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Impact of Nutritional Status and Placental Malaria Infection on Pregnancy Outcome in a Population of Nigerian Maternal-Infant Dyads

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IMPACT OF NUTRITIONAL STATUS AND PLACENTAL MALARIA INFECTION ON PREGNANCY OUTCOME IN A POPULATION OF NIGERIAN MATERNAL-INFANT DYADS

by

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University of Nebraska, 2023

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Nigeria is the most populous country in Africa and accounts for one-quarter of the newborn mortality on the continent. Most newborn deaths are from prematurity (33%), infections (33%), and intrapartum complications (31%). Using an adapted analytical framework for the study of child survival in developing countries, this dissertation aimed to look at the pregnancy outcome of a population of Nigerian women and their offspring by looking at proximate determinants of health from nutritional status and environmental conditions. In Chapter 1, we characterized the micronutrients of the mother-infant dyads, looking specifically at concentrations of vitamin D and its metabolites. In Chapter 2, we looked at omega-3 fatty acids derived specialized pro-resolving mediators (SPMs). Finally, in Chapter 3, we characterized the prevalence of placenta malaria infection in a population of asymptomatic pregnant women in an endemic malaria region by looking at the presence of different Plasmodium species. Descriptive statistics were used. Spearman correlation was used to compare continuous variables, Mann-Whitney for dichotomous variables, and Kruskal-Wallis for two or more groups. Salient findings in Chapter 1 were: high cord percent 3-epi-25(OH)D_3 levels were positively associated with newborn evaluation for sepsis ($p = 0.036$) while maternal and cord 25(OH)D and 24,25$(OH)_2D_3$ levels were not. Further studies are needed to reproduce our findings and better understand the biology

behind our observed association; in Chapter 2. maternal Nigerian RvD1 and RvD2 levels were significantly lower than that of the US cohort (p = 0.002 and p = 0.004, respectively). Maternal Nigerian RvD2 was negatively associated with maternal BMI and newborn length. (r= -0.288; p+ 0.033, r= -0.281; p = 0.028, respectively). Studies are needed in populations with more different diets and supplementation habits to understand the biology behind these SPM dynamics better. In Chapter 3, we report the presence of *P. knowlesi* in the African region in mixed placental malarial infections in asymptomatic pregnant women. This finding highlights the need to use PCR assays to characterize single and mixed infections better. A strong research collaborative team with our country partner and alignment of research priorities will help us continue to explore our research findings.

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BMI	Body Mass Index
CGA	Corrected Gestational Age
EMR	Electronic Medical Record
GA	Gestational Age
HIV	Human Immunodeficiency Virus
IOM	Institute of Medicine
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MeSH	Medical Subject
NICU	Newborn Intensive Care Unit
PCR	Polymerase Chain Reaction
UATH	University of Abuja Teaching Hospital
UNMC	University of Nebraska Medical Center
REDCap	Research Electronic Data Capture
RDT	Rapid Diagnostic test
SAS	Statistical Analysis System
SDOH	Social Determinants of Health
SPMs	Specialized Pro-resolving Mediators
SPSS	Statistical Package for Social Sciences
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
VTI	Vertically Transmitted Infections
WHO	World Health Organization

INTRODUCTION

Nigeria is the most populous country in Africa and accounts for one-quarter of the newborn mortality on the continent. Most newborn deaths are from prematurity (33%), infections (33%), and intrapartum complications (31%). [1, 2] The Nigerian Federal Ministry of Health developed a Roadmap to Action to help reduce maternal newborn morbidity and mortality by working on increasing funding for health and access to care. [3] Over the past decade, there has been a slow decline in neonatal mortality in the country; the neonatal mortality rate has gone from 39 deaths per 1,000 live births in 2011 to around 35 deaths per 1,000 live births as of 2021. [4] To tackle these dismal statistics, the International Foundation Against Infectious Disease in Nigeria (IFAIN, www.ifain.org) was established over a decade ago and built a surveillance platform that helps determine the etiologic agents of infections in Nigerian children. IFAIN has a unique position as a Nigerian research enterprise that is in Abuja, the Federal Capital Territory, in Central Nigeria, and operates a nearby research unit at the University of Abuja Teaching Hospital (UATH) in Gwagwalada, which is a peri-urban settlement that serves a large population of mothers and children at varying risks of infectious diseases. As such, IFAIN can run large population-based studies and is well suited to run exploratory studies to address neonatal morbidity and mortality.

Between April 2016 and March 2018, IFAIN conducted a large study on vertically transmitted infections and newborn outcomes called VTI at UATH. Collaborating with experts in Nigeria and the US at the University of Nebraska Medical Center (UNMC), we identified local research priorities addressing maternal health status and associated newborn outcomes that could be incorporated into the VTI study and secured funding for three small pilot studies. The overall goal of this dissertation was to look at the pregnancy outcome of a population of Nigerian women and their offspring by:

1) Characterizing the nutritional status of the mothers, looking specifically at concentrations of vitamin D and its metabolites and omega-3 fatty acids derived specialized pro-resolving mediators (SPMs) at the time of delivery.
2) Characterizing the prevalence of placenta malaria infection in a population of asymptomatic pregnant women in an endemic malaria region by looking specifically at the presence of different *Plasmodium* species.

In the following sections of this introduction, we will discuss the overall conceptual framework we used for these studies and present an overview of the importance of each of the chosen research areas that we will further discuss in the following three chapters of this thesis.

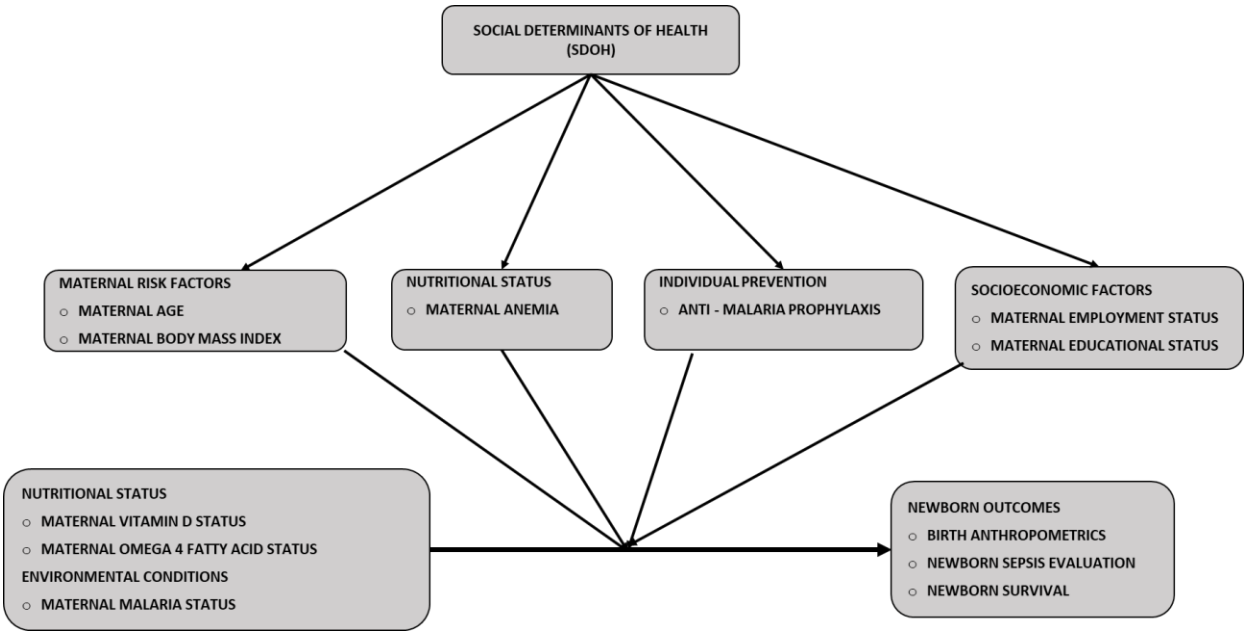
Conceptual framework

Addressing child health and survival, particularly in regions of the world with limited resources, warrants consideration of the social, environmental, economic, and biological determinants of health. On these premises, Henry Mosley and Lincoln Chen, in *An Analytical Framework for the Study of Child Survival in Developing Countries*, proposed a conceptual framework for research in child survival. [5] In their article, they described the use of proximate determinants in the study of child survival. They justified this approach based on the understanding that newborn survival, with survival at least through infancy and early childhood, is affected by social, economic, environmental, and biological conditions. Furthermore, failure to thrive and child mortality are the results of the cumulative impact of these different interactions on disease processes. [5] Mosley and Chen described a set of 5 categories of proximate determinants that can directly affect newborn survival. They included maternal risk factors, environmental contamination, nutrient deficiency, injury, and personal illness control. They selected these determinants because studies have shown them to directly impact pregnancy

outcomes. [5] The maternal risk factors they presented included age, parity and birth spacing, and environmental contamination, which referred to routes through which infectious agents are transmitted, such as air, food, water, finger, and insect vectors. Nutrient deficiency refers to maternal calorie, protein, and micronutrient intake, while injury refers to physical injury, burns, and poisoning. The last proximate determinant they proposed was personal illness control, which included measures taken to prevent disease, such as immunizations and prophylactic medications. [5] Finally, in their model, Mosley and Chen proposed a dependent variable that they would refer to as health status of "sick" that can lead to death directly or failure to thrive and then death. This allowed the capture outcomes at the different stages of a disease process and not just mortality, as studies that focus on mortality would have to enroll a very large population because it is a rare event. [5]

Figure 1 depicts the conceptual framework of social determinants of health on newborn outcomes that we developed for our research by adapting the model proposed by Mosley and Chen to our study setting. In our adapted model, we chose four of the five proximate determinants of health described by Mosley and Chen based on the research scope of our three studies. The following determinants were selected based on studies that have shown that they directly impact newborn outcomes in Nigeria: maternal risk factors, environmental conditions, nutritional status, and individual prevention.

Figure 1. Conceptual framework of social determinants of health on newborn outcome. Our proposed framework depicted four proximate determinants of health that directly impact newborn outcomes: maternal risk factors, environmental conditions, nutritional status, and individual prevention. We included in this framework socioeconomic factors, which are independent variables that impact newborn health outcomes through their influence on the proximate determinants. The outcome we assessed, our dependent variables, included birth anthropometrics, newborn sepsis evaluation, and newborn survival. This model was adapted from An Analytical Framework for the Study of Child Survival in Developing Countries by Henry Mosley and Lincoln Chen. [5]



We looked at maternal risk factors described as significantly associated with pregnancy outcomes, including maternal age, weight, and gestational age. [6, 7] The environmental condition we explored in our study was the impact of malaria endemicity, a disease propagated by an insect vector, on pregnancy outcomes. [8] For nutritional status, our study looked specifically at two micronutrients, vitamin D and omega-3 fatty acid, and how their concentration in the mother affected pregnancy outcomes. [9] Finally, for individual prevention, we looked at how the use of malaria prophylaxis impacted pregnancy outcomes. [10] Unlike Mosley and Chen. in our studies, we did not look at injuries or include this determinant in our model. We added components to our conceptual model: socioeconomic factors and independent variables that can impact newborn health outcomes by influencing our selected proximate determinants. The socioeconomic factors we were tracking included maternal educational level and employment status. [11] Mosley and Chen described socioeconomic factors in their paper but did not include them in their conceptual model. [5] Finally, in our framework, we selected the following dependent variables as our newborn outcome based on the dynamics of our list of socioeconomic and proximate determinants of health: birth anthropometrics, newborn evaluation for sepsis, and survival of the infant during the newborn period, which correspond to the first 28 days of life. [12]

From our conceptual framework, we selected three proximate determinants of health to focus on in our three studies. The first two were in nutritional status; we looked at characterizing the concentration of two micronutrients, vitamin D and its metabolites and omega-3 fatty acids derived specialized pro-resolving mediators (SPMs) on pregnancy outcomes. The third one was in environmental conditions; we looked at living in a malaria endemic region and pregnancy outcomes. The remaining proximate determinates and socioeconomic factors remained in our study for analysis considerations with our main findings. In the following section we will discuss

the importance of choosing these specific proximate determinants in our studies and the knowledge gaps in our population of interest.

Micronutrients and newborn outcomes

Essential vitamins and minerals required to sustain normal cellular and molecular functions are called micronutrients, and the most common micronutrient deficiencies reported in many populations, including in the African region, are vitamin A, folate, and iron. [13-15] The World Health Organization (WHO) recognizes these deficiencies and recommends, in addition to the use of a balanced diet with protein, that pregnant women take supplements with vitamin A, iron and folic acid, particularly in populations where deficiencies are prevalent in order to improve pregnancy outcomes. [16] Furthermore, micronutrients can impact outcomes from infectious diseases, for example, vitamin A treatment in children with measles. [17]

Most micronutrient deficiencies can be reversed by the timely and adequate intake of supplements to address the specific deficiency. [18] Pregnant women and their children, especially 5 years of age and younger, are the most vulnerable population subgroups affected by micronutrient deficiencies. [19] Evidence-based approaches to determine the prevalence of micronutrient deficiencies are necessary to inform effective intervention prevention and treatment strategies that take into consideration the social environment and living conditions of at-risk populations. [18]

Vitamin D and its metabolites

Vitamin D and its metabolites play a role in bone health and innate and adaptive immunity. [22] Low maternal and cord 25(OH)D (the biomarker of vitamin D status) concentration have been associated with adverse newborn outcomes that include poor fetal

growth, low birth weight, poor skeletal health, a weaker immune system that may lead to an increased susceptibility to infection which can then affect neonatal survival. [23] Additional studies looking at two vitamin D metabolites we specifically analyzed in our research, 3-epi-$25(OH)D_3$ and $24,25(OH)_2D_3$, have also shown their impact on fetal growth and skeletal health. [24, 25] Accurate vitamin D and metabolite measures in the newborn period require the use of liquid chromatography with tandem mass spectrometry assay which is not readily available in Nigeria. [26] No studies reported in the literature have looked at associations of maternal-infant vitamin D and metabolite level with pregnancy outcomes in mother-infant dyads in Nigeria.

Omega-3 polyunsaturated fatty acids and related metabolites

During pregnancy changes occur in the woman's body that allows for a controlled inflammatory milieu to favor implantation and fetal growth; studies have shown that excessive inflammation can lead to preterm birth, fetal growth restriction, and pre-eclampsia. [27, 28] Omega-3 polyunsaturated fatty acids have been shown to have several health benefits during pregnancy that are likely associated with their anti-inflammatory properties, and maternal supplementation with omega-3 fatty acids has been associated with preventing complications such as pre-eclampsia, preterm delivery, and newborn admission to the intensive care unit. [27, 28] Docosahexaenoic acid (DHA), one of three main omega-3 fatty acids, serves as a precursor for the biosynthesis of specialized pro-resolving mediators (SPMs), two of which are resolvin D1(RvD1) and resolvin D2 (RvD2). RvD1 and RvD2 additionally have been found to have antimicrobial properties in studies involving activated macrophages. [29, 30] Furthermore, SPMs levels in human breast milk have been found to be up to 100-fold higher in the first month of lactation than the levels observed in adults. [31] Data, however, on the SPMs levels in mother-infant dyads and their associated pregnancy outcomes in Africa in general and in Nigeria in

particular have not been reported. The World Health Organization (WHO) has recommendations for a balance diet in pregnancy, but they do not list omega-3 fatty acid supplementation as part of their guidelines. [16] Studies that have reported the benefits of supplementation with omega-3 fatty acid in improving pregnancy have been conducted in high- and upper-middle-income countries. [32, 33]

Endemic infectious diseases and newborn outcome

Nigeria has one of the largest complication rates associated with malaria infection in pregnancies, ranging from 8.4% to 58.1% of pregnancies. [34] These complications can lead to maternal anemia, prematurity, low birth weight in the newborn, and even fetal demise or newborn death. [35-38] The use of barriers such as screens and bed nets, prophylactic antimicrobials, intermittent screening and treatment during pregnancy are essential to decreasing adverse pregnancy outcomes from malaria. [39] However, there are some special considerations with malaria infection during in pregnancy. In malaria-endemic regions, there can be asymptomatic infection that complicates malaria eradication programs but also complicates pregnancy outcomes for the asymptomatically affected mother. [40] The physiology of malaria infection during pregnancy highlights the importance of addressing asymptomatic infections. When there is a malaria infection, the circulating infected erythrocytes are sequestered in the placenta where they not only affect the transfer of nutrients to the fetus but also where the parasite can accumulate to reach levels of parasitemia that are much higher than in the peripheral blood. [41] Furthermore, in endemic regions, most diagnostic methods use thin and thick peripheral blood smear microscopy or rapid diagnostic tests that do not capture accurately placental infections. [42] In these cases, polymerase chain reaction (PCR)-based methods are allow the detection parasites at densities that are not captured in peripheral blood and help with

the accurate identification of parasite species and the identification of mixed infections. [43] There are no studies reported in the literature on looking at malaria infection in the placenta of asymptomatic pregnant women in Nigeria using PCR assays.

In the following three chapters we addressed the overall goal of our thesis using the following main objectives in each chapter:

Characterizing the nutritional status and pregnancy outcomes

- Chapter 1: The primary objective was to assess the association of newborn sepsis evaluation to maternal and cord vitamin D and metabolite levels, specifically 25(OH)D, 3-epi-25(OH)D3, and 24,25$(OH)_2D_3$ levels, at the time of delivery in a population of maternal-infant dyads in Nigeria.
- Chapter 2: The primary objective was to characterize the omega-3 fatty polyunsaturated fatty acids derived specialized pro-resolving mediators, resolvin D1 and resolvin D2 levels, in maternal and cord samples of a population of maternal infant dyads in Nigeria.

Characterizing the placenta malaria burden in an endemic region and pregnancy outcomes

- Chapter 3: The primary objective was to determine the prevalence of different *Plasmodium* species in the placenta of a population of asymptomatic pregnant Nigerian women at the time of delivery.

CHAPTER 1: VITAMIN D METABOLITES IN MOTHER-NEWBORN DYADS AND ASSOCIATED CLINICAL OUTCOMES IN A POPULATION OF NIGERIAN WOMEN

One of the causes of high neonatal mortality in Nigeria is neonatal sepsis. Interventions that could potentially decrease this adverse outcome are crucial as part of a multiprong approach to improve neonatal survival in the region. In this chapter, we explore the important extra-skeletal functions of vitamin D as an innate and adaptive immune response modulator. Studies have shown that low levels of vitamin D in both maternal and cord blood were significantly associated with neonatal sepsis and vitamin D supplementation for pregnant women and their newborns could decrease the incidence of neonatal sepsis. This study is the first of its kind to look not only at the status of vitamin D and its metabolite levels in a population of pregnant Nigerian and their newborn but also their association with evaluation for newborn sepsis in addition to other risk factors and clinical outcomes.

The research conducted in this chapter was funded by the Edna Ittner Pediatric Research Support Fund at the University of Nebraska Medical Center, a research grant from the Department of Pediatrics, University of Nebraska Medical Center, and a research grant from the office of the Vice Chancellor for Research at University of Nebraska Medical Center.

This chapter will be submitted for publication at the journal *Nutrients* in January 2024.

ABSTRACT

Low levels of vitamin D in maternal and cord blood have been associated with neonatal sepsis. This study assessed the association of vitamin D metabolites (25(OH)D, 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$) levels in maternal and cord blood with newborn sepsis evaluation in Nigerian mother-infant dyads. Maternal and cord blood from 534 mothers and 536 newborns were processed using liquid chromatography-tandem mass spectrometry. Spearman correlation was used to compare continuous variables, Mann-Whitney for dichotomous variables, and Kruskal-Wallis for two or more groups. High cord percent 3-epi-25(OH)D_3 levels were positively associated with newborn evaluation for sepsis (p = 0.036), while maternal and cord 25(OH)D and 24,25$(OH)_2D_3$ levels were not. Being employed was positively associated with maternal and newborn 3-epi-25(OH)D_3 concentrations (p = 0.007 and p = 0.005, respectively). The maternal 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3 were positively associated with vaginal delivery, (p = 0.013 and p = 0.012, respectively). Having a weight-for-age Z-score ≤-2 was positively associated with newborn percent 3-epi-25(OH)D_3 levels (p = 0.004), while a weight-for-length Z-score ≤-3 was positively associated with maternal and newborn percent 3-epi-25(OH)D_3 levels (p= 0.044 and p = 0.022, respectively). Our study highlights the need to further investigate the biological role of 3-epi-25(OH)D_3 and its clinical significance in fetal growth and newborn outcome.

INTRODUCTION

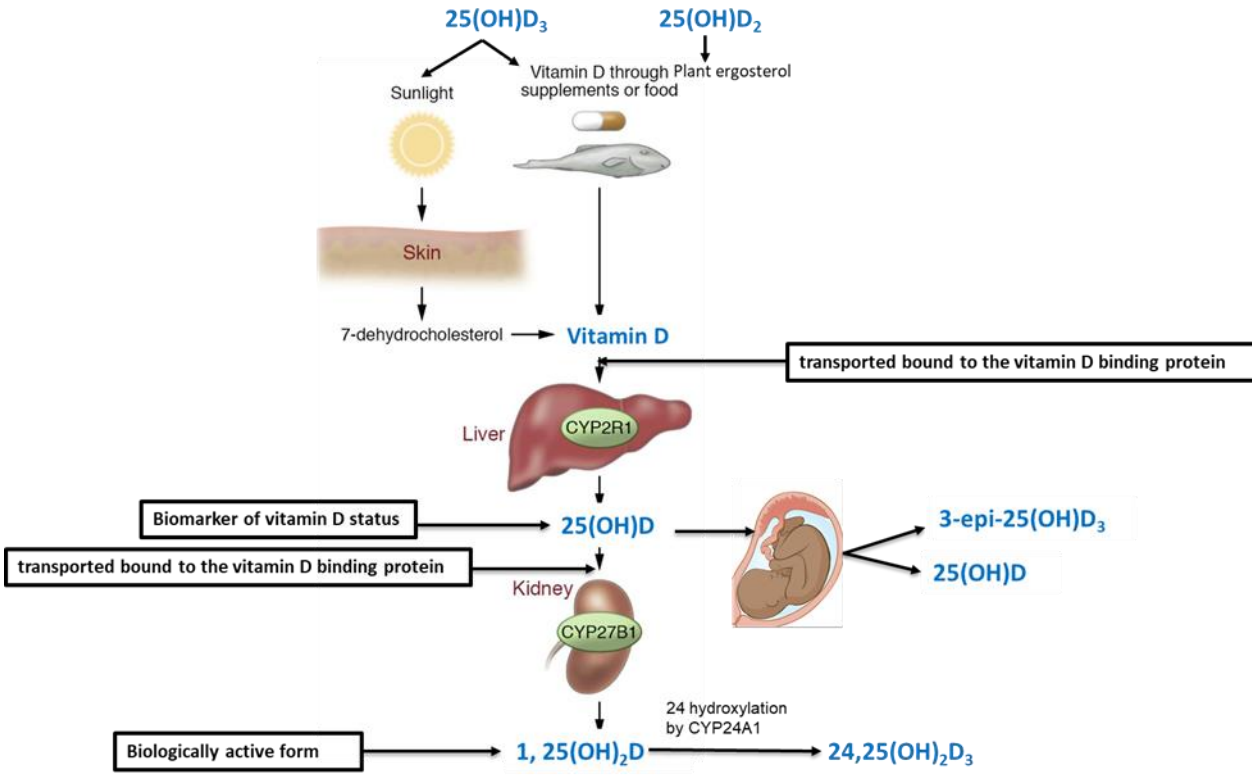
Nigeria accounts for nearly one-quarter of Africa's newborn deaths (death in the first 28 days of life) and has the 8th highest neonatal mortality rate in the world at 35 per 1,000 live births. [4] A third of newborn deaths are due to infections, exploring different approaches to reduce infection-related neonatal morbidity and mortality is crucial in the region. While mitigating programs such as maternal vaccinations[44] and screening for vertically transmitted infections[45] are important, concomitantly addressing other factors such as maternal nutrition and micronutrient status can help improve newborn health and increase survival. One such micronutrient, vitamin D, has been studied for its key role in bone metabolism and calcium homeostasis but it also has important extra-skeletal functions as an innate and adaptive immune response modulator. [22]

Vitamin D physiology

Sunlight, diet, and supplements are major sources of vitamin D which comes in two major forms: vitamin D2, 25(OH)D_2, and vitamin D3, 25(OH)D_3. [22] Figure 2 shows a graphical depiction of key steps in vitamin D physiology. While 25(OH)D_2 is synthesized from plant ergosterol, mostly mushroom, and yeast, 25(OH)D_3 is synthesized in the skin by the reaction of 7-dehydrocholesterol with UVB radiation and is also found in selected animal products such as fatty fish and fish oil. Once vitamin D enters the circulation (as 25(OH)D_2, 25(OH)D_3 or both), it is transported to the liver bound to the vitamin D binding protein (VDBP) where it is metabolized to 25(OH)D by the vitamin D-25-hydroxylase (CYP2R1).[46, 47] 25(OH)D is the main circulating form of vitamin D and is a biomarker of vitamin D status per the IOM.[48] 25(OH)D, bound to the VDBP, is then transported to the kidney where it is metabolized by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) to its biologically active form, 1,25$(OH)_2$D

