (n= 173)	76.3	Serum iron <8.9 µmol/L	53.4
Yeast	23.5	Folic acid, nmol/L	13.3 (6.3 – 45.4)
Diplococcus (n= 173)	21.4	Folic acid <10 nmol/L 54	26.4
Intestinal nematodes		Vitamin D, nmol/L	44.9 ± 15.6
Ascaris (n=101)	32.7	Vitamin D <50 nmol/L	62.6
Hookworm (n= 101)	59.4	Vitamin A, µmol/L (n= 172)	1.1 (0.4 – 2.9)
Trichuris (n= 101)	13.9	Vitamin A <1.05 µmol/L (n= 172)	41.9

Abbreviations: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MMN, multiple micronutrients; RBC, red blood cells; RDW-CV, red cell distribution width-coefficient of variation; RDW-SD, red cell distribution width-standard deviation; sTfR, serum transferrin receptors; UTI, urinary tract infection; WBC, white blood cells

Table 2 Comparison of maternal characteristics by symphysis-fundal height (SFH) classification

SFH classification – per category	<3rd VSGA	≥3rd - <10th SGA	≥10th -≤90th AGA	>90th LGA	P
n = 174	**66**	**22**	**70**	**16**	
BMI corrected for fetal weight	23.9 ± 3.5[b]	25.0 ± 3.0[ab]	26.1 ± 3.9[a]	24.8 ± 4.0[ab]	0.006
Gestational age	33 (16.3-40.8)[a]	35.1 (16.7-42)[a]	30.2 (16.7-40.7)[ab]	24.7 (17.6-36.7)[b]	0.007
Wood smoke exposure	35.6%	12.1%	37.9%	6.9%	0.048
Inflammation indicators					
CRP (mg/L)	3.6 (0.1-19.4)	2.7 (0.5-21.5)	3.5 (0.4-27.2)	4.4 (0.1-17.4)	0.790
Cytokines (pg/L) (n=173) Median (min-max)	66	22	69	16	
IL1β	1.1 (0.07-19)	5.8 (0.07-32.5)	2.3 (0.07-95.7)	1.3 (0.07-18.9)	0.095
IL4	3.2 (0-79.5)[b]	11.6 (0.03-134.2)[a]	3.5 (0.03-76.0)[b]	17.6 (0.2-125.3)[a]	0.045
IL6	1.6 (0.2-72.7)[b]	6.5 (0.4-58.2)[a]	2.5 (0.2-42.8)[b]	7.3 (0.4-48.9)[ab]	0.025
IL10	1.6 (0-24.9)	2.3 (0-45.7)	1.6 (0-24.2)	0.07 (0-21.1)	0.616
IL12	0.7 (0-90.1)[b]	19.4 (0-203.4)[a]	1.7 (0-332.9)[ab]	3.5 (0.02-54.8)[ab]	0.018
IL13	1.6 (0.03-23.7)	4.6 (0.06-50.2)	1.6 (0.06-25.8)	1.6 (0.08-15.7)	0.499
IL17	1.6 (0.02-24.7)[b]	9.5 (0.02-66.6)[a]	1.6 (0.01-37.8)[b]	1.2 (0.02-18.3)[b]	0.024
INFγ	1.7 (0.05-73.9)	9.3 (0.05-44.1)	6.4 (0.05-47.9)	3.2 (0.05-34.2)	0.127
TNFα	2.1 (0-22.6)[b]	11.5 (0-36.9)[a]	7.8 (0-27.8)[ab]	7.6 (0-29.6)[ab]	0.010
Hepcidin, µg/L	8.9 (1.9-37.3)[a]	6.7 (0.3-27.9)[ab]	6.7 (0.8-67.4)[b]	7.1 (2.2-11.3)[ab]	0.012
Nutrients					
Vitamin B_{12}, pmol/L	106 (53-376)[a]	91.5 (62-191)[ab]	93.5 (54-241)[b]	90 (57-176)[ab]	0.042
Vitamin B_{12} <150 pmol/L	58 (e59)	20 (e20)	63 (e62)	14 (e14)	0.965
RBP, mg/L (n= 173)	34.9 (9.7-412.2)[b]	56.4 (22.3-327.1)[ab]	43.8 (8.6-302.4)[ab]	88.4 (13.0-288.8)[a]	0.017
RBP <30 mg/L (n= 173)	14.4%	2.3%	11.6%	0.6%	0.048

