ASSOCIATION OF VITAMIN D WITH MEASLES VIRUS AND DIPHTHERIA TOXOID SPECIFIC VACCINE ANTIGEN IMMUNOGLOBULIN G RESPONSES IN MOTHER - CHILD PAIRS AT CHULAIMBO SUB-COUNTY HOSPITAL IN WESTERN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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DEDICATION

To my family, friends and the University fraternity for making this research a success.

ABSTRACT

Vitamin D is a lipid soluble vitamin that acts as a hormone as well as a micronutrient. Through vitamin D receptors (VDR) expressed on immune cells, vitamin D polarizes Th1/Th2 cytokine balance in pregnancy regulating humoral immune responses triggered by B cell antigens. How plasma concentrations of 25-hydroxyvitamin D affect the quantity of anti-measles virus and anti-diphtheria toxoid vaccine-antigen antibodies during pregnancy in early life years remain unclear. This study assumed that vitamin D affects antibody responses to infections and therefore investigated its association with IgG levels following measles virus and diphtheria toxoid vaccination during childhood. Specifically, the study quantified plasma concentrations of 25 (OH) D, anti measles virus and anti diphtheria toxoid IgG, and computed the association between 25(OH) D and the vaccine-induced IgG responses in mother-child pairs. This was a hospital-based prospective cohort study of pregnant mothers from their first trimester to delivery; and mother-infant pairs from delivery/birth up to 18 months old. Mothers attending antenatal care at Chulaimbo Sub-County hospital were enrolled in their first trimester and followed up to delivery. Infants were followed up from delivery to 18 months old at 6 months interval. Plasma was freshly processed from the blood samples, and stored at -80^{o}C before bio-assaying. Plasma concentration of 25(OH) D was measured by enzyme immuno-sorbent assay technique (EIA) *in vivo* at ANC1,2,3 and 4/delivery in mothers; in cord blood, at 6, 12 and 18 months in children. Anti-measles virus and anti-diphtheria toxoid vaccine IgG antibody levels were quantified using commercially available ELISA kits at delivery in mothers; in cord blood, at 6, 12 and 18 months in children. Data was analysed from 47 mother-child pairs. Vitamin D quantifications showed that, at delivery, 8.51% (4) of the mothers were deficient (<25nmol/L), 27.66% (13) insufficient (<50nmol/L) and 63.83 % (30) were sufficient (≥75nmol/L). At delivery, 6, 12 and 18 months, 27.66% (13), 10.64% (5), 12.77% (6) and 10.64% (5) infants were deficient and 17.02% (8), 25.53% (10) and 21.28% (10) infants were insufficient respectively. Vaccine-induced IgG antibody profile data showed that, 10.64% (5) of the mothers at delivery; 19.15% (9), 57.15% (27), 4.26% (2), 2.13% (1) infants had low(≤ 0.5) anti-measles virus vaccine-induced IgG levels; 65.96% (31) mothers and their infants, 36.17% (17), 53.19% (25) and 46.81% (22) infants had low(≤ 1.6) anti-diphtheria toxoid vaccine-induced IgG levels at delivery and subsequent follow up time points. Maternal plasma 25(OH) D concentrations were correlated with infants' anti-measles virus (r^2=0.71) and anti-diphtheria toxoid (r^2=0.56) vaccine-induced IgG antibody levels at delivery. These findings add knowledge about vitamin D status and anti measles virus and anti-diphtheria vaccine-antigen IgG antibody responses. Pregnant women and their infants from regions with constant sunny climatic conditions have varying vitamin D status including; deficient (<50nmol/L), insufficient (≥50<75nmol/L) and sufficient (≥75nmol/L) status. Similarly, proportions of these participants depict high, medium and low vaccine-induced IgG antibody responses with regards to the respective vaccine standard references. Maternal vitamin D status is associated with the infant's vitamin D status early in life, besides, maternal vitamin D concentrations at delivery are associated with the infant's vaccine-induced IgG antibody levels at birth, thus influencing the child's passive immunity. This study therefore recommends maintenance of adequate maternal plasma vitamin D concentrations for sufficient infant plasma vitamin D levels at birth and optimum vaccine-induced IgG responses in their infants in early life years.

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LIST OF ABBREVIATIONS AND ACRONYMS

25, (OH) D	25, Hydroxy-Vitamin D
ANC	Anti-natal Care Clinic
ATCC	American Type Culture Collection
CB	Cord Blood
DPT	Diphtheria Toxoid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno-sorbent Assay
HB	Haemoglobin
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
Ig A	Immunoglobulin A
Ig E	Immunoglobulin E
Ig G	Immunoglobulin G
LAZ	Length for Age z score
MCH	Maternal Child Health
MHC	Major Histo-compatibility Complex
MOH	Ministry of Health
MS	Measles
MTCT	Maternal-to Child Transmission
OD	Optical Density
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween

RTQPCR	Real Time Quantitative Polymerase Chain Reaction
SGS	School of Graduate Studies
Th	T helper cells
TMB	Tetramethylbenziddine
VB	Venous Blood
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
WAZ	Weight for Age z-score
WHO	World Health Organization
WLZ	Weight for length z score

DEFINITION OF TERMS

Gestational age: A period from conception to delivery time normally divided into trimester 1, 2, 3 and 4

Hypovitaminosis D: Vitamin D deficiency (<25nmol/L)

Humoral Immunity: This refers to immune responses mediated by antibodies secreted by the B lymphocytes in to the extracellular fluids.

Impact: This refers to the association between vitamin D concentration/status and vaccine induced antibody levels as measured by plasma 25(OD) D nmol/L and anti-measles virus and anti-diphtheria toxoid vaccine antigen IgG levels (OD).

Lymphoproliferative response: Refers to a heterogeneous group of expanding, monoclonal or oligoclonal lymphoid cells that occur in the setting of an immune response.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Vitamin D is important in the maintenance of a functional immune system (Prietl et al., 2013). It is involved in the regulation of cell growth and differentiation, proliferation, apoptosis and modulation of the immune system (Di Rosa et al., 2011). By virtue of these, vitamin D remains an essential micronutrient during rapid growth in infants and young children, adolescents and pregnant women (Di Rosa et al., 2011). Vitamin D receptor (VDR) expression on immune cells including; dendritic cells, monocytes, macrophages and lymphocytes make vitamin D an important molecule in antigen recognition and ultimate mounting of optimum innate and adaptive immune responses (Di Rosa et al., 2011; Prietl et al., 2013;Khoo et al., 2011; Mora et al., 2008). Vitamin D polarizes Th1 responses towards more regulatory Th2 responses important in maintenance of pregnancy (Raghupathy 2001; Hyppönen 2011). Polarization of Th1/Th2 cytokine balance regulates immunoglobulin isotype production influencing antibody class switching (Harwood and Batista 2010). In essence, Th1 cytokines promote IgG immunoglobulin isotype production whereas Th2 cell cytokines induce the production of IgE (Snapper et al., 1997). Given that Th1/Th2 balance directly influences humoral immunity (Khoo et al., 2011; Mora et al., 2008), it is likely that this Th1/Th2 polarization by vitamin D micronutrient affects subsequent antibody responses during pregnancy and even early childhood.

Vitamin D status is defined at different plasma concentrations of 25 Hydroxy-vitamin D (25 (OH) D. Deficiency state is defined at plasma concentrations of <50nmol/L; vitamin D insufficiency is defined as plasma concentrations of <75 nmol/L serum concentrations. Severe deficiency is defined as plasma concentrations of < 25nmol/L (Kennel et al., 2010; Ginde et al.,

2009). Plasma values of (≥75nmol/L) reflect sufficient concentrations (Alvarez-Rodriguez et al., 2012; Holick et al., 2012; Rosen 2011). Regular exposure to direct sunlight provides the required hydroxylation to produce systemic bioactive vitamin D molecules. Humans acquire much of their vitamin D through a photosynthetic reaction in the skin on direct exposure to direct sunrays with a small amount through digestive intake. This is then stored in the liver and storage fats. Limited direct sunlight exposure, poor dietary intake and low stores for vitamin D pose a great risk factor for its deficiency (Khoo et al., 2011). Dark-skinned people with high melanin concentration and reduced vitamin D storage capacity have significantly low serum vitamin D levels due to reduced vitamin D binding protein (VDBP) and hence less bioactive vitamin D (Health Quality Ontario 2010a). Vitamin D deficiency predisposes individuals to infectious diseases such as tuberculosis and autoimmune disease such as type 1 diabetes mellitus and multiple sclerosis due to dysfunction of the immune system's physiological process (Bikle 2011).

Vitamin D deficiency, also known as hypovitaminosis D, is of great public health importance in humans from areas with adequate sunshine mainly attributed to poor dietary intake and inadequate sunshine exposure (Allali et al., 2009; Gannagé-Yared et al., 2000). Optimum concentration of vitamin D during pregnancy is essential for bone mineralization of the developing fetus, despite the adequate high level of sunshine in equatorial countries, the prevalence of maternal hypovitaminosis D is on the rise (Ergür et al., 2009; Halicioglu et al., 2012; Karras et al., 2014; Parlak et al., 2014). This may lead to severe unwanted pregnancy outcomes as well as skeletal and non-skeletal health complications among the offspring (Dawodu and Wagner 2007b; Bodnar et al., 2013a and b; Thandrayen and Pettifor 2010). Previous findings have reported a high burden of vitamin D deficiency among pregnant women and their

infants at delivery in low socio-economic status populations with great risk attributed to their lifestyle and nutritional status (Halicioglu et al., 2012; Parlak et al., 2014). Some studies have looked at the status of vitamin D in infants born in different conditions. For instance, infants born before term are at a higher risk of vitamin D deficiency compared to those born at full term (Burris et al., 2013). Indoor exclusive breastfeeding is a major risk factor for vitamin D deficiency in infants aged 1-6 months (Choi et al., 2013a). In addition, although majority of healthy children at the age of 12 months are vitamin D sufficient, there is need for supplementation and use of fortified foods besides breastfeeding to reduce the risk of vitamin D insufficiency (Thorisdottir et al., 2014). Maternal vitamin D is an important factor in determining the infant's vitamin D status and their risk of developing vitamin D deficiency associated complications (Dawodu and Wagner 2007b; Thandrayen and Pettifor 2010; Walker and Modlin 2009). Recent reviews report a great global burden of vitamin D deficiency in adults advocating for better supplementation strategies. Evidence shows that, current supplementation recommendations for pregnant and lactating women are inadequate to ensure vitamin D sufficiency in these groups (Thandrayen and Pettifor 2012; Kovacs 2008). Maintenance of adequate levels of vitamin D through supplementation and sunlight exposure is one of the essential factors of prophylaxis for infectious diseases such as tuberculosis and parasitic infections such as malaria (Pludowski et al., 2013). Most Africans with dark skin complexion and high melanin concentration are at a risk of vitamin D deficiency. There was need to focus on defining vitamin D status in vulnerable populations hence, this study sought to investigate vitamin D status in pregnant mothers through their gestation period to delivery time in western Kenya, a region with holoendemic malaria transmission. An infant's vitamin D status is highly dependent on the mother's general knowledge about its biological importance and sources (Choi,

Kim, and Jeong 2013). Infants get vitamin D from their mothers through breast milk and exposure to direct sunlight (Choi, Kim, and Jeong 2013). Exclusively breast-fed children resting indoors without ample direct sunlight exposure, register the highest rate of deficiency defined at ≤50nmol/L in serum (Choi, Kim, and Jeong 2013). This suggests that, breast-fed children born to mothers with vitamin D deficiency that are un-supplemented and get little exposure to direct sunlight are at a high risk of vitamin D deficiency. Given the current implementation of exclusive breast feeding for six months, there are increased risks of vitamin D deficiency in mothers and their children. Hence, the current study investigated vitamin D status in mothers and their children from western Kenya.

Antibody responses to anti-measles virus, a live attenuated vaccine antigen, following B-lymphocytes activation (Plotkin 2001; Savy et al., 2009; Plotkin 2010) are highly influenced by human genetic and environmental factors (Kizito et al., 2013). Natural infections and recurrent disease outbreaks affect vaccine efficacy. Recent findings in Uganda show that malnutrition along with malaria and HIV co-infection during pregnancy lead to reduced antibody response to measles immunization in infancy (Kizito et al., 2013). Despite adequate measles vaccine coverage, cases of measles infections in young children are still evident in western Kenya (WHO report, 2011: IFRC report, 2011). Vaccine protection is partly dependent on the quantity of the specific vaccine induced protective immune correlate. In this case, this study quantified anti-measles virus vaccine antigen IgG antibody responses in mothers and their children in western Kenya, an area with holoendemic malaria transmission.