(calcitriol).[22] The enzyme CYP27B1 is expressed in many tissues such as activated macrophages, parathyroid glands, and the colon, for example. When $1,25(OH)_2D$ is formed in these tissues, it exerts its autocrine and paracrine role by binding to the vitamin D receptor (VDR) in the nucleus to regulate different gene expressions.[49, 50] Additionally, $1,25(OH)_2D$ regulates its own metabolism by feedback mechanisms using the CYP24A1 gene to convert $1,25(OH)_2D$ into 1, $24,25(OH)_3D_3$ as well as conversion of 25(OH)D into $24,25(OH)_2D_3$. [50]

Figure 2 Vitamin D physiology Adapted from Demay, MB "The good and the bad of vitamin D inactivation" [20] and normal placenta image [21]



During pregnancy, 25(OH)D freely crosses the placenta and is the main source of circulating vitamin D for the developing embryo; typically, cord blood 25(OH)D levels are approximately 50–75% of the maternal levels. [51, 52] Vitamin D metabolism yields the previously described metabolites during pregnancy and in the newborn period, however, the functionality of the CYP24A1 enzyme is reduced, thereby suppressing the metabolism of 25(OH)D. [53] Both $25(OH)D_3$ and $1,25(OH)_2D$ can be alternatively metabolized through a C-3 epimerization pathway that parallels the standard metabolic pathway. [54] The concentration of the product of this epimerization process, 3-epi-$25(OH)D_3$, increases at the end of the pregnancy and decreases over the first year of life of the offspring and the presence of this metabolite can affect the accurate measurement of maternal and neonatal vitamin D status. [49, 50] Singh et al. reported that in a group of 172 infants <1 year of age with detectable $25(OH)D_3$ $25(OH)D_3$ 22.7% had C3 epimers that ranged between 8.7% and 61.1% of the total 25(OH)D, leading to an overestimation of 25(OH)D levels, and the epimer percentage was inversely correlated to the infants age ($r = 0.48$; $p < 0.002$). [55-57] Therefore, in infants < 1 year of age, an assay that allows accurate detection of 25(OH)D in the presence of its C-3 epimers such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) method should be used. [58]

Effects of *Vitamin D on the immune system and infections*

In the innate immune system, when an infection occurs, activated macrophages and monocytes strongly express the enzyme CYP27B1 which then converts 25(OH)D into its active form, $1,25(OH)_2D$. This conversion further increases the antimicrobial properties of the activated macrophages and monocytes, leading to an enhancement of their antimicrobial activities, and helping them stimulate the production of an endogenous antimicrobial, cathelicidin, that can act against invasive bacteria, fungi, and viruses. [58] In the adaptive immune system, dendritic cells

and activated T cells express the enzyme CPY27B1. Therefore 25(OH)D can exert an effect on them when it is converted to its active form, $1,25(OH)_2D$, by modulating immune activation and inflammation.[58]

Low maternal and newborn 25(OH)D levels have been associated with an increased susceptibility to neonatal infections. [59] Several studies have shown that 25(OH)D deficiency at birth was associated with an increased risk of lower respiratory tract infections. [51, 52] In a 2021 Nigerian study, a low vitamin D level in children aged 1-59 months was associated with an increased risk of acute pneumonia. [60] In addition to increased susceptibility to infections, studies have shown an association between maternal vitamin D deficiency and pregnancy complications such as intrauterine growth restriction, increased caesarian sections, and preterm birth. [61] Maternal risk factors that have been associated with maternal 25(OH)D deficiencies include the type of maternal employment and educational level. [62] Studies from lower middle- and high-income countries have shown that lack of a college education was associated with a higher risk of 25(OH)D deficiency in the mother. [63]

Vitamin D metabolites and associated outcomes

The prevalence of 25(OH)D deficiency in African settings can vary extensively based on the population, study design, and methods; in a systematic review and meta-analysis on the prevalence of vitamin D deficiency in Africa, by Mogire et all, the authors found that the mean serum 25(OH)D concentrations were lower in populations living in northern African countries or South Africa compared to other African regions. [58] Furthermore, the study found that urban areas had low 25(OH)D levels compared with rural areas, women compared to men, and newborns compared to their mothers. [58] While there was sunlight almost 12 hours a day, most people were dark-skinned, and often women extensively practiced head covering for religious

reasons. [58] This may have led to decreased sun exposure and a higher risk for 25(OH)D deficiency, which is of increased concern in pregnant women. The following studies conducted on pregnant women in Nigeria have looked at the prevalence of 25(OH)D deficiency in pregnancy. A 2018 study by Owie et al. showed that though the prevalence of maternal 25(OH)D deficiency was 4.8%, the associated newborn 25(OH)D deficiency was 29.5%. [64] A 2019 study by Oluwole showed that the prevalence of serum 25(OH)D deficiency was higher among women with preterm delivery than among those with term delivery. [65] No study, however, has assessed the impact of 25(OH)D, and associated metabolites status on pregnancy and newborn outcomes in Nigeria. [61, 66, 67] No studies with the Nigerian populations have looked at associations of maternal-infant vitamin D and metabolite level with the above risk factors and outcomes.

In contrast to 25(OH)D, 3-epi-25(OH)D_3 does not efficiently transfer across the placenta, high levels observed in infants are likely due to post-natal epimerization of 25(OH)D. [49, 68] Furthermore, 3-epi-25(OH)D_3 has a lower affinity for the VDR than 25(OH)D; this could mean that its biological activity may be lower; this is important, as 25(OH)D may be overestimated if the assay used does not separate 3-epi-25(OH)D_3. when measuring 25(OH)D status. [25] Though its affinity for the VDR may be lower, 3-epi-25(OH)D_3 is reported as potent of a suppressor of parathyroid hormone (PTH) as the biologically active 1,25-$(OH)_2$D. [24] Furthermore, 3-epi-25(OH)D_3 has also been reported to possess significant activity in stimulating surfactant phospholipid synthesis in alveolar type II cells. [69] Another metabolite, 24,25$(OH)_2D_3$, the first metabolite in the process of inactivation of 25(OH)D, is thought to help stimulate growth plate development, bone or cartilage mineralization, bone fracture repair or suppression of PTH. The effect of 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$ on suppressing PTH is particularly important for premature infants who are at higher risk of osteopenic bone disease. [22, 25] There are no

reports of these metabolites being associated with the risk of sepsis in the newborn, as seen with 25(OH)D. Additionally, there are no reported studies looking at these metabolites in Nigerian mother-infant dyads.

The primary objective of this study was to assess the association of newborn sepsis evaluation to maternal and cord 25(OH)D, 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$ levels in a population of pregnant Nigerian women and their offspring. The secondary objective was to look at associated maternal risk factors and newborn outcomes based on these maternal and cord metabolite levels.

METHODS

Recruitment

This pilot study was conducted within a larger observational cohort study on vertically transmitted infections and neonatal outcomes that was being conducted at UATH from April 2016 to March 2018. UATH serves a peri-urban settlement located in Gwagwalada, Nigeria near Abuja, the Federal Capital Territory. A total of 2,586 pregnant women ≥18 years of age were enrolled in the large observational cohort over the course of the study, after informed consent, along with their offspring which accounted for 1,033 newborn males and 963 newborn females. The samples analyzed in our pilot study were collected at the time of delivery at UATH between April 2016 to February 2017 and represented 534 mothers and 536 newborns. Reasons for not obtaining a sample were not always documented, but when information was reported, most of the reasons were that the delivery occurred at another healthcare facility/home and that, due to the timing of delivery, there was no available research personnel.

Ethical approval

Ethical approval for the study was granted by the ethics committee at UATH. Additionally, a separate IRB approval was obtained at the University of Nebraska Medical Center (UNMC) and Queen's University, given some of the investigators, and the grant funding came from that institution.

Sample and Data Collection

A sample of 2.5mL of maternal blood and a sample of 2.5mL of cord blood were collected at the time of delivery from each mother who consented to participate in the study along with their newborn. Inclusion criteria were maternal age ≥18 years and newborn gestational age ≥24 weeks and infant age at the time of enrollment could be between 0 to 7 days, though all mother and infant dyads were enrolled at or right before delivery. Additional inclusion criteria were an absence of heavy peripartal vaginal bleeding and the ability to provide written informed consent, no specific exclusion criteria were listed for the study. Maternal infant demographic and clinical data were prospectively collected and entered in UNMC's research electronic data capture tool termed (REDcap). Clinical data collected included maternal age, body mass index (BMI) at the time of delivery, hemoglobin, gestational age, birth anthropometrics, gender, and Apgar scores (1-min and 5-min). Infant clinical outcome data collected included 7-day and 28-day follow-up phone interviews conducted with participating mothers to assess infant well-being, interval illness or hospitalization for newborn sepsis evaluation. Neonatal sepsis, a leading cause of morbidity and mortality, was defined as a bloodstream infection of infants in the first 28 days of life. [70] In our study, we also looked at early neonatal death (birth to < 7 days of age) and late newborn death (>7 to 28 days). [71]

Biochemical Analysis

All blood samples were protected from direct exposure to sunlight by placing them in a brown after collection and then stored at - 80°C. They were batched and shipped every 3 months to UNMC on dry ice. From there all samples were then stored again at 80°C and shipped to Queens University, Ontario, Canada where concentrations of 25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_3, and 24,25$(OH)_2D_3$ were measured using the liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based method involving derivatization with DMEQ-TAD29.[26] 25(OH)D was calculated by adding 25(OH)D_3 and 25(OH)D_2. The Queen's University Vitamin D Laboratory LC-MS/MS method had been accredited by DEQAS for the last 3 annual cycles at the time of the study.

Clinical Outcome

On days 7 and 28 of life, study personnel contacted the mother to assess whether the infant was alive or deceased and whether the newborn had been evaluated for sepsis since birth. The primary endpoint for statistical analysis was the association of neonatal sepsis evaluation by day 28 of life with maternal and newborn 25(OH)D and metabolite levels.

Growth Outcome

Birth anthropometric parameters were measured for infant birth weight, length, and head circumference using international standards from the INTERGROWTH-21st Project and reported in percentiles. [72] Birth growth parameter z-scores were constructed using the World Health Organization (WHO) Child Growth Parameter's Anthro software for SPSS (SPSS Inc., Chicago, IL, USA).

Statistical Analysis

Data were summarized using descriptive statistics to include mean, standard deviation, median, minimum, and maximum for continuous variables while counts and percentages were used to display categorical data. Maternal and newborn 25(OH)D levels were categorized as deficient (<20 ng/mL), insufficient (20-29 ng/mL), or sufficient (≥30 ng/mL) based on the Endocrine Society guidelines. [73]. Spearman correlation coefficients were calculated to look at the association of mother and cord serum measurements and serum measurement with continuous participant characteristics. The Mann-Whitney test was used to compare median serum levels between dichotomous participant characteristics. The Kruskal-Wallis (KW) test was used to compare the median serum levels between types of maternal head covering. If the KW test was significant, pairwise comparisons for head covering distribution were adjusted using Dunn's test. Continuous vitamin D and metabolite values were also analyzed to look for correlations with maternal age, body mass index, gestational age, and newborn anthropometrics using Spearman correlation coefficients. Logistic regression was used to adjust for maternal age as a potential confounder. Statistical significance was set at $p \leq 0.05$. SAS version 9.3 (SAS Institute, Cary, NC, USA) was used for all analyses.

RESULTS

A total of 534 mothers and 536 infants were included in the study. Vitamin D analysis was completed in 525 maternal and 526 cord samples available. The baseline clinical characteristics of the mother-baby pairs are listed in Table 1. Maternal age ranged from 18-45 years with a mean age of 30 ($\pm$5). The mean newborn gestational age was 38.5 weeks ($\pm$2.2). During the newborn period, 34 of 492 babies were evaluated for sepsis with 10 newborns lost to

follow up. Of the 536 newborns enrolled in this study, we had survival data for 464 in the first 28 days of life; 5 newborns died within the first 7 days of life, and 4 babies between days 8 and 28 of life.

Table 1 Maternal-newborn dyad characteristics

Maternal Characteristics			Newborn Characteristics		
Continuous variables	N	Mean (SD)	*Continuous variables*	N	Mean (SD)
Age (years)	534	30 (5)	Gestational age (weeks)	536	38.4 (2.2)
BMI (Kg/m2)	535	30 (5.34)	**Birth anthroprometrics**		
Hemoglobin (g/dL)	426	11.4 (1.1)	Birth weight (g)	536	3088.1 (527.2)
			Birth length (cm)	536	49.7 (4)
			Birth head circumference (cm)	533	34.5 (2.8)
Categorical variables	N (%)		*Categorical variables*	N (%)	
Education level			**Gender**		
Grade 1-12	208 (47.5)		Female	261 (49)	
Tertiary	309 (49.5)		Male	275 (51)	
Employed			**Apgar at 1 minute**		
Yes	341 (64.2)		≥ 7	470 (78.8)	
No	190 (35.8)		<7	48 (14.1)	
Type of head covering			Unknown	18 (7.1)	
None reported	96 (18.4)		**Apgar at 5 minutes**		
Hijab/Niqab	131 (25.2)		≥ 7	500 (93.3)	
Head tie or handkerchief	294 (56.4)		<7	19 (3.5)	
HIV status			Unknown	17 (3.2)	
Yes	22 (4.3)		**Newborn evaluation for sepsis**		
No	487 (95.7)		Yes	34 (6.3)	
Malaria status			No	492 (91.8)	
Yes	21 (3.9)		Unknown	10 (1.9)	
No	62 (11.6)		**Newborn alive at 7 days**		
Unknown	453 (84.5)		Yes	450 (83.9)	
Mode of delivery			No	5 (1)	
Vaginal	398 (74.3)		Unknown	81 (15.1)	
Caesarean Section	138 (25.7)		**Newborn alive at 28 days**		
			Yes	446 (83.2)	
			No	9 (1.7)	
			Unknown	81 (15.1)	

Table 2 Newborn clinical outcomes associated with maternal and newborn vitamin D and metabolite levels

Maternal and newborn vitamin D and metabolites levels vs newborn evaluation for sepsis					
Evaluation for Sepsis	**Yes**		**No**		
	Median	IQR	Median	IQR	p value
Mother (N = 525)					
25(OH)D ng/mL	32.82	15.44	37.09	16.26	p = 0.112
24,25(OH)2D3	1.47	0.96	1.75	1.27	p = 0.212
3-epi-25(OH)D3 ng/mL	1.24	0.72	1.28	0.69	p = 0.679
3-epi-25(OH)D3 %	3.78	1.13	3.58	1.36	p = 0.213
Cord (N = 526)					
25(OH)D ng/mL	22.36	7.79	24	10.29	p = 0.308
24,25(OH)2D3	0.96	0.65	1.03	0.77	p = 0.529
3-epi 25(OH)D3 ng/mL	1.34	0.82	1.3	0.8	p = 0.524
3-epi 25(OH)D3 %	**6.18**	**2.16**	**5.68**	**2.12**	**p = 0.036**
Maternal and newborn vitamin D and metabolites levels vs newborn survival at 28 days					
Newborn survival at 28 days	**Yes**		**No**		
	Median	IQR	Median	IQR	p value
Mother (N = 455)					
25(OH)D ng/mL	36.7	16.74	31.61	10.03	p = 0.06
24,25(OH)2D3	1.73	1.31	1.36	0.93	p = 0.205
3-epi-25(OH)D3 ng/mL	1.27	0.71	1.03	0.57	p = 0.212
3-epi-25(OH)D3 %	3.57	1.34	3.76	1.55	p = 0.944
Cord (N = 456)					
25(OH)D ng/mL	23.48	10.44	23.35	7.15	p = 0.574
24,25(OH)2D3	1.02	0.81	0.99	0.59	p = 0.870
3-epi 25(OH)D3 ng/mL	1.3	0.83	0.69	0.43	p = 0.377
3-epi 25(OH)D3 %	5.65	2.06	2.5	2.62	p = 0.626
Maternal and newborn vitamin D and metabolites levels vs weight for age Z-score ≤-2					
Weight for age z-score ≤-2	**Yes**		**No**		
	Median	IQR	Median	IQR	p value
Mother (N = 524)					
25(OH)D ng/mL	33.36	14.73	37.15	16.79	p = 0.156
$24,25(OH)_2D_3$	1.76	1.01	1.74	1.28	p = 0.592
3-epi-25(OH)D3 ng/mL	1.17	0.7	1.29	0.7	p = 0.698
3-epi-25(OH)D3 %	3.6	1.4	3.59	1.35	p = 0.554
Cord (N = 525)					
25(OH)D ng/mL	22.16	7.25	24.07	10.29	p = 0.139
$24,25(OH)_2D_3$	0.97	0.76	1.03	0.77	p = 0.77
3-epi 25(OH)D3 ng/mL	1.3	0.83	1.31	0.79	p = 0.502
3-epi 25(OH)D3 %	**6.17**	**2.29**	**5.65**	**2.06**	**p = 0.004**
Maternal and newborn vitamin D and metabolites levels vs weight for length Z-score ≤-3					
weight for for length Z-score ≤-3	**Yes**		**No**		
	Median	IQR	Median	IQR	p value
Mother (N = 471)					
25(OH)D ng/mL	35.75	14.84	37.55	16	p = 0.282
$24,25(OH)_2D_3$	1.7	1.25	1.75	1.3	p = 0.641
3-epi-25(OH)D3 ng/mL	1.42	0.87	1.28	0.65	p = 0.358
3-epi-25(OH)D3 %	**3.76**	**2.01**	**3.58**	**1.31**	**p = 0.044**
Cord (N = 470)					
25(OH)D ng/mL	24.53	11.69	23.58	9.93	p = .795
$24,25(OH)_2D_3$	1.11	0.79	1.01	0.71	p = .79
3-epi 25(OH)D3 ng/mL	1.44	0.86	1.3	0.76	p = .192
3-epi 25(OH)D3 %	**6.25**	**2.21**	**5.64**	**1.97**	**p = 0.022**

Vitamin D metabolites and associated newborn outcomes

The association between maternal and newborn 25(OH)D and metabolite level and newborn outcomes are listed in Table 2. Maternal and infant median levels of 25(OH)D_3, 24,25$(OH)_2D_3$, and 3-epi-25$(OH)D_3$ were not significantly associated with newborn sepsis evaluation. However, the median values of percent 3-epi-25$(OH)D_3$ were higher in the neonates who were evaluated for sepsis than in those who were not, and this difference was statistically significant. (6.18 vs. 5.68, $p = 0.036$). Using logistic regression, cord 3-epi-25$(OH)D_3$ levels remained significantly associated with sepsis evaluation after adjusting for maternal age (p= 0.0053). The median 25(OH)D_3, 24,25$(OH)_2D_3$, and 3-epi-25$(OH)D_3$ levels of infants who were not alive at 28 days from birth were not significantly lower when compared with the levels in infants who were alive at 28 days.

There was no significant difference between the median maternal and infant levels of 3-epi-25$(OH)D_3$ or percent 3-epi-25$(OH)D_3$ with weight for age Z-score ≤-2 and weight-for-length Z-score. However, newborn median percent 3-epi-25$(OH)D_3$ was associated with weight-for-age Z-score ≤-2 (6.17 vs. 5.65, $p = 0.004$) while both maternal and infant median percent 3-epi-25$(OH)D_3$ were associated with weight-for-length Z-score ≤-3 (3.76 vs. 3.58, $p = 0.044$ and 6.25 vs. 5.64, $p = 0.022$, respectively).

Vitamin D metabolites and associated maternal risk factors and outcomes

The association between maternal and newborn 25(OH)D and metabolite level and maternal risk factors, employment status, education level, and outcomes, mode of delivery, are listed in Table 3. Mothers who were employed had a higher median maternal 25(OH)D level than mothers who were unemployed and the difference was significant. (37.65 ng/mL vs. 34.97 ng/mL, $p = 0.041$) Mothers who were employed had a higher median newborn 25(OH)D level

than mothers who were unemployed and the difference was also significant. (24.56 ng/mL vs. 23.04 ng/mL, $p = 0.01$)

Table 3 Maternal factors and outcomes associated with maternal and newborn vitamin D and metabolite levels

Maternal newborn vitamin D and metabolites status vs employment status

Employment status	**Yes**		**No**		
	Median	IQR	Median	IQR	p value
Mother (N = 521)					
25(OH)D ng/mL	**37.65**	**15.47**	**34.97**	**17.71**	**p = 0.041**
24,25(OH)2D3	1.74	1.18	1.74	1.42	p = 0.122
3-epi-25(OH)D3 ng/mL	**1.24**	**0.72**	**1.19**	**0.77**	**p = 0.007**
3-epi-25(OH)D3 %	3.61	1.25	3.57	1.67	p = 0.2
Cord (N = 522)					
25(OH)D ng/mL	**24.56**	**10.29**	**23.04**	**9.84**	**p = 0.01**
24,25(OH)2D3	**1.06**	**0.72**	**0.98**	**0.75**	**p = 0.03**
3-epi 25(OH)D3 ng/mL	**1.37**	**0.76**	**1.26**	**0.88**	**p = 0.005**
3-epi 25(OH)D3 %	5.78	2.13	5.66	2.21	p = 0.394

Maternal newborn vitamin D and metabolites status vs education level

Education level	**Grade 1-12**		**Tertiary**		
	Median	IQR	Median	IQR	p value
Mother (N = 517)					
25(OH)D ng/mL	38.89	14.1	35.66	17.69	p = 0.07
24,25(OH)2D3	1.84	1.03	1.67	1.36	p = 0.092
3-epi-25(OH)D3 ng/mL	**1.4**	**0.68**	**1.21**	**0.73**	**p < 0.0001**
3-epi-25(OH)D3 %	**3.76**	**1.52**	**3.5**	**1.24**	**p < 0.0001**
Cord (N = 518)					
25(OH)D ng/mL	24.37	9.83	23.41	10.52	p = 0.332
24,25(OH)2D3	1.05	0.67	1	0.82	p = 0.252
3-epi 25(OH)D3 ng/mL	**1.41**	**0.76**	**1.26**	**0.79**	**p = 0.001**
3-epi 25(OH)D3 %	**6.29**	**2.19**	**5.39**	**1.99**	**p < 0.0001**

Maternal newborn vitamin D and metabolites status vs mode of delivery

Mode of delivery	**Vaginal**		**C-section**		
	Median	IQR	Median	IQR	p value
Mother (N = 525)					
25(OH)D ng/mL	37.01	15.31	36.44	18.11	p = 0.675
$24,25(OH)_2D_3$	1.77	1.26	1.59	1.27	p = 0.249
3-epi-25(OH)D3 ng/mL	**1.32**	**0.69**	**1.16**	**0.7**	**p - 0.013**
3-epi-25(OH)D3 %	**3.68**	**1.46**	**3.45**	**1.07**	**p - 0.012**
Cord (N = 526)					
25(OH)D ng/mL	23.44	10.47	24.42	8.83	p = 0.717
$24,25(OH)_2D_3$	1.03	36	1.02	0.67	p = 0.629
3-epi 25(OH)D3 ng/mL	1.33	0.8	1.27	0.8	p = 0.385
3-epi 25(OH)D3 %	5.77	2.19	5.63	1.97	p = 0.413

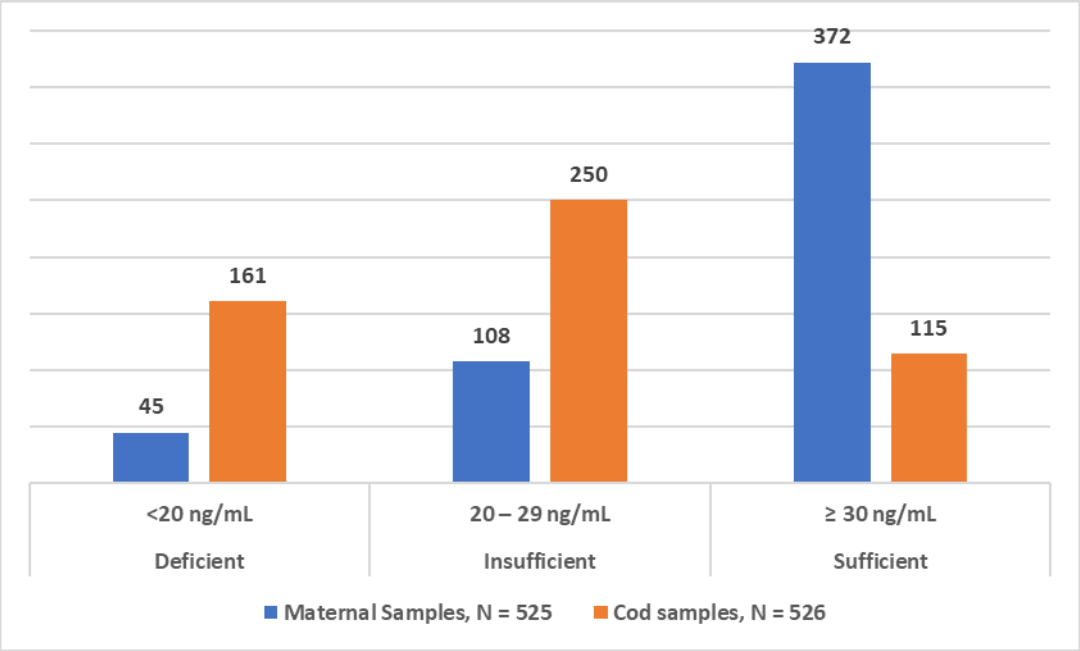
There was no evidence of a difference between the infant median concentrations of 3-epi-25(OH)D_3 and the median percent 3-epi-25(OH)D_3 with maternal employment status, however, mothers who were employed had higher median maternal and newborn 3-epi-25(OH)D_3 than mothers who were unemployed and the difference was statistically significant. (1,24 ng/mL vs. 1.19 ng/mL, p = 0.007 and 1.37 ng/mL vs. 1.26 ng/mL, p = 0.005 respectively). Overall, the only significant association observed for 24,25$(OH)_2D_3$ concentration with maternal risk factors was that the median infant 24,25$(OH)_2D_3$ levels were higher for infants of mothers who were employed compared to the mothers who were not. (1.06 vs 0.98, $p = 0.03$).