Abbreviations: AGA, adequate for gestational age; BMI, body mass index; CRP, C-reactive protein; LGA, large for gestational age; RBP, retinol-binding protein; SGA, small for gestational age; VSGA, very small for gestational age

Table 3 Multiple logistic regression models for symphysis-fundal height (SFH) <10th centile

A. SFH <10th centile[1]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Weight by height classification[3]	0.58 ± 0.16	0.054	0.34, 1.01	n= 172 P= 0.0006 Pseudo R2= 0.073 VIF= 1.01 Condition number= 11.68 Goodness of fit test: P= 0.560
Months on iron	1.12 ± 0.08	0.118	0.97, 1.28	
Hepcidin, µg/L (category)[4]	2.08 ± 0.51	0.003	1.29, 3.36	
Constant	0.61 ± 0.47	0.529	1.31, 2.83	
B. SFH ≥3rd and <10th centiles[2]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Weight by height classification[3]	0.74 ± 0.35	0.529	0.29, 1.88	n= 173 P <0.0001 Pseudo R^2= 0.239 VIF= 1.17 Condition number= 21.37 Goodness-of-fit test, P= 0.487
Age classification (<19, 19-30, >30 yr)	0.39 ± 0.16	0.025	0.17, 0.89	
IL10 pg/mL (category)[4]	0.27 ± 0.15	0.023	0.09, 0.83	
Serum iron, µmol/L	1.08 ± 0.03	0.016	1.01, 1.15	
Lymphocytes, number	4.05 ± 2.29	0.013	1.34, 12.27	
IL17 pg/mL	1.12 ± 0.03	0.001	1.05, 1.19	
Constant	0.21 ± 0.40	0.415	0.005, 9.15	

[1] Variables that were included but had P> 0.15: vitamn D<50 nmol/L, presence of *Lactobacillus*, TNFα, IL17, presence of *Bacteroides/Gardnerella*, vitamin B_{12} (pmol/L), CRP (mg/L)
[2] Variables that were included but had P> 0.15: IL13, vitamin D (nmol/L), fieldwork exposure, NLR, months on iron supplements
[3] Weight by height classification according to PAHO reference tables for under-weight, normal and overweight pregnant women [26].
[4] Ordinal variable with three categories: <25th, ≥25th – ≤75th and >75th centiles
SFH, symphysis-fundal height

Table 4 Multiple logistic regression for symphysis-fundal height (SFH) <3rd centile

A. SFH <3rd centile, model without intestinal nematodes[1]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Weight by height classification[3]	0.79 ± 0.24	0.452	0.43, 1.45	n= 172 P <0.0001 Pseudo R^2= 0.160 VIF= 1.03 Condition number= 20.65 Goodness-of-fit test: P= 0.372
Pulse pressure, mmHg	0.94 ± 0.02	0.014	0.40, 0.99	
Hepcidin, µg/L (category) [4]	2.02 ± 0.53	0.008	1.20, 3.39	
TNFα, pg/mL	0.93 ± 0.02	0.006	0.89, 0.98	
RBP, mg/L (category) [4]	0.59 ± 0.15	0.039	0.36, 0.97	
Vitamin B_{12}, pmol/L (category) [4]	1.96 ± 0.52	0.012	1.16, 3.31	
Constant	3.02 ± 3.85	0.387	0.25, 36.8	
B. SFH <3rd centile, model with intestinal nematodes[2]	**OR ± SE**	**P**	**95% CI**	**Overall model**
Weight by height classification	0.43 ± 0.17	0.037	0.19, 0.95	n= 100 P= 0.0006 Pseudo R^2= 0.162 VIF= 1.04 Condition number= 18.38 Goodness-of-fit test: P= 0.489 MCAR test: 0.575
Eosinophils, number	0.09 ± 0.11	0.044	0.008, 0.94	
Hepcidin, µg/L (category)	2.02 ± 0.68	0.037	1.04, 3.89	
Bacteroides/Gardnerella, presence	0.11 ± 0.14	0.084	0.01, 1.34	
Trichuris, presence	4.86 ± 3.43	0.025	1.22, 19.38	
Constant	14.13 ± 24.52	0.127	0.47, 424.09	