Antibody responses to diphtheria toxoid, a dead bacterial vaccine antigen following B lymphocyte activation, are highly dependent on the host's immune status at the time of vaccination (Plotkin 2001; Savy et al., 2009; Plotkin 2010). Like anti-measles virus vaccine

antigen antibody responses, anti-diphtheria toxoid vaccine antigen antibody responses are affected by genetic and environmental factors including natural infections and recurrent disease outbreaks (Grassly et al., 2015). Despite sound diphtheria toxoid vaccine coverage, cases of specific vaccine associated illness are still evident in western Kenya (WHO report, 2011: IFRC report, 2011). This could be attributed to introduction of a new biotype of *Corynebacterium diphtheria,* accumulation of susceptible individuals, migrating populations and recurrent parasitic infections such as malaria. Vaccine protection is partly dependent on the quantity of the specific vaccine induced protective immune correlate. This study therefore quantified anti-diphtheria toxoid vaccine antigen IgG antibody responses in mothers and their children in western Kenya with holoendemic malaria transmission.

Vitamin D deficiency leads to decreased innate immunity due to impaired macrophage activation, decreased T cell numbers and function (Savy et al., 2009). Many studies have investigated the effects of vitamin D on the dynamics of humoral immunity, for example, (Pincikova et al., 2011) described an inverse relationship on the levels of serum total IgG with serum vitamin D concentration in cystic fibrosis. Serum concentrations of 25(OH) D in the elderly have been shown to be directly proportional to low IgG2 and IgG4 levels in serum, and inversely proportional to increasing IgG1 and IgA levels (Sakem et al., 2013a). On the other hand, some findings show no significant role with less observed increase or reduction in antibody levels following vitamin D supplementation. Kriesel and Spruance (1999) reported that, vitamin D co-administered with influenza vaccine did not enhance humoral immunity in humans. On the contrary, it has been shown that 1, 25 Hydroxy vitamin D_3 (1, 25 (OH) D_3), another biologically active form of vitamin D present in plasma, suppresses the proliferation and immunoglobulin production by normal human peripheral blood mononuclear cells activated *in*

vitro by mitogens (Lemire et al., 1984). In addition, serum 25 (OH) D levels are associated with enhanced pneumococcal antibody levels in individuals with asthma (Lee et al., 2011). Other studies also show contradicting information on the actual role of vitamin D on protective immunity induced by vaccine antigens. While some study findings show inverse correlation, for instance, an association of reducing vitamin D levels with frequent serological response to influenza vaccine (Chadha et al., 2011; Principi et al., 2013), some findings depict no correlation at all (Kriesel and Spruance 1999). As such, a previous study concluded that there was need to investigate and ascertain the immuno-regulatory role of vitamin D during infection and vaccine-induced protective humoral immunity (Nair et al., 2007a). Optimum vaccine efficacy requires a full set of immunological responses starting with antigen recognition and presentation through to memory cell generation and antibody production or priming of cellular responses (Beverley 2002). Given the immunological role of vitamin D in the polarization of Th1/Th2 cytokine responses (Raghupathy 2001; Hyppönen 2011), and limited information about the association of vitamin D and the magnitude of antibodies in circulation (Pletsityĭ et al., 1997; Lee et al., 2011; Sakem et al., 2013b), this study determined the association between vitamin D status and anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody profiles in mothers at delivery and their children 0-18 months (at six months interval) from western Kenya.

1.2 Statement of the Problem

There is an increased burden of hypovitaminosis D among pregnant women and young children in tropical countries (Karras et al., 2014; Parlak et al., 2014; Halicioglu et al., 2012; Ergür et al., 2009). Despite high vaccine coverage, there are notable increasing incidences of re-emerging vaccine preventable infections in children and adults in tropical countries (Li et al., 2012; Brennan 2005**).** Incidences of measles and diphtheria cases have been shown among young

children in western Kenya (WHO report, 2011: IFRC report, 2011). Previous review reports have shown that there is limited information about the association of vitamin D and the magnitude of antibodies in circulation (Pletsityĭ et al., 1997; Lee et al., 2011; Sakem et al., 2013b). Hence, this study determined vitamin D status in pregnant mothers through their gestation period to delivery and in their children from birth up to 18 months at six months interval. Furthermore, this study determined the association between vitamin D status and childhood anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody responses in mother child pairs from western Kenya.

1.3 Study Objectives

1.3.1 General Objective

To evaluate the association between vitamin D status and antibody responses to attenuated measles virus and diphtheria toxoid vaccine antigens in mother-child pairs attending maternal child health (MCH) services at Chulaimbo Sub-County hospital in western Kenya.

1.3.2 Specific Objectives

1. To determine 25(OH) D status in mothers from their first trimester to delivery and their children from birth at 0, 6,12 and 18 months seeking health care from Chulaimbo sub-County hospital in western Kenya.
2. To determine anti-measles virus specific vaccine antigen IgG antibody levels in plasma from mothers at delivery and their children at 0, 6, 12 and 18 months seeking health care from Chulaimbo sub-County hospital in western Kenya.
3. To determine diphtheria toxoid specific vaccine antigen antibody levels in plasma from mothers at delivery and their children at 0, 6, 12 and 18 months seeking health care from Chulaimbo sub-County hospital in western Kenya.

4. To establish the association between maternal and fetal vitamin D and specific vaccine antigen IgG antibody responses at delivery time and subsequent follow up time points in mother-child pairs seeking health care from Chulaimbo sub-County hospital in western Kenya.

1.3.3 Research Questions

1. What are the plasma concentrations and status of 25(OH) D in mothers through their gestation period and their children at 0, 6, 12 and 18 months seeking health services from Chulaimbo sub-County hospital in western Kenya?
2. What are the anti-measles virus specific vaccine antigen IgG antibody levels in plasma from mothers at delivery and their children at 0, 6, 12 and 18 months seeking health services from Chulaimbo sub-County hospital in western Kenya?
3. What are the anti- diphtheria toxoid specific vaccine antigen IgG antibody levels in plasma from mothers at delivery and their children at 0, 6, 12 and 18 months seeking health services from Chulaimbo sub-County hospital in western Kenya?
4. What is the association between maternal and fetal vitamin D and specific vaccine antigen IgG antibody responses at delivery time and subsequent follow up time points in mother-child pairs seeking health care from Chulaimbo sub-County hospital in western Kenya?

1.4 Significance of the Study

The ability of vaccines to induce protection is dependent on a number of factors including the hosts' nutritional status, the vaccine dose administered, the quantity and quality of the immune correlates induced characterized by the affinity and avidity of vaccine antigen specific antibodies. The ability to induce sufficient naïve immune cells into antigen specific effective and

rapidly reactivated immune memory cells is an important determinant for the induction of long-term protection. Vitamin D deficiency down regulates the body's ability to regulate its immune physiology. The large population of adults and children with micronutrient deficiency, coupled with a gradual increase in the incidences of re-emerging vaccine preventable infectious diseases in developing countries, could explain the limited vaccine efficacy in these countries. This study investigated the association between vitamin D status and antibody responses to attenuated measles virus and diphtheria toxoid vaccines induced responses in mother-child pairs attending maternal child health (MCH) services at Chulaimbo Sub-County hospital in western Kenya. Findings depicted proportions of mothers and their children with deficient and insufficient vitamin D states. In addition, different proportions of mothers and their children had varying levels of specific vaccine antigen IgG antibody responses. Vitamin D deficiency and insufficiency states were associated with medium and low specific vaccine antigen IgG antibody responses. These findings indicate an association between vitamin D and anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody responses.

These study findings provide a good basis for formulating public health interventions using vitamin D supplements in enhancing maternal and child specific vaccine antigen immune responses. Understanding the association between vitamin D micronutrient and vaccine efficacy is vital in designing intervention strategies aimed at micronutrient supplementations that boost specific vaccine antigen immune response in mothers and children.

CHAPTER TWO

LITERATURE REVIEW

2.1. Vitamin D and its Biological Importance

Vitamin D is a commonly used collective term for a family of closely related seco-steroids. Upon exposure to sunlight, 7-dehydro-cholesterol, located deep in the actively growing layers of the epidermis, undergoes photolytic cleavage of the "B" ring to yield pre-vitamin D_3, which is isomerized to vitamin D_3 (cholecalciferol). Vitamin D_3 and vitamin D_2 (ergocalciferol) may also be obtained by dietary supplementation or from a limited number of foods. Vitamin D_2 is metabolized in a similar way to vitamin D_3. Vitamin D is stored in adipose tissue and enters the circulation bound to vitamin D binding protein (VDBP) and albumin. In the liver, vitamin D is hydroxylated to give 25-hydroxyvitamin D (25-OH D), which also circulates as a complex with VDBP. A small proportion of the 25-OH D is further hydroxylated in the kidney, under direct regulation by parathyroid hormone and ionized calcium levels, to form the biologically-active calcitropic hormone 1, 25 di-OH D. Further, hydroxylation and metabolism of vitamin D produces compounds that are water soluble and readily excreted. Hepatic vitamin D 25 hydroxylase activity is not tightly regulated, and changes in cutaneous production of vitamin D_3, or ingestion of vitamin D (D_3 or D_2), which results in changes in circulating levels of 25-OH D (Norman et al., 1989).

Plasma concentration of 25-OH D is considered to be the most reliable measure of overall vitamin D status and thus can be used to determine whether a patient is vitamin D sufficient (Holick, 1996). Vitamin D status is defined at different plasma 1, 25-OH D concentrations in nmol/L. A person is considered to have vitamin D deficiency at <50 nmol/L of 25(OH)-D and insufficiency at <75 nmolL serum concentrations. Severe Vitamin D deficiency is defined at <

25nmol/L (Kennel et al., 2010; Ginde et al., 2009). Concentrations (≥75 nmol/L) reflect sufficient concentrations (Alvarez-Rodriguez et al., 2012; Holick et al., 2012; Rosen 2011). Vitamin D remains essential during rapid growth in infants, young children and pregnant women (Hossein-nezhad and Holick 2013). Adequate concentration of vitamin D during pregnancy is essential for bone mineralization of the developing fetus (Raghupathy 2001)

Vitamin D and its metabolites have been associated with calcium and phosphate metabolism important in bone mineralization (Bendik et al., 2014; Wacker and Holick 2013). The importance of vitamin D and its regulation of calcium and phosphorus in ensuring optimal pregnancy outcomes have been examined ever since its discovery in the early 1900s (Roth 2011; De-Regil et al., 2012; Harvey et al., 2014). Many of these studies initially focused on the prevention of osteomalacia and eclampsia (Wacker and Holick 2013; Hossein-nezhad and Holick 2013; Khazai et al., 2008; Gennari 2001). For instance, studies have shown that a diet supplemented with calcium, phosphorus, and vitamin D could treat osteomalacia in China and India (De-Regil et al., 2012). Adequate provision of vitamin D during pregnancy is essential for bone mineralization of the developing fetus (Hossein-nezhad and Holick 2013; Bendik et al., 2014). It is estimated that approximately 25–30g of calcium are transferred to the fetal skeleton by the end of pregnancy, most of it during the last trimester (Jacquemyn et al., 2013; Mahadevan et al., 2012; Olmos-Ortiz et al., 2015). The role of vitamin D during pregnancy may be indicated by an increase in the concentrations of the active form, 1.25-dihydroxyvitamin D—50%–100% over the non-pregnant state in the second trimester and over 100% in the third trimester (Barrett and McElduff 2010; Bodnar, et al., 2013b). Increase in concentrations potentially accounts for changes such as greater transfer of calcium to the fetus (Hossein-nezhad and Holick 2013; Khazai et al., 2008). Poor vitamin D status may lead to severe unwanted pregnancy outcomes involving either skeletal

(growth restriction) or non-skeletal health complications, including pre-eclampsia, gestational diabetes, preterm birth, and low birth weight (Bodnar et al., 2013; Bodnar et al., 2010).

Typically, *in-vivo* vitamin D metabolism is mainly dependent on direct exposure of the skin to ultraviolet (UV) rays, which is required for the isomerization of endogenous 7-dehydrocholesterol in the formation of cholecalciferol (Roth 2011; De-Regil et al., 2012; Harvey et al., 2014). Vitamin D deficiency, hypovitaminosis D, is of great public health importance in sunny regions mainly attributed to increased urbanization, seasonal climatic changes, skin pigmentation, clothing style, use of sun-screen and poor dietary intake (Allali et al., 2009; Gannagé-Yared et al., 2000). Despite the high level of sunshine in equatorial countries, the prevalence of maternal hypovitaminosis D is on the rise (Karras et al., 2014; Parlak et al., 2014; Halicioglu et al., 2012; Ergür et al., 2009). This may lead to severe unwanted pregnancy outcomes, skeletal and non-skeletal health complications among the offspring (Thandrayen and Pettifor 2010; Dawodu and Wagner 2007a). Previous findings have reported a high burden of vitamin D deficiency among pregnant women and their infants at delivery in low socio-economic status populations with great risk attributed to their lifestyle and nutritional status (Halicioglu et al., 2012; Parlak et al., 2014). Some studies have looked at the status of vitamin D in infants born with different conditions. For instance, infants born prematurely, before term are at a higher risk of vitamin D deficiency compared to those born at full term (Burris et al., 2013). Exclusive breastfeeding in-doors is a major risk factor for vitamin D deficiency in infants aged 1-6 months (Choi et al., 2013). In addition, although majority of healthy children at the age of 12 months are vitamin D sufficient, there is need for supplementation and use of fortified foods besides breastfeeding to reduce the risk of vitamin D insufficiency (Thorisdottir et al., 2014). Dark-skinned people with high melanin concentration and reduced vitamin D storage capacity have

significantly low serum vitamin D levels due to reduced vitamin D binding protein (VDBP) and hence less bioactive vitamin D (Health Quality Ontario 2010). Low vitamin D concentrations deregulate immune responses leading to enhanced disease development including infectious vaccine preventable pathogen associated diseases (Bikle 2011). Additionally, vitamin D deficiency has been shown to play a role in susceptibility to bacterial and viral infections, such as tuberculosis (Anty et al., 2014; Park et al., 2017) and human immunodeficiency virus (HIV) (Sabetta et al., 2010; Martineau and Jolliffe 2014). Further maternal vitamin D deficiency and parasitic infections during pregnancy have also been associated with an increased risk of anemia and iron deficiency (Finkelstein et al., 2012). With the dark skin complexion, high melanin concentration and exposure to infectious diseases such as malaria and other vaccine preventable infections, populations in western Kenya with low socio-economic status are at a high risk of vitamin D deficency-associated complications. As such, this study measured maternal plasma 25(OH) D through the different trimesters to delivery and in child's cord blood at birth up to 18 months old at six months interval in a cohort of pregnant women seeking antenatal care services at Chulaimbo Sub-County hospital in western Kenya.