Mothers with a grade 1-12 educational level had higher median maternal and newborn 3-epi-25(OH)D_3 levels than mothers who had a tertiary level of education (1.4 ng/mL vs. 1.21 ng/mL, $p < 0.0001$ and 1.41 ng/mL vs. 1.26 ng/mL, $p < 0.001$, respectively). Mothers with a grade 1-12 educational level had higher median maternal and newborn percent 3-epi-25(OH)D_3 levels than mothers who had a tertiary level of education and the difference was significant. (3.76 vs. 3.5, $p < 0.0001$, and 6.29 vs. 5.39, $p < 0.0001$, respectively). Mothers who had a vaginal delivery had higher median maternal 3-epi-25(OH)D_3 and median percent 3-epi-25(OH)D_3 than mothers who had a cesarean-section and the difference was statistically significant. (1.16 ng/mL, $p = 0.013$ and 3.68, $p = 0.012$, respectively)

Comparison of maternal-infant dyad categories of 25(OH)D levels

Most mothers, 70.86% (372/525), had sufficient levels of 25(OH)D, while only 21.9% (115/526) of the newborns had sufficient levels. Most newborns had insufficient levels at 47.52% (250/526). There was a significant difference in the categorized concentrations of 25(OH)D levels between maternal and cord blood levels ($p < 0.001$). (Figure 2.)

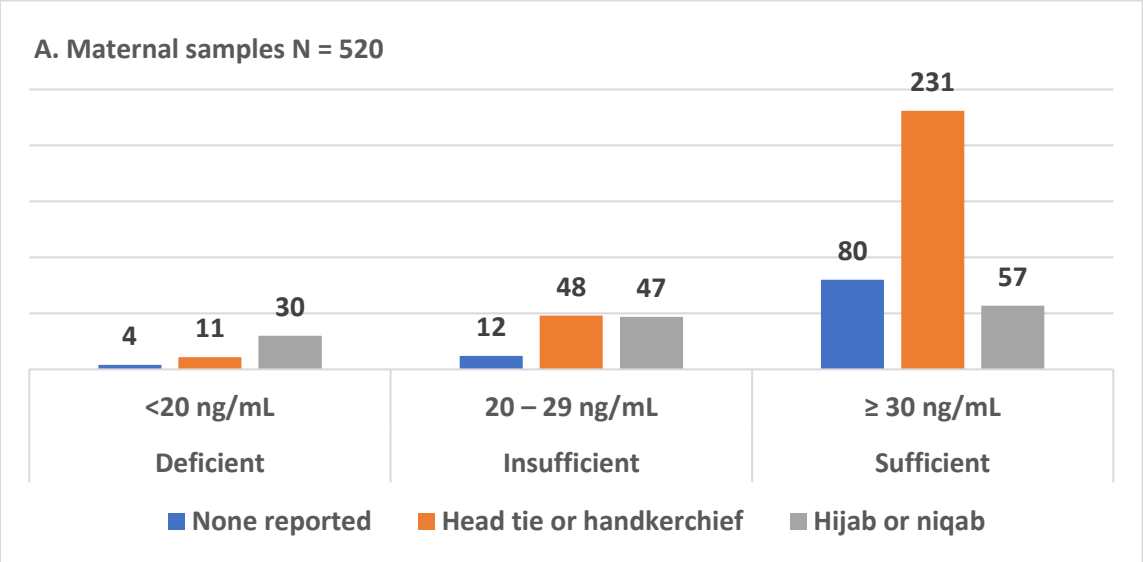
Figure 3 Maternal and newborn 25(OH)D categorized levels. Levels are categorized as deficient, insufficient, or sufficient based on the Endocrine Society guidelines. [73] Data show a significant difference between maternal and cord blood. ($p < 0.001$).

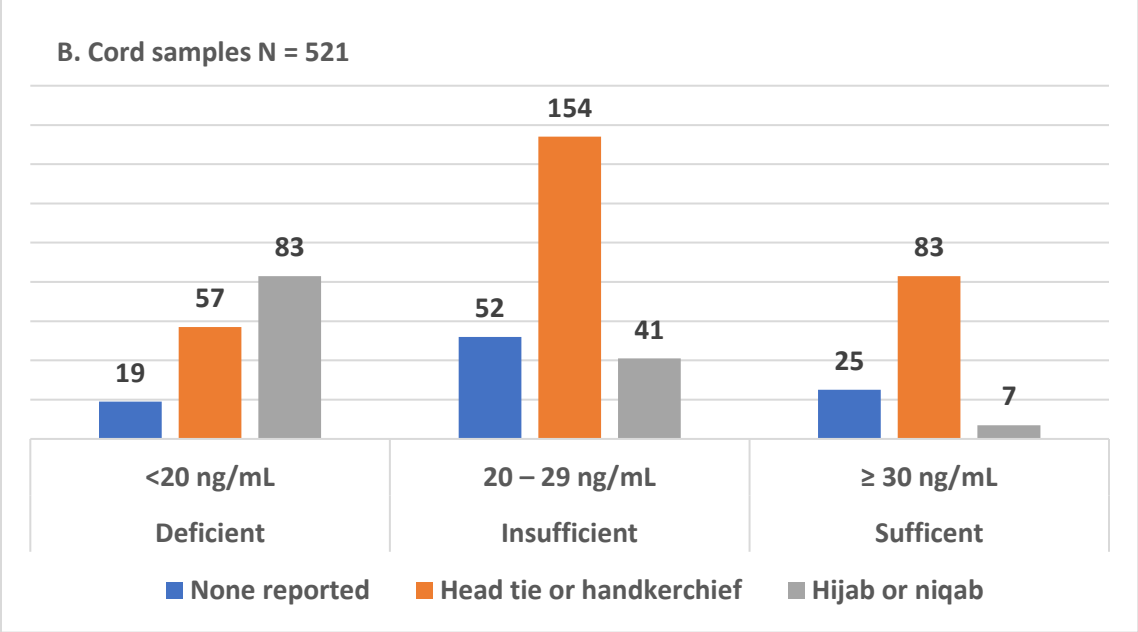


Type of maternal head covering and 25(OH)D status in mother-infant dyads.

Figure 3 shows the association of type head covering by maternal and infant 25(OH)D levels. In Figure 3A, most mothers with sufficient levels of 25(OH)D wore a head tie 79.7% (231/290) while most mothers with deficient 25(OH)D levels wore a hijab 52.2% (83/159). In Figure 3B, most infants who had deficient levels of 25(OH)D had mothers who wore a hijab 52.2% (83/159) while most infants who had insufficient levels of 25(OH)D wore a head tie 62.3 (154/247). There was a difference in the median maternal and cord 25(OH)D, 3-epi-25(OH)D_3, and 24,25$(OH)_2D_3$ levels when comparing mothers who wore a head tie versus a hijab and those who wore no head garment versus a hijab with $p < 0.0001$ for both pairwise combinations.

Figure 4 Head covering versus maternal and cord 25(OH)D category levels. Comparing the type of head covering used to maternal and cord vitamin D levels. **A.** There is a significant difference in the median maternal 25(OH)D levels between mothers who used a head tie versus a hijab, and between mothers who used no head covering versus wearing a hijab. ($p < 0.0001$). **B**. There is a significant difference in median cord 25(OH)D levels between mothers who used a head tie versus a hijab, and between mothers who used no head covering versus wearing a hijab. ($p < 0.0001$)





Correlations associated with maternal-infant dyad 25(OH)D and metabolite levels

The correlation of the mean maternal-newborn 25(OH)D metabolite levels is listed in Table 4. The mean maternal 25(OH)D level at 36.72 (±11.6) ng/mL was higher than the mean newborn 25(OH)D level at 24.52 (±8.14) ng/ml. The mean 3-epi-25(OH)D_3 in maternal samples at 1.33 (±0.61) ng/mL accounted for 3.8% (±1.14) of the total 25(OH)D concentration. In cord blood samples, absolute levels of 3-epi-25(OH)D_3 were like maternal levels at 1.39 (±0.59) ng/mL, but the percentage of 25(OH)D comprised by 3-epi-25(OH)D_3 was higher at 5.9% (±1.6). Mothers had higher 24,25$(OH)_2D_3$ means at 1.77 (±0.9) compared to infants who were at 1.09 (±0.57). Maternal and cord 25(OH)D levels, 24,25$(OH)_2D_3$ were positively correlated (r = 0.7, p < 0.0001; r = 0.81, p < 0.0001). Concentrations and the percentage of 3-epi-25(OH)D_3 in maternal and cord samples were positively correlated as well (r = 0.84, p < 0.0001; r = 0.66, p < 0.0001, respectively).

Correlation of continuous mother and infant characteristics with 25(OH)D, 24,25$(OH)_2D_3$ and 3-epi-25(OH)D_3 levels and percent 3-epi 25(OH)D_3 showed a positive association between maternal age with cord 24,25$(OH)_2D_3$ levels (r = 0.1, p = 0.03) and gestational age and cord percent 3-epi 25(OH)D_3 (r = -0.1, p = 0.02). Birthweight was positively associated with maternal 25(OH)D levels and cord 24,25$(OH)_2D_3$ levels.(r = 0.1 & p = .03 and r = 0.11 & p = 0.01 respectively) while it was negatively associated with cord 25(OH)D levels.(r =-0.12 & p = 0.005) There was no other significant correlation between 25(OH)D levels with maternal age, BMI, gestational age, hemoglobin, birth weight, birth length, and head circumference.

Table 4 Correlation of the mean maternal and newborn 25(OH)D and metabolite levels

Measurements	Mother N = 525	Newborn N = 526	*r*	*p value*
25(OH)D_2, ng/mL	2.64 ±1.46	1.3 ±0.57	0.91	< 0.0001
25(OH)D_3, ng/mL	34.08 ±11.6	22.28 ±8.03	0.7	< 0.0001
25(OH)D, ng/mL	36.72 ±11.6	24.52 ±8.14	0.7	< 0.0001
3-epi-25(OH)D_3, ng/mL	1.33 ± 0.57	1.39 ±0.59	0.84	< 0.0001
3-epi-25(OH)D_3 %	3.8 ±1.14	5.9 ± 1.6	0.66	< 0.0001
24,25$(OH)_2D_3$	1.77 ±0.90	1.09 ±0.57	0.81	< 0.0001

DISCUSSION

This study is the first to evaluate 25(OH)D, 3-epi 25(OH)D_3, 24,25(OH) $_2D_3$ and associated risk factors and pregnancy outcomes in a population of Nigerian women and their newborn at the time of delivery using the highly sensitive LC-MS/MS method.

Primary outcome

Our study showed that high cord median percent 3-epi-25(OH)D_3 was significantly associated with being evaluated for newborn sepsis (p = 0.036). Maternal median 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3, as well as cord median 3-epi-25(OH)D_3, were not associated with sepsis evaluation. Median maternal and cord 25(OH)D and 24,25$(OH)_2D_3$ were also not associated with sepsis evaluation. There was also no significant association between the maternal and cord levels of these three metabolites and newborn death within the first 28 days of life.

A 2021 systematic review and meta-analysis of 18 studies by Workneh Bitew et al, reported that low levels of 25(OH)D in maternal and cord blood were associated with newborn sepsis. [59] A 2022 meta-analysis of 42 randomized controlled trials by Liu et al showed that vitamin D supplementation during pregnancy was associated with a lower risk of intrauterine or neonatal death (RR, 0.69; 95% CI, 0.48-0.99). [74] The lack of a significant association between 25(OH)D levels and newborn sepsis evaluation and newborn death could be due to the low incidence of these events in our study population compared to the larger trials described by Workneh Bitew et al., and Liu et al.

A review of the literature did not reveal other studies that have investigated the association of 3-epi-25(OH)D_3, percent 3-epi-25(OH)D_3, or 24,25$(OH)_2D_3$ with newborn sepsis. Percent 3-epi-25(OH)D_3 represents the percentage of 25(OH)D that is in the epimer form. [75]

This means a higher 3-epi-25(OH)D_3 indicates more circulating 3-epi-25(OH)D_3 and lower circulating 25(OH)D. Our findings of an association between evaluation for newborn sepsis and high 3-epi-25(OH)D_3 may be associated with these low 25(OH)D stores in the newborn which is known to predispose newborns to adverse outcomes including sepsis.

Vitamin D and metabolite status

Maternal and cord 25(OH)D, 3-epi 25(OH)D_3, 24,25$(OH)_2D_3$ levels were all positively correlated with ($r = 0.7$, $r = 0.84$ and $r = 0.81$ respectively with $p < 0.0001$ for all). This is consistent with what has been previously described.[76, 77] Most mothers, 70.86%, had sufficient levels of 25(OH)D. In contrast, only 21.9% of the newborns had sufficient levels of 25(OH)D. The prevalence of maternal 25(OH)D deficiency and insufficiency was 8.6% and 20.6%, respectively. The prevalence of neonatal 25(OH)D deficiency and insufficiency was 30.6 % and 47.5%, respectively. These observations suggest that there may not be an efficient transfer of 25(OH)D to the newborn or that there is an independent regulation of 25(OH)D in the mother and the newborn. [76, 77] These observations between sufficient maternal 25(OH)D levels and deficient newborn 25(OH)D levels have been reported in several studies, [78, 79]

The cord 25(OH)D mean concentrations observed in our study were similar to those reported in a Lagos study by Owie et al. where the prevalence of newborn 25(OH)D deficiency and insufficiency were 28.3% and 46.1%, respectively. [64] In the same study, the prevalence of maternal 25(OH)D deficiency was about half our values at 4.8%, while the prevalence of insufficient 25(OH)D levels was about 50% higher. Lagos is a large metropolitan area, while UATH serves a more peri-urban and rural settlement; the differences in geographic settings would not clearly explain the varying results. Furthermore, it would also seem that even with the differences in maternal stores of 25(OH)D in both study populations, a similar distribution of

newborns with 25(OH)D deficient and insufficient levels was noted, Owie et al used an enzyme-linked immunosorbent assay (ELISA) method to assess vitamin D while we used LC-MS/MS assay. The prevalence of deficient and insufficient 25(OH)D levels in the newborn population may be higher in the Owie study if the measurement of 25(OH)D included the concentration of 3-epi-25(OH)D_3 based on their study assay; we avoided this problem by using the LC-MS/MS method. In a study by Oluwole et al. also conducted in Lagos, the authors reported that 14.1% of the mothers had deficient vitamin D levels. However, the authors defined deficient at levels of <30 ng/ml. We defined deficient as <20 ng/mL and insufficient 20-29 ng/mL per the Endocrine Society guidelines. [73] If we had used a similar cut-off as in the Oluwole team, our prevalence of deficient levels would have increased to 29.2% which would then reflect a higher prevalence of 25(OH)D deficiency. [80] In the Oluwole study newborn levels were not assessed. In another study in Lagos by Gbadegesin et al, maternal vitamin D levels were only collected at an antenatal visit between gestational age 10 and 28 weeks. The authors reported a prevalence of vitamin D deficiency and insufficiency of 29% and 10.4% which are higher than what we observed in our study. Gbadegesin et al did not repeat maternal serum sampling at delivery. We, however, collected samples at delivery, and most deliveries were term, with a mean gestational age of 38.4. Moreover, knowing that vitamin D levels progressively decline with pregnancy, the prevalence of vitamin deficiency and insufficiency in the Gbadegesin et al study might be higher. [64, 65] To have more reliable data on the prevalence of vitamin D deficiency and insufficiency in Nigeria, it would be important to have reference levels at different stages of a pregnancy and that consistent specific guidelines for 25(OH)D level categorization be followed.

We also measured the metabolites 3-epi-25(OH)D_3 and 24,25(OH)$_2D_3$ in our study; normal levels for these metabolites have not yet been determined. With regards to 3-epi-25(OH)D_3, however, distinguishing between 25(OH)D and 3-epi 25(OH)D_3 is of biological

relevance particularly in infants; 3-epi-25(OH)D_3 accounts for a significant proportion of the circulating total 25(OH)D and increases at the end of the pregnancy and decreases over the first year of life. [61] Access to an adequate assay method such as LC-MS/MS can help provide an accurate measurement. In our study, the 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3 in the newborn were generally higher than the maternal 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3 except for the infant who was not alive at 28 days from birth where the newborn values were lower for both 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3 than for the mothers. These data suggest that the fetus may contribute significantly to 3-epi-25(OH)D_3 production. Some studies have suggested that 3-epi-25(OH)D_3 may not transfer efficiently across the placenta and that it may be generated endogenously by the fetus from maternal 25(OH)D stores. [55-57] Other studies have shown that after birth, 3-epi-25(OH)D_3 may be generated from exogenous sources such as specific C3 epimerized vitamin D_3 supplements. [25, 68] A randomized controlled trial of premature infants receiving specifically 25 (OH)D_3 supplementation, showed an increase of their 3-epi-25(OH)D_3 from 6–8% of total 25(OH)D_3 to 30–45% of total 25(OH)D_3 after 4 and 8 weeks of 25(OH)D_3 supplementation. [81] In this study, 25(OH)D2 levels were insignificant, so serum 25(OH)D_3 instead of 25(OH)D was used to assess vitamin D status. An epimerization pathway only active in the first year of life has also been suggested as a possible way of endogenous production of 3-epi-25(OH)D_3. [55-57]

With regards to 24,25$(OH)_2D_3$, the metabolism of 25(OH)D into 24,25$(OH)_2D_3$ decreases in pregnancy to maintain persistently elevated serum 1,25$(OH)_2$D, the biologically active form of 25(OH)D.[82] Data from nonpregnant populations suggest that serum 24,25$(OH)_2D_3$ and the ratio of serum 24,25$(OH)_2D_3$ to 25(OH)D are useful indicators of 25(OH)D deficiency, and they have been reported to help predict the response to 25(OH)D supplementation. [81] In the previously reported randomized trial by Hanson et al, premature infants who received 25(OH)D_3

supplementation had an increase in 24,25(OH)2D3 values over time, and there was a high correlation between concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$. Furthermore, the same study observed a positive association between the ratio of $25(OH)D_3$:$24,25(OH)_2D_3$ and PTH concentrations ($r = 0.52$, $p = 0.02$) which was not observed at 4 weeks. This ratio has also been observed to increase linearly during times of rapid linear growth where there is an increased demand for calcium and phosphorus. The utility of these indicators during pregnancy needs further investigation. [82] In our study, we did not calculate the $25(OH)D_3$:$24,25(OH)_2D_3$ for our participants.

Birth anthropometrics

Our study did not show an association of maternal or newborn 25(OH)D levels with newborn outcomes such as weight-for-age, or weight-for-length. A critical review showed that though 25(OH)D is essential for fetal growth and has been associated with intrauterine growth restriction in some studies, some investigations, however, have failed to establish any association between 25(OH)D levels and fetal birth weight. [49] Newborn higher percent 3-epi-$25(OH)D_3$ levels were positively associated with weight-for-age Z-score **≤-**2 ($p = 0.004$) while both maternal and infant percent 3-epi-$25(OH)D_3$ were positively associated with weight-for-length Z-score **≤-**3 ($p = 0.044$ and $p = 0.022$, respectively), these positive associations with percent 3-epi-$25(OH)D_3$ for some growth parameters could be explained but the role percent 3-epi-$25(OH)D_3$ calcium homeostasis and as a suppressor of PTH. [83] Finally, the median cord $24,25(OH)_2D_3$ level was positively associated with higher birthweight ($p = 0.01$), which could be explained by the role of $24,25(OH)_2D_3$ in helping stimulate growth plate development and bone mineralization. [22, 25]

Maternal risk factors and newborn outcomes

Maternal 25(OH)D levels decline as pregnancy advances due to progressive fetal physiological demands; our study design did not allow us to compare predelivery and delivery levels 25(OH)D that may have affected newborn outcomes. [84] Though we did not look at maternal vitamin D supplementation like Liu et al, we also did not ask mothers if they were taking vitamin D supplements on their own. Most of our mothers, 70.86% (372/525), however, had sufficient levels of 25(OH)D based on the Endocrine Society guidelines; the mean level was 36.72ng/ml (+/- 11.6). [73] There are recommendations on the amount of vitamin D that should be supplemented during pregnancy and there is evidence that supplementation early on during pregnancy leads to better newborn outcomes; the ideal maternal 25(OH)D concentration throughout pregnancy for best outcome is not yet determined.

Luxwolda et al. in a study with Tanzanian tribes, reported that pregnant mothers had mean levels of 25(OH)D that were 60ng/ml, while non-pregnant women had mean levels of 46ng/ml. [85] Holles et al. used these data to develop a mathematical model that recommended circulating levels of 25(OH)D should be >40ng/ml during pregnancy. [86] There is no consensus on what the normal pregnancy values should be during pregnancy; the IOM recommends a level of 20ng/ml as sufficient, while the Endocrine Society guidelines recommend 30ng/ml. [73] Our study showed that even when most mothers had sufficient 25(OH)D levels, most newborn 25(OH)D levels were in the insufficient and deficient categories. This would imply that sufficient maternal 25(OH)D stores were not enough in our study population for the newborns to achieve sufficient 25(OH)D levels as well. Studies looking at maternal 25(OH)D levels throughout pregnancy, with and without vitamin D supplementation, and collecting cord 25(OH)D levels as

well, could help determine what the ideal 25(OH) levels during pregnancy should be to achieve sufficient cord 25(OH)D levels and better newborn outcomes.

Our study showed significant associations between maternal 25(OH)D levels and the type of head covering used. The median 25(OH)D maternal levels between mothers who used a head tie versus a hijab, and between mothers who used no head covering versus wearing a hijab. ($p < 0.0001$). The median 25(OH)D cord levels between mothers who used a head tie versus a hijab, and between mothers who used no head covering versus wearing a hijab. ($p < 0.0001$). Most mothers who had 25(OH)D deficiency wore a hijab while most mothers with 25(OH)D insufficiency wore a head tie. Mothers who did not use a type of head covering had higher 25(OH)D levels, due to increased exposure to the sun that may be further influenced by working outdoors versus indoors. These observations were consistent with previous reports on the effects of covering styles on 25(OH)D levels. [78, 79] A higher proportion of infants born to mothers who wore a head tie, or a hijab had deficient or insufficient vitamin 25(OH)D levels compared to infants of mothers who used a head covering. In our study, the percent of mothers who had sufficient levels of 25(OH)D was higher than the percent with insufficient or deficient levels, regardless of the type of head covering. This finding did not translate to a similar proportionate distribution in the newborn 25(OH)D levels. Further studies are needed in the region to better understand the multifactorial contributions of diet, dressing styles and 25(OH)D supplementations and work to help maintain optimal maternal stores of 25(OH)D that can lead to sufficient levels for the newborn. Of note in our study population, 25(OH)D supplementation was not part of routine prenatal care, and we did not ask mothers if they were independently taking any. Supplementation, along with covering style and residing in a metropolitan versus rural area have been shown to affect maternal 25(OH)D levels in Nigerian populations in Owie et al. and Oluwole et al. reports. [64, 65]

In our study, maternal and newborn 25(OH)D levels were both significantly higher in mothers who were employed versus unemployed. (p = 0.041 and p = 0.01, respectively). Kofi-Amegah et al., in a study in Ghana, looked at factors that influenced dietary and non-dietary 25(OH)D intake among pregnant women and noted that women who were employed had a higher exposure to sunlight ($p < 0.0001$). Since Ghana is in geographic proximity to Nigeria, our study findings showing an association between 25(OH)D levels and employment status could also be potentially explained by sunlight exposure. [80] Owie et al and Oluwole articles on 25(OH)D levels in pregnant women in Nigeria did not report maternal employment status in relationship to 25(OH)D levels. [64, 65] El-Khateeb et al. in a study conducted in Jordan, found that low 25(OH)D levels were associated with women being unemployed ($p < 0.001$).[87] Brian et al. found in a study of pregnant women in Nigeria that the workplace location was associated with 25(OH)D status; women who worked indoors had lower 25(OH)D levels than those who worked outdoors ($p < 0.031$). [61] However, we did not specifically ask our study participants about the amount of sunlight exposure they had daily, nor did we inquire about the details of their jobs if they were employed.

Other significant associations observed regarding maternal employment status: mothers who were employed mothers had higher median maternal and newborn 3-epi-25(OH)D_3 than unemployed mothers (p = 0.007 and p = 0.005 respectively) The median cord 24,25$(OH)_2D_3$ level was positively associated with being employed (p = 0.03), Given that both 3-epi-25(OH)D_3 and 24,25$(OH)_2D_3$ are metabolites of 25(OH)D, the likelihood of higher maternal levels of 25(OH)D could lead to high levels of these substrates in a category of such as employment. Working outdoors, which may be more likely in the peri-urban setting of our study population, could explain this finding. However, though we asked about employment, we did not inquire about the type of employment which could have helped our interpretation of these findings.