[1] Variables that were included but had P> 0.15: number of lymphocytes, presence of *Lactobacillus* and *Bacteroides/Gardnerella*, CRP (mg/L), vitamin D (category)

[2] Variables that were included but had P> 0.15: TNFα, B_{12} category, pulse pressure, basophils number, lymphocyte number, vitamin D category, presence of *Lactobacillus*, RBP category, presence of *Ascaris*, CRP (mg/L), presence of hookworm

[3] Weight by height classification according to PAHO reference tables for under-weight, normal and overweight pregnant women [26].

[4] Ordinal variable with three categories: <25th, ≥25th – ≤75th and >75th centiles

SFH, symphysis-fundal height

Table 5 Logistic regression models for symphysis-fundal height (SFH) <3rd centile and interactions A. between B_{12} classification and low protein status (RBP <30 mg/L) and B. between B_{12} classification and eosinophilia (eosinophils count >0.6 x10^3/mm^3)

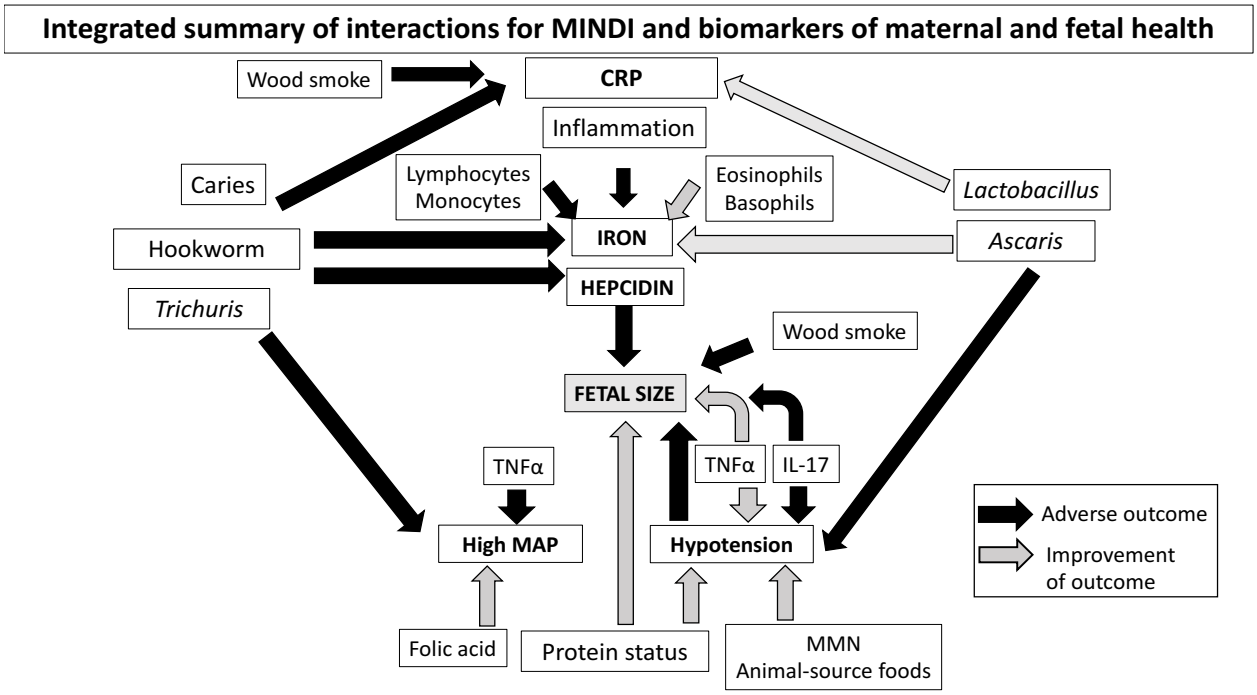
SFH <3rd centile and term interactions					
A. B_{12} (pmol/L) [1]	**RBP (mg/L)**	**OR ± SE**	**P**	**95% CI**	**Overall model**
<82	<30	1.86 ± 1.29	0.372	0.48, 7.23	n= 173 P= 0.0389 Pseudo R^2= 0.039 VIF= 1.01 Condition number= 6.58
≥82, ≤127	≥30	1.90 ± 0.96	0.201	0.71, 5.10	
≥82, ≤127	<30	4.28 ± 2.44	**0.011**	1.40, 13.10	
>127	≥30	3.22 ± 1.83	**0.040**	1.05, 9.84	
>127	<30	9.28 ± 8.71	**0.018**	1.47, 58.47	
	Constant	0.27 ± 0.11	0.002	0.12, 0.62	
B. B_{12} (pmol/L) [1]	**Eosinophils (nx10^3/mm^3)**	**OR ± SE**	**P**	**95% CI**	**Overall model**
<82	>0.6	0.75 ± 0.88	0.806	0.07, 7.44	n= 174 P= 0.132 Pseudo R^2= 0.037 VIF= 1.01 Condition number= 6.31
≥82, ≤127	≤0.6	1.98 ± 0.83	0.103	0.87, 4.49	
≥82, ≤127	>0.6	2.14 ± 1.46	0.263	0.56, 8.14	
>127	≤0.6	4.09 ± 2.16	0.008	1.45, 11.5	
>127	>0.6	1.5 ± 1.18	0.607	0.32, 7.03	
	Constant	0.33 ± 0.11	0.002	0.17, 0.66	