2.2. Measles Virus Vaccine antigen Antibody Responses

Antibody responses to anti-measles virus, a live-attenuated vaccine antigen, are highly influenced by exposure to natural infections, recurrent disease outbreaks and nutritional status of the host individuals (Kizito et al., 2013). Previous study findings have shown that persistence of maternal antibodies and young ages affect the quantity and quality of vaccine-induced neutralizing antibodies (Nair et al., 2007). Age dependent differences exist in IgG isotype avidity induced by measles vaccine received during the first year of life (Nair et al., 2007). Children show excellent antibody responses to measles virus vaccine, but few generate detectable

lymphoprolifarative responses to measles virus antigen, partly contributing to varied specific vaccine-induced responses (Bautista-López et al., 2001). Kizito et al., (2013) reported that, malaria and HIV infections alongside malnutrition in mothers and their neonates during pregnancy may result in reduced antibody responses to measles immunization in infancy. Earlier studies by Heilmann et al., (2006) reported varying measles virus vaccine antigen specific antibody responses in vaccinated individuals. Despite good measles vaccine coverage in western Kenya WHO report, 2011: IFRC report, 2011). Vaccine protection is partly dependent on the quantity of the specific vaccine induced protective immune correlate. Hence, this study quantified anti-measles virus specific vaccine antigen IgG antibody responses in mothers and their children in western Kenya, an area holoendemic for *P. falciparum* malaria transmission.

2.3. Diphtheria Toxoid Vaccine antigen Antibody Responses

Antibody responses to diphtheria toxoid, a dead bacterial vaccine antigen is highly dependent on the host's immune status at the time of vaccination (Plotkin 2001; Savy et al., 2009; Plotkin 2010). Like anti-measles antibody responses, anti-diphtheria toxoid vaccine-induced vaccine antigen responses are affected by an array of environmental and endogenous factors.. These include natural exposure to infections, recurrent disease outbreaks and nutritional status of the host individuals (Grassly et al., 2015). Maternal factors including the presence of high maternal diphtheria toxoid specific antibody levels obscure generation of the specific vaccine-induced antibody responses in infants following vaccination (Edwards 2015). Despite sound diphtheria toxoid vaccine coverage, cases of specific vaccine associated illness are still evident in western Kenya (WHO report, 2011: IFRC report, 2011). This could be attributed to introduction of a new biotype of *Corynebacterium diphtheria*, accumulation of susceptible individuals, migrating populations and recurrent parasitic infections such as malaria. Vaccine protection is partly

dependent on the quantity of the specific vaccine induced protective immune correlate. This study quantified anti-diphtheria toxoid specific vaccine antigen IgG antibody responses in mothers and their children in western Kenya with holoendemic malaria transmission.

2.4. Vitamin D and Immunity

Vitamin D status appropriately determined by plasma concentrations of 25 hydroxy-vitamins D (25-OH D), the most stable vitamin D metabolite in the circulatory system, may considerably impact on the bioavailability of active vitamin D metabolites vital in immune-regulation. Recent findings showing the expression of vitamin D receptors (VDR) on body cells unlimited to skeletal health suggest a role in cell growth, differentiation and proliferation (Sun 2010; Bendik et al., 2014). VDR expression on dendritic cells, monocytes, macrophages and lymphocytes make vitamin D an important molecule in the regulation of both innate and adaptive immune responses (Di Rosa et al., 2011; Prietl et al., 2013). The ability of vitamin D to modulate Th1/Th2 cytokine balance makes it a vital molecule in the regulation of both cellular and humoral immune responses (Khoo et al., 2011; Mora et al., 2008). Interactions between antigen stimulated B cells and MHC class II associated helper T cells yield more variable and functionally versatile antibody molecules (Mora et al., 2008). In addition, the regulation of the Th1/Th2 cytokine balance has an impact on immunoglobulin isotypes, influencing antibody class switching (Harwood and Batista 2010). In essence, Th1 cytokines promote IgG2a and IgG3 where as Th2 cell cytokines induce the production of IgG1 and Ig E (Snapper et al.,1997). Vitamin D polarizes Th1 responses towards more regulatory Th2 responses which are important especially in the maintenance of pregnancy (Raghupathy 2001; Hyppönen 2011). Vitamin D deficiency is associated with decreased innate immunity due to impaired macrophage activation decreased T cell numbers and function (Hewison 2010). All other factors constant, with the use

of stable, potent vaccines, re-emergence of vaccine preventable infections among populations may be well explained by reduced levels of vaccine antigen specific immune correlates early in life. To adequately assess effects of vitamin D on the immune system, it was appropriate to study its effects on antigen specific immune responses such as antibody responses to vaccines. Given the immunological role of vitamin D in the polarization of Th1/Th2 cytokine responses (Raghupathy 2001; Hyppönen 2011), and limited information about the association of vitamin D and the magnitude of antibodies in circulation (Pletsityĭ et al., 1997; Lee et al., 2011; Sakem et al., 2013b), this study determined the association between vitamin D status and anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody profiles in mothers and their children from western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

This study recruited and followed up pregnant women attending antenatal clinic (ANC) and seeking postnatal care with their children at Chulaimbo sub-County hospital in Kisumu District of western Kenya (Figure 3.1) from June 2011 to January 2014. Chulaimbo sub-County hospital is a public rural health facility located within the Lake Victoria basin, an area distinctly characterized by stable holoendemic malaria transmission (Jenkins et al., 2015). Chulaimbo sub-County hospital is located in Kisumu County, Kisumu West sub County, Maseno division, North-west Kisumu location, Kisumu rural constituency, Marera sub-location (Figure 3.1). Apart from Maseno Division, the hospital serves patients from other neighboring divisions including Winam and Luanda Division. The facility serves approximately 19,230 patients annually. It has few hospital beds limiting the number of in-patients to only 26. This holoendemic malaria transmission area experiences moderate temperature changes and receives reliable rainfall throughout the year with alternating dry and wet seasons (Minakawa et al., 2002). The climatic characteristics form very suitable conditions for mosquito breeding. *Anopheles gambiae* mosquitoes are the main species responsible for transmitting *P. falciparum* malaria commonly found within this lake region (Jenkins et al., 2015). Despite excellent vaccine coverage, there is gradual increase in specific vaccine preventable infections among individuals in these areas. Despite sound vaccine coverage in western Kenya, the prevalence of measles and diphtheria infections are evident among children in their early life years.

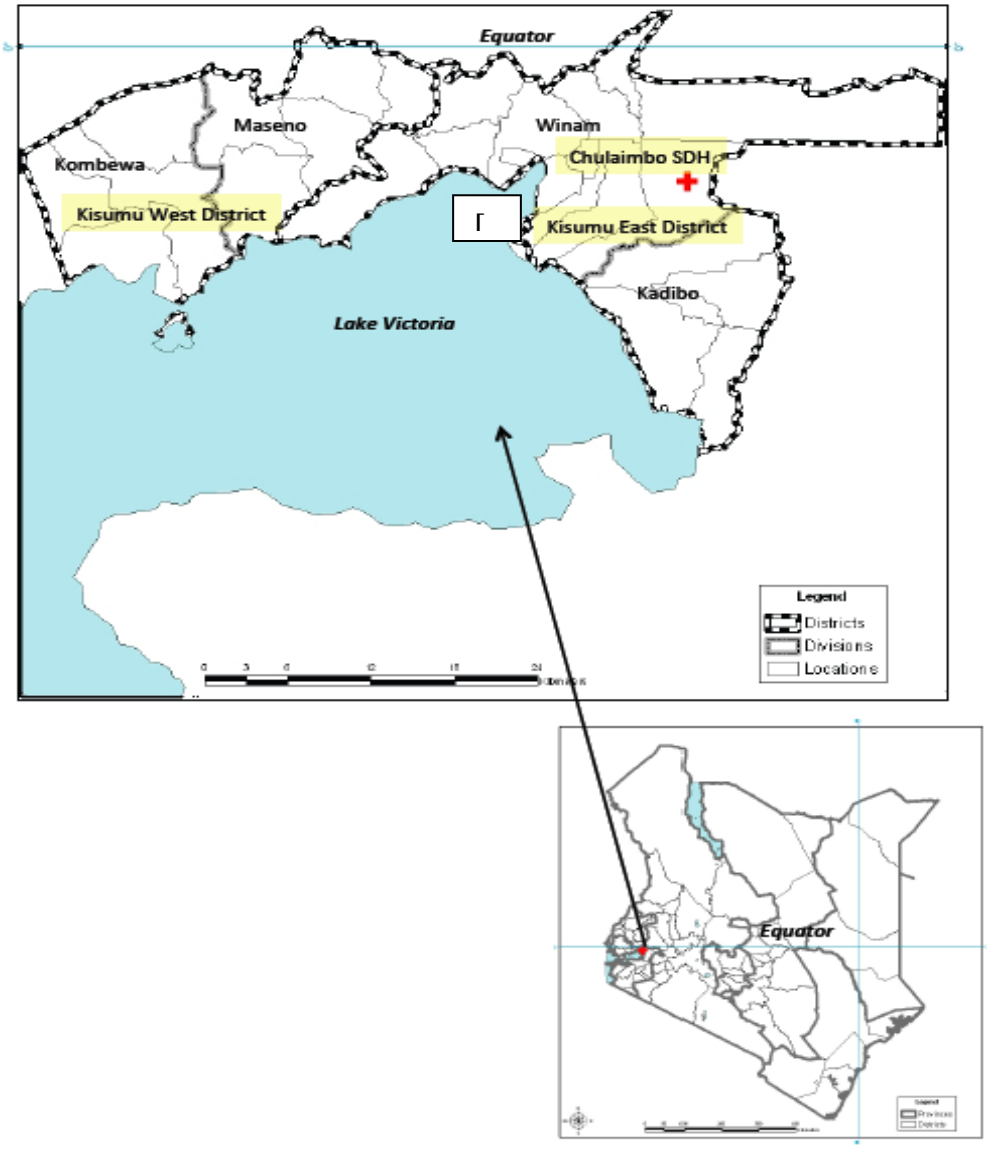


Figure 3.1: A Map of Chulaimbo Sub-County Hospital and its Catchment Areas

3.2 Study Design

This was a prospective cohort study best suited to address the general and specific objectives that needed follow up data at constant intervals from the first trimester to delivery for mothers and from delivery to 18 months at 6 months interval for children (Figure. 3.2).