Unlike other studies, we showed no association between maternal or newborn 25(OH)D levels to maternal educational level. [55-57] Mothers with a grade 1-12 educational level had higher median maternal and newborn 3-epi-25(OH)D_3 than mothers who had a tertiary level of education (both with $p < 0.0001$). Some of the associations above could reflect situations where a mother is likely to have increased sun exposure based on employment status and educational level, though our study did not specifically investigate the amount of sun exposure based on employment and educational attainment.

Looking at the mode of delivery, we found no association with 25(OH)D levels. Observational reports have described an increased risk of cesarean section with vitamin D deficiency. A systematic review of 25(OH)D levels and pregnancy outcomes showed that there was an increased risk of caesarian sections in mothers with 25(OH)D deficiency and insufficiency likely due to reduced muscle mass and strength in the pelvic floor. [88] The prevalence of cesarean sections in our study population was 25.7%, which is because our study was conducted at a referral teaching hospital that has a high caseload of complicated obstetric cases that may require cesarean section for improved maternal and/or fetal outcomes. Mothers who had a vaginal delivery had higher medial maternal 3-epi-25(OH)D_3 and median percent 3-epi-25(OH)D_3 than mothers who had cesarean sections. ($p = 0.013$ and $p = 0.012$, respectively) While studies have shown that 3-epi-25(OH)D_3 can be elevated in pregnancy, the reason for this mechanism is unclear and there are no reports on an association of 3-epi-25(OH)D_3 and mode of delivery. [89] In a 2022 study by Mao et al, where the authors looked at maternal and cord 3-epi-25-OH-D_3 levels and pregnancy outcomes, they reported that pregnancy with a male fetus, ambient solar radiation and maternal glycemia were associated with maternal 3-epi-25-OH-D_3 levels. [49] When examining the cord blood 3-epi-25(OH)D_3 levels, the study found that they were associated with higher maternal age, multiparity, maternal gestational weight gain, maternal

glycemia, and earlier gestational age at delivery. Of the associations Mao et al looked at in his research, in our study, there was a positive association between maternal age and cord 24,25(OH)$_2$D$_3$ levels (r = 0.1, p = 0.03) and gestational age and cord percent 3-epi 25(OH)D$_3$ (r = -0.1, p = 0.02). Further studies conducted in diverse populations are needed to further explore the associations observed with 3-epi-25(OH)D$_3$ and 24,25(OH)$_2$D$_3$.

Limitations

Our study had several limitations. As our study was conducted within a larger study, it was not powered to detect the association of neonatal sepsis with 25(OH)D deficiency that has been observed in a systematic review. [59] Mothers enrolled in our study came from a tertiary referral institution in a major metropolitan area of Nigeria; given that a significant number of deliveries in the region occur in the home, the population studied was not reflective of the community's pregnant population. [84] Furthermore, the population may also not be representative of the country's diverse diet, climate, clothing styles, and urban versus rural regions, all factors that can affect 25(OH)D levels. Finally, a lack of detailed information on specific dietary intake and 25(OH)D supplementation that could affect the levels of 25(OH)D and metabolites obtained collected from the study participants.

CONCLUSIONS

Our study is the first study to highlight a significant positive association between the cord percent 3-epi-25(OH)D_3 with neonatal sepsis evaluation. Furthermore, 3-epi-25(OH)D_3 and percent 3-epi-25(OH)D_3 were associated with important maternal factors such as employment status, educational level, and mode of delivery, newborn outcomes associated with this metabolite include weight-for-age and weight-for-length. Larger prospective studies may help better characterize the biological and clinical significance of 3-epi-25(OH)D_3 and the percent 3-epi-25(OH)D_3 levels in pregnant mothers and their offspring.

CHAPTER 2: SPECIALIZED PRO-RESOLVING MEDIATORS AND CLINICAL OUTCOMES IN A POPULATION OF NIGERIAN AND U.S. MATERNAL INFANT DYADS

One of the causes of high neonatal mortality in Nigeria is prematurity. Interventions that could potentially allow to prolong gestation, thus allowing for fetal growth and improved birth anthropometrics, are crucial as part of a multiprong approach to improve neonatal survival in the region. Recent studies suggest that products from polyunsaturated fatty acids called specialized pro-resolving mediators (SPMs) may mediate inflammatory physiology in the intrauterine environment and thus playing a vital role in maternal-fetal health. In this chapter, we explore the first of such studies in Nigeria by looking at the status of SPMs, resolvin D1, and resolvin D2, levels in both maternal and cord blood of a population of pregnant women and their newborns. Associations to maternal and birth outcomes will be reported.

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ABSTRACT

Omega-3 fatty acids derived specialized pro-resolving mediators (SPMs), resolvin D1 (RvD1) and resolvin D2 (RvD2) attenuate inflammatory response and supplementation with omega-3 fatty acids during pregnancy have been associated with lower risk of preterm birth. This study was conducted first to assess the dynamics of SPMs production in Nigerian maternal infant dyads. The Nigerian cohort was then compared to United States (US) mother-infant dyads. Maternal and cord blood samples were collected at time of delivery in pregnant women in Abuja, Nigeria, and in Midwestern city in the United States (US). Descriptive statistics were calculated and correlations between variables were evaluated using Spearman tests. *P* values < 0.05 were significant. Mean maternal RvD1 (307.2 pg/ml) and RvD2 (313.92 pg/ml) levels in the Nigerian cohort were significantly lower than the mean maternal RvD1(16995.92 pg/ml) and RvD2(3499.03 pg/ml) in the US cohort ($p = 0.002$ and $p = 0.004$, respectively) Mean cord RvD1 (216.91 pg/ml) and RvD2 (329.22pg/ml) levels in the Nigeria cohort were higher than the mean cord RvD1 (181.1 pg/ml) and RvD2 (144.06 pg/ml) in the US cohort, however, the differences were not significant ($p = 0.788$ and $p = 0.091$, respectively). Maternal RvD2 in Nigerian levels were negatively correlated with maternal BMI and infant length. There was no difference between maternal and cord mediators in the Nigerian population. Variations in dietary customs, supplementation intake and underlying inflammatory processes between the two populations may account for the differences observed.

INTRODUCTION

Neonatal mortality in Nigeria is 35 per 1,000 and around 23.1 % of newborn deaths (death in the first 28 days of life) are associated with prematurity (live birth occurring at < 37 weeks gestational age). [4, 90, 91] In low-income settings, simple and cost-effective prevention strategies are needed to reduce the incidence of prematurity and subsequently improve neonatal mortality. Many intervention strategies to promote newborn health can be addressed through maternal nutrition. Maternal nutritional status before and during pregnancy plays a key role in providing the necessary nutrients for optimal fetal growth; adequate maternal nutrient intake can reduce the risk of preterm birth. [92] A modifiable and adaptable intervention, such as micronutrient supplementation to pregnant mothers, can be an important strategy in tackling prematurity. Given the importance of maternal nutrition on newborn outcomes, the WHO has recommended the use of a balanced diet with protein as well as supplements with iron and folic acid, in addition to calcium and vitamin A, particularly in populations where deficiencies are prevalent to improve newborn outcomes. [16]

One micronutrient not included in the WHO recommendations are omega-3 fatty acids. Supplementation with omega-3 fatty acids in high- and upper-middle-income countries has been associated in some reviews with a reduction in pre-term birth and more adequate birth anthropometric. [32, 33] while some reviews have not found these benefits. [93] Data, however, on their use as supplements during pregnancy or their baseline levels in maternal blood have not been reported in Africa in general nor specifically in Nigeria.

The three main omega-3 polyunsaturated fatty acids are alpha-linolenic acid (ALA), found mainly in plant oils, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) both found mainly in fish and other seafood, and dietary supplements. [94] EPA and DHA serve as

substrates for the biosynthesis of specialized pro-resolving lipid mediators (SPMs), that have potent anti-inflammatory and immunoregulatory effects, including resolvin D1 (RvD1) and resolvin D2 (RvD2).[95] RvD1 and RvD2 additionally have been found to have antimicrobial properties in studies involving activated macrophages. [29, 30]

During pregnancy, changes occur in the woman's body that allow for a controlled inflammatory milieu to favor implantation and fetal growth; studies have shown that excessive inflammation can lead to preterm birth, fetal growth restriction, and pre-eclampsia.[27, 28] Given that almost half of all preterm births are triggered by an inflammatory process at the level of the feto-maternal interface [96], omega-3 fatty acids and SPMs may potentially impact pregnancy outcomes through the regulation of inflammation during implantation, placentation, and parturition. [94, 97]

Mozurkewich et al. observed that SPMs precursors, upstream metabolite 17-hydroxy docosahexaenoic acid concentrations, in cord blood were higher than in maternal blood; this could be explained by the production and transfer of SPMs from the placenta to the fetus and/or synthesis by the fetus, the exact mechanism is not yet clear. [27, 98] During the first month of lactation, the levels of SPMs in human breast milk are up to 100-fold higher than what is found in adult serum and infants who transition from a soybean oil-based lipid emulsion to a fish oil-based lipid emulsion high in n-3 fatty acids have a higher SPMs production. [28, 99] Though the mechanism of this health benefit are not yet clear, these observations show that omega-3 fatty acids have an important role in infant health. [27]

The primary aim of this study was to assess the docosahexaenoic acid DHA-derived SPMs resolvin D1 and D2 (RvD1 and RvD2, respectively) levels in the plasma of pregnant Nigerian women and the cord blood of their newborns at the time of delivery. The secondary aim is to

compare RvD1 and RvD2 levels in 2 differing populations of maternal/infant dyads: one in an urban setting in a low-income country, Nigeria, and the other an urban setting in a high-income country, the United States (US), and their associated newborn outcomes.

METHODS

Recruitment

This pilot study was conducted within a larger observational cohort study on vertically transmitted infections and neonatal outcomes, called VTI, that was conducted at UATH from April 2016 to March 2018. UATH serves a peri-urban settlement located in Gwagwalada, Nigeria near Abuja, the Federal Capital Territory. A total of 2,586 pregnant women ≥18 years of age were enrolled in the VTI cohort over the course of the study, after informed consent, along with their offspring which accounted for 1,033 newborn males and 963 newborn females. The samples analyzed in our pilot study were collected at the time of delivery at UATH between April and May 2016 represented only 99 mother infant dyads, which were part of the first batch of samples sent from Nigeria to the US for quality assurance. At the time of our study, 2 parent pilot studies looking at other micronutrients used a portion of the samples for analysis. By the time our study was started only 78 mother samples, and 58 infant samples were available for RvD1 and RvD2 testing. In the US, pregnant women 18 years of age and older were enrolled with their newborn at delivery in the Nebraska Medicine Labor and Delivery Unit and the Newborn Nursery in the US Midwestern city of Omaha, Nebraska, where the delivery population had a racial and ethnic demographical profile that reflected that of the U.S. population. The samples were collected in the US around the same time of our Nigerian study. We had access to 116 maternal samples were tested for RvD1 and 117 for RvD2 and for the US infant samples: 95 tests for RvD1 and 111 for RvD2.

Ethical Approvals

Ethical approval for the study in Nigeria was granted by the ethics committee at UATH. Additionally, a separate IRB approval for the Nigerian study was obtained at the University of Nebraska Medical Center (UNMC), given some of the investigators, and the grant funding for the study came from that institution. A separate IRB approval was obtained from the UNMC for participant enrollment in the U.S.

Sample and Data Collection

This study, conducted both in Nigeria and in the US, was part of larger observational cohort studies looking at maternal-fetal outcomes, and thus the sample size studied was a convenient sample and not powered to test for SPMs in the populations being studied. Samples of both maternal and cord blood were collected from both populations from those who consented to participate at the time of delivery. In Nigeria, blood (ethylene diamine tetraacetic acid plasma (EDTA plasma)) was collected from 78 mothers, and cord blood (EDTA plasma) samples were collected from 58 infants at the time of delivery. In the US, blood (EDTA plasma) was collected from 136 mothers, and cord blood (EDTA plasma) samples were collected from 138 infants at the time of delivery. Inclusion criteria for the Nigerian population included maternal age ≥18 years of age, gestational age ≥24 weeks, infant age 0–7 days at enrollment (however, all mother/infant dyads were enrolled at or just before delivery), and absence of heavy peripartal vaginal bleeding. Inclusion criteria for the US population were all live births at the Nebraska Medicine Labor and Delivery Unit and the Labor and Delivery Nursery to mothers aged ≥19 years of age and able to give informed consent in English or with a medical interpreter. Exclusion criteria for the U.S. population included congenital abnormalities, gastrointestinal, liver, or kidney disease, or inborn errors of metabolism in the infant or the mother. Demographic

and clinical data from the Nigerian subjects were collected and entered an electronic capture tool, termed REDCap. Pertinent demographic and clinical data were collected from the U.S. population's electronic medical records.

SPMs Measurement

Maternal and cord plasma levels of RvD1 and RvD2 were measured using commercially available enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA) performed according to the manufacturer's directions. Assay results calculations were performed using the Cayman Chemical-provided enzyme immunoassay data analysis computer spreadsheet.

Clinical and Growth Outcome

Clinical data collected in both study populations included maternal age in years, body mass index (kg/m^2), maternal hemoglobin (g/dL), mode of delivery (vaginal versus cesarean), gender, prematurity (<37 weeks' gestation) and presence of chorioamnionitis (acute inflammation of the membranes and chorion of the placenta). Birth anthropometric parameters were measured for infant birth weight (g) length (cm) and head circumference (cm)

Statistical Analysis

Data were summarized using means, standard deviations, medians, ranges, counts, and percentages. Spearman correlation was used to look at the association of maternal and cord blood measurements, as well as the correlation of RvD1 and RvD2 with select clinical risk factors and outcomes that included maternal age, maternal body mass index, maternal hemoglobin level, gestational age, and newborn anthropometrics. Independent t-test or Fisher's exact test were used to compare maternal and cord RvD1 and RvD2 levels between populations for

sociodemographic, risk factors and newborn outcomes. Statistical significance was set at $p \leq 0.05$. IBM SPSS Statistics (Version 29) was used for all analysis.

RESULTS

Baseline Characteristics

Our study populations included 99 Nigerian and 137 US mother-infant pairs. The participant's characteristics are displayed in Table 5.

Nigerian and US Mothers

The median age for the Nigerian mothers was 31 and ranged from 18 to 40 while the median age for US mothers was 29 and ranged from 19 to 43. The Nigerian mothers on average were older than the US mothers and the difference was statistically significant (p = 0.002). The Nigerian mothers had a mean at delivery BMI of 31Kg/m^2(±4.2). which was significantly higher than the US mothers' mean pre-pregnancy BMI of 26.8Kg/m^2(±6.1), (p < 0.001). There was no statistical difference between the hemoglobin levels of the mothers in both study populations. In terms of outcomes for the mothers, there was no difference between the cohorts for mode of delivery. The US cohort, however, had 9 episodes of chorioamnionitis, while there were no cases reported in the Nigerian cohort and this difference was statistically significant. (p = 0.03)

Table 5 Demographic characteristics of the Nigerian and US study participants

0	Nigerian Mothers		US Mothers		*p* value
Continuous Variables	**N**		**N**		
		Median (Range)		**Median (Range)**	
Maternal Age (years)	98	31 (18-40)	137	29 (19-43)	
		Mean (SD)		**Mean (SD)**	
		31.1 (4.7)		28.9 (5.6)	0.002
BMI (kg/m2)	99	31 (4.2)	84	26.8 (6.1)	< 0.001
Hemoglobin (g/dL)	63	11.4(1.3)	135	11.7 (1.5)	0.08
Categorical Variables		**N (%)**		**N (%)**	
Mode of delivery					
Vaginal delivery		67 (67.7)		93 (67.9%)	0.97
Caesarean section		32 (32.3)		44 (32.1%)	
Chorioamnionitis Diagnosis					
Yes		0		9 (6.6)	0.03
No		81 (100)		128 (93.4)	

	Nigerian Infants		US Infants		*p* value
Continuous Variables	**N**	**Mean (SD)**	**N**	**Mean (SD)**	
Gestational Age at Delivery (weeks)	99	38.4 (2.4)	137	38.2 (3.2)	0.6
Infant Birth Anthropometrics					
Birth weight (g)	99	3086.2 (479.1)	137	3166.7 (719.9)	0.33
Birth length (cm)	99	49.3 (3.8)	137	48.5 (4.9)	0.19
Birth head circumference (cm)	97	34.4 (2.4)	137	33.6 (2.8)	0.04
Categorical Variables		**N (%)**		**N (%)**	
Premature					
Yes		15 (15.2)		22 (16)	0.85
No		84 (84.8)		115 (84)	
Gender					
Male		50 (50.5)		70 (51)	0.93
Female		49 (49.5)		67 (49)	

BMI was pre-pregnancy for US mothers and at delivery for Nigerian mothers;.

Nigerian and US newborns

There was no statistical difference between the gender, gestational age, birth weight and birth length of the Nigerians and US newborns. The mean head circumference of the Nigerian newborns, 34.4cm (±2.4), was significantly higher than the mean US newborn head circumference, 33.5cm (±2.8). ($p = 0.04$) Prematurity occurred in 15.2% of Nigerian births, while in the US births, it was at 16%, and there was no significant difference between the groups.

SPMs levels by study population

Of the 99 Nigerian mother-infant pairs we had in our overall study population, only 78 mothers and 58 infants had delivery plasma samples analyzed for RvD1 and RvD2. Of the 137 US mother-infant pairs, only 116 maternal and 95 infant samples were tested for RvD1 and 111 for RvD2. The mean, median, interquartile (IQR) range and overall range of plasma levels of mother and infant RvD1 and RvD2 are displayed for both populations in Table 6. The mean maternal levels of RvD1 and RvD2 were 307.8 pg/mL and 313.9 pg/mL, respectively in the Nigerian samples. The mean maternal levels of RvD1 and RvD2 were 16,996 pg/mL and 3,499 pg/mL in the US samples. The Nigerian mean RvD1 and RvD2 levels in were lower than the US mean RvD1 and RvD2 levels and the difference was statistically significant ($p = 0.002$ and $p = 0.004$ respectively)

Table 6 Comparison of Mean Nigerian and US Maternal/Infant RvD1 and RvD2 values

	Nigerian Samples				US Samples				
	N	Mean pg/mL	Median pg/mL (IQR)	Range pg/mL	N	Mean pg/mL	Median pg/mL (IQR)	Range pg/mL	*p*-value
Maternal RvD1	78	307.82	224.5 (204)	47 - 2052	116	16995.92	2616.5 (6343.5)	232 - 265355	0.002
Maternal RvD2	78	313.92	232 (253)	20 - 1170	117	3499.03	819 (1873)	3 - 74740	0.004
Infant RvD1	58	216.91	208 (102)	74 - 421	95	181.1	61 (49.7)	7.6 - 9882	0.788
Infant RvD2	58	329.22	268.5 (218)	107 - 872	111	144.06	6.4 65.5)	1.1 - 8404	0.091

The mean cord levels for RvD1 and RvD2 were 216.9 pg/mL and 329.2 pg/mL, respectively in the Nigerian samples compared to 181.1 pg/mL and 144.1 pg/mL in the US samples and the difference between study populations was not statistically significant.

Table 7 shows the comparisons between the mean RvD1 and RvD2 levels within the Nigerian and US mother infant dyads. The mean Nigerian mother RvD1 level was significantly higher than the mean Nigerian cord RvD1 level. ($p = 0.04$). There was no significant difference between the mean Nigerian mother and cord RvD2 levels. Both the mean US mother RvD1 and RvD2 levels were significantly higher than the mean US cord RvD1 and RvD2 levels with $p < 0.001$ in each case.

Table 7 Comparing the mean RvD1 and RvD2 levels within the Nigerian and US Mother Infant Dyads

	Maternal RvD1	Infant RvD1	p value	Maternal RvD2	Infant RvD2	p value
Nigerian Samples	N = 78	N = 58		N = 78	N = 58	
Mean pg/mL	307.82	216.91	0.04	313.92	329.22	0.68
US Samples	N = 116	N = 95		N = 117	N = 111	
Mean pg/mL	16995.92	181.1	< 0.001	3499.03	144.06	< 0.001

Table 8 displays the correlation between maternal infant RvD1 and RvD2 levels within the Nigerian and the US study populations. In the Nigerian population, maternal RvD1 levels were positively correlated with maternal RvD1 (r = 0.738), cord RvD1 (r = 0,318) and cord RvD2 (0.343) with p <0.001, p = 0.009 and p = 0.02, respectively. Additional Nigerian maternal RvD2 levels were positively correlated with infant RvD1 levels (r = 0.316, p = 0.032) but not with cord RvD2 levels. Cord RvD1 levels were positively correlated with Cord RvD2. (r = 0.697, p < 0.001)

In the US cohort, maternal RvD1 and RvD2 plasma levels were positively correlated with r = 0. 639 and p < 0.001. Maternal RvD2 was negatively correlated with cord RvD2, r = -0.205 with p = 0.043. In the cord RvD1 and RvD2 were also positively correlated with r = 0.380 and p < 0.001. Finally, cord RvD1 was positively correlated with cord RvD2 with r = 0.38 and p < 0.001.

Table 8 Correlation of Maternal Infant RvD1 and RvD2 levels in Nigerian and US Samples

Nigerian Samples

	Maternal RvD1 N = 78		Maternal RvD2 N = 78		Infant RvD1 N = 58		Infant RvD2 N = 58	
	r	***p*-value**	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
Maternal RvD1	NA		0.738	<.001	0.381	0.009	0.343	0.02
Maternal RvD2	0.738	<.001	NA		0.316	0.032	NS	
Infant RvD1	0.381	0.009	0.316	0.032	NA		0.697	<.001
Infant RvD2	0.343	0.02	NS		0.697	<.001	NA	

US Samples

	Maternal RvD1 N = 116		Maternal RvD2 N = 117		Infant RvD1 N = 95		Infant RvD2 N = 111	
	r	***p*-value**	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
Maternal RvD1	NA		0.639	<.001	NS		NS	
Maternal RvD2	0.639	<.001	NA		NS		-0.205	0.043
Infant RvD1	NS		NS		NA		0.38	<.001
Infant RvD2	NS		-0.205	0.043	0.38	<.001	NA	

NA not applicable, NS not significant

Table 9 displays the correlation of maternal-infant RvD1 and RvD2 levels with maternal risk factors and newborn outcomes in both the Nigerian and US cohorts. In the Nigerian mother-infant group, maternal BMI was negatively correlated with maternal RvD2 levels with r = -0.288, *p* = 0.033, and birth length was also negatively correlated with maternal RvD2 with r = -0.281, *p* = 0.028. Maternal and cord RvD1 and cord RvD2 in the Nigerian samples did not show any significant correlation with any other of our maternal risk factors or outcome measures.

In the US cohort, birth weight was negatively correlated with RvD2 with r = -0.200, p = 0.019 and birth head circumference was negatively correlated with maternal RvD2 for and r = -0.254, p = 0.006. Maternal and cord RvD1 and cord RvD2 in the US samples did not show any significant correlation with any other of our maternal risk factors or outcome measures.