[1]Vitamin B_{12} categories based on <25^{th}, ≥25^{th} – ≤75^{th}, and >75^{th} centiles: <82, ≥82 and ≤ 127, >127 pmol/L
RBP, retinol-binding protein

General discussion

Study findings from our MINDI Cohort highlight the complexity of nutrition-infection interactions in remote marginalized communities and the need to better understand the conditions that affect biomarkers of health status used in contexts where multiple infections, nutrient deficiencies and inflammation coexist. Our results showed a range of unexpected associations between MINDI and maternal and fetal biomarkers that have implications for assessing maternal and fetal health status:

Figure B Final conceptual framework containing summary of main results



We observed that CRP was indeed, elevated in our population with multiple infections, but given that 95% of the women had two or more infections, we expected a higher prevalence of inflammation than the 20.8% found. It is possible that inflammation had not a higher prevalence because of to the chronic nature of infections leading to a low-grade inflammation, which was not captured by the conservative cut-offs used to define inflammation in pregnancy [146]. However, our multiple linear regression showed that CRP was higher with stressors such

as wood smoke, and with oral, vaginal and intestinal infections that are associated with pro-inflammatory Th1 cytokines (caries, *Diplococcus* and hookworm), but it was lower in presence of infections that elicit a Th2 response such as *Ascaris* [147] and bacteria that are part of normal (*Lactobacillus*) or pathological (*Gardnerella vaginalis*) vaginal microflora [148]. That bidirectional associations of CRP were both positive or negative depending on the type of infection require important considerations in both a clinical context and in population studies when evaluating the response to treatments/interventions that include the use of antimicrobial or anti-parasitic medications that could favor a stronger pro-inflammatory or anti-inflammatory response and the dominance of one pathogen over another in the MINDI context.

Importantly, this pattern of positive and negative associations of MINDI in pregnant women was repeatedly observed with other biomarkers. A Th1 type of immune response was associated with lower iron status and with higher blood pressure measurements leading to risk of HDPs, higher IL17 was associated with lower erythropoiesis indicated by sTfR and with lower pulse pressure, whereas a Th2 type of immune response was associated with higher iron status and better peripheral perfusion indicated by pulse pressure. In particular, divergent direction of associations between outcome variables with intestinal nematodes, *Ascaris* and hookworm, was consistent across studies. We propose that these parasite-specific responses may be due to their different modes of transmission, stage of life cycle inside the host and specially, the way adult parasites feed from the host; *Ascaris* is less-invasive and is able to stay in human intestinal lumen braced against the intestinal wall helped by its neuro-muscular system [149, 150], whereas hookworm is capable of penetrating the intestinal mucosa producing bleeding and considerable mucosal damage [151]. More recent research has also demonstrated that *Ascaris* is able to down-regulate transcriptional pro-inflammatory, Th1 and antigen processing pathways [152], whereas hookworm excretory/secretory proteins affect host cellular and humoral immune responses, inducing a non-healing Th-1 immune response mediated by IL12 [153]. These findings clearly indicate that studies looking at the influence of infections on biomarkers of health, controlling only for the “presence of infections” or “presence of intestinal