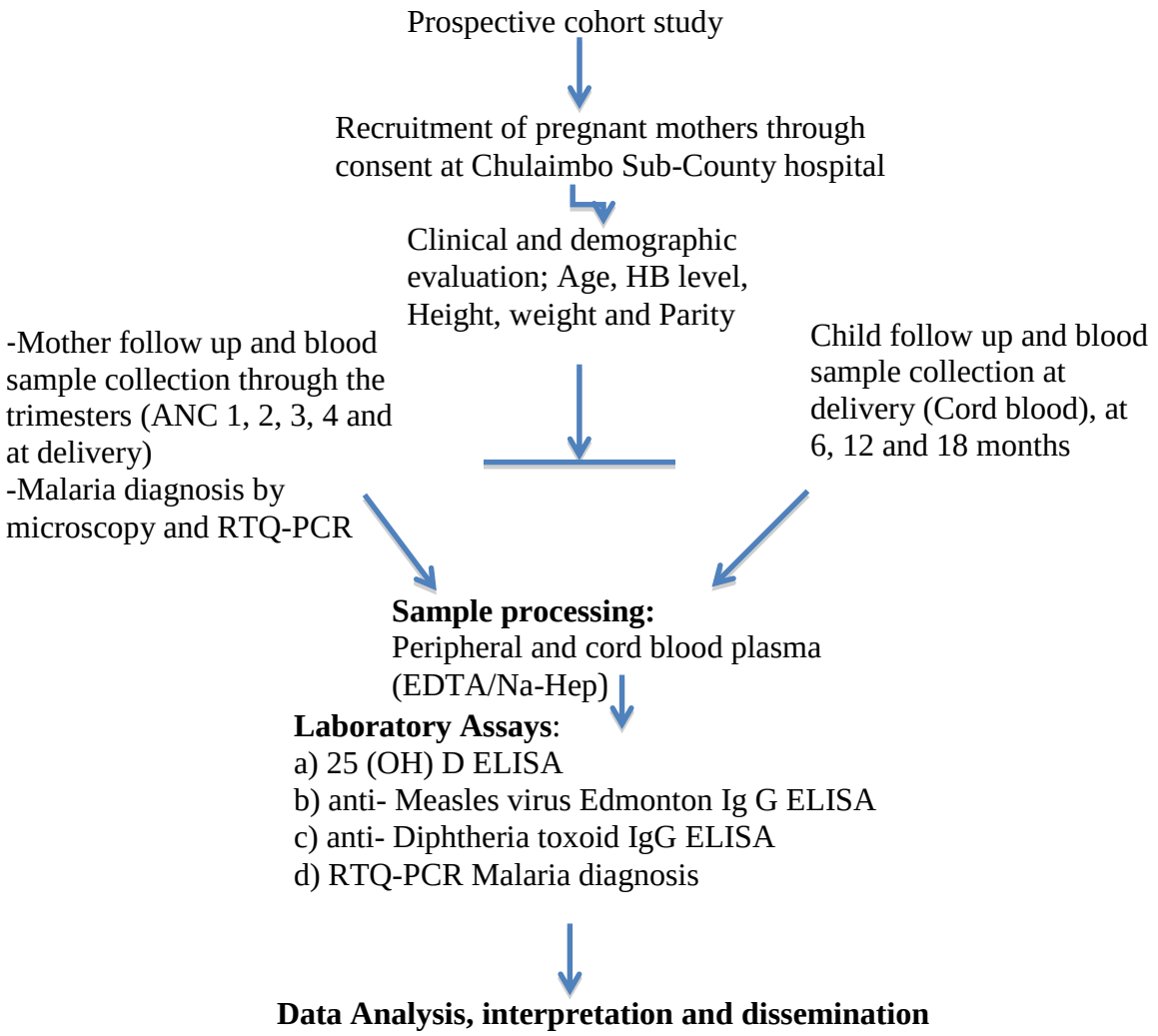


Figure 3.2: Study Flow Chart

3.3. Study Population

The study population comprised of all the pregnant women through their gestation period and their children on delivery, seeking maternal child healthcare at Chulaimbo Sub-County hospital in western Kenya, recruited from June 01st to November 30th, 2011 and followed up to 22nd January 2014 upon delivery.

3.3.1. Inclusion Criteria

Healthy pregnant mothers of all gravidities with less than thirty weeks gestation age, normal complete blood count, HIV negative, residing within 10km radius from the hospital and ready to participate voluntarily on signing the consent form and return to the facility for subsequent antenatal and postnatal follow-up clinical procedures were enrolled.

3.3.2. Exclusion Criteria

Pregnant mothers with complicated, non-communicable infections were not included into this study.

3.4. Sample Size Calculation

The sample size for this study was determined to detect a 50% decline in the mean specific vaccine antigen IgG response for mother – child pairs from birth to 18 months follow-up, assuming 84% power, 95% confidence, 5% margin of error and a standard deviation of 1.25 for both measles virus and diphtheria toxoid specific vaccine antigen IgG responses. Using the formula by (Charan and Biswas 2013), the minimum sample size at 18 months follow-up should have been 39. This prospective study had a high risk of participant dropout rate (39%), assuming a 40% non-response rate (Charan and Biswas 2013), the final sample size including only participants with complete follow up data (n=47) was adequate to determine an association between maternal 25 (OH) D and specific vaccine antigen IgG responses.

Formula for calculation of sample size for prospective studies with quantitative outcomes (means) (Charan and Biswas 2013).

$$N = 2 \frac{(SD^2 (Z_\beta + Z_\alpha)^2}{D^2}$$

Where;

N – Sample size

SD – Standard deviation of outcome variable

Zα – Level of statistical significance (1.96)

Zβ – Desired study power (84%)

D – Effect size difference in means

$$N = 2 \frac{(1.12^2 (0.84 + 1.96)^2}{0.5^2}$$

$$N = 39$$

Plus 40% (16) non-response rate, N = 39+16 = 55

3.5. Data Collection

3.5.1. Collection of Demographic and Anthropometric Data

All the information regarding maternal age, area of residence, parity, body weight, Hb levels were collected in to maternal prenatal (ANC) visit forms (Appendix 1). Similarly, child anthropometric data including birth-weight, length, head circumference and Apgar test score was collected at delivery and subsequent follow up time points from delivery to 18 months was collected in to child follow up visit forms (Appendix 2).

3.5.2. Blood Sample Collection and Processing

At enrollment of mothers at ANC 1 and subsequent ANC visits at every trimester, ~200µl of finger prick blood was collected into EDTA tubes. Thin and thick blood smears were prepared

for malaria diagnosis by microscopy. Hemoglobin (HB) measurements were done using a portable β-hemoglobin photometer (Hemocue AB Angelholm, Sweden). Within 24 hours after delivery, ~200µl of peripheral blood sample was collected from the mother. Cord blood was also aseptically collected at delivery. At subsequent child follow up time points at six months interval from birth to 18 months, ~200µl of peripheral blood was collected into EDTA tubes.

All blood samples collected in to EDTA tubes and stored in cool boxes were transported to KEMRI/SUNY laboratory located at the Center for Global Health Research, KEMRI in Kisumu for processing within 1 hour. Each EDTA whole blood sample was then aliquoted into two portions. One for DNA extraction was stored at 4°C. The other portion was processed immediately for plasma by centrifugation (Jouan C412) at 1500rpm (420g) and aliquots stored at -80^0C until use for laboratory bioassays.

3.5.3. Laboratory Assays

3.5.3.1. Malaria Diagnosis

The study area being a malaria holoendemic zone, thick and thin blood smears were prepared for malaria diagnosis by microscopy (Appendix 3). DNA was also extracted from EDTA whole blood for molecular diagnosis of malaria (Appendix 4). Mothers were diagnosed positive by either of the diagnostic techniques with RTQ-PCR as the confirmatory test due to its high sensitivity compared to microscopy.

3.5.3.2. Vitamin D Measurement by Enzyme-linked Immunoassay (EIA)

Vitamin D micronutrient levels in plasma remain stable (El-Khoury and Wang 2012; Newens, Filteau, and Tomkins 2006) following infection and treatment, hence suitable for immunological investigations. Commercially available 25-hydroxy Vitamin D (25(OH) D) enzyme Immunoassay kit, (Immunodiagnostic systems, IBL International GMBH, D-22335 Hamburg, Germany) was used to quantify vitamin D concentrations in peripheral blood plasma from

mothers and children (on enrolment and subsequent follow up time points) as per the manufacturer's instructions. This is an enzyme-immunoassay (EIA) kit for the quantification of 25(OH) D and other hydroxylated metabolites in serum or plasma. Just before the assay, plasma samples stored at -80°C and the kit reagents at 4°C were brought to room temperature on a clean laboratory working surface. All ELISA pipette aids were well calibrated; pipette tips and the polypropylene tubes for preparing the reaction mix were kept clean well in advance. Calibrators, controls and samples were diluted with biotin labeled 25-(OH) D. The diluted samples were then incubated in micro plate wells coated with a highly specific sheep 25-OH D antibody for 2 hours at room temperature before aspiration and washing. Enzyme (horseradish peroxidase)-labeled avidin was added, this binds selectively to complexed biotin and, following a further wash step, color was developed using a chromogenic substrate (TMB). Absorbance of the reaction mixtures in nanomoles per liter (nmol/L), was read in a micro-titer plate reader (Opsys MR, Dynex Technologies) at 450nm wavelength against a four parameter standard curve (Appendix 5).

3.5.3.3. Measles Vaccine Antigen IgG Levels by In-House Conventional ELISA Technique

Measles vaccine IgG levels were quantified in mothers' peripheral blood and their children cord blood at delivery, and in children's peripheral blood at 6, 12 and 18 months. ELISA plates (Dynatech, Kloten, Switzerland) were coated with 50µls measles virus Edmonston vaccine antigen (American Type Culture Collection, ATCC, No.VR24) and incubated overnight at 4^{o}C. Washing was done using phosphate buffered saline (PBS) to remove excess pre-coating reagent. Un-pre-coated spaces were blocked using blocking buffer [5% milk/PBST (5 % dry milk/ 1 x PBS/ 0.05% Tween 20)] to avoid subsequent non-specific binding of the test antigens at 37°C for 1 hour. After washing, 50µl diluted plasma samples and controls (blanks) (1:100) were introduced to each well and incubated for 1 hour to allow for antigen– antibody immune complex formation. Excess of the sample was then washed off using phosphate buffered saline –

Tween-20 (PBST). At this time, the IgG conjugated secondary antibodies specific to measles virus Edmonston vaccine antibody were added and incubated at 37^{o}C for 1 hour. After washing with PBST, a tetramethylbenzidine (TMB) substrate was then added and incubated at room temperature in the dark to react with the enzyme on the secondary antibody and produce a colour change. A stop solution, HCL was then introduced to stop the reaction and optical densities read with the aid of an ELISA reader (Softmax Pro. ELISA reader) at 450nm wavelength. The ODs generated corresponded to the antibody levels in each reaction (Appendix 6).

3.5.3.4. Diphtheria Toxoid Vaccine Antigen IgG Levels by Conventional ELISA Technique

Diphtheria toxoid vaccine antigen IgG levels were quantified in mothers' peripheral blood and their children's cord blood at delivery, and in children's peripheral blood at 6, 12 and 18 months. Immunol ELISA plates (Dynatech, Kloten, Switzerland) were coated by adding 0.5µg diphtheria toxin (CRM 197 biological laboratories #149) antigen in 100µl of carbonate buffer pH=9.6. After incubation overnight at 4^{o}C, these plates were washed three times with 0.01M PBS pH 7.2 containing tween 20(T). Blocking was done with 0.5% gelatin in 0.06M carbonate buffer (pH 9.6) at 37°C for 1 hour. Wells were washed as before and 100µl of plasma diluted 1:100 in BSA PBS-T containing 0.5% gelatin added. Each dilution was run in duplicate. After 2 hours incubation and washing, the wells were filled with 100µl of anti-human IgG /alkaline phosphatase conjugate antibody (Jackson Immuno labs#109-055-098) diluted 1:1000 in BSA PBST-20 and incubated for 1 hr. After washing, 100µl of alkaline phosphatase substrate was added and the reaction observed for colour development within 15 minutes. Wells without the test sample (plasma) referred to as blanks, were used as controls for the conjugate. Intra-plate variations were minimized by running pre and post vaccination plasma samples obtained from one individual in the same plate. This enzymatic reaction was stopped with HCL after color development within 15 minutes. The optical density was then read at 405nm with the aid of a

Softmax Pro. ELISA reader. The ODs generated correspond to antibody levels in each reaction (Appendix 7).

3.6. Measurement of Variables

Vitamin D concentrations measured in nanomoles per litre (nmol/L) was the main exposure variable and the outcome variables included anti-measles virus and anti-diphtheria toxoid vaccine antigen IgG antibody levels expressed as optical densities (OD). Maternal plasma vitamin D levels were measured at every trimester and at delivery. Child vitamin D levels were quantified at birth in cord blood, at 6, 12 and 18 months in peripheral blood plasma. Vitamin D insufficiency was defined as plasma concentrations <75≥50nmol/L and deficiency as plasma concentrations <50≥25nmol/L serum concentrations. Severe deficiency were defined at plasma concentrations < 25nmol/L (Kennel et al., 2010; Ginde, Liu, and Camargo 2009), values ≥(75 nmol/L) indicated sufficient vitamin D concentrations (Alvarez-Rodriguez et al., 2012; Holick et al., 2012; Rosen 2011). Child birth weight was taken in grams and underweight defined at less than 2500g. Gestational age at delivery was defined in weeks and preterm delivery defined at less than 34 weeks gestation period. Hemoglobin levels were measured in g/dL and anemia diagnosed at levels less than 13g/dL for pregnant mothers. The anti-vaccine antibody responses including anti-measles IgG and anti-diphtheria toxoid IgG antibody concentrations were expressed as optical density (OD) values obtained after correction of the readings according to the mean variation of the reference plasma at 2.5-3.0. Anti-measles virus specific vaccine antigen IgG antibody responses were classified as high (≥2.5), medium (≥1.1<2.5) and low (≤0.5); while anti_diphtheria toxoid vaccine antigen antibody responses were classified as high (≥3.4), medium (≥2.9<3.4) and low (≤1.6).