Table 9 Correlation of Maternal Infant RvD1 and RvD2 levels with demographics & outcome in Nigerian and US samples

Nigerian Samples

	Maternal RvD1 N = 78		Maternal RvD2 N = 78		Infant RvD1 N - 58		Infant RvD2 N - 58	
	R	*p*-value	R	*p*-value	R	*p*-value	R	*p*-value
Maternal Age	NS		NS		NS		NS	
Maternal BMI	NS		-0.288	0.033	NS		NS	
Gestational Age	NS		NS		NS		NS	
Infant birthweight	NS		NS		NS		NS	
Infant Length	NS		-0.281	0.028	NS		NS	
Infant Head Circumference	NS		NS		NS		NS	

US Samples

	Maternal RvD1 N = 116		Maternal RvD2 N = 117		Infant RvD1 N = 95		Infant RvD2 N = 111	
	R	*p*-value	R	*p*-value	R	*p*-value	R	*p*-value
Maternal Age	NS		NS		NS		NS	
Maternal BMI	NS		NS		NS		NS	
Gestational Age	NS		NS		NS		NS	
Infant birthweight	NS		-0.2	0.019	NS		NS	
Infant Length	NS		NS		NS		NS	
Infant Head Circumference	NS		-0.254	0.006	NS		NS	

R = correlation coefficient, NS not significant

DISCUSSION

Comparing SPM levels between the Nigerian and US cohorts

Comparing the SPM values between populations, we observed that the mean levels of maternal RvD1 and RvD2 were significantly lower in the Nigerian cohort compared to the US cohort. ($p = 0.002$ and $p = 0.004$ respectively). Though the difference was not statistically significant, the Nigerian newborns had higher RvD1 and RvD2 levels than the US newborns at delivery. Additionally, we found that mean maternal RvD1 and RvD2 in the US cohort were significantly higher than US cord RvD1 and RvD2, respectively, with $p < 0.001$ for both, whereas in the Nigerian cohort, only the mean maternal RvD1 levels were significantly higher than the mean Nigerian cord levels. ($p = 0.04$). This finding has been described previously with a similar US cohort but has not been described with a Nigerian cohort. [28] Furthermore, the Nigerian RvD1 levels varied from 47 to 2552 pg/ml, and the RvD2 varied from 20 to 1170 pg/ml, while in the US cohort, RvD1 levels varied from 232 - 265355pg/ml and the RvD2 varied from 3 - 74740pg/ml. These observations could suggest that at baseline the Nigerian diet provides overall about the same levels of omega-3 fatty acid. Though we did not collect data on the ethnicity of the participants in the Nigerian cohort, we know that the study was conducted in an ethnically diverse community served by a tertiary referral hospital. Another consideration would be that across ethnicities in our study setting, food preparation seems to provide around the same content of omega-3 fatty acid because it is more likely made of the local readily available ingredients even if certain dishes are prepared differently. Studies on diet diversity in Nigeria have shown that ethnic foods are diverse but there are some staple base items like the Nigerian stew. [100] The US-based cohort was also diverse, with the population attending a midwestern hospital representing the surrounding community. the wide variations could reflect differing supplemental intake and baseline dietary practices that rely less on common food staples,

compared to the Nigerian cohort, and could be strongly influenced by socio-economic status. [101, 102]

Another possible explanation for the higher RvD1 and RvD2 levels observed in the US pregnant cohort compared to the Nigerian one could be due to differences in the inflammatory status of both populations. Given that RvD1 and RvD2 have anti-inflammatory properties, when there is inflammation, their levels increase. [27, 28] The US cohort could have had a higher pregnancy inflammatory baseline status compared to the Nigerian cohort. More studies would be needed to further elucidate the reasons for these differences. With such high SPM levels in the US maternal cohort, proportionately high US cord SPM levels would be expected to reflect an efficient maternal-fetal transfer or efficient production of SPM by the fetus using maternal SPMs as substrate, however, the US infant cord levels were even lower than the Nigerian levels. This observation suggests that the source of cord SPMs may be independent of the maternal SPM production. This observation might suggest that beyond a certain level of maternal RvD1 and RvD2 there might no longer any further placental transfer of RvD1 or RvD2 to the newborn near or at delivery and the newborn source of RvD1 and RvD2 may not be synthesizing its SPM from maternal placental stores but will rather shift to human breast milk, especially given that the levels of SPMs in human breast milk are up to 100-fold higher than what is found in the adult serum during the first month of lactation. [28, 99]

Furthermore, US and Nigeria SPM levels could be explained by genetic differences in the ability to metabolize omega-3 fatty acids to SPMs; studies have shown that genetic variations of the fatty acid desaturase (FADS) locus on chromosome 11 are associated with the capacity of tissues to synthesize long-chain polyunsaturated fatty acids. [103, 104] Furthermore, this is the

first study comparing SPM levels between two populations of mother-infant dyads, one based in a low-income country and the other in a high-income country, the differences in SPMs levels could be related to dietary customs coupled with socio-economic status, however, the dietary intake in the US populations is low, we do not have data on the Nigerian populations.

SPMs levels and maternal risk factors and birth anthropometrics

Maternal RvD2 levels in both study populations were the only SPM levels that were significantly associated with maternal risk factors and birth anthropometrics in our study. In terms of maternal risk factors, there was a negative correlation between maternal BMI and RvD2 levels while this was not observed in the US cohort. Higher BMIs in young women have been associated with lower omega-3 fatty acid levels. [105] However, while the BMI from the US cohort was based on the pre-pregnancy weight, the BMI information collected from the Nigerian cohort was measured at delivery, and this is a limitation in our ability to adequately interpret this information between our two study populations. Obtaining pre-pregnancy data was difficult as most mothers in the region do not usually know their pre-pregnancy weight, and this has been an issue with studies conducted that require pre-pregnancy weight values.[106]

Additional interesting findings from our study, in the Nigerian cohort, there was a significant negative correlation between the RvD2 levels and the newborn length ($r = -0.281$, $p = 0.028$) while in the US cohort, there was a significant negative correlation between the RvD2 levels and the newborn weight ($r = -0.2$, $p = 0.019$) and birth head circumference ($r = -0.54$, $p = 0.006$). The levels of these SPMs are dependent both on the amount of available substrate, omega-3 fatty acid from supplementation or diet, and the inflammatory conditions and associated risk factors during pregnancy, [28] In a review of meta-analyses of randomized trials on the effects of omega-3 polyunsaturated fatty acids supplementation in pregnancy, lactation, and infancy, supplementation was found to be significantly associated with a reduced risk of low

birth weight and improved head circumference measurements. [107] In another systematic review of randomized controlled trials on the impact of omega-3 fatty acid intake in pregnancy on maternal health and birth outcomes, the authors reported that supplementation was associated with increased birth weight and decreased risk of low birth weight.

Finally, another study looking at the association between first-trimester omega-3 fatty acid supplementation and fetal growth trajectories found no significant associations in the weekly differences in measured fetal femur and humerus length in our study, though we did not observe any association between newborn length with RvD1 and RvD2 levels. So far, studies show that there are mixed results on the role that omega-3 fatty acids may play in newborn growth measures. These studies also show that repeated measures are necessary to monitor trends in fetal and infant growth and development to see if omega-3 fatty acids are beneficial. In our study, the associations that we observed with anthropometrics in both the Nigerian and US populations are more of a snapshot, and repeated measures, particularly since our findings involved a correlation, would have helped us better understand the correlations observed in our population.

Participant characteristics

Nigerian and US mothers

Overall, our two populations had significant differences in their demographics and outcome characteristics. The Nigerian mothers were older on average ($p = 0.002$) and had a significantly higher BMI ($p < 0.001$). There was no statistical difference between maternal hemoglobin levels between both groups, and on average the mothers of both groups were not anemic based on WHO classification as the mean hemoglobin level for both groups was ≥11gm/dL. [108]

When analyzing pregnancy outcomes between both groups, there was no difference in the mode of delivery. However, chorioamnionitis occurred significantly more times in the US population whereas there were no episodes in our Nigerian cohort. Of note, however, we only had documented data for 81 of the 99 Nigerian mothers. 18 mothers had no recorded information regarding a chorioamnionitis diagnosis. Chorioamnionitis amnionitis is not a rare occurrence in pregnancies in Nigeria and when it is diagnosed, it is usually based on clinical findings. The clinical findings may underestimate the true prevalence of chorioamnionitis as compared to the histologic findings. A study in Enugu showed that of the 136 deliveries in the teaching hospital, 50% of them had histopathologic findings of chorioamnionitis but only 16% of those cases were diagnosed clinically. [109] In our study population, they would have only found clinical chorioamnionitis locally, while in the US, access to histopathology would make diagnosing chorioamnionitis more reliable and consistent. Given that chorioamnionitis is an inflammatory process, a more accurate report of the cases of chorioamnionitis in the Nigerian cohort, would allow us to assess its association with the levels of RvD1 and RvD2, which have anti-inflammatory properties.

Nigerian and US babies

When analyzing the birth outcomes between both study populations, there was no difference in gender, gestational age, and birth anthropometrics except that the Nigerian newborns had a significantly larger mean head circumference than the US newborns. ($p = 0.04$) this difference is due to phenotypic differences between populations. In a study by Vafai et al, looking at the association between first-trimester omega-3 fatty acid supplementation and fetal growth trajectory, a significant increase in head circumference through the pregnancy was observed for the mothers who reported omega-3 fatty acid supplementation. [110] The Nigerian diet, in general, according to a systematic review by Petrikova et al, looking at literature with

mainly household-level survey data describing the common Nigerian diet, has shown that it is high in total polyunsaturated fatty acids, fish, plant omega-3 fatty acids. [111] Another important limitation was that we did not survey the Nigerian mothers specifically on their diet, nor we did not specifically ask our Nigerian population about omega-3 fatty acid supplement intake.

Omega-3 fatty acids and cost

A cost-effective strategy is important when considering interventions in low-income countries. Cost and commercial availability of these supplements in low-income countries may be one of the reasons the WHO recommendations do not include omega-3 fatty acids supplementation. A South African study looking at the omega-3 fatty acid content of South African fish oil supplements found that to meet the International Society for the Study of Fatty Acids and Lipids recommendations of 500 mg EPA + DHA per day for the prevention of cardiovascular disease, it comes at a considerable cost to the average South African consumer. [112] Additional concerns were the lack of regulatory structure and quality assurance for the potency and purity of the supplements. [112] However, there are current research studies looking at alternative cost-effective and sustainable pathways for omega-3 fatty acid production, none of which involves using microalgae cultivation. [113] A narrated review of alternative natural sources of bioavailable omega-3 DHA for the promotion of mental health in East Africa also identified microalgae. [114] The authors proposed It as a better alternative than local fish oil due to concerns of heavy metal and antibiotic contamination in the region. [114] Our study did not look specifically look at the cost of supplements and accessibility of these to our study population, but given that the Nigerian diet in the aforementioned systematic review is rich and there are reports on the continent on alternative sustainable ways to produce them for supplementation, continued research in further characterizing the dynamic of SPM in mother infant dyads is useful and may have a locally sourced options.

Limitations

During the discussion we mentioned some limitations to our study. In addition, our sample size, particularly for the Nigerian cohort, was very small and our study was not powered to detect differences between study populations. Furthermore, an especially important limitation was that we had many missing variables in our data collection which may have impacted our results within each study population and across the populations as well. An additional important limitation is that about 60% of the births in the region occur in the home. This means our study population is missing participants from a more rural setting that may have different dietary practices that could have influenced the SPM profile of our Nigerian study population. In addition to obtaining a detailed diet intake, sampling levels over time pre-delivery could have also provided a better picture of the dynamic of the SPMs during pregnancy and how it could have affected newborn outcomes.

CONCLUSION

Maternal RvD1 and RvD2 concentrations were significantly lower in the Nigerian population compared to the US population while there were no significant differences between the cord concentrations of RvD1 and RvD2 between the two populations. Variations in dietary customs between the two populations may account for the major differences, but there could also issues with how the different populations metabolism of omega-3 fatty acids. Furthermore, cord RvD1 and cord RvD2 may not be dependent of maternal levels at the time of delivery given that high maternal RvD1 and RvD2 values in the US cohort did not proportionate maternal RvD1 and RvD2 values. This could indicate that near delivery, infant may be preparing to rely more on exogenous sources of omega-3 fatty acids for their growth via human breastmilk which has significantly high levels of omega-3 fatty acids during the first month of lactation. Obtaining a

detailed diet intake in our Nigerian study population, along with environmental, geographic, and economic factors, and sampling SPM levels over time during pregnancy and at birth could have provide a better picture of the dynamic of the SPMs during pregnancy and help better characterize these levels with newborn outcomes. Collecting these data will help inform locally relevant evidence-based strategies for the Nigerian population on omega-3 fatty acid consumption, based on local diet, and supplementation, that could promote healthy pregnancy and newborn outcomes.

CHAPTER 3: CHARACTERIZING THE PREVALENCE OF *PLASMODIUM SPECIES* PLACENTAL INFECTIONS IN A POPULATION OF ASYMPTOMATIC PREGNANT NIGERIAN WOMEN

Newborn health relies on mother for many of the nutrients needed for growth as we have seen in the previous chapters with vitamin D and polyunsaturated fatty acids. The vehicle for this transfer is the placenta, therefore a healthy placenta is crucial. In a region with high malaria transmission like in Nigeria, pregnant women, are particularly susceptible to malaria as the parasite can be sequestered in the placenta this affecting fetal outcome. Intermittent preventative treatment of malaria for pregnant women in Nigeria in addition to measure like the use of insecticide impregnated nets have decrease the incidence of malaria. The new malaria vaccine R21/Matrix-M vaccine approved in April 2023 will further reduce the incidence of infection. However, of note the current measures are target Plasmodium falciparum, the most common strain, however, there has been a rise in non-falciparum strains. Further understanding of the epidemiology of the non-falciparum strains is crucial for a comprehensive malaria elimination program. In this chapter, we explore the prevalence of different plasmodium species in the placenta of asymptomatic pregnant Nigerian women. The results will provide more data to inform a multiprong approach to improve newborn survival.

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ABSTRACT

In 2020, 46.1 million of the 121.9 million pregnancies that occurred in areas of the world with endemic malaria transmission occurred in Africa. in Africa, Nigeria has one of the largest rates of complications associated with malaria infection in pregnancies, ranging from 8.4% to 58.1%. These include preterm births, low birthweight, and maternal and fetal demise. Efforts to better understand the epidemiology of malarial infections are crucial to develop evidence-based interventions to decrease adverse outcomes. The purpose of this study was to determine the prevalence of different *Plasmodium species* in the placenta of asymptomatic pregnant women > 18 years of age at the time of delivery at the University of Abuja Teaching Hospital in Gwagwalada, Nigeria between September and October of 2016. Chi-square was used to examine the association between two independent categorical variables, Spearman correlation coefficients to compare the means of two continuous variables, and Pearson correlation one dichotomous categorical with a continuous variable. In total, 104 placental samples and 106 cord blood were analyzed. *Plasmodium species* were identified by immunohistochemical staining and by nested PCR assays. Of the samples stained for malaria 7% (7/99) were positive. Malaria nested PCR assays were positive for at least one infection in 38.5% (40/102) of the specimens tested. The most detected species were *P. ovale wallikeri* (27.9%; 29/104), *P. knowlesi* (21.2%; 22/104) and *P. falciparum* (5.8%; 6/104). There were 16 cases of single infection with *P. ovale wallikeri* (9), *P. falciparum* (4), *P. vivax* (2), *P. ovale curtisi* (1). Dual species infections occurred in 22 cases and triple species infections in 2 cases. *P. knowlesi* was detected only in mixed infections, while *P. malariae* was not detected at all. Malaria PCR positivity was not significantly associated with maternal anemia, gestational age, use of sulfadoxine and pyrimethamine prophylaxis, placenta weight, placenta malarial stain, chorioamnionitis, funisitis, prematurity, birth anthropometrics, newborn survival at 7 or 28 days. This is the first report of *P. knowlesi* in

the African region. Larger studies looking symptomatic and asymptomatic participants are needed to assess the clinical significance of this finding.

INTRODUCTION

In 2020, the African region accounted for 46.1 million of the 121.9 million pregnancies that occurred in areas of the world with endemic malaria transmission. [115] Malaria infection during pregnancy poses serious risks to the mother, the fetus, the newborn baby, and the infant child as it can lead to maternal death, maternal anemia, stillbirth, preterm birth, low birth weight, and neurodevelopmental delays in infants. [35-38] In Africa, Nigeria has one of the largest complication rates associated with malaria infection in pregnancies ranging from 8.4% to 58.1% of pregnancies. [34] Thus, efforts to better understand the epidemiology of malaria infections in the country and the spectrum of species involved, in addition to current preventative measures such as screening, diagnosis, and treatment with most recent vaccinations, are crucial to decreasing adverse pregnancy outcomes. [39]

Epidemiology of Plasmodium species

Malaria infection occurs due to the protozoan parasite *Plasmodium* which has five species known to infect humans: *P. falciparum*, *P. vivax*, *P. ovale* spp.*, P. malariae*, and *P. knowlesi*. [116] *P. falciparum* and *P. vivax* are the most prevalent species worldwide with *P. falciparum* being the major species in the African region, while *P. vivax* is more globally distributed. [117, 118] Global efforts to eliminate malaria have reduced *the P. falciparum burden in many regions. However, there has been an increase in malarial infections associated with P. vivax* in areas where both species coexist. [119] The highest rates of complications and mortality are associated with *P. falciparum* while *P. vivax* is less fatal but can cause relapsing infections; they have differing life cycles, whereas P. vivax has dormant liver stage hypnozoites that can activate and invade the bloodstream weeks to years following an initial infection, leading to recurrent malarial infections. [117, 118] Of the two, P. falciparum is the most common in

pregnancy; P. vivax, though less common, can also lead to adverse outcomes in pregnancy, such as maternal anemia and low birth weight. [120] *P. ovale* spp. (subspecies *P. ovale curtisi* and *P. ovale wallikeri*), and *P. malariae* have globally low prevalence and lower disease severity as compared to *P. falciparum* and *P. vivax*. [121] *P. malaria* is prevalent in individuals of all age groups. However, *P. ovale spp.*, which has dormant liver-stage hypnozoites like *P. vivax*, was found to be more prevalent in pregnant women than adults and children. [121] Diagnosis of *P. malariae* and *P. ovale* spp. is often complicated as the species are often misidentified as *P. falciparum* and *P. vivax,* respectively. *P. knowlesi* is a zoonotic malaria parasite endemic in Southeast Asia whose primary host is the macaque and infections in humans are considered as accidents in the parasite life cycle. *P. knowlesi* can lead to a broad spectrum of clinical disease from asymptomatic infection to severe malaria and death. [122] Though *P. knowlesi* infection is relatively rare during pregnancy, it may still be, however, associated with adverse maternal and pregnancy outcomes such as anemia. [123]

Malaria diagnosis

Malaria control with disease management and surveillance requires early and accurate diagnosis followed by effective treatment. The World Health Organization (WHO) recommends using microscopy or a rapid diagnostic test (RDT), which detects parasite histidine-rich proteins (HRP), prior to initiating treatment in all patients suspected of malaria, as misdiagnosis can lead to significant morbidity and mortality. [124] In endemic countries, the examination of thick and thin blood film using microscopy is considered the gold standard for malaria diagnosis due to its high sensitivity, low cost, and ability to quantify parasitemia and identify the species. [125] There are some important limitations, however, as it requires skilled microscopists and strict quality control that are often unavailable in many at risk low resource settings. Furthermore, in cases of

low parasitemia or mixed infections, the accuracy of microscopic-based detection decreases. The main advantage of RDTs is their simplicity to use and interpret with a short time to results (~ 5–20 min). However, in low transmission settings, RDTs do not reliably detect low-density parasitemia. [125] They are unable to detect parasites lacking HRP. [126] RDT sensitivity is highest in detecting first P. falciparum and secondarily in detecting *P. vivax* infections. [127] A systematic review looking at the performance of RDTs for the detection of *P. malariae*, *P. ovale* spp., and *P. knowlesi*, found their performance to be suboptimal. [128] Polymerase chain reaction (PCR)-based methods are important tools to diagnose malarial infections as they allow the detection of parasites even at low density, the accurate identification of parasite species and the identification of mixed infections. [43] Significant limitations include access to PCR equipment, trained technicians, and increased time to perform the test compared to microscopy and RDT, which decreases the utility of PCR-based detection for clinical diagnosis. [43]

Malaria in pregnancy

Women during pregnancy have an increased susceptibility to malaria infection, and their risk of severe disease is based on the patterns of transmission in the region where they reside and their parity. [129] When a pregnant woman is infected with *P. falciparum*, the circulating infected erythrocytes are sequestered in the placenta and then infiltrate the immune cells within its intervillous spaces. Deposition of malarial pigment in the placenta turns it black in color with the thickening of its basement membranes, which affects the effective transfer of nutrients to the fetus for its development. [41] Three major structures form the placenta: the placental disc, the chorioamniotic membrane, made up of the chorion, the outer layer and the amnion the inner layer that surrounds the developing fetus, and the umbilical cord. When an inflammatory process affects the placenta, which can occur with infections, neutrophils infiltrate these

structures leading to inflammation of the chorioamniotic membrane, known as chorioamnionitis, and inflammation of the umbilical cord, known as funisitis. [130] Malaria infection has been associated with histologic findings of epithelial degeneration and denudations consistent with chorioamnionitis. [131]

The increased susceptibility to malaria infections during pregnancy leads to high parasitic infiltration of the placenta vasculature, and parasitemia is much higher in the placenta compared to peripheral blood. Primigravid women are more susceptible to infection than those who have had several pregnancies as each pregnancy confers added protection due to the development of placental-specific immunity that increases with every pregnancy. [132] In areas of low malaria transmission, infections are infrequent, and subsequently, the susceptible population has low immunity. This means that should there be an infection, there is a higher likelihood of rapidly progressing to severe disease. [129, 133] In contrast, in areas of high malaria transmission, the susceptible population, which often gets infected multiple times, develops stronger immunity and thus shows few or no symptoms with each infection. [129, 133] Additionally, in these areas of high transmission, when a woman's first malaria infection occurs during pregnancy, the symptoms are the most severe, with higher rates of adverse outcomes such as miscarriage, premature delivery, newborns with low birth weight, and even neonatal death. [134] Women with immunity due to prior infections, though they have fewer symptoms when they contract malaria, during pregnancy they still remain susceptible to adverse pregnancy outcomes such as anemia and low birth weight. [129, 133] Moreover, first-time pregnant women are at high risk of severe malaria due to *P. falciparum* even if they lived in a high transmission area, had long-term exposure to the parasite, and developed protective antibodies; once they become pregnant for the first time, they are susceptible again to infection with a high risk of severe malaria. [135]

Asymptomatic malaria infection

Subclinical *P. falciparum* infections in pregnant women, which are more frequent than symptomatic infections in Africa, can cause placental infections that lead to adverse maternal-fetal outcomes. [136] Subclinical infections often have low parasitemia making conventional diagnostic methods such as microscopy and RDT less sensitive, especially in programs aiming at screening pregnant women. [136] Several studies using PCR assays have documented the prevalence of asymptomatic malaria in pregnant women in various African regions: 17.2% in Mozambique, 18.1% in Ethiopia, and 19% in the Congo. [136-138] There is one Nigerian report from 2009 where the prevalence of asymptomatic malaria in pregnant women in Lagos was 7.7%, however, in this study, blood smears stained with Giemsa and microscopy was used for diagnosis. [139] Since the sensitivity of microscopy in asymptomatic infection with low parasitemia is low, the true prevalence in this setting may be higher. There are no studies reported on asymptomatic pregnant women in Nigeria using PCR assays.

The purpose of this pilot study was to determine the prevalence of different *Plasmodium* species in the placenta of a population of asymptomatic pregnant women at the time of delivery at the University of Abuja Teaching Hospital (UATH) in Gwagwalada, Nigeria.

METHODS

This pilot study was conducted within a larger observational cohort study on vertically transmitted infections and neonatal outcomes that was being conducted at UATH from April 2016 to March 2018. Ethical approval for the study was granted by the ethics committee at UATH. Additionally, a separate IRB approval was obtained at the University of Nebraska Medical Center (UNMC) given some of the investigators and the grant funding came from that institution.

UATH serves a peri-urban settlement located in Gwagwalada, Nigeria near Abuja, the Federal Capital Territory. A total of 2,586 pregnant women ≥18 years of age were enrolled in the large observational cohort, after informed consent, along with their offspring which accounted for 1,033 newborn males and 963 newborn females. The samples analyzed in our pilot study were obtained during the months of September and October of 2016, which corresponds to the rainy season in the region, and during that time, 267 enrollment visits occurred. The consent for our pilot study was included in the overall informed consent for the larger observational cohort and most mothers agreed to the study in the months our pilot study samples were collected with only 3 mothers refusing participation. The study inclusion criteria were that the participants had to be asymptomatic which was defined as being without any clinical symptoms of malaria at the time of delivery. Based on the timing of delivery of previously consented participants and study personnel availability, 104 placental samples were collected between September and October, and 35 samples between November and December of 2016.

Data Collection

At enrollment clinical and sociodemographic data were collected from mothers and stored in the electronic data capture, REDCap, and include maternal age in years, body mass index (BMI) in kg/m^2, gestational age in weeks, hemoglobin levels in g/dl, employment status, educational level, HIV status and use of malaria prophylaxis. Anemia in pregnancy was defined using World Health Organization (WHO) classification of < 11g/dl.[108] Malaria prophylaxis in the study consisted of the use of intermittent presumptive treatment in pregnancy (IPTp) of malaria using sulfadoxine-pyrimethamine per National Nigerian Pregnancy Guidelines. [140] Pregnancy outcome data included placental weight in grams (g), birth weight in kilograms (kg), birth length in centimeters (cm), birth head circumference in cm. Birth anthropometric

parameters were calculated for infant birth weight, length, and head circumference using international standards from the INTERGROWTH-21st Project and reported in percentiles. [72] Birth growth parameter z-scores were constructed using the WHO Child Growth Parameter's Anthro software for SPSS (SPSS Inc., Chicago, IL, USA). Additional data collected for the newborn were gender, mode of delivery (vaginal or caesarean section) and gestational age in weeks. Prematurity was defined < 37 weeks gestational age per WHO guidelines. [91] Data collected on the placenta included placenta weight (g), immunohistochemical stain and PCR for malaria, placental blood bacterial cultures, and reports on histologic evidence of chorioamnionitis and funisitis. On days 7 and 28 of life, study personnel conducted follow-up phone interviews with mothers to assess infant well-being, interval illness or hospitalizations for sepsis evaluation since birth.

Immunohistochemical staining

Plasmodium spp. detection was performed using two different methods. The first was histological evaluations of fixed placental specimens. From each placenta at delivery, a section of the umbilical cord and a full-thickness large core were taken from the central disc 1 cm from the center/cord insertion site to include the covering membranes and maternal surface. Sections were placed in one tissue cassette and stored at room temperature in a 50 mL vial with formalin-free FineFIX (Milestone Srl, Sorisole, Torre Boldone, Italy). [141] Placenta and cord sample were then packaged and shipped to the UNMC Tissue Science Facility (TSF) for processing, embedding, sectioning, and hematoxylin and eosin (H&E) staining per standard protocols. The study pathologist reviewed each placenta/cord case. Results were reported as positive or negative immunohistochemical stain for malaria. Sections were evaluated for evidence of acute chorioamnionitis, and other histologic evidence of ascending or transplacental infection. Those

cases with H&E changes suggestive of the former had immunoperoxidase staining performed by the TSF for cytomegalovirus (CMV), herpes simplex virus (HSV), toxoplasma, and syphilis.