nematodes" is not sufficient, given the great variability in the immune response induced by different types of infections.

The bidirectional associations of infections and inflammation are of particular relevance in the study of iron status of populations, in which our MINDI studies provide new insights for the scientific community.

(1) *First*, serum iron has been largely excluded from clinical and population-research practices as an iron indicator on the basis of its negative association with inflammation, whereas ferritin has been promoted despite its positive association with inflammation [154]. In an effort to control ferritin concentrations for inflammation, CRP in combination with another acute-phase protein, α-1-acid-glycoprotein (AGP), and for the presence of malaria, a regression-correction approach, using the following formula:

$$(\text{Ferritin}_{\text{adjusted}} = \text{ferritin}_{\text{unadjusted}} - \beta_1 (\text{CRP}_{\text{obs}} - \text{CRP}_{\text{ref}}) - \beta_2 (\text{AGP}_{\text{obs}} - \text{AGP}_{\text{ref}}) - \beta_3 \text{malaria}$$

[155] has been proposed. However, limitations of this approach in our population included the observations that CRP was not associated with ferritin, that we did not measured AGP, and that not malaria but other multiple infections were present, some of which lowered CRP, in particular *Ascaris* and vaginal bacteria. Moreover, ferritin was strongly associated with higher hepcidin, which in turn was associated with CRP and hookworm infection. Therefore higher ferritin could be indicating either good iron status or iron restriction due to inflammation. By using the regression-correction approach a 2- to 3-fold increase in the estimated prevalence of iron deficiency could result, which is not desirable since it would lead to supplementing women in whom iron should be withheld given the presence of inflammation.

Using the combination of ferritin (positive acute-phase reagent) and serum iron (negative acute-phase reagent) as biomarkers of iron status, a prevalence of 78% iron deficiency was detected in our population, and both biomarkers showed associations in the same directions: serum iron and ferritin were positively associated with a higher Th2 response (*Ascaris* and higher eosinophil count), whereas lower iron status was associated with Th1 inflammation-

related indicators (hookworm and higher monocyte count). These findings together show that by measuring serum iron, the parallel rise of ferritin with hepcidin and CRP could be balanced, providing an alternative way to adjust for inflammation in populations with MINDI.

(2) Second, measuring hepcidin helped to better understand the impact of inflammation on biomarkers of iron status. Positive associations of hepcidin with higher CRP and ferritin but not serum iron, indicate that hepcidin would be an interesting tool for measuring inflammation in MINDI populations. High concentrations of hepcidin alerted for the presence of iron restriction due to inflammation in at least 70% of the population, which has important implications regarding the safety of systematic provision of iron supplements to all pregnant women and may help to explain the lack of response to iron supplementation in pregnant women in our study. Moreover, positive associations of more severe UTI with higher ferritin and lower sTfR provide evidence and may support growth of intra-cellular bacteria during iron supplementation, which has been experimentally observed for uro-pathogenic *Escherichia coli* [156]. This is clinically important given that UTI are associated with increased risk of preterm delivery, LBW [157] and preeclampsia [158] [6] in studies in developing countries, all of which would be favored by providing iron supplementation.