3.7. Statistical Analysis

All statistical analyses were performed using Stata, IC software (13.1, Stata Corp LP, College Station, TX) and Graph Pad Prism 5 software setting 2-tailed alpha for significant results at $p \leq 0.05$. As continuous variables, descriptive analysis was performed to establish the mean plasma vitamin D concentrations, specific anti-vaccine antigen IgG antibody responses and anthropometric measurements. Wilcoxon Sign Rank test was used to compare median maternal vitamin D concentrations during gestation in the different trimesters until delivery. Median plasma vitamin D concentrations and anti-vaccine antigen IgG antibody responses in the children at birth, 6, 12, and 18 months were also compared using Wilcoxon Sign Rank test. Vitamin D concentrations and anti-vaccine antibody responses in mothers and children at delivery were compared using Kruskal Wallis test. Vitamin D concentrations were categorized as deficient, insufficient and sufficient; and anti-vaccine antigen IgG antibody responses defined as low, medium and high. Differences in proportions were evaluated using Chi-square analysis. Spearman's correlation and regression analysis was used to evaluate the association between maternal vitamin D at delivery and child's specific anti-vaccine antigen IgG antibody responses at birth, 6,12, and 18 months.

3.8. Proposal and Ethical Approval

This study was approved by the School of Graduate Studies (SGS), Maseno University (Appendix 8); both Kenya Medical Research Institute and State University of New York (SUNY) Upstate Medical University Ethical Review Boards [KEMRI SSC Protocol number 1910 (Appendix 9)]. To guarantee ethical approval, a written informed consent was obtained from each mother before participation spelling out confidentiality, benefits and potential risks and protection of participants' rights (Appendix 10). All mothers were also tested for human immune-deficiency virus (HIV) infection status as part of Maternal-to-Child-Transmission

(MTCT) of HIV programs in accordance with the Kenya Ministry of Health (MOH) national guidelines. This being a malaria holo-endemic area, their malaria status was confirmed and those diagnosed with the infection treated with Artemether (20mg) and Lumefantrine (120mg), a first line anti-malarial drug recommended for non-severe malaria and quinine for severe complicated malaria infections by the Kenya Ministry of Health (MOH). During the second trimester of the gestation period, all the expectant mothers were given sulfadoxine pyrimethamine (SP) for malaria prophylaxis, as recommended by the Kenya Ministry of Health (MOH). At the first ANC visit, stool samples were collected and analyzed for the presence of helminthes; infected mothers were treated with Albendazole in accordance with the Kenyan MOH. Vitamin D supplementation is not part of the Kenyan standard of care during pregnancy, and was therefore not administered as part of this study.

CHAPTER FOUR

RESULTS

4.1 Baseline Characteristics of the Study Participants

General clinical and demographic characteristics of the study participants are shown in Tables 4.1 and 4.2 below. A total of 47 mother-child pairs took part in this study. At enrolment, the mean age of mothers was 24±6.78 years with the oldest mother being 46 years and the youngest mother, 15 years old. Their mean weight and height were 62.3±7.88Kg and 163.3±8.38cm, respectively; with a mean body mass index of 22.9±2.9kg/m. Mean gestation age in weeks was 19.9±5.7. Quantification of HB levels revealed that mothers had a mean of 11.23±1.7g/dL; 49% (23/47) of the mothers were at risk of anemia (HB levels <11.0g/dl), 8.5 % (4/47) had severe anemia (HB levels <8.5g/dl) and 42.6% (20/47) were normal (HB levels ≥11.0g/dL). Microscopy and RTQ-PCR malaria diagnosis depicted that 40.4% (19/47) of the mothers had either symptomatic or asymptomatic gestational malaria infections, while 59.6% (28/47) had no malaria infection through the entire gestation period. Maternal gestation age in weeks was recorded at every visit during the gestation period. At ANC 1, mothers had 19.9 ± 5.7 weeks, ANC 2, mothers had 23.6±5.7 weeks, at ANC 3, 27.2±6.2 weeks, at ANC 4, 30.4±5.7 weeks and at delivery time, they had 39.2 ± 2.8 weeks, respectively (Table 4.1).

Table 4.1: Maternal Characteristics at Enrollment, Through Gestation to Delivery

Variable	Mean ± SD or % (n)	Median
Maternal characteristics at enrolment ANC -1 (N=47)		
Maternal Age, years	24 ± 6.78	22
Maternal height, cm	163.19 ± 8.38	163
Maternal weight, kg	62.34 ± 7.88	61
Gestational age, weeks	19.9 ± 5.7	21
Body Mass Index,kg/m	22.9±2.9	22
Hb levels, g/dL	11.23±1.7	11.55
Anemia (Hb<11.0g/dL)	49% (23)	
Severe Anemia (Hb<8.5g/dL	8.5% (4)	
Maternal malaria infection (by qPCR)	61.7% (29)	
ANC-2 Visit		
Gestational age, weeks	23.6±5.7	24
ANC-3 Visit		
Gestational age, weeks	27.2±6.2	29
ANC-4		
Gestational age, weeks	30.4±5.7	34
At Delivery		
Gestational age, weeks	39.2 ± 2.8	40

qPCR: quantitative polymerase chain reaction; Data presented were generated by descriptive analysis.

Gender characterization of the children at birth showed that 42.6% (20/47) were female while 57.4 % (27/47) were male. Mean children's gestation age was 39.2±2.8 weeks; 17% (8/47) children were born before term (<37weeks gestation) while 82.9% (39/47) were normal (>37weeks). Children had mean birth weight of 3.61±2.39kg, mean length, 48.3±4.51cm, mean head-circumference, 35.63±1.8cm and mean Apgar score, 9.4±0.72 (Table 4.2).

Table 4.2: Child Characteristics at birth

Variable	Mean ± SD or % (n)	Median
Newborn characteristics at birth (N = 47)		
Female	42.6% (n=20)	
Male	57.4% (27)	
Gestational age, weeks	39.2 ± 2.8	40
Preterm (<37 weeks of gestation)	17% (8)	
Birth weight, kg	3.61 ± 2.39	3.2
Length, cm	48.3 ± 4.51	49
Head circumference, cm	35.63 ± 1.8	36
Apgar Score,	9.4±0.72	9.5

Data presented were generated by descriptive analysis

4.1.1. Study Participants

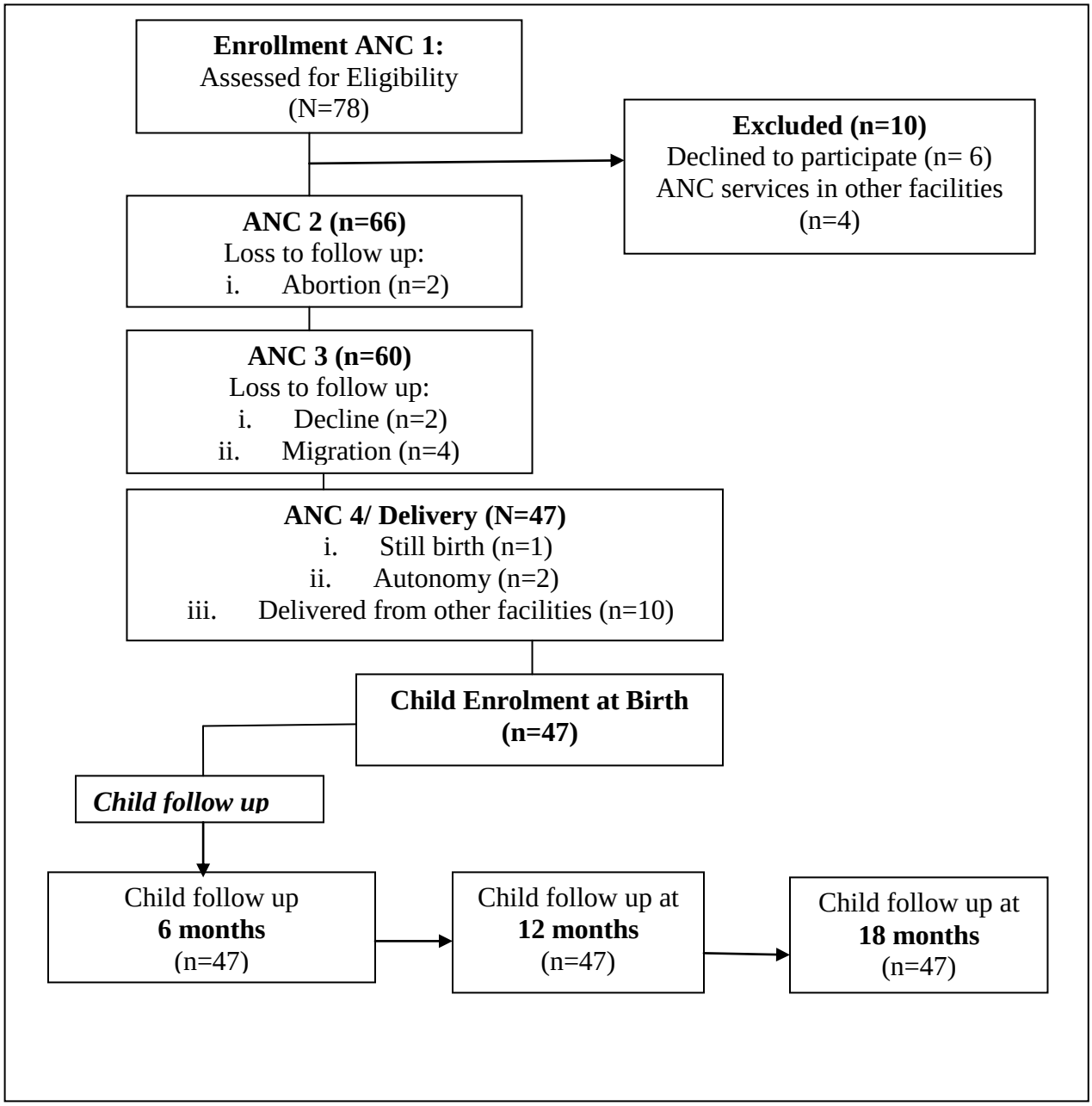


Figure 4.1: Consort Flow Diagram

To investigate the association between maternal vitamin D and the child's vitamin D status, anti-measles virus and anti-diphtheria toxoid antibody responses early in life in malaria holo-endemic areas, this hospital study recruited 78 pregnant women with consent by convenience sampling on first come, first served basis in their first antenatal care (ANC1) visit during their first trimester. Six of these pregnant women declined to participate while four decided to go for ANC services from other hospital facilities. At ANC 2, during their second trimester, two of these pregnant women had abortion, while. At ANC 3, during their third trimester, two pregnant women declined while 4 migrated to join their spouses in other distant locations. At ANC 4/delivery, one woman had still birth, two of these pregnant women made an informed decision not to participate and ten delivered in other hospital facilities. Overall, only 47 mothers delivered at Chulaimbo Sub-County hospital and enrolled their children for the 18 months follow up at 6 months interval (Figure 4.1)

4.2 Status of Vitamin D (25(OH) D) in Mothers and their Children

4.2.1 Mothers' Vitamin D concentration during Gestation period to delivery

Mothers had 87.7±31.86nmol/L mean plasma 25(OH) D concentration at enrolment (ANC 1). Maternal mean plasma 25 (OH) D concentrations were recorded at every visit during the gestation period. At ANC 2, they had 92.7±32.3nmol/L, at ANC 3, 96.6±29.5nmol/L, at ANC 4, 101.3±28.1nmol/L and at delivery 87.87 ± 31.86, respectively. These concentrations were then compared at all time points. Although, there was a general trend of improving vitamin D concentrations in pregnant mothers with decreased variation from conception to delivery time, median concentrations were not significantly different, Wilcoxon Sign Rank test with Dunn's multiple comparison, $p = 0.1890$ (Figure 4.2).

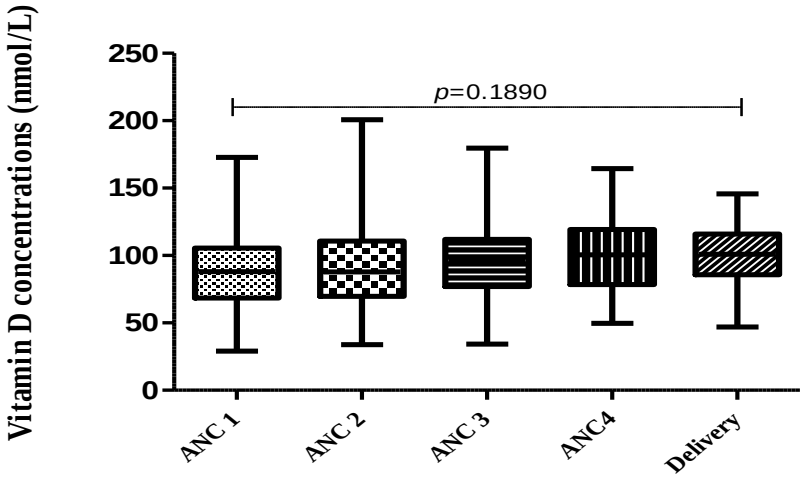


Figure 4.2: Mean maternal plasma vitamin D Concentration Over the Gestation Period

4.2.2 Maternal Vitamin D Status is Independent of Malaria Infection Status

Most mothers (61.7%) had had gestational malaria. There was no significance difference in the concentration of vitamin D between mothers with malaria and mothers without malaria, Kruskal Wallis test, p=0.829 (Figure 4.3).