Nested PCR

The second method of detection was using a qualitative nested polymerase chain reaction (PCR) of placental samples to detect the 5 *Plasmodium* species that infect humans (*P. falciparum*, *P. vivax*, *P. ovale curtisi, P. ovale wallikeri*, *P. malariae*, and *P. knowlesi*). As a negative control, placenta tissue was obtained from 3 malaria-negative donors in Omaha, Nebraska. Paraffinized placental samples were sectioned using a microtome at the UNMC TSF. For each sample, four sequential sections, each 5 µm thick, were pooled into a 1.5 mL tube and delivered to the Ng lab at UNMC for further analyses. Placental samples were deparaffinized in a non-toxic manner by the addition of 300 µl mineral oil (Acros Organics) and incubation at 95°C for 20 min [142]. Uninfected red blood cells (RBC) were purchased from the American Red Cross (Omaha, NE). DNA was isolated from placental samples and red blood cells according to the manufacturer's protocol using DNeasy Blood and Tissue Kit (QIAGEN, Venlo, Netherlands), and DNA concentration was determined using Nanodrop 2000 (ThermoFisher).

Nested PCR was performed with slight modifications. [143, Komaki-Yasuda, 2018 #388] Briefly, the first PCR reaction used primers (p1 and p2) that recognized the 18S small subunit ribosomal ribonucleic acid (18S SSU rRNA) of all six *Plasmodium* species that infect humans. For the first PCR, we used 25 ng DNA template (placental samples or RBC) or 0.2 ng plasmid DNA, 0.2 µM each of p1 and p2 primers, 200 µM dNTPs, 2.5 mM $MgCl_2$, and 1.25 U Taq polymerase in a 25 µL reaction. Cycling conditions were: 92°C, 2 min; 25 cycles of (92°C, 30 sec; 58°C, 30 sec; 62°C, 25 sec); 62°C, 5 min. PCR products of the first reaction were diluted 1:1000 in DNA-grade water (Fisher Scientific, Hampton, NH) and 2 µL was used as the template for the subsequent species-specific PCRs with Dream Taq Green PCR 2X Master Mix (ThermoFisher Scientific,

Waltham, MA). Amplification was performed using 0.2 µM each of p1 and the species-specific primers, in a 25 µL reaction. Cycling conditions were 92°C, 2 min; 25 cycles of (92°C, 45 sec; 60°C, 15 sec; 60°C, 5 sec); 62°C, 5 min [43]. A fifth (5 µL) of the PCR product was separated on a 2% agarose gel electrophoresis. If faint bands were questioned, a re-PCR was performed with 2 µL of the PCR product as a template for further confirmation.

Plasmids encoding partial sequences of 18S SSU rRNA genes from *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, and *P. knowlesi* were kindly provided by Dr. Shigeyuki Kano (Department of Tropical Medicine and Malaria, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan). The primers ordered from Integrated DNA Technologies (IDT, Coralville, IA) included universal primers designed for the conserved sequence of the SSU rRNA genes from the *Plasmodium* species and inner primers designed for the specific sequences of genes from *P. falciparum* (Pf), *P. vivax* (Pv), *P. ovale curtisi* (Poc), *P. ovale wallikeri* (Pow), *P. malariae* (Pm), and *P. knowlesi* (Pk). [43] To verify that these PCR conditions could be replicated in the Ng lab and with their modifications to the protocol, plasmids encoding each of the five *Plasmodium* spp. (Pf, Pv, Poc, Pow, Pm, Pk) and uninfected red blood cells (u1, u2) were used as templates for the first PCR (p1+p2), which recognizes the 18S SSU rRNA of these *Plasmodium spp*.

Using primers p1 and p2, a PCR was performed using 0.2 ng plasmid DNA encoding *P. falciparum* 18S SSU rRNA as a template, with the conditions described above. 2.2 ng of the PCR product was calculated to be equivalent to a plasmid copy number of 2×10^{10}. Ten 10-fold dilutions of 2.2 ng PCR product were titrated in DNA-grade water to the lowest concentration of 2×10 copy numbers. PCR with primers p1 and p3 was performed with these serially diluted templates, using conditions described above. 5 µL of the nested PCR products were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis

The primary outcome was the prevalence of different *Plasmodium* species in the placenta of a population of asymptomatic pregnant Nigerian women. The study population, whose samples were analyzed, was part of a larger observational cohort looking at newborn outcomes from vertically transmitted infections, and thus, the sample size used was a convenient sample; therefore, the study was not powered for the proposed primary outcome. Data collected were summarized using descriptive statistics to include mean, standard deviation, median, minimum, and maximum for continuous variables while counts and percentages were used to display categorical data. Chi-square was used to examine the association between two independent categorical variables. Spearman correlation coefficients to compare the means of two continuous variables. Pearson correlation was used to measure the relationship of continuous variables. Categorical variables included maternal employment status, educational level, HIV status, anemia status, use of malaria prophylaxis and survival at delivery. Categorical variables for placenta evaluation included placenta malaria staining and PCR results, as well as the presence or absence of chorioamnionitis and funisitis and whether placental blood cultures were positive or not. Newborn categorical variables included gender, mode of delivery, Z-scores for birth anthropometrics, evaluation for sepsis, and survival at 7 and 28 days. Continuous variables were maternal age, body mass index, hemoglobin, gestational age, placenta weight, and newborn anthropometrics. Statistical significance was set at $p \leq 0.05$. IBM SPSS Statistics (Version 29) was used for all analyses.

RESULTS

After collection and processing, 104 placental samples and 106 cord samples, there were 2 twin births, were viable upon arrival at UNMC and sent for analysis. Table 9 lists the

sociodemographic, risk factors, and outcomes of the maternal infant pairs, as well as malaria histologic staining and overall PCR results of the placentas. The mean maternal age was 30 (±4.77), 60.8% (62/102) of mothers were employed, and 66.9% (69/102) had tertiary school education. 30.1% (22/53) were anemic, 98% (97/99) were HIV-negative, and all mothers remained alive at 28 days follow-up. The gender of the newborns was equally distributed between male and female, with 68.9% (73/106) of the newborns being delivered vaginally and only 9.4% (10/106) were preterm births.

Table 10 Sociodemographic and clinical profile of asymptomatic mothers and their newborns

Maternal factors and outcomes N = 104		**Infant factors and outcomes N = 106**	
Continuous	Mean (SD)	*Continuous*	Mean (SD)
Age (years)	30 (4.77)	Weight (Kg)	3.1 (.525)
BMI (Kg/m2	28.73 (5.53)	Length (cm)	51 (2.85)
Haemoglobin (gm/dL)	11.3 (.89)	Head Circumference (cm)	34 (1.93)
estational Age at delivery (weeks)	39 (1.99)	Gestational Age (weeks)	39 (2.85)
Placenta weight (grams)	607.21(132.82)		
Categorical	N (%)	*Categorical*	N (%)
Employment Status		Gender	
Yes	62 (60.8%)	Male	53 (50%)
No	40 (29.2%)	Female	53 (50%)
Educational Level		Mode of delivery	
Grade 1-12	34 (33.1%)	Vaginal	73 (68.9%)
Tertiary	69 (66.9%)	Ceasarean section	33 (31.1%)
HIV status		Prematurity (<37 weeks)	
Positive	2 (2%)	Yes	10 (9.4%)
Negative	97 (98%)	No	96 (90.6%)
Anemia		Stillborn	
Yes	22 (30.1%)	Yes	-
No	51 (69.9%)	No	106 (100%)
Malaria prophylaxis		Abortion	
Yes	94 (90.4%)	Yes	-
No	10 (9.6%)	No	106 (100%)
Malaria Placenta Staining		length-for-age-Z score <-2	
Positive	7 (7.7%)	Yes	6 (5.7%)
Negative	92 (92.9%)	No	100 (94.3%)
Malaria species PCR		Weight-for-age-Z score <-2	
Positive	40 (38.5%)	Yes	6 (5.7%)
Negative	62 (61.5%)	No	100 (94.3%)
Placenta Bacterial Cultures		Weight-for-length-Z score <-2	
Positive	32 (36%)	Yes	34 (33.3%)
Negative	57 (64%)	No	68 (66.6%)
Chorioamnionitis		Sepsis evaluation	
Positive	27 (28.1%)	Yes	-
Negative	69 (71.9%)	No	106 (100%)
Funisitis		Alive at 7 days	
Positive	18 (18.4%)	Yes	85 (98.8%)
Negative	80 (81.6%)	No	1 (1.2%)
Maternal alive at delivery		Alive at 28 days	
Yes	104 (100%)	Yes	87 (97.8%)
No	-	No	2 (2.3%)

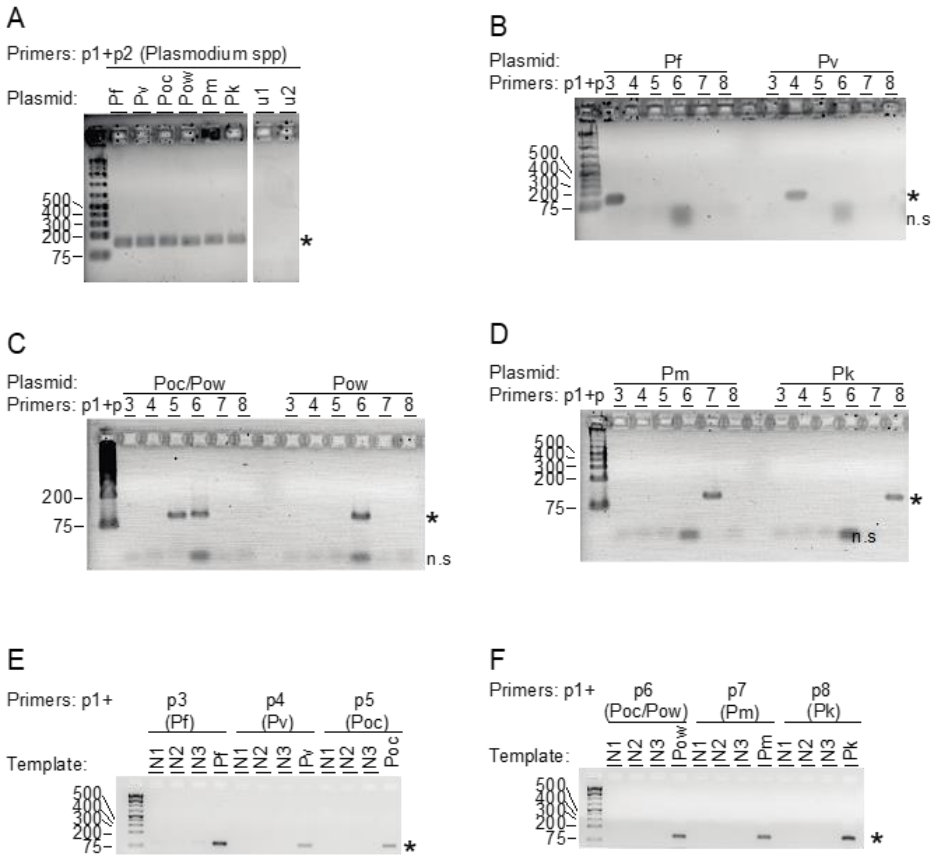
Of the placenta specimens stained for malaria, 7% (7/99) were positive. Of the placental specimens tested, 38.5% (40/102) of samples were positive for at least one infection as detected by nested PCR. Using the primers listed in Table 10, confirmation of PCR conditions using plasmid DNA encoding 18S SSU rRNA from *Plasmodium spp*. are shown in Figure 4. Input with plasmid DNA amplified an expected band at ~150 bp (Figure 5A). A sequential PCR with species-specific primers, as outlined in Table 2, yielded bands only with the matching template/primer pair (Figure 1B-D). Nested PCR using known malaria-negative placental samples (N1, N2, N3) and plasmid DNA demonstrate that no bands are detected in placental samples uninfected with malaria (Figure 1E, F). Note that PCR reactions with p1+p6 detect both *P. ovale wallikeri* and *P. ovale curtisi*, whereas reactions with p1+p5 only detect *P. ovale curtisi*. Therefore, detection of *P. ovale wallikeri* was made by inference i.e., if there is a positive band in a PCR with p1+p6 but not p1+p5.

Table 11 Primer sequences used in this study. [43]

Name	Sequence	Specificity	PCR product length * (bp)
p1	5'-ACGATCAGATACCGTCGTAATCTT-3'	*Plasmodium spp.*	-
p2	5'-GAACCCAAAGACTTTGATTTCTCAT-3'	*Plasmodium spp.*	143 (Pf), 145 (Pv), 151 (Pm), 154 (Pk), 145 (Poc/Pow)
p3	5'-CAATCTAAAAGTCACCTCGAAAGATG-3'	*P. falciparum*	102
p4	5'-CTAAGAATAAACTCCGAAGAGAAAATTC-3'	*P. vivax*	101
p5	5'- CTAAGAAATTTCCCCGAAAGGAATTCTC-3'	*P. ovale curtisi*	100
p6	5'- CAATCTAAGAAATTTCCAAAAGGAATTTTC-3'	*P. ovale wallikeri and P. ovale*	104
p7	5'- CTAAAAGAAACACTCATATATAAGAATGTC-3'	*P. malariae*	106
p8	5'- GTTCTAATCTCCGGAGAGAAAAGAAAAAC-3'	*P. knowlesi*	103

* PCR product length calculated when p1 is paired with p2-p8.

Figure 5 PCR conditions using plasmid DNA encoding 18S SSU rRNA from *Plasmodium* spp.



The most common detected *Plasmodium* species/subspecies was *P. ovale wallikeri* (27.9%; 29/104) followed by *P. knowlesi* (21.2%; 22/104) and *P. falciparum* (5.8%; 6/104). *P. malariae* was not detected in this population (Figure 6.) There were 16 cases of single infection with *P. ovale wallikeri* (56.2%; 9/16) being the most common species detected. Dual species infections occurred in 22 cases and triple species infections in 2 cases. *P. knowlesi* was only detected in mixed infections and was detected with concomitant P. ovale wallikeri infections in 87% (20/23) of the cases (Table 11). Details of the results of the nested PCR to determine each different plasmodium species are shown in Figures 7-12.

Figure 6 Number of *Plasmodium* spp. Detected by Nested PCR in 104 Placental Specimens

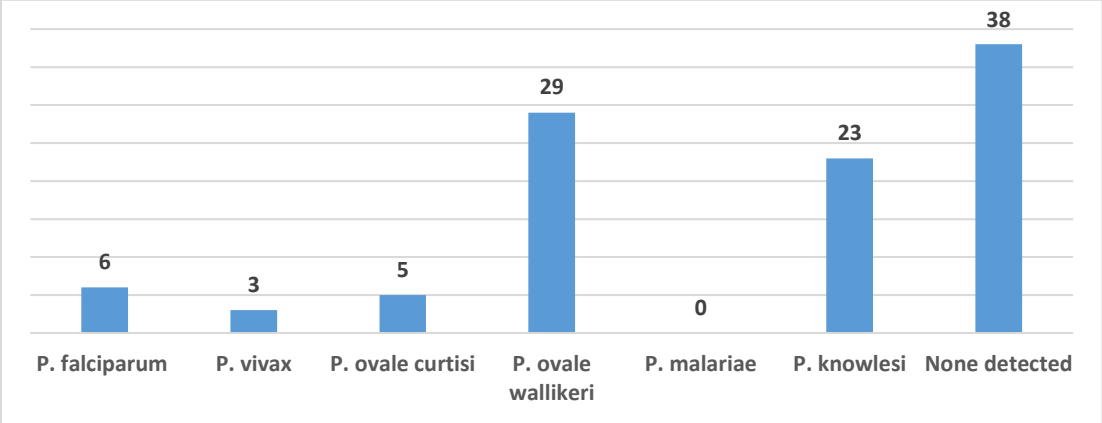


Table 12 Distribution of malaria species infection in 104 placental specimens by type of infection and type of *Plasmodium* species

Types of infection	Positive specimens N (%)	*Plasmodium spp.* detected by PCR	Positive specimens (N, %)
Single species infections	16 (15.4%)	*P. falciparum*	4 (3.8)
		P. vivax	2 (1.9)
		P. ovale wallikeri	9 (8.7%)
		P. ovale curtisi	1 (0.9%)
Double species infections	22 (21.2%)	*P. knowlesi, P. ovale wallikeri*	20 (19.2%)
		P. knowlesi, P. ovale curtisi	2 (1.9)
Triple species infections	2 (1.9%)	*P. falciparum, P. ovale curtisi, P. vivax*	1 (0.9%)
		P. falciparum, P. ovale curtisi, P. knowlesi	1 (0.9%)

Figure 7 Results of nested PCR to detect *Plasmodium spp*. in specimens 1-8. None of these specimens were positive for malaria infection. n.s. indicates non-specific primer dimer bands.

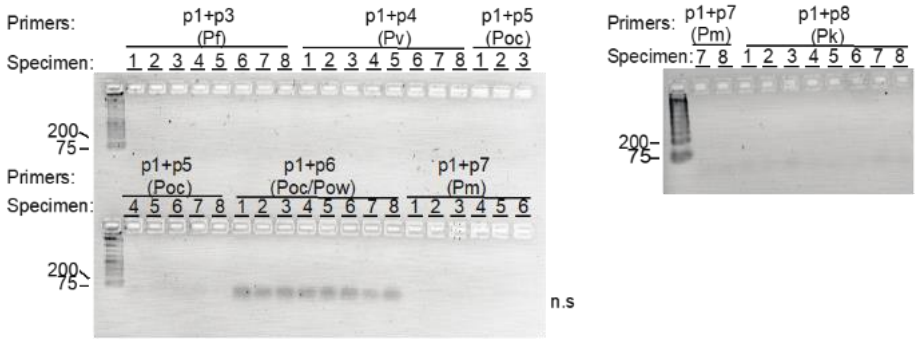


Figure 8 Nested PCR detected *P. falciparum* species. Specimens examined were (A) 9-40, (B) 41-78, (C) 79-88, and (D) 89-104. (E) Faint bands in panels A-D, indicated by #, were confirmed by performing a further amplification using the nested PCR products as templates and primers p1 + p3, as indicated. *denotes bands at 102 bp, indicating specimens were positive for P. falciparum.

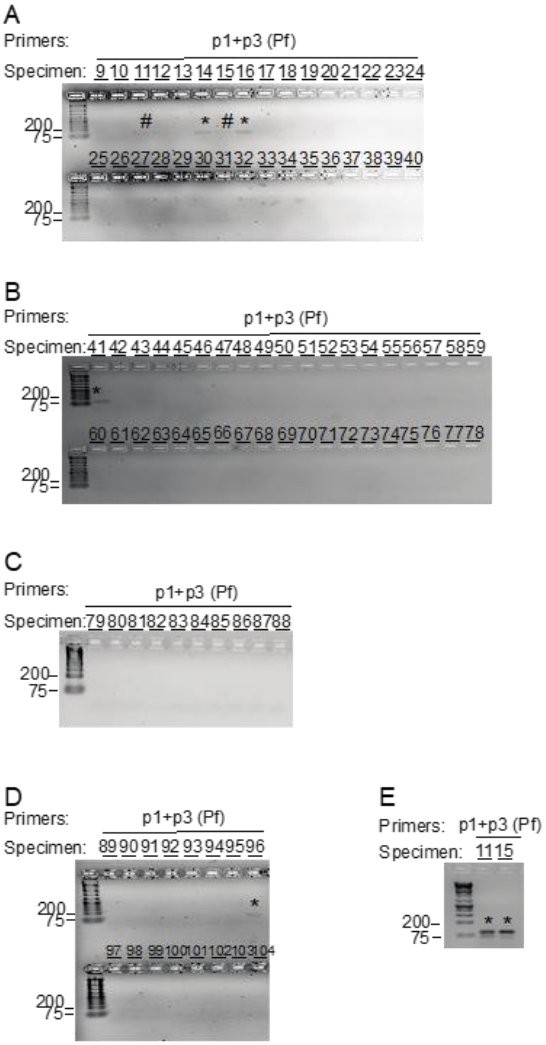


Figure 9 Nested PCR detected *P. vivax* species. Specimens examined were (A) 9-40, (B) 41-78, (C) 79-88, and (D) 89-104. (E) Faint bands in panels A-D, indicated by #, were confirmed by performing a further amplification using the nested PCR products as templates and primers p1 + p4, as indicated. *denotes bands at 101 bp, indicating specimens were positive for P. vivax. n.s. indicates non-specific primer dimer bands.

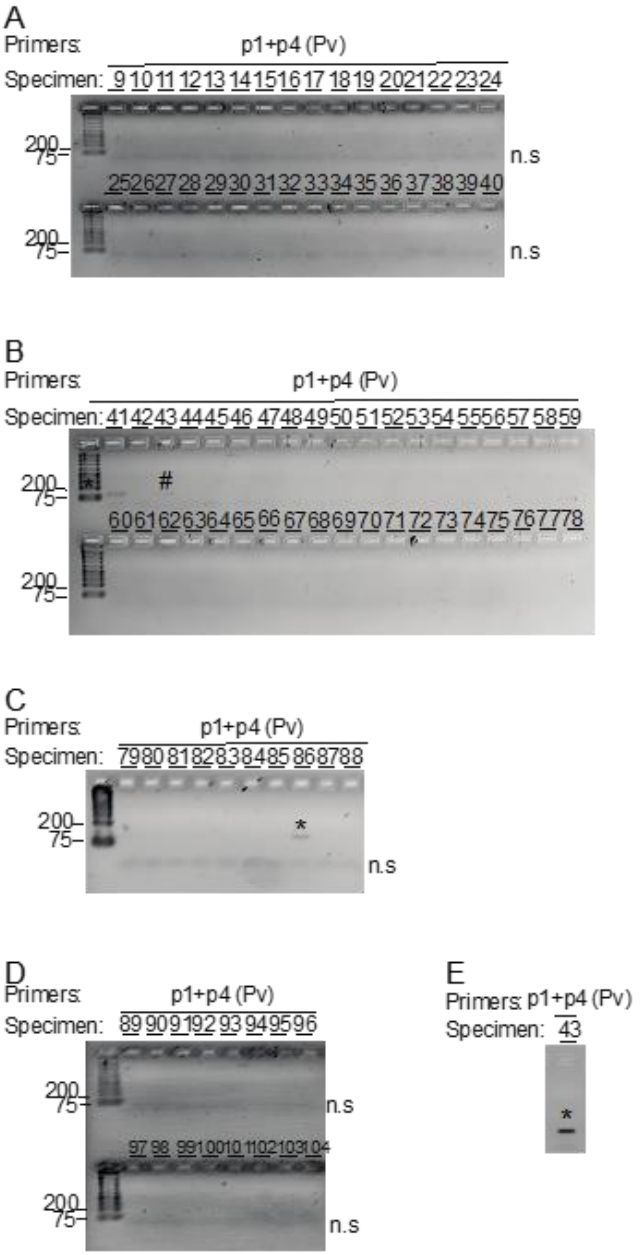


Figure 10 Nested PCR products detected *P. ovale curtisi* species. Specimens examined were (A) 9-40, (B) 41-78, (C) 79-88, and (D) 89-104. (E) Faint bands in panels A-D, indicated by #, were confirmed by performing a further amplification using the nested PCR products as templates and primers p1 + p5, as indicated. *denotes bands at 100 bp, indicating specimens were positive for P. ovale curtisi. n.s. indicates non-specific primer dimer bands.

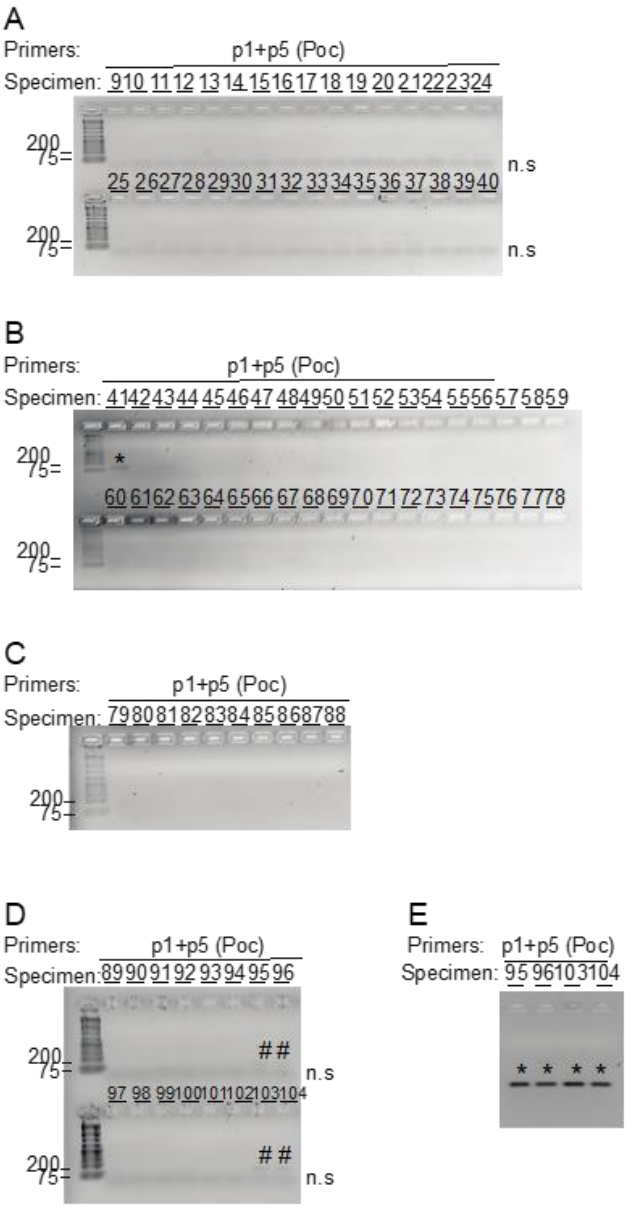


Figure 11 Nested PCR products detect either *P. ovale curtisi* or P. *ovale wallikeri* species.