(3) Third, neither hemoglobin nor sTfR were good indicators of iron deficiency in our population. One of the more concerning issues, the observation of Hb being negatively associated with protein status, suggested that hemo-concentration rather than the physiologic hemodilution may be occurring in these pregnant women. This is a risk for adverse pregnancy outcomes itself, given that mild anemia and hemodilution have been associated with better placental perfusion [159]. Moreover, since hemoglobin cut-offs during pregnancy are intended to account for physiologic hemodilution, therefore, under protein-deficiency, we might be under-estimating the prevalence of anemia. These observations, together with associations of anemia with lower folic acid and vitamin A concentrations, and lower Hb being associated with higher CRP and with infection by *Trichuris*, show that Hb as biomarker is a reflection of a sum of

factors, difficult to separate from its whole. Therefore in MINDI populations, anemia and iron deficiency need to be addressed and treated as different entities.

Regarding sTfR, false negative results for iron deficiency in 72% of women who had both low serum iron and low ferritin, uncovered great limitations in its use as iron status indicator in MINDI populations. We also showed that sTfR was bidirectionally associated with cytokines (IL13, IL17), infections (caries, hookworm, UTI), and negatively associated with vitamin A and D. This is far from the traditional concept of sTfR as the biomarker of iron deficiency that is less affected by inflammation, while sTfR has been disregarded in its role as erythropoiesis indicator. Having in mind the known dampening effect of inflammation [160], and of deficiencies in protein [161], vitamin A [162] and D [163] on erythropoiesis, sTfR should be cautiously interpreted in populations with MINDI.

Our findings on anemia and iron deficiency indicate that, despite their high prevalence, iron supplementation may not be indicated in most women given the presence of iron restriction due to inflammation in the majority of the study population, and that public health policies need to be re-oriented towards comprehensively treating infections, particularly those shown to increase a Th1 immune response, before providing iron. A step further could include the screening and identification of women with iron deficiency due to inflammation using hepcidin as supported by others [164], which poses challenges in terms of technical implementation and cost-effectiveness in field settings. Our findings also show the importance of improving women's diet in order to impact on protein, folate vitamin A and vitamin D status in women with anemia, which may require a more complex approach than just providing MMN supplements.

Other indicators of maternal - fetal health status: Blood Pressure and Symphysis-Fundal Height

Indicators of maternal and fetal health showed interesting similarities and contrasts. In addition to iron status indicators, other common indicators of maternal and fetal health also showed bi-

modal associations with MINDI, and by doing so, we provide insight of their usefulness in remote settings where technology is not available. We observed that calculating MAP and PP from systolic and diastolic BP may allow clinicians to detect women at risk of HDPs and hypoperfusion, respectively. Of particular relevance, we observed that hypotension, usually overlooked in pregnancy in ambulatory settings, may be just as important as the detection of elevated blood pressure, given the association of low PP, which was in turn associated with *Ascaris* infections, with small fetuses in our MINDI cohort.

The use of SFH, although considered not an accurate indicator of fetal growth [29], provided us with a meaningful way of studying SGA while lacking sonography, when applying the new INTERGROWTH standards [28]. Whereas only the presence of *Trichuris* was associated with increased odds of VSGA, other infections did not enter our SFH models. In contrast, associations of infections with maternal CRP, iron status and BP, demonstrated that multiple mild-moderate infections could affect fetal growth via inflammation, as shown by the emergence of maternal hepcidin associated with increased odds of very small fetuses (SFH <3rd centile). Others have failed to demonstrate an association between higher hepcidin in cord blood and SGA [165], or a difference in maternal hepcidin concentrations between pregnant women with preeclampsia with normal fetuses, preeclampsia with IUGR and controls [166]. To our knowledge, the link between multiple chronic infections, inflammation, higher hepcidin and SGA has not been shown before in a MINDI setting and is novel.

It was also very interesting to observe that both nutrient and cytokines were differentially associated with maternal BP and SFH. It is known that an appropriate protein status is needed to maintain adequate volemia and perfusion [167, 168], and that placental perfusion is disturbed in both, HDPs and IUGR [169]. In our study, higher intake of animal-source foods and of MMN reduced the odds of hypotension, was also associated with higher PP and MAP (but not with elevated MAP), and both low pulse pressure and low maternal protein status were associated with lower SFH. Those findings point to a role of low protein status in hypoperfusion and impaired fetal growth.