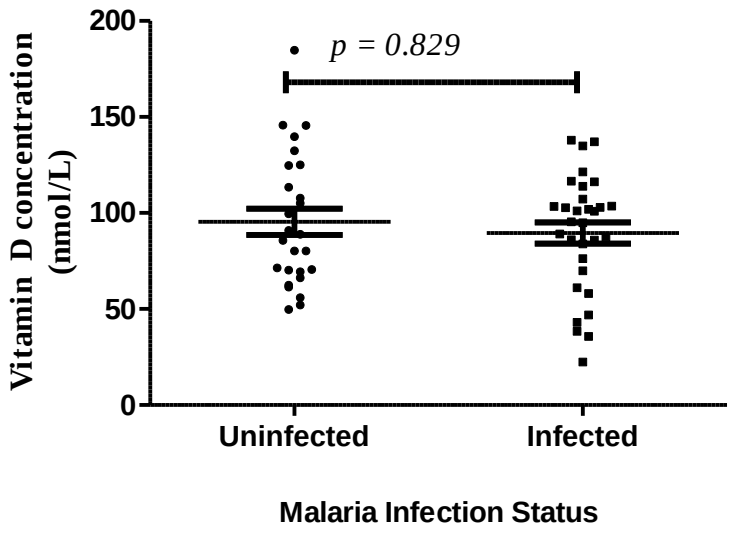


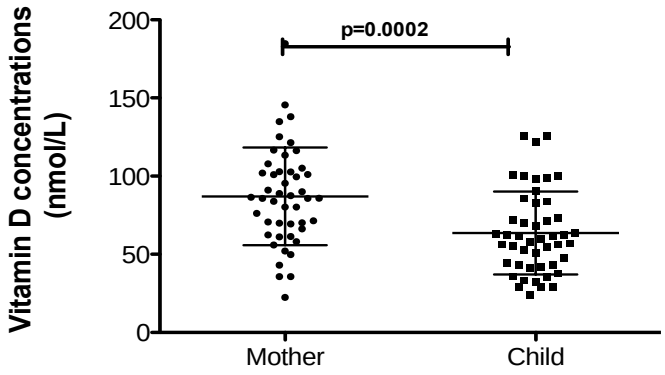
Figure 4.3: Comparison of Mean Vitamin D Concentrations in the Different Malaria Infection Status

4.2.3 Maternal and Child Vitamin D Concentrations at Delivery

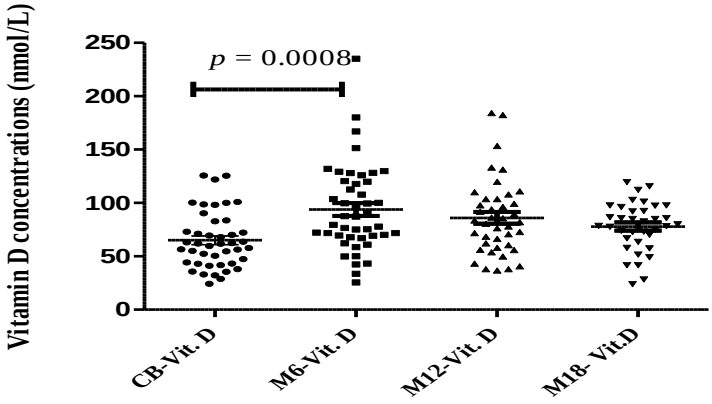
Plasma 25(OH) D concentrations were measured at birth and subsequent child follow-up time points. This quantification revealed that children had 65.04±26.4nmol/L at birth; 93.82±40.72nmol/L at 6 months; 85.25±35.36nmol/L at 12 months; 77.84±23.03nmol/L at 18 months**.** At delivery, Wilcoxon Sign Rank test for paired samples at delivery time showed that median vitamin D concentrations in mother's venous blood was high compared to their children as measured in cord blood, p=0.0002. However, concentrations of vitamin D between mothers and their children at 6 months, 12 months and 18 months (p=0.1532, p=0.9421, p=0.4523, respectively) were statistically insignificant. Subsequent comparison of median vitamin D measurements in mothers and their children at delivery, 6, 12 and 18 months were different, p=0.0003, Wilcoxon Sign Rank test with Dunn's multiple comparison. Significant differences in concentrations were depicted in maternal venous blood versus the childen's cord blood at

delivery/birth; maternal versus child's venous blood at six months; child's cord blood versus the child's venous blood at twelve months (Figure 4.4 a, b, and c).

(a) At delivery



(b) Child at delivery, 6,12 and 18 months



(c)

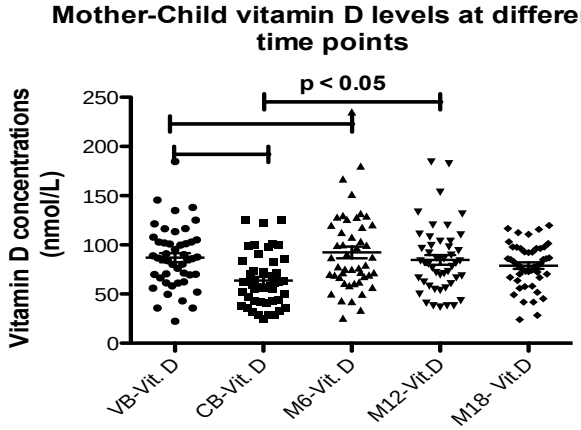


Figure 4.4 (a), (b) and (c) : Comparison of Median Plasma Vitamin D Concentrations in Mothers and their Child at Different Follow up months (M)

4.2.4. Vitamin D Deficiency, Normal and Insufficiency States among Mothers and their Children Early in Life

Vitamin D was considered as a categorical variable. According to the WHO standards, 8.5% (n=4) of the mothers were deficient, 27.66% (13/47) insufficient and 63.83 % (30/47) normal at delivery. Vitamin D concentration measurements in children at birth in cord blood plasma showed that; 27.66% (13/47) were deficient, 17.02% (8/47) were insufficient and 55.32% (26/47) were normal. Successive follow ups showed that; at 6 months, 10.64% (5/47) were deficient, 25.53% (12/47) were insufficient and 63.83% (30/47) were normal; at 12 months 12.77% (6/47) were deficient, 25.53% (12/47) were insufficient and 61.7% (29/47) were normal; at 18 months, 10.64% (5/47) were deficient, 21.28% (10/47) were insufficient and 68.09% (32/47) were normal (Table 4.3) Children had the highest proportion of deficiency (≤50≥25nmol/L) and insufficiency (≤75≥50nmol/L) at birth as measured in their cord blood but the least normal (≥75nmol/L).

Table 4.3: Proportions of Vitamin D Deficient, Insufficient and Normal Mothers and their Children

Variable	Levels of concentrations (nmol/L)	Mother at delivery n (%)	Child at birth n (%)	Children at 6 months n (%)	Children at 12 months n (%)	Children at 18 months n (%)
Vitamin D	**Deficient ≤50**	4 (8.51)	13 (27.66)	5 (10.64)	6 (12.77)	5 (10.64)
	Insufficient ≤51<75nmol/L	13 (27.66)	8 (17.02)	12 (25.53)	12 (25.53)	10 (21.28)
	Normal ≥75nmol/L	30 (63.83)	26 (55.32)	30 (63.83)	29 (61.70)	32 (68.09)

Data presented were generated by descriptive analysis

Mean vitamin D concentrations in children born to mothers with varying vitamin D status were computed at delivery in cord blood, at 6, 12 and 18 months. Children born to vitamin D deficient mothers had mean plasma concentrations of 32.74nmol/L at birth, 44.77nmol/L at 6 months, and 54.94nmol/L at 12months and 56.59nmol/L at 18 months old. Those born to vitamin D insufficient mothers had mean plasma concentrations of 58.73nmol/L at birth, 103.15nmol/L at 6 months, and 99.68nmol/L at 12 months and 88.03nmol/L at 18 months old. Children born to vitamin D sufficient mothers had mean plasma concentrations of 75.24nmol/L at birth, 103.19 nmol/L at 6 months, and 92.77nmol/L at 12 months and 85.37nmol/L at 18 months old. Children born to vitamin D deficient mothers remained with relatively low vitamin D levels early in life compared to those born to insufficient and sufficient mothers (Table 4.4).

Table 4.4: Mean Vitamin D Concentrations for Children Born to Vitamin D Deficient, Insufficient and Sufficient Mothers

Mother's vitamin D status	CB-vit. D	M6-vit. D	M12-vit.D	M18-vit.D
Deficient (n=4)	32.74	44.77	54.94	56.59
Insufficient (n=13)	58.73	103.15	99.68	88.03
Sufficient (n=30)	75.24	103.19	92.77	85.37

Deficient≤50nmol/L; Insufficient≤51<75nmol/L; Normal ≥75nmol/L; Data presented was generated by Descriptive analysis; CBVit.D-Vitamin D Concentrations in cord blood; M6 Vit.D - Vitamin D Concentrations at six months; M12Vit.D-Vitamin D Concentrations at 12 months; M18Vit.D-Vitamin D Concentrations at 18 months

4.3. Anti-Measles Virus Specific Vaccine antigen IgG Antibody Responses

4.3.1 Mean Anti- Measles Virus Specific Vaccine antigen IgG Antibody Responses in mothers and their children

Anti-measles virus antibody responses were expressed as optical density values and treated as continuous as well as categorical variables. Mean ± SD anti-measles antibody responses were

calculated at all follow up time points for mother-child pairs. At delivery, the mothers had 1.54±1.04 OD anti-measles virus IgG levels. Mean anti-measles virus specific vaccine antigen antibody responses were measured and calculated in cord blood plasma for children. Results showed that children had 1.64±1.09 OD at birth; 0.55± 0.71 OD at six months; 1.71± 0.81 OD at 12 months; 1.78± 0.79 OD at 18 months. Anti-measles specific vaccine antigen IgG levels showed a decline from birth and an increase as the children approached 18 months. Variations were evident in median anti-measles responses, p=0.0001, with significant differences from birth to 6 months old, 6 months to 12 months and 6 months to18 months following Wilcoxon Sign Rank test with Dunn's post-test comparing all pairs of columns. There was no significant difference in measles virus specific vaccine antigen IgG antibody response levels between the mother and child at delivery, p=0.7167, Wilcoxon Sign Rank test (Table 4.5).

Table 4.5: Anti- Measles Virus Specific Vaccine antigen IgG Antibody Responses from Delivery at Six Months Interval to 18 months In Mothers and their Children

Variable	Mean ± SD or % (n)	Median	P value
Maternal Vaccine antigen IgG Levels through gestation to Delivery (N=47)			
Anti-Measles IgG levels, OD	1.54 ± 1.04	1.75	
Newborn Vaccine antigen IgG Levels at birth, 6,12 and 18 months (N = 47)			*P*=0.0001[a,]
At birth			*P*=0.0001[b]
Anti-Measles IgG levels, OD	1.64±1.09	1.36	*P*=0.0001[c]
6 months			*P*=0.07167[d]
Anti-Measles IgG levels, OD	0.55 ± 0.71	0.61	
12 months			
Anti-Measles IgG levels, OD	1.71± 0.81	1.75	
18 months			
Anti-Measles IgG levels, OD	1.78 ± 0.79	1.81	

OD-Optical density: Descriptive Analysis: Wilcoxon Sign Rank test with Dunn's post-test comparing all pairs of columns; [a] p=value comparing anti-measles virus vaccine antigen IgG response levels in children between birth and 6 months; [b] p=value comparing anti-measles virus vaccine antigen IgG response levels between 6 and 12 months; [c] p=value comparing anti-measles virus vaccine antigen IgG response levels between birth and 18 months: **Wilcoxon Sign Rank test;** [d] p=value comparing anti-measles virus vaccine antigen IgG response levels between mothers and their children at birth.

Median anti-measles virus specific vaccine antigen IgG levels varied significantly in mothers and their children at 6 months, p=0.0001. No significant differences were observed between mothers and their children at birth, 12 months, and 18 months, p = 0.9612, p=0.7821, p=0.3132, respectively (Table 4.6).

Table 4.6: Paired Analysis Between Mother Child Pairs Measles Virus Specific Vaccine antigen IgG Concentration

Variable		CBMS	M6MS	M12MS	M18MS
VBMS	P-value	0.9612	0.0001	0.7821	0.3132
	Z-test	-0.05	3.26	-0.28	-1.01

VB-MS- Maternal anti-measles vaccine antigen IgG antibody levels at delivery in venous blood; CB-MS- Child anti-measles vaccine antigen IgG antibody levels at delivery in cord blood; M6-MS-Child anti-measles vaccine antigen IgG antibody levels in venous blood at six months, M12-MS - Child anti-measles vaccine antigen IgG antibody levels in venous blood at 12 months; M18-MS-Child anti-measles vaccine antigen IgG antibody levels at 18 months.