Specimens examined were (A) 9-40, (B) 41-78, (C) 79-88, and (D) 89-104. (E) Faint bands in panels A-D, indicated by #, were confirmed by performing a further amplification using the nested PCR products as templates and primers p1 + p6, as indicated. *denotes bands at 104 bp, indicating specimens were positive for P. ovale curtisi or P. ovale wallikeri. Specimens that yielded positive bands here were compared with that of Fig. 5 to identify specimens that were positive for P. ovale wallikeri infection. n.s. indicates non-specific primer dimer bands.

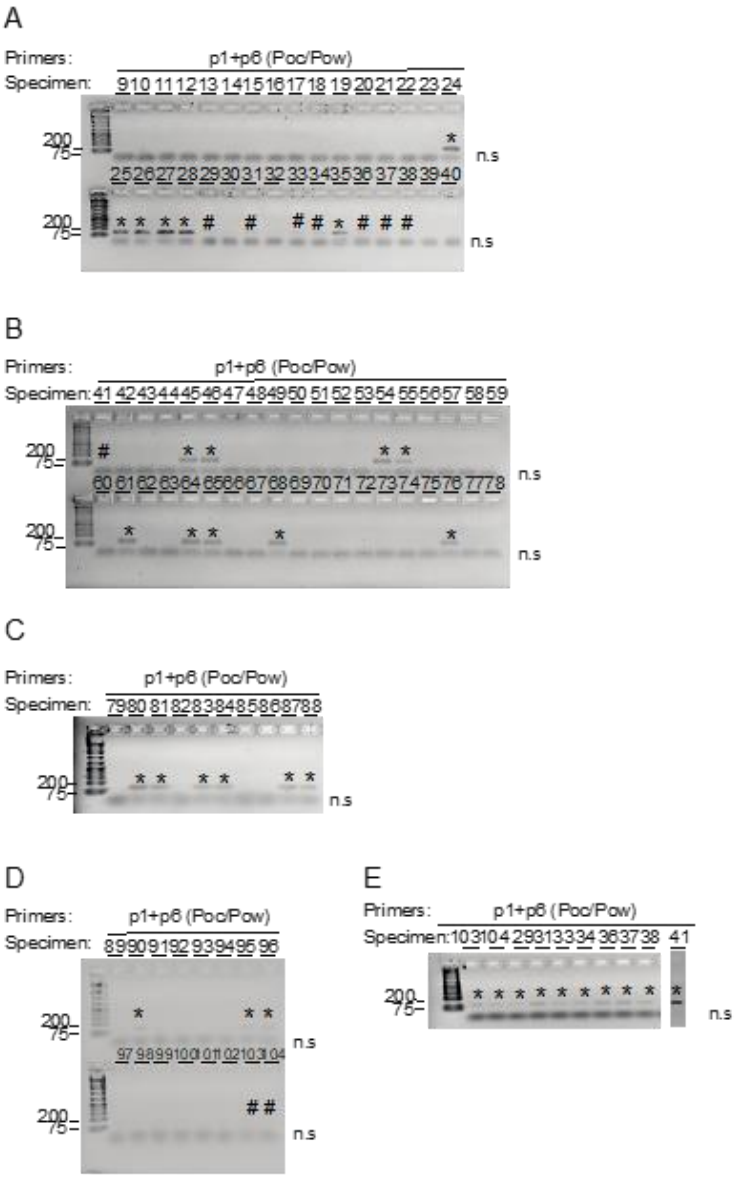


Figure 12 Nested PCR products to detect *P. malariae* species. In specimens (A) 9-40, (B) 41-72, (C) 73-88, and (D) 89-104. No bands were detected at 106 bp, indicating specimens were negative for P. malariae.

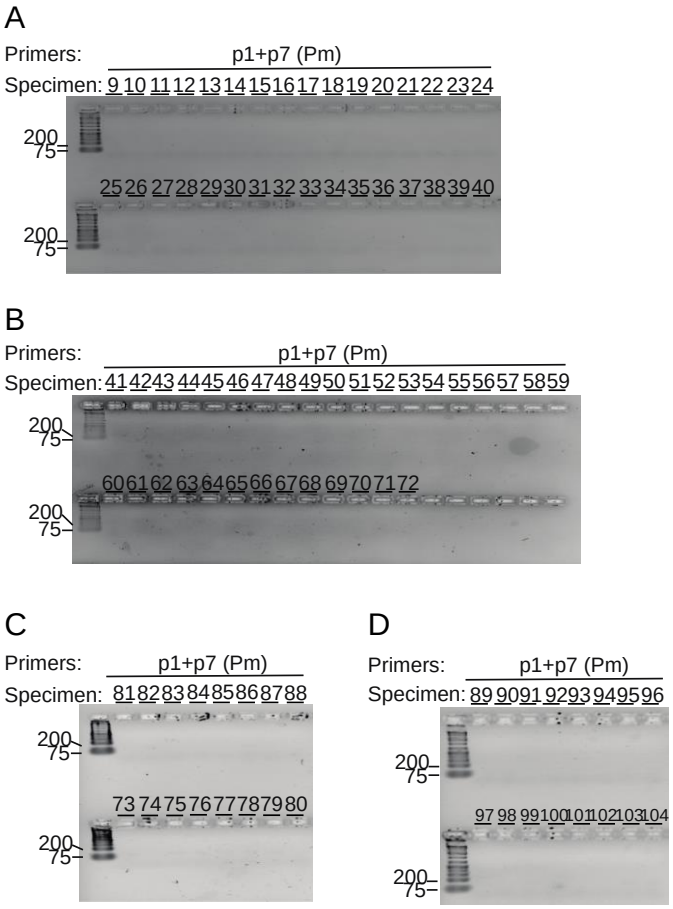
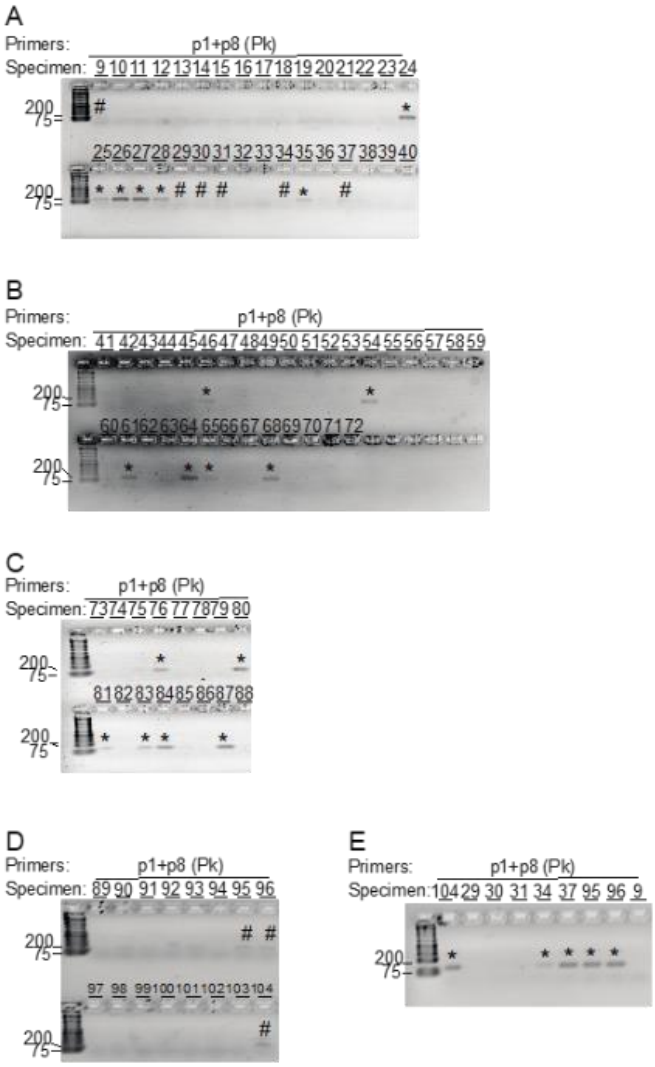


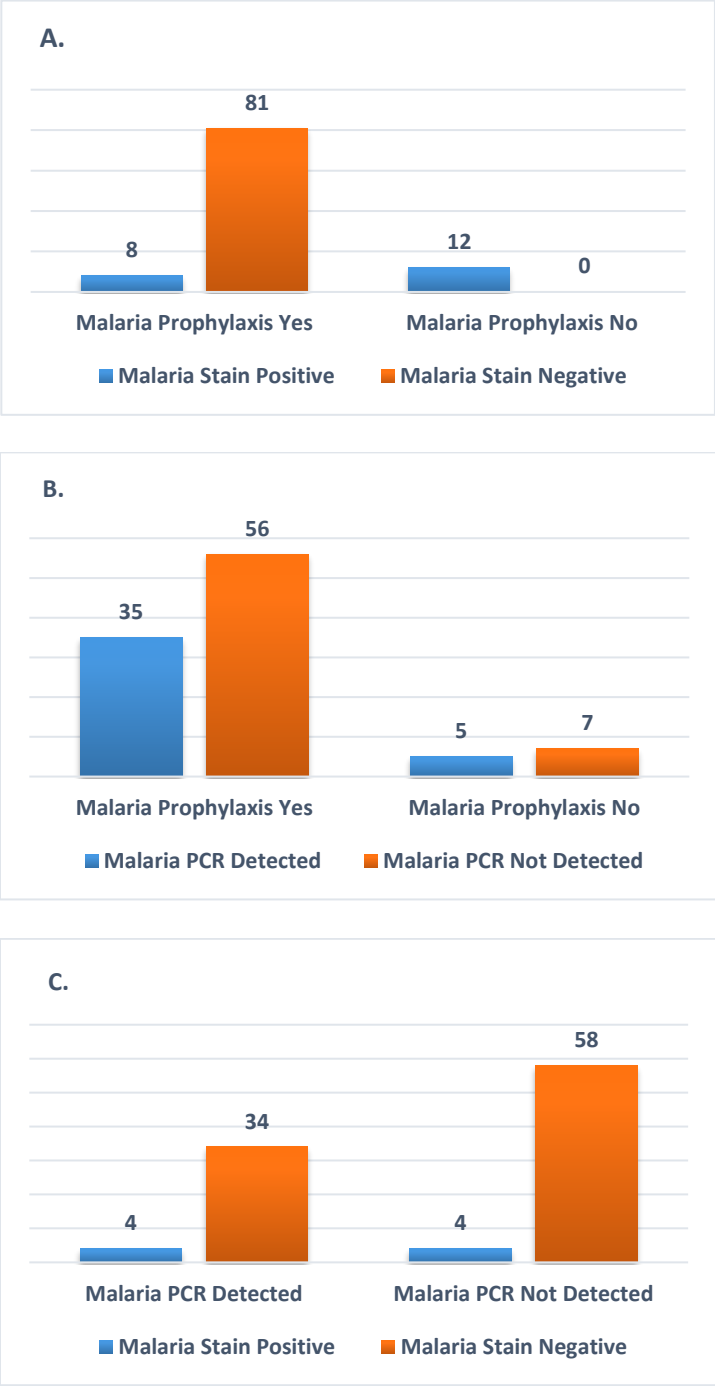
Figure 13 Nested PCR products detected *P. knowlesi* species. Specimens examined were (A) 9-40, (B) 41-72, (C) 73-88, and (D) 89-104. (E) Faint bands in panels A-D, indicated by #, were confirmed by performing a further amplification using the nested PCR products as templates and primers p1 + p8, as indicated. *denotes bands at 103 bp, indicating specimens were positive for P. knowlesi.



In the population examined for this study, RDT or peripheral thin and thick smears were not routinely recorded and 90.5% (94/104) were on intermittent preventive treatment of malaria with sulfadoxine and pyrimethamine. There was no significant association between malaria PCR results and the presence of maternal anemia. (p = 0.13) There was no significant association between the use of anti-malarial prophylaxis and the results of positive malaria immunohistochemical stain or positive malaria PCR results of the placentas (p = 0.34 and p = 0.83, respectively) (Figure 13a and b). There was also no significant association between the malaria staining and the malaria PCR results. (p = 0.36) (Figure 13c.)

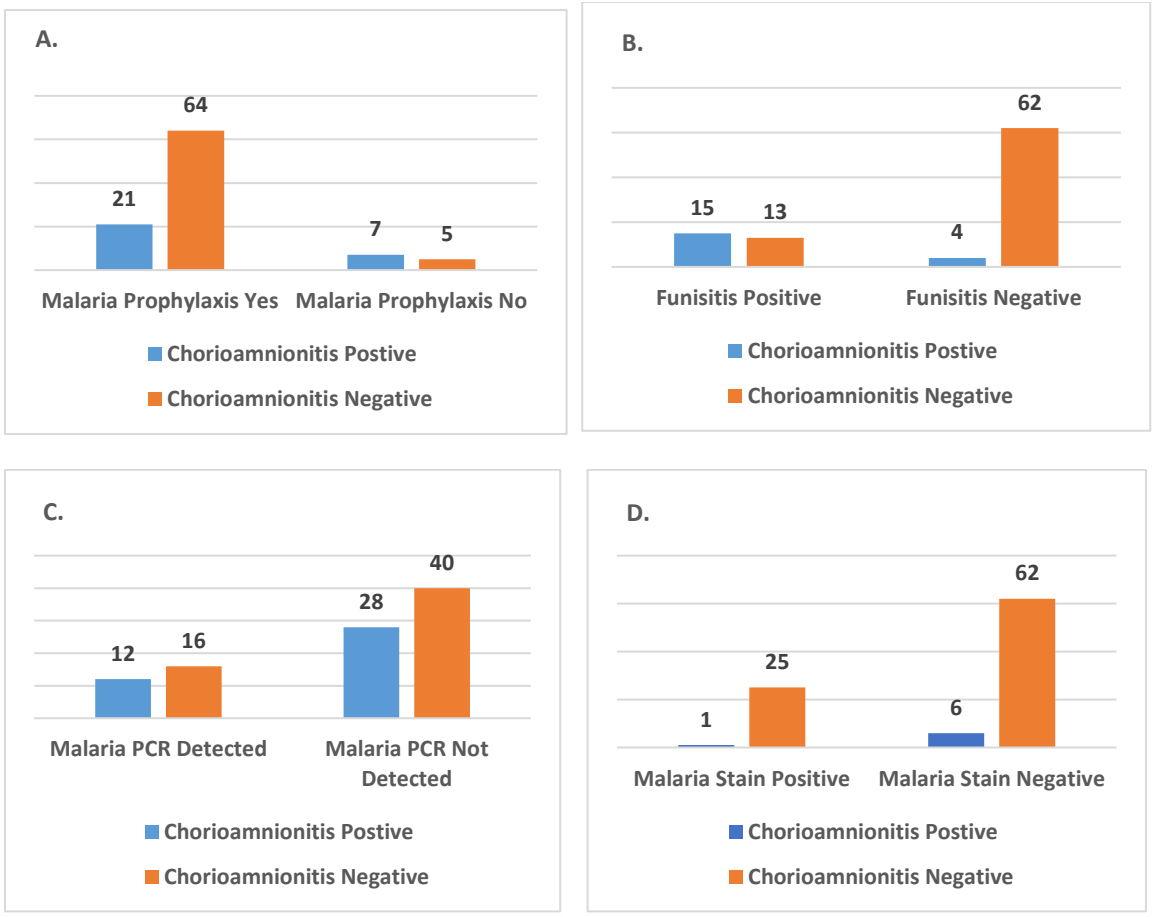
Malaria-positive Giemsa stain and PCR results were each not significantly associated with maternal hemoglobin, maternal anemia, maternal gestational age, use of sulfadoxine and pyrimethamine prophylaxis, placenta weight, chorioamnionitis, funisitis, mode of delivery, gender, prematurity, birth anthropometrics, newborn evaluation for sepsis, newborn survival at 7 or 28 days of life.

Figure 14 Malaria Prophylaxis Use and Malaria Placenta Testing Results. In **A.** malaria prophylaxis use was not significantly associated with negative malaria Giemsa stain. (p = 0.34) In **B.** malaria prophylaxis use was not significantly associated with negative malaria PCR testing. (p = 0.83) In **C.** malaria PCR results were not significantly associated with malaria Giemsa stain. (p = 0.36)



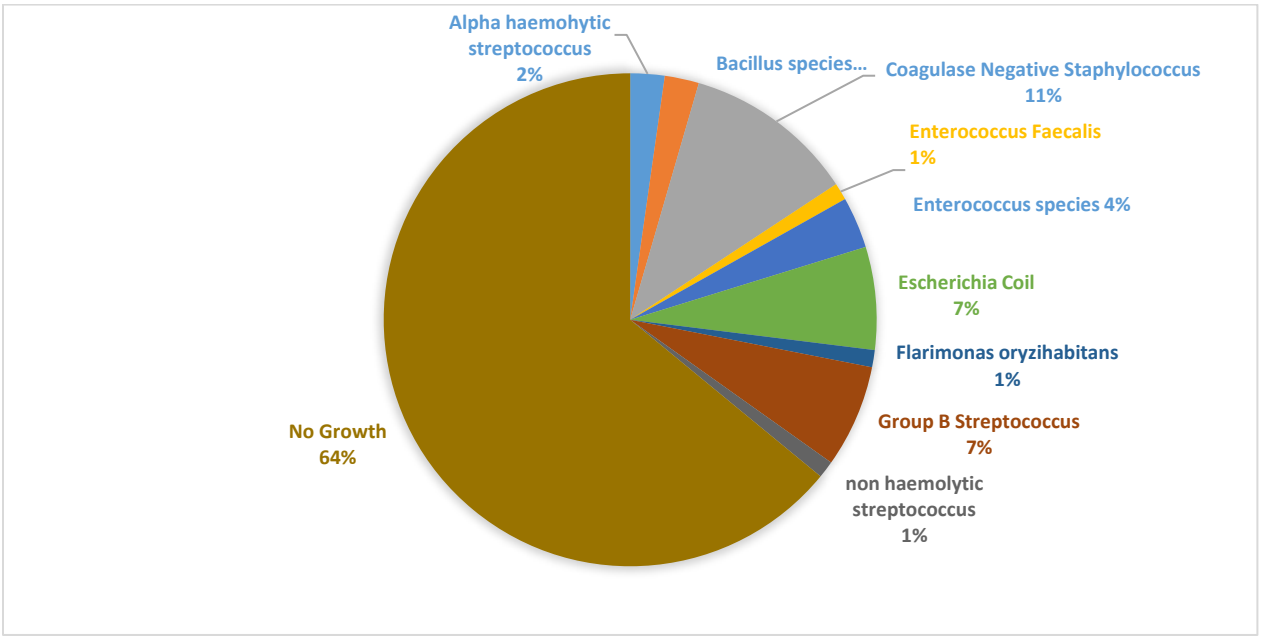
Placenta specimens were reviewed for evidence of chorioamnionitis, and cord specimens were reviewed for funisitis and chorioamnionitis was present in 28.1% (27/96) of the cases, while funisitis was seen in 18.4 % (18/98) of the cases (Table 5). Placenta specimen samples with concerns for chorioamnionitis were stained for HSV, CMV, syphilis, and toxoplasmosis; all results were negative. The absence of chorioamnionitis was significantly associated with the use of anti-malarial prophylaxis ($p = 0.023$) while the presence of chorioamnionitis was significantly associated with funisitis ($p < 0.001$). (Figure 14a and b) There was no significant association between chorioamnionitis and malaria Giemsa stain ($p = 0.88$) or malaria detection of at least 1 *Plasmodium species* infection by PCR ($p = 0.37$). (Figure 14c and d)

Figure 15 Evidence of Chorioamnionitis based on Malarial Prophylaxis Use and Placenta Malaria Testing Results. In **A.,** the use of malaria prophylaxis was significantly associated with no histopathologic changes of chorioamnionitis (p = 0.023) In **B.,** a significant association was observed between histopathologic evidence of chorioamnionitis and funisitis. (p < 0.001). In **C. and D.,** no significant associations were observed between histopathologic evidence of chorioamnionitis and malaria PCR results or malaria Giemsa stain. (p = 0.88 and p = 0.37, respectively)



Placenta blood was also sent for cultures, and 36% (32/99) of the cultures were positive for bacterial organisms (Table 1) *Coagulase-negative staphylococcus* were the most isolated organisms at 11.2%, while *Escherichia coli* and *Group B Streptococcus* both came in second at 6.7% each (Figure 15). No significant associations were observed with placenta blood culture positivity and the use of anti-malarial prophylaxis, malaria Giemsa staining and PCR results, or the presence or absence of chorioamnionitis or funisitis.

Figure 16 Distribution of Placental Blood Cultures Organisms of N = 89 samples



DISCUSSION

Our study showed the prevalence of different *Plasmodium species* in the placenta in a population of asymptomatic pregnant mothers seen at the time of delivery at UATH in Gwagwalada, Nigeria, to be 7% by immunohistochemical staining and 38.5% by nested PCR for at least 1 *Plasmodium species* infection with no significant association between the malaria staining and the malaria PCR results. (p = 0.36) Moreover, anemia, usually observed during pregnancy due to placental sequestration of parasites, though present in 30.1% of our study population, it was not significantly associated with malaria infection. (p = 0.13)

Malaria staining versus PCR

The more robust detection of malaria when examined by PCR compared to microscopy has been documented in the literature. A study conducted in the Congo to detect *P. falciparum* infection in asymptomatic pregnant women found that 7% of individuals harbored asymptomatic infection by peripheral microscopy vs. 19% when detected by PCR. [138] In that same report, microscopy only detected *P. falciparum,* and all specimens identified by microscopy were identified by PCR, in contrast to our study where there were we had more cases of non-falciparum malaria species that are often not easily identified by microscopy. Additionally, using WHO classification for anemia, 51.5% of their study participants were anemic, and this was significantly associated with *P. falciparum* infection, likely due to *P. falciparum* being more virulent of the species. [144] We had 30.1% of participants with anemia in our study, though anemia was not significantly associated with malaria PCR results. However, we had a higher prevalence of non-falciparum malarial species, which are associated with less anemia. A different study also in the Congo looked at maternal, placental, and cord blood samples via microscopy and found a prevalence of 7.3% in the peripheral blood, 2.7% in the placenta, and no

infection in the cord blood in contrast, when they used PCR analysis, the prevalence was 25.4%, 16.7%, and 9.4% respectively. [145] This study also looked specifically at *P. falciparum* and only samples negative by microscopy were sent for PCR analysis. Though microscopy is the gold standard for *P. falciparum*, species-level identification may be difficult in low parasitemia, which occurs in asymptomatic infections. [146] The finding of malaria infections being captured more by PCR versus microscopy was also observed in other regions of the continent. In a study conducted in Ethiopia, the prevalence of asymptomatic *Plasmodium species* infection was 9.1% and 18.1% using microscopy and PCR, respectively. However, in contrast to our study, they used peripheral blood and not placenta blood samples and they only tested for *P. falciparum* and *P. vivax* by PCR. [137] A report on pregnant women enrolled longitudinally in 3 maternity clinics in Benin found that microscopy detected infection in 16% of the women, and PCR detected 40% of the infections. [147] The study focused only on detecting *P. falciparum,* unlike our study, and they did not use the concept of asymptomatic infection but rather "submicroscopic infection," which they defined as present when the peripheral thick smear was negative but the PCR was positive. While we only looked at the malaria status at delivery, they looked at the status at enrollment and follow-up, which allowed them to show that submicroscopic infection was associated with adverse outcomes such as maternal anemia, premature births, and low birth weight. Our study was not designed to look at longitudinal outcomes, our sample size was 10 times smaller than theirs, and they recruited from several centers which gave them a more geographically diverse pool of participants. In contrast, we had a single center study in a region where most births do not occur in the hospital setting; this further limited the diversity of our study participants.

Plasmodium species detection

The most detected malaria species/subspecies in our study was *P. ovale wallikeri* (27.9%; 29/104) followed by *P. knowlesi* (21.2%; 22/104). *P. falciparum* was the third in prevalence with 5.8% (6/104). *P. malariae* was not detected in this population. This study is the first to report *P. knowlesi* infection in an African cohort of pregnant women. *P. falciparum* is the majority species in the African region, while *P. vivax* is found more globally. [117, 118] *P. ovale* spp*. (P. ovale curtisi and P. ovale wallikeri)*, and *P. malariae* have globally low prevalence. [117, 118] A systematic review and meta-analysis of global trends of *P. ovale* spp*.* and *P. malariae* infections between 2002-2020 has shown however, an increase in both species in the African and Latin American regions that was likely due to the increased usage of diagnostic PCR assay. The overall prevalence reported as 2.01% for *P. malariae* infections and 0.77% for *P. ovale spp.* with a note that the African region is most affected by both species. [121] In our study, we detected *P. ovale* spp. at a higher rate than *P. malariae* which was not found at all. Our study population included participants who were receiving antimalaria prophylaxis 90.4% (94/104) which could explain the low prevalence of *P. falciparum* and furthermore given that the treatment for P malaria is like that of *P. falciparum*, the anti-malarial prophylaxis could have affected the detection of *P. malaria*e which was already at low prevalence. Furthermore, anti-malarial prophylaxis does not treat *P. ovale spp.*, given its propensity to be dormant in the liver stage for prolonged periods of time evading elimination. [121] This could explain the higher prevalence of *P. ovale spp.* in our study.