Another interesting bi-directional observation was the association of higher TNFα with blood pressure and fetal growth. On one hand, TNFα, a cytokine known for its role of HDPs was associated with higher MAP and also with higher SFH, whereas IL17, known to be produced in response to placental hypo-perfusion, was associated with lower pulse pressure and with lower SFH. TNFα has been proposed as an one of several biomarkers (together with CRP, IL6 and IL8) for predicting HDPs, based on significant and consistent positive association with this condition [170], however, TNFα (together with IL-6 and IL-8) was found unaltered in IUGR [171], which supports our findings. In contrast, it has been experimentally demonstrated that Th17 cells from animals with reduced uterine perfusion pressure induced IUGR [172]. IL17 is known to increase in response to angiotensin II [173], and to be in part responsible for the placental oxidative stress and the vascular response to angiotensin II observed in HDPs [174]. We hypothesize that, as compensatory mechanism of placental hypoperfusion, TNFα increased blood pressure, putting women at future risk developing HDPs but at the same time contributing to improved placental perfusion and fetal growth.

Novel therapies for treatment of HDPs include the use of TNFα blockers [175], although reviews of available information have revealed that women under therapy for auto-immune diseases had smaller babies, which authors have attributed to underlying disease [176]. The use of TNFα blockers may not be recommended during the third trimester, due to possible effects on the newborn immune system [177]. However, our findings provide evidence of a direct association of TNFα with both maternal and fetal health, which may caution against the use of such type of medications in MINDI populations, where both HDPs and SGA have the highest prevalence.

As observed, traditional interpretation of maternal/fetal biomarkers may not apply in populations with MINDI, and a more holistic approach should be taken when doing research or providing health services in impoverished and vulnerable communities.

Final conclusion and summary

In summary, regarding biomarkers in MINDI populations, our study suggests that

- The presence of common mild-moderate infections such as caries, hookworm and diplococcal infection should alert clinicians to the possible presence of inflammation that can lead to adverse pregnancy outcomes, but also to consider that other infections (*Ascaris* and vaginal *Lactobacillus* and *Bacteroides/Gardnerella*) may be dampening the inflammatory response, and that by treating them, CRP could rise.
- Anemia should be seen as a different problem from iron deficiency, and needs to be addressed in agreement with the multiple etiological factors: infections leading to bleeding, infections leading to inflammation, iron deficiency but also folic acid and vitamin A deficiency. Adjustment of Hb concentrations for low protein status may need be considered.
- For the study of iron status, the combined use of ferritin and serum iron could be helpful. Ideally, hepcidin would facilitate the identification of women in whom iron supplements is contraindicated due to inflammation. In absence of this biomarker, clinicians should be attentive and actively looking for chronic/asymptomatic infections before providing iron supplements.
- A more strict pregnancy follow-up/referral may be needed for women with elevated MAP or low PP, both early indicators of possible pregnancy complications in asymptomatic pregnant women.
- SFH was able to detect an important proportion of SGA fetuses using New INTERGROWTH standards, and was able to capture important and modifiable factors associated with SGA, which helps validating the use of SFH in pregnant women with MINDI.

Finally, several critical points where public health measurements could be implemented were identified:

1. Measures to change biomass fuels for cooking would decrease adverse pregnancy outcomes from the exposure to wood smoke.
2. Treating of Th1-inducing infections (caries, hookworm, vaginal trichomoniasis) previous to iron supplementation, may help to decrease inflammation.
3. Adapting iron and MMN supplementation policies to MINDI context would imply the systematic evaluation and treatment of infections and/or the measurement of combined biomarkers to determine protein and iron status, and iron restriction due to inflammation. Cost-effectiveness of evaluations given the impact on reducing morbidity and mortality in the population need to be taken in account.
4. Deworming campaigns or preventive use of shoes, water and sanitation infrastructure targeting decrease hookworm and other intestinal nematode infection in pregnant women are needed.
5. Finally, the improvement of diet quality in impoverished populations by ensuring that pregnant women consume foods with balanced protein-energy contents, and fruits and vegetables according with local produce and traditions may help decreasing the high prevalence of adverse pregnancy outcomes.

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