4.3.2. High, Medium and Low Anti-Measles Virus Vaccine -Induced IgG Antibody Response States among Mothers and their Children Early in Life

Anti-measles virus specific vaccine antigen antibody response levels were treated as categorical variables. With reference to WHO standards, varying proportions of the mothers and children had low antibody response levels. Most (53.19%) mothers had medium anti-measles virus vaccine antigen IgG antibody responses at delivery time. Most (63.83%) children had medium anti-measles antibody responses at 12 months old. Medium responses were observed in children at 6 months old (Table 4.7).

Table 4.7: Proportions of Mothers and their Children with Varying Anti-Measles Virus Specific Vaccine Antigen IgG Antibody Response Status at Different Time Points

Vaccine Antigen	Antibody response status	Mother at delivery n (%)	Child at birth n (%)	Children at 6 months n (%)	Children at 12 months n (%)	Children at 18 months n (%)
	Low	**5**	**9**	**27**	**2**	**1**
	(≤0.5)	**(10.64)**	**(19.15)**	**(57.45)**	**(4.26)**	**(2.13)**
Measles	Medium	25	19	12	30	26
virus	(≥1.1<2.5)	(53.19)	(40.43)	(25.53)	(63.83)	(55.32)
	High	17	19	8	15	20
	(≥2.5)	(36.17)	(40.43)	(17.02)	(31.91)	(42.55)

Data presented were generated by descriptive analysis.

4.4 Anti-Diphtheria Toxoid Specific Vaccine antigen IgG Antibody Responses

4.4.1 Mean Anti-Diphtheria Toxoid Specific Vaccine antigen IgG Antibody Responses in mothers and their children

Anti-diphtheria toxoid IgG antibody responses were expressed as optical density values and treated as continuous as well as categorical variables. Mean ± SD anti-diphtheria toxoid IgG antibody responses were calculated at all follow up time points for mother-child pairs. At delivery, the mothers had 1.01±0.84 OD anti-diphtheria toxoid IgG levels. Mean anti-diphtheria specific vaccine antigen IgG anti-body responses were measured and calculated in cord blood plasma for children, results showed that children had 1.02±0.09 OD at birth; 1.59±1.02 OD at six months; 1.38± 0.69 a OD at 12 months; 3.42±12.5 OD at 18 months. There was an increase in anti-diphtheria toxoid specific vaccine antigen IgG levels from birth and these declined as the

children approached 12 months of age, the levels increased rapidly as the child approached 18 months old. Anti-diphtheria antibody responses were measured in matched pairs, that is, mothers' venous blood and the children's cord blood at delivery. Comparison was done by Wilcoxon Sign Rank test. There was no significant difference in diphtheria toxoid specific vaccine antigen IgG antibody response levels between the mother and child at delivery, *p*=0.9693, Wilcoxon Sign Rank test (Table 4.8).

Table 4.8: Anti- Diphtheria Toxoid Specific Vaccine antigen IgG Antibody Responses from delivery at Six months Interval to 18 months in mothers and their children

Variable	Mean ± SD or % (n)	Median	P value
Maternal Vaccine antigen IgG Levels through gestation to Delivery (N=47)			
Anti-Diphtheria IgG levels, OD	1.01±0.84	1.03	
Newborn Vaccine antigen IgG Levels at birth, 6,12 and 18 months(N = 47)			
At birth			
Anti-Diphtheria IgG levels, OD	1.02±0.09	1.01	*P*=0.0228[a]
6 months			*P*=0.9693[b]
Anti-Diphtheria IgG levels, OD	1.59±1.02	1.62	
12 months			
Anti-Diphtheria IgG levels, OD	1.38 ± 0.69	1.41	
18 months			
Anti-Diphtheria IgG levels, OD	3.43±12.5	3.51	

OD-Optical density, Data presented was generated by Descriptive Analysis and Wilcoxon Sign Rank test with Dunn's post-test for comparison; [a] p=value comparing anti-diphtheria toxoid induced IgG median response levels in children between birth and 6 months **Wilcoxon Sign Rank test;** [b] p=value comparing anti-diphtheria toxoid induced IgG median response levels between mothers and their children at birth.

Although there was no significant difference in the diphtheria toxoid specific vaccine antigen IgG antibody levels between the mother and their children at delivery/birth, $p = 0.6531$, a significant difference was depicted at 6 months ($p = 0.0312$), 12 months ($p = 0.0423$), and 18 months ($p = 0.0013$), Wilcoxon Sign Rank test, ($p \leq 0.05$; Table 4.9).

Table 4.9: Paired Analysis Between Mother Child Pairs Diphtheria Virus Vaccine Concentration

Variable		CBDPT	M6DPT	M12DPT	M18DPT
VBDPT	P-value	0.6531	0.0312	0.0423	0.0013
	Z-test	0.46	-2.23	-2.06	-2.94

VB-DPT- Maternal anti-diphtheria toxoid vaccine antigen IgG antibody levels at delivery in venous blood; CB-DPT- Child anti-diphtheria toxoid vaccine antigen IgG antibody levels at delivery in cord blood; M6-DPT-Child anti-diphtheria toxoid vaccine antigen IgG antibody levels in venous blood at six months, M12-DPT - Child anti-diphtheria toxoid vaccine antigen IgG antibody levels in venous blood at 12 months; M18-DPT-Child anti-diphtheria toxoid vaccine antigen IgG antibody levels at 18 months.

4.4.2. High, Medium and Low Anti-Diphtheria Toxoid Specific Vaccine antigen IgG Antibody Response States among Mothers and their Children Early in Life

Anti-diphtheria toxoid specific vaccine antigen IgG anti-body response levels were treated as categorical variables. With reference to WHO standards, varying proportions of the mothers and children had low antibody response levels. Most (65.96%) mother-child pairs had low anti-diphtheria antibody responses at delivery. Subsequent child follow up showed that 36.17% (17/47), 53.19% (25/57) and 46.81% (22/47) of the children had low anti-diphtheria toxoid specific vaccine antigen IgG antibody responses at 6, 12 and 18 months respectively. Medium responses were observed in 48.94% of the children at 6 months of age (Table 4.10).

Table 4.10: Proportions of Mothers and their Children with Varying Anti-diphtheria toxoid Specific Vaccine antigen IgG Antibody Response Status at Different Time Points

Vaccine Antigen	Antibody response status	Mother at delivery n (%)	Child at birth n (%)	Children at 6 months n (%)	Children at 12 months n (%)	Children at 18 months n (%)
Diphtheria	**Low**	**31 (65.96)**	**31 (65.96)**	**17 (36.17)**	**25 (53.19)**	**22 (46.81)**
toxoid	Medium	6 (12.77)	10 (21.28)	23 (48.94)	15 (31.91)	13 (27.66)
	High	10 (21.28)	6 (12.77)	7 (14.89)	7 (14.89)	12 (25.53)

All the above were generated by descriptive analysis.

4.5 Vitamin D Status Influence Vaccine antigen IgG Antibody Responses

4.5.1 Vaccine antigen IgG Antibody Responses at Varying Vitamin D Status in Mothers and their Children

Maternal and child anti-measles and anti-diphtheria specific vaccine antigen IgG antibody responses were evaluated based on plasma vitamin D status, that is, deficient (<50nmol/L), insufficient (<75nmol/L) and normal (>75nom/L). Maternal and child mean anti-measles antibody responses at delivery varied among the different vitamin D status categories, Kruskal Wallis test, $p<0.0001$. No significant mean antibody differences were observed in children at 6 months, Kruskal Wallis test $p=0.9043$, 12 months Kruskal Wallis test, $p=0.6925$ and 18 months Kruskal Wallis test, $p=0.1301$ (Figure 4.5).

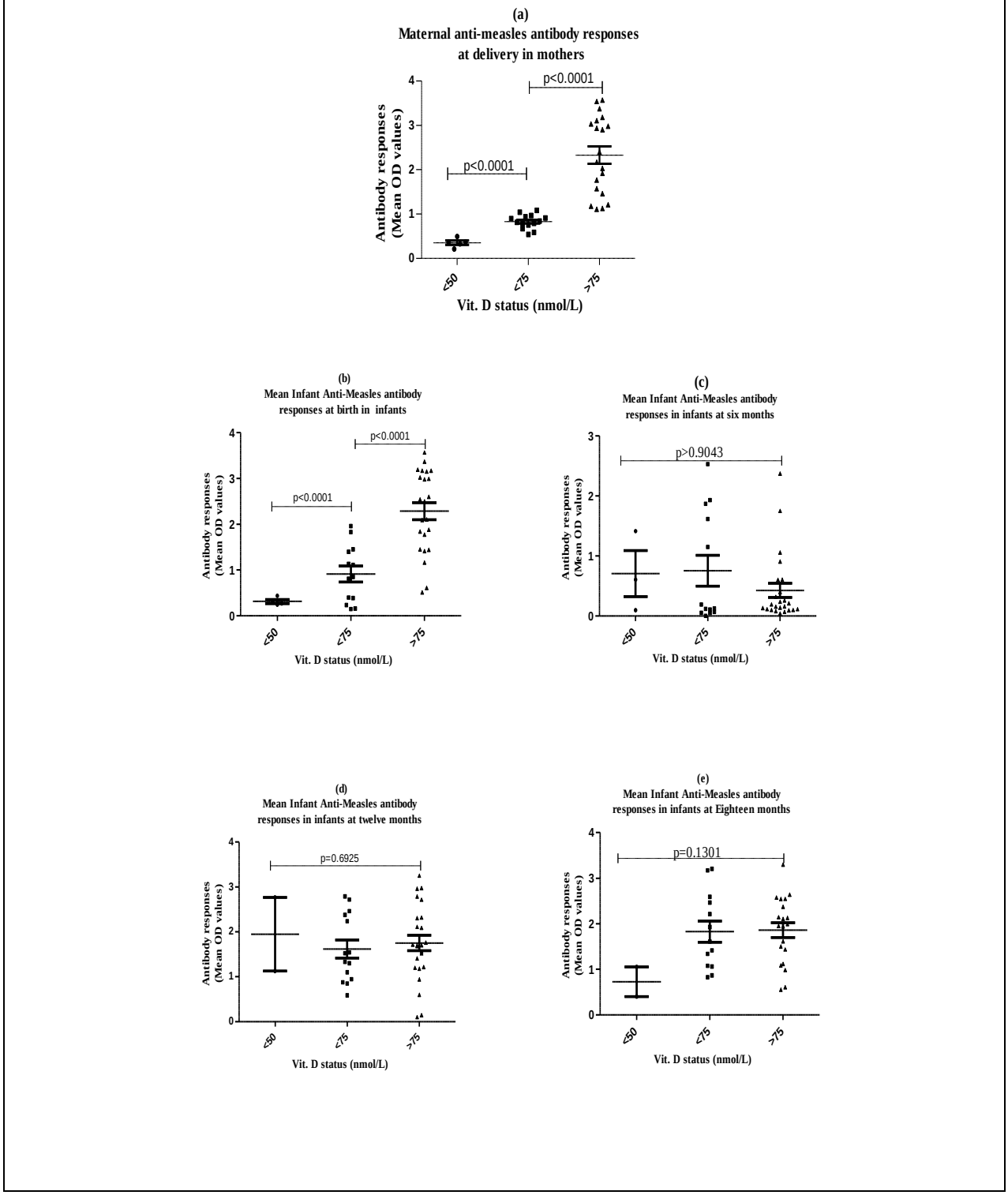


Figure 4.5: Anti-Measles Virus Vaccine IgG Antibody Responses in Mothers and Children With Varying Vitamin D Status; At (a) delivery in mothers venous blood (b) Birth in children cord blood (c) Six months (d) 12 months (e) 18 months.

Mean maternal and child anti-diphtheria toxoid specific vaccine antigen IgG antibody responses remained the same at all follow up time points, Kruskal Wallis test, $p < 0.05$, Maternal and children at delivery/birth, p=0.4356 and 0.1839, respectively. Childs at six months, p=0.2306, at 12 months, p=0.6399 and at 18 months, p=0.1124 (Figure 4.6).