P. knowlesi is endemic to Southeast Asia and is the most common zoonotic malaria in humans. It can lead to severe and fatal disease. [122] In areas where *P. knowlesi* is co-endemic with *P. falciparum* and *P. vivax*, there appears to be a lower risk of infection for pregnant women with *P. knowlesi*. [122] The reason is uncertain but may likely be due to changes in the work

environment when pregnant; this lower risk of infection has made it more difficult to ascertain maternal and newborn outcomes from *P. knowlesi* infection during pregnancy, though some case reports have described anemia, preterm delivery, and newborn death as adverse outcomes. [122] *P. knowlesi* is also frequently misdiagnosed by microscopy as *P. falciparum* or *P. vivax;* thus, it may be underdiagnosed, and its true incidence may be underestimated. PCR testing is necessary to detect *P. knowlesi* as RDT is unreliable. [148] There have been no reports in the literature of *P. knowlesi* in the African region; most surveillance studies, even using PCR techniques, have not systematically tested for the organism. Most recently, a couple of studies in West Africa used PCR assays that included detection for *P. knowlesi*. However, the organism was not identified in the populations where the studies were conducted, Cameroon [149] and Ghana. [150] In the study in Cameroon, which recruited 119 children and adults and no pregnant women, *P. ovale curtisi* and *P. malariae* were also not detected as single infections. [149] Though the enrollment included asymptomatic and symptomatic participants, all non-falciparum infections in this study were symptomatic. [149] The Ghana study, which recruited 164 asymptomatic and symptomatic children, did not find *P. vivax* or *P. ovale* spp. [150] Though these two studies did not identify *P. knowlesi*, they had small sample sizes, like our study, and did not include pregnant women in contrast to our study. Both studies, like ours, identified co-infections. Our finding of *P. knowlesi* in the placenta of asymptomatic pregnant Nigerian women should warrant further and broader studies where PCR assays or deep sequencing systematically include the possibility of identifying *P. knowlesi* to better characterize the true prevalence of the organism. These studies should furthermore include a mix of symptomatic and asymptomatic participants across ages and pregnancy status to better characterize the clinical significance of *P. knowlesi* infection.

Mixed malarial infections

This study is the first to report mixed malarial infection in pregnancy associated with asymptomatic mothers in Nigeria. There were 16 cases of single infection, with *P. ovale wallikeri* (56.2%) being the most common species detected in this scenario. Dual species infections occurred in 22 cases, and triple species infections in 2 cases. *P. knowlesi* was only detected in mixed infections and was detected with concomitant *P. ovale wallikeri* infections in 87% of the cases. In a multi-country study conducted in Burkina Faso, The Gambia, Ghana, and Mali, blood samples were collected at the first antenatal clinic attendance of women enrolled in a trial of intermittent screening and treatment of malaria in pregnancy. [151] Using nested PCR testing to detect *P. falciparum*, *P. vivax*, *P. malariae,* and *P. ovale*, they found mixed *P. falciparum* and *P. non-falciparum* infections with *P. malariae* being the most frequent non-falciparum infection identified. [152] Mixed malarial infections have also been detected in non-pregnant populations; studies in Cameroon [153] and Ghana [153] that were discussed in the previous section had co-infections when using the PCR assays. In the Cameroon study, there were three *Plasmodium* spp. infections with *P. falciparum*, *P. ovale curtisi* and *P. vivax* and two co-infections, one was *P. falciparum* and P. *vivax,* while the other was *P. falciparum* and *P. ovale curtisi*. [149] The Ghana study had 9 cases of co-infection with *P. falciparum* and *P. malariae*. [150] Though both of these studies included asymptomatic and symptomatic participants, all participants with non-falciparum infections in the Cameroon study were symptomatic. In a multisite study of febrile children and adults in Ethiopia that excluded pregnant women, PCR assays detected *P. falciparum* and *P. vivax* co-infections. [152] Another Ghanian study in asymptomatic school children reported co-infections with *P. falciparum*, *P. malariae, P. ovale curtisi*, and *P. ovale wallikeri* when using PCR assays. [153] In this study, even after three weeks of treatment with artemisinin-combination therapy, there was persistent parasitemia; one speculation was that chronic, asymptomatic malarial infections may not elicit immune responses that would help with

drug-induced parasite clearance. [153] A systematic review by Kotepui *et al.* looking at *Plasmodium spp*. mixed infection leading to severe malaria found that these infections were often not recognized as only a low percentage are detected by microscopy due to low parasitemia or lack of technical expertise. [154] There is also a concern that treating single species infection while having mixed infections could worsen parasitemia with the untreated species, particularly if the single species are *P. vivax* and *P. falciparum*. [154]

The clinical findings of mixed infections in all the studies mentioned in the previous section varied based on the region where the study was conducted and on the characteristics of the population enrolled in the study. The clinical findings have ranged from asymptomatic infections to severe disease without a clear pattern or species association. In our study, malaria infection was not significantly associated with maternal anemia, birth anthropometric, maternal, and newborn survival. Based on our small sample size and even smaller number of mixed infections, we could not determine if mixed infections were associated with adverse maternal or newborn outcomes. Broader surveillance studies that are inclusive of asymptomatic and symptomatic pregnant women in addition to children and adults should be conducted in the region to understand better the clinical significance of co-infections with different *Plasmodium* species. Studies in pregnant women should also include sampling of the placenta; this is particularly important to characterize better, given the higher susceptibility of pregnant women to malaria infection.

Clinical outcomes with asymptomatic malarial infections

Our study did not show a significant association between placenta malaria infection and maternal anemia, premature birth, or low birthweight at delivery. This finding could be due to the lower prevalence of *P. falciparum*, the most virulent species. The prevalence of chorioamnionitis in our study population was 28.1% (27/106) and was not significantly associated with culturable bacterial nor parasitic infection of the placenta, additionally, HSV and CMV staining for all samples with chorioamnionitis were negative. Chorioamnionitis was significantly associated with funisitis ($p < 0.001$), which can be expected given the anatomical proximity of the umbilical cord to the chorioamniotic membrane. Chorioamnionitis was also found to be significantly associated with the use of anti-malarial prophylaxis. ($p = 0.023$) The use of sulfadoxine–pyrimethamine for anti-malarial prophylaxis has been associated with a decrease in preterm birth. [155] A possible mechanism for this finding could be that by decreasing the likelihood of malaria infection, there is less inflammation of the placental structures, which includes the chorioamniotic membranes. This leads to a decreased likelihood of chorioamnionitis. Further studies designed to look directly at the impact of sulfadoxine–pyrimethamine use on chorioamnionitis are needed to confirm this association.

Limitations

The study had several limitations. Given that the study participants were conveniently sampled from an ongoing longitudinal observational cohort, and that we do not have data for the prevalence of asymptomatic placenta malaria in the region, the study could not be powered to detect differences in the prevalence of malaria species and associated pregnancy outcomes. The prevalence of different *Plasmodium* species in this study may not reflect the epidemiologic pattern of species in the region, given the high prevalence of home births in the country at 41%.

[156] Furthermore, when pregnant participants were enrolled at the time of delivery, the effects of prior clinical or subclinical parasitemia earlier in the pregnancy could not be assessed as we were not collecting data over time, which could have affected the point prevalence of our findings. Participants were asymptomatic and peripheral RDT, thin and thick smears were not routinely performed on peripheral blood, which could have provided more information on common diagnostic tools for comparison. Furthermore, we did not collect fetal blood to check for anemia; while maternal anemia may not have been significantly associated with placental malaria infection, fetal anemia may have been.

CONCLUSIONS

This is the first study to report human *P. knowlesi* infection in the African region. This study is also the first to present various mixed *Plasmodium* species infections in pregnant women in Nigeria. Malaria elimination programs will need to leverage PCR testing or deep sequencing to better describe the epidemiology of the different *Plasmodium species* and better understand the burden of disease and the impact on health outcomes particularly in pregnant women and their offspring at higher risk of complications in malaria endemic regions.

DISCUSSION

We presented the results of our pilot studies in three manuscripts. In the following sections, we will first highlight the most salient study results for each chapter, then going stepwise through our study method, we will discuss some of the strengths and limitations in our research. We will conclude with our considerations for the next steps beyond this research endeavor.

Overall contribution to scientific advancement in maternal child health in Nigeria

The overall findings of our research revealed new details on vitamin D and specialized pro-resolving mediators' status in a cohort of Nigerian maternal-infant dyads, along with their associated newborn outcomes in birth anthropometrics and newborn morbidity from sepsis. Additional salient study findings include new details on the epidemiology of asymptomatic placental malaria infections in pregnant Nigerian women and newborn outcomes.

Research results highlighted by chapter

Chapter 1

We are the first to describe a significant positive association of cord percent 3-epi-25(OH)D_3 levels with neonatal sepsis evaluation (p = 0.036). We did not, however, observe a positive association with maternal or cord 25(OH)D blood that has been reported.[59] A review of the literature did not reveal other studies that have investigated the association of percent 3-epi-25(OH)D_3 with newborn sepsis. Our findings may suggest that a higher percent 3-epi-25(OH)D_3 means more circulating 3-epi-25(OH)D_3 and lower circulating 25(OH)D. Thus, there is a possibility that our findings could be an alternative way of showing that low 25(OH)D stores may predispose newborn to sepsis but that the percent epimerized level and not just 25(OH)D

provides a more accurate picture. Further studies are needed to reproduce our findings and better understand the biology behind our observed association.

Chapter 2

This is the first study to quantify the SPMs in a population of Nigerian mother-infant dyads by looking at both maternal and cord blood levels of resolvin D1(RvD1) and resolvin (RvD2). We compared our Nigerian cohort to a midwestern US-based mother/infant dyads and observed that the maternal Nigerian RvD1 and RvD2 levels were significantly lower than that of the US cohort; this could be due to differences in diet and omega-3 fatty acid supplementation between populations. (p = 0.002 and p = 0.004, respectively). However, the significantly higher US RvD1 and RvD2 levels did not lead to proportionately high US cord RvD1 or RvD2, in fact the Nigerian cord RvD1 and RvD2 had slightly higher levels than the US cord levels, however, the difference was not statistically significant. This could suggest that after a certain level of maternal RvD1 or RvD2, there is no increased benefit or transfer to the newborn. Further studies are needed with populations with different diets and supplementation habits to better understand the biology behind these SPM dynamics.

Chapter 3

Our study is the first to report human *P. knowlesi* infection in the setting of low prevalence of *P. falciparum* in a cohort of asymptomatic pregnant Nigerian mothers at delivery. Our findings suggest that intermittent preventive treatment for malaria in this population of pregnant women helped decrease the prevalence of *P. falciparum*. But our study also highlights the need to use PCR assays to better quantify the presence of single and mixed infections in asymptomatic pregnant women, who are at higher risk of disease and who may be infected by *Plasmodium* species that are not responsive to *P. falciparum* focused treatment strategies.

Limitations and strengths of our studies

To integrate the discussion of all three studies, we will review the different stages of our clinical research method, starting with study conditions, recruitment, data collection, specimen processing, cost and team expertise, data analysis, and interpretation, and then we will conclude with next steps.

Study conditions

Successful clinical research studies conducted in resource-limited regions of the world rely on strong collaboration with local partners that span across the research enterprise, from local clinical expertise, local technical personnel, and good infrastructure. These conditions were met with our partnership with the International Foundation Against Infectious Disease in Nigeria, IFAIN, as the organization is led and run by Nigerians. This helped secure local buy-in and trust in the research project with access to a research unit at the University of Abuja Teaching Hospital (UATH) and that was one of our strengths. However, the reality is that funding agencies based in high-income countries have historically favored providing more indirect research support to US-based institutions or international but US-run programs; this has impacted the ability of low-income countries to build research infrastructure. [157] IFAIN, during our studies, had a partnership with UNMC but has since transitioned to independence, which has allowed it to reinforce its research capacity.

Recruitment

The consent process was conducted by study research personnel during an enrollment visit at UATH in either the antenatal clinic area or the labor and delivery ward. All pregnant women who came to the hospital for care were invited to participate in the VTI study and our three studies were conducted simultaneously within the larger VTI study, which was a strength

as we only had to administer one consent. Table 13 lists the three studies discussed in this dissertation in addition to the larger cohort study from which they were conducted.

Table 13 List of the different studies, their timeline, and enrollment

Study	Study Duration	Study Enrollment	
Vertically Transmitted infections (VTI)	April 2016 to March 2018	2,586 mothers	1996 infants
Vitamin D	April 2016 to February 2017	525 mothers	526 infants
Specialized pro-resolvin mediators	April to May 2016	78 mothers	58 infants
Placental Malaria	October to December 2016	104 mothers	106 infants

Most mothers approached to participate in the VTI consented except for 10. During the time that corresponded to our vitamin D study, 1308 mother-infant dyads were enrolled. 231 samples were not collected from mother-baby pairs, with the most common reasons noted: the delivery was missed, or the mother delivered outside the hospital. Our study population may not have reflected the overall susceptible population because the study was only conducted at UATH, a tertiary hospital, and the rate of homebirths in Nigeria is high at around 60%. [158] An alternative would have been to collaborate with local community-based health workers/midwives for enrollment to capture a broader base of participants; trained midwives have been shown to increase health facility birthing. [159] This approach would have increased the cost of the study but would have provided a more representative sample that would have strengthened our three studies. Furthermore, the newborns of mothers who gave birth at home were at higher risk of newborn sepsis or dying in the neonatal period. [160] Since we did not capture this population at higher risk of morbidity and mortality in our study, this could have

impacted our findings, especially for the vitamin D and metabolite study as evaluation for sepsis and newborn death were two of our study outcomes.

The advantage of being embedded into a larger study was that only one overall consent was administered, however, the limitations are the length of the consent process and the difficulty with consistently documenting all the sections of the study intake form. One important element that was not well documented and could have impacted the interpretation of our research findings was participant ethnicity. One of our strengths was that we had research team members who spoke several of the common languages in the regions, however, participant ethnicity was not consistently captured. Nigeria, in general, and our study site near Abuja is ethnically diverse. Studies in Nigeria have shown that child health outcomes vary by ethnicity due to differing cultural practices. [161, 162] Studies have also shown that dietary customs vary across ethnicities and that understanding these differences is crucial to designing effective nutritional interventions. [100] Having ethnicity data in our vitamin D and SPM studies would have helped to further contextualize the results. Understanding the differences between ethnic practices, such as parity and birth spacing or adherence to malaria prophylaxis and treatment, could have also helped with our placenta malaria study, as reports have shown, for example, that there is a higher level of malaria parasitemia with lower parity. [163-165]

Data collection

For all our studies, some additional key data that were not collected could have strengthened our findings. For our evaluation of maternal micronutrient status, we did not ask if mothers were taking vitamin D or omega-3-fatty acid supplements; this information would have been useful to interpret the concentration levels that we observed in the mothers and their newborns. Additionally, during the VTI study, a subset of the study participants was enrolled in a

parent pilot study on describing their daily food intake using a food frequency questionnaire. The data management system did not allow us to retrieve the food questionnaire report and micronutrient level blood sampling for each participant. To assess maternal risk of anemia with asymptomatic malaria infection, hemoglobin was not routinely collected for all study participants at delivery. With the limited data we have, there was no significant association of malaria infection with the presence of maternal anemia. In contrast, studies have shown that even with an asymptomatic infection in pregnancy, there is a higher risk of maternal anemia. [166] In addition to collecting hemoglobin levels on all mothers, our study would have been strengthened if the newborn had also been checked for anemia; when a mother contracts malaria during pregnancy, it can lead to maternal anemia with the newborn possibly developing anemia as well. [167]

We knew that most of our study participants were on malaria prophylaxis (90.4%). However, additional data not collected that would have helped to further strengthen our study included results of peripheral thin and thin smear microscopy, peripheral rapid diagnostic testing, and information on recent malaria treatment, all of which are important data to correlate to our placenta malaria infection findings. Furthermore, collecting maternal peripheral blood, placenta, and cord blood for malaria PCR would have completed a cross-sectional snapshot of malaria status in our population and help provide more data points to strengthen our findings.

The data collected in the VTI study included maternal rectovaginal swabs to assess for bacterial colonization at delivery, and the newborn surface swabs, including skin, throat, and ear canal samples were obtained; this sampling was not painful. Maternal blood samples were collected, and for the baby, cord blood samples were obtained; this was not painful for the newborn, but for the mother, the venipuncture could have led to refusal to participate in the

study. This is important because two of our pilot studies required larger volumes of serum, which is an added burden and due to this, we were unable to obtain samples for all the participants for whom we had sociodemographic data. This particularly affected our SPM, where we were able to collect only 78 maternal sample and 58 newborn samples for analysis of specialized pro-resolving mediators due to low sample volumes. Though sampling cord blood is not viewed as painful per se, there is an important cultural component that could explain why in many cases enough blood was not collected from the cord or placenta and why, from the VTI study enrollment that included 264 for the placenta malaria study, only 139 samples were collected, with 104 resulting viable to be processed. There were 125 missed biopsies from participants who did not withdraw consent for the VTI study but from whom placenta biopsy specimens were not obtained. The study registry did not document the reasons for not obtaining a biopsy. Aside from the timing of deliveries with possible unavailability of study personnel, and mothers delivering at another facility or at home, there are cultural considerations to handling a placenta after birth in the West African region. [83] For some ethnic groups in Nigeria, the placenta is considered as the deceased twin of a newborn; after delivery, it is collected and buried under a tree, considered the tree of life that connects the child to the earth. [168] This may explain why mothers would prefer not to have a placenta biopsy as it would no longer remain intact; our study procedure was to cut up sections of the placenta and the umbilical cord. This cultural practice may have affected recruitment to our placenta malaria study as we could not capture a more representative segment of the pregnant population. Furthermore, distinct cultural practices may also mean different environmental risk factors for malaria based on the type of dwelling people live in and their adherence to preventative medication.

Our study finding of detecting *P. knowlesi* infection in our study population highlights

the need to continue to further characterize the epidemiology of the varying *Plasmodium* species infection in the placenta of asymptomatic pregnant women using more sensitive diagnostic tools, such as PCR. One procedure that would leave placentas more intact after sampling and be more acceptable to local cultural practices is minimally invasive tissue sampling (MITS). [169] MITS is a needle-based postmortem procedure used to find the cause of death that has been particularly useful in low- and middle-income countries where complete diagnostic biopsies are rarely performed. [169] This procedure is relatively simple, does not require particular infrastructure, and can be performed by a non-pathologist health professional and an assistant.[170] With support from the Bill & Melinda Gates Foundation a MITS surveillance alliance was established to support the generation of cause of death data and capacity building through the program to perform this procedure has been conducted across in several countries across the globe, including Nigeria. [170] There are no reports, however, on the acceptability of this procedure in Nigeria. On the continent, a study in Mozambique looked at the acceptability of MITS in hypothetical and real case scenarios and found that families were willing to consent to MITS especially if it would help prevent future deaths and a significant barrier was concern for confidentiality. [171] A study in Kenya on the acceptability of the procedure showed that 40% of study participants would have preferred MITS over conventional autopsy while 81% of those who refused conventional autopsy would have accepted a MITS procedure if it had been offered. [172]

Specimen processing, cost, and team expertise

Our study collaborative comprised of a team in Nigeria, Nebraska, and Canada with leading experts in nutrition science, pathology, microbiology, and infectious diseases. Our study required, however, technical components that may have affected the outcomes of our three studies. For the study in Chapter 1, the vitamin D and metabolite analysis, samples had to be

shipped from Nigeria to the US and then to Canada for access to the liquid chromatography mass spectrometry (LC-MS/MS) assays which came at a high cost and impacted our ability to continuously collect data for the duration of the VTI study.[26] Additionally, our study would have benefited from collecting data at more than one time point per participant (before delivery, at delivery, and during infancy), with or without vitamin D supplementation, to observe changes in levels over time and their associated outcomes. Our study showed that percent 3-epi-25(OH)D_3 was associated with sepsis in addition to some of the birth anthropometric; knowing the maternal stores of 25(OH)D before delivery would have given us an insight into the amount of substrate available that would cross the placenta to make for the fetus to make its own 3-epi-25(OH)D_3. [173] Furthermore, studies have shown that the concentration of 3-epi-25(OH)D_3 decreases over the first year of life, so assessing the vitamin D metabolite levels after birth would have allowed for a more comprehensive view of its association with infant morbidity due to sepsis. [173]

For Chapter 2, looking at SPMs in maternal-infant dyads sampling at multiple time points would have also provided more information in addition to collecting dietary and supplement intake information. Capturing more information about the levels of SPM precursors in the blood during pregnancy, at and after delivery would also allow us to see the variations of their concentration over time while comparing maternal and infant levels. This is particularly important as our studies showed a significant difference between the amount of SPM in maternal blood between US and Nigeria mothers, while for infants from both countries, the concentrations were not statistically significant. The lack of observed difference could have been due to the very small sample size of Nigerian infant samples available for the study. Alternative approaches would require more sampling which would have been cost prohibitive. Though employment status was obtained, details about the type of employment were not obtained,

neither were the location of employment, amount of time outside under direct sun in addition to type of overall clothing beyond just head covering. All these data would have helped strengthen and contextualize our study findings.

Our third study looked at the placenta and the umbilical cord at the time of delivery; in addition to the cultural considerations surrounding the handling of the placenta in West Africa, one key component that also affected our findings came from actual specimen collection and processing prior to shipment to the US for analysis. Technical histopathology support at UATH was limited for the size of our study, so we relied on the training of study personnel for simple blood sampling and to collect biopsies of the placenta and the cord. Our pathology team in the US provided us with training material that included handouts, and an onsite training prior to initiating consent was performed by the senior microbiologist at IFAIN using the US pathology team instructions. The area within the delivery ward where the sampling occurred was small, and the placenta and cord could not be processed outside of the ward as they had to be close to the mother for the cultural reasons we explained. This led to an inconsistent quality of the specimen in terms of sampling of the placenta, which could have affected the biopsy and placenta blood culture results. At the beginning of the study, blood cultures were discarded due to contaminations from the collection process. Further training helped decrease these collection errors, however, the overall process remained tedious.

Moreover, specimen processing and storage prior to shipment were added complications after specimen collection. Given the lack of locally trained personnel to handle the biopsies, specimens had to be shipped in small vials with FineFIX, a formalin-free water-based concentrate we used to avoid the issues with formalin pigment that make it difficult to detect malaria pigment. [141, 174] Given the inconsistencies in the size of the placenta specimen collection, the vials often contained too many sample pieces with too little fixing agent. Upon

arrival at UNMC, they were not viable and had to be discarded. After receiving the first batch of placenta and cord specimens, the collection of placenta specimens was suspended due to inconsistent specimen quality and increasing cost. The specimens we analyzed in our study were viable; however, we could only collect data for a brief period. When assessing the effects of living in a region with endemic malaria, sample collection should occur over several seasons; the central region of Nigeria has a dry season (December to March) and a rainy season (April to September). An accurate epidemiologic profile of *Plasmodium* species should reflect the local environmental conditions.

Furthermore, there were limitations in our approach to sample analysis that could affect the interpretation of our findings. The placenta histologic studies were only conducted in the US samples were not kept in Nigeria for Giemsa staining for malaria due to cost and lack of personnel availability. Since our histologic evaluation included more than just staining for malaria, we also included staining for CMV, HSV, syphilis, and toxoplasmosis in addition to assessing for chorioamnionitis and funisitis. All of these additional tests were not available at the lab in Abuja. However, locally, there is expertise in looking at blood smears for malaria; our findings would have strengthened had we been able to compare malaria Giemsa staining interpretation in Nigeria and the US, it would have also strengthened our study to have the staining done by at least two blinded pathologists on the US side, but it was not possible due to lack of funding and lack of local US based expertise. Finally, malaria PCR testing was not part of the original protocol and was performed only on the viable fixed specimens. Had we anticipated PCR testing to be done at the time of the initial protocol, placenta blood samples would have been collected for this study. This would have provided more study samples, and we could have looked at blood versus tissue PCR testing. Our study is the first to report a plasmodium species that is not seen in this region of the world, *P. knowlesi*; an approach with two blinded

parasitologists to analyze the specimens via PCR would have strengthened our findings. This was not done due to lack of funding and available US-based local expertise.

Next Steps

From this global health research experience, we have learned the importance of assembling a strong collaborative team with our country partner and the significance of aligning our research priorities with the needs of our country partner. We will continue our collaboration with IFAIN. and expand local interprofessional collaborations to include community health workers and midwives to perform a larger and more representative population-based study. This time, we would design a longitudinal interventional study to evaluate the impact of vitamin D and omega-3 fatty acid supplementation during pregnancy on newborn and infant outcomes in a region with endemic malaria, more specifically, looking to assess if placental malaria infection can affect the potential benefits of maternal micronutrients supplementation to their offspring. The results of this study would provide crucial data to inform recommendations on micronutrient supplementation and additional malaria screening and prevention strategies for pregnant Nigerian women.

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