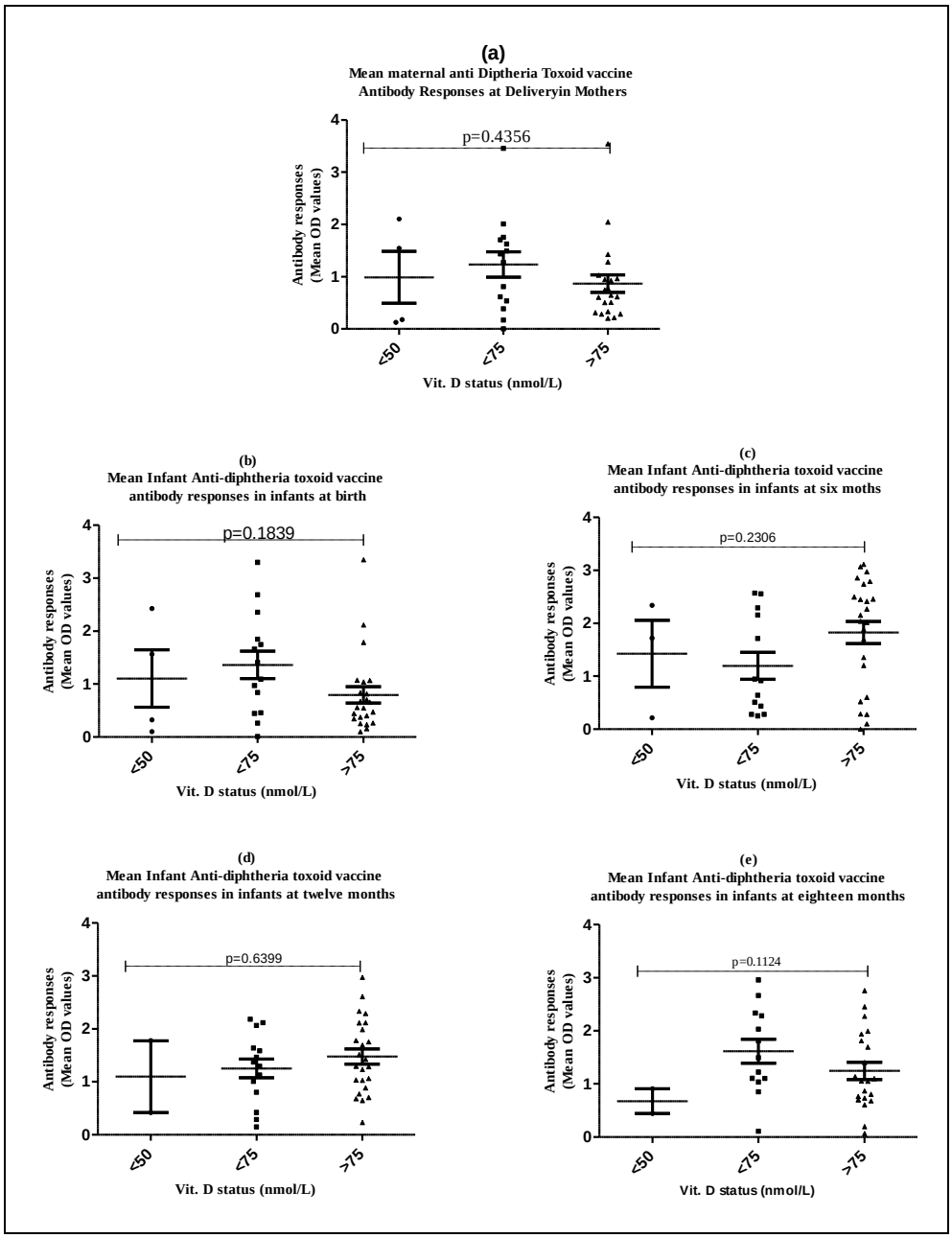


Figure 4.6: Anti-Diphtheria Toxoid Vaccine Antibody Responses in Participants with Varying Vitamin D Status: At (a) delivery in mothers venous blood (b) Birth in children's cord blood (c) Six months (d) 12 months (e) 18 months.

4.5.2 Association of Maternal Plasma Vitamin D Concentrations with Anti-Measles and Anti-Diphtheria Specific Vaccine antigen IgG Antibody Responses in Mothers and their Children at Delivery

The spearman correlation and regression model using graph pad prism 5 included maternal vitamin D as the independent variable and anti-vaccine antigen IgG antibody responses as the dependent outcome variables in children at delivery, 6, 12 and 18 months. There was a significant, positive strong and moderate association between maternal vitamin D concentrations and the child's anti-measles and anti-diphtheria toxoid specific vaccine antigen IgG responses respectively. These analysis showed that maternal vitamin D concentration is a significant predictor for both anti-measles and anti-diphtheria toxoid specific vaccine antigen IgG antibody responses at birth (p<0.001), r^2=0.71, r^2=0.56, respectively. This means that 71% of the children's anti-measles specific vaccine antigen IgG antibody responses as the outcome variable is explained by maternal vitamin D concentration, 29% is explained by other factors. Similarly, 56% of the children's anti-diphtheria toxoid specific vaccine antigen IgG antibody responses as the outcome variable is explained by maternal vitamin D concentration, 44% is explained by other factors (Figure 4.7).

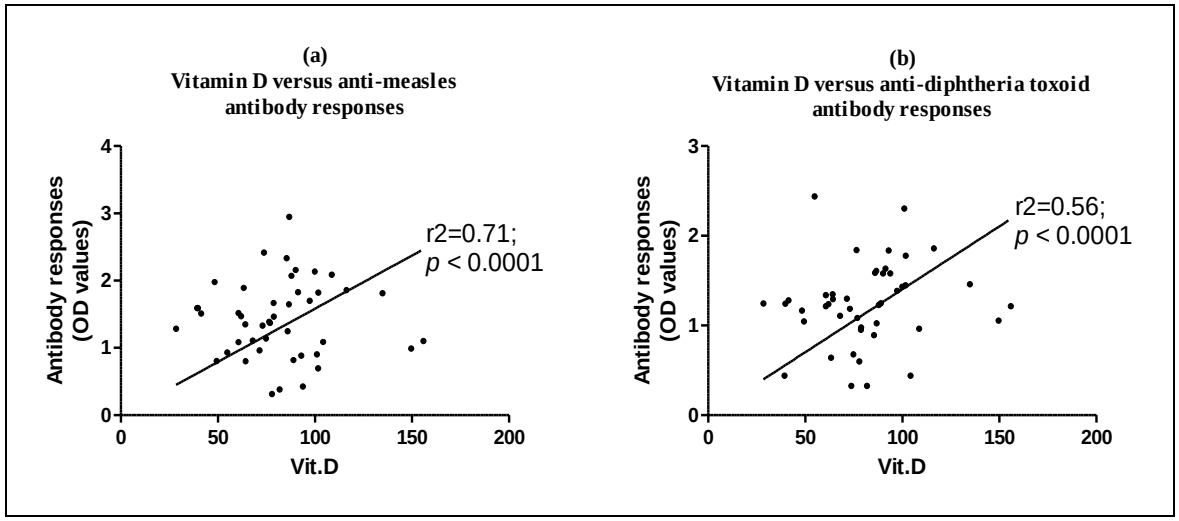


Figure 4.7 Mean Mother-Child Vaccine Antibody Responses Versus Vitamin D Concentrations: (a) Vitamin D versus anti-measles antibody responses (b) Vitamin D versus anti-diphtheria toxoid antibody responses.

Spearman correlation and regression analysis showed a significant, positive, weak association between maternal vitamin D concentrations at delivery and anti-measles virus specific vaccine antigen IgG antibody responses at 6 months (p=0.05; r^2 = 0.42); a significant, weak negative association between maternal vitamin D concentrations at delivery and the child's anti-measles virus specific vaccine antigen IgG antibody levels at 12 months and 18 months (p=0.05, r^2 =1). These analyses also showed statistically significant, weak, positive correlation between maternal vitamin D concentrations at delivery and the child's anti-diphtheria toxoid specific vaccine antigen IgG antibody levels at 18 months (p=0.05, r^2= 0.19). Collectively, these results show that there was a significant, weak, negative association between maternal vitamin D concentrations at delivery and the child's anti-diphtheria toxoid specific vaccine antigen IgG antibodies at six and 12 months.

CHAPTER FIVE

DISCUSSION

5.1 Varying Maternal and Child Plasma 25(OH) D Concentrations

5.1.1 Varying Maternal Plasma 25(OH) D Concentrations

Areas with stable sunny climatic conditions bear a high burden of reduced vitamin D concentrations posing a high risk of residents developing vitamin D associated complications (Allali et al., 2009; Gannagé-Yared et al., 2000; Arabi et al., 2010). Similar to the current study setting, studies have reported that high dark skin pigmentation with high melanin concentration obscure penetration of UV rays required for hydroxylation to produce synthetic bioactive vitamin D molecules (Health Quality Ontario 2010). In the current study, plasma 25(OH) D concentrations were quantified in Kenyan mothers at delivery and their children from birth to 18 months at 6 months interval. Although majority (63.83%) of the mothers had sufficient vitamin D concentrations (≥75nmol/L), 27.66% had insufficient (≥50<75nmol/L) and 8.51% had deficient (<50nmol/L) concentrations. Some (27.66%) of the children born to mothers enrolled in this study had deficient plasma 25(OH) D concentrations and 17.02% had insufficient concentrations. Although work on vitamin D status in Kenya has not been done, the current study findings are in line with findings in studies reporting high prevalence of vitamin D hypovitaminosis D Middle East and North Africa with similar climatic conditions (Bassil et al., 2013). These observations could be attributed to reduced exposure to direct sunshine due to lifestyle events such as indoor activities and poor dietary habits on non-fortified foods (Halicioglu et al., 2012; Parlak et al., 2014; Wagner et al., 2012). In addition, advancing age, female gender, multiparty, seasonal climatic changes and low socioeconomic status could be the other contributing vitamin D deficiency risk factors. Furthermore, genetic variation in vitamin D

biosynthesis may have a great bearing in the observed differences in findings. Variations in vitamin D concentrations could also have been attributed to other independent factors affecting vitamin D bioavailability, which were not explored in the current work. Owing to the reported role of vitamin D in skeletal and non-skeletal complications (Dawodu and Wagner 2007b; Bodnar et al., 2013a and b; Thandrayen and Pettifor 2010), pregnant mothers with vitamin D deficiency are at a high risk of developing skeletal complications like osteomalasia and rickets; and non-skeletal disorders such as rheumatoid disorders, elevated viral loads and interleukin levels in hepatitis C, increased BMI lipids and insulin sensitivity, increased blood pressure, heart failure and mortality (Bassil et al., 2013).

5.1.2 Varying Child Plasma 25(OH) D Concentrations

Child vitamin D status is dependent on the mothers' vitamin D status at birth. Findings have reported that children born prematurely , before term, are at a high risk of vitamin d deficiency compared to term children (Burris et al., 2013). Exclusive breastfeeding in-doors is a major risk factor for vitamin D deficiency in children aged 1-6 months (Choi et al., 2013). In addition, although majority of healthy children at the age of 12 months are vitamin D sufficient, there is need for supplementation and use of fortified foods besides breastfeeding to reduce the risk of vitamin D insufficiency (Thorisdottir et al., 2014). Current study findings demonstrated significant paired analysis between maternal and child cord blood plasma 25(OH) D concentrations at delivery/birth, p =0.0002. Comparison of median vitamin D measurements in mothers and their children at the different follow up time points depicted significant differences at six and twelve months (p=0.0003). This finding is similar to a previous one in which there was a strong correlation between maternal serum and umbilical cord blood in multiethnic populations conducted in similar study design (Burris et al., 2013; Jacquemyn et al., 2013). Both the current

study and previous studies used prospective cohort study design which recruited pregnant women and followed them through their gestation period to delivery. Throughout the gestation period to delivery/birth, the developing fetus gets its' vitamin D from the mother via transplacental transfer. Most children below age of six months are exclusively breast fed indoors thus increasing the risk of vitamin D insufficiency. With increasing age, the child starts to engage in outdoor activities hence gets exposure to direct sunlight and starts feeding on their food staffs which increase their sources of vitamin D. Following current implementation of exclusive child breast feeding for six months, there is likely to be increased cases of vitamin D deficiency in children. Due to poor dietary habits without vitamin D supplementation and limited exposure to sunlight children are at a risk of developing vitamin D deficient complications such as rickets.

5.2 Varying Anti-Measles Virus Specific Vaccine Antigen IgG Responses in Mothers and Their Children

Antibody responses to anti-measles virus vaccine antigen, a live-attenuated vaccine antigen, are highly influenced by exposure to natural infections, recurrent disease outbreaks and nutritional status of the host individuals (Kizito et al., 2013). Previous study findings have shown that persistence of maternal antibodies and young age affects the quantity and quality of vaccine-induced neutralizing antibodies (Nair et al., 2007). Age dependent differences in IgG isotype avidity are induced by measles vaccine received during the first year of life (Nair et al., 2007). In the current study, multiple measurements of anti-measles virus antigen-specific vaccine antigen IgG antibody responses were done in mothers at delivery and their childs from delivery to 18 months old. The current findings report significant proportions of mothers and their children with low levels of measles virus specific vaccine antigen IgG antibody responses vital for the children's immunity early in life years. There was a general trend of reducing anti-measles IgG

antibody profiles from birth to 9 months and an increase from 9 months to 18months. Previous findings have shown that many children show excellent antibody responses to measles virus vaccine, but few generate detectable specific lymphoproliferative responses to measles virus antigen, contributing to varied responses (Bautista-López et al., 2001). Earlier studies have reported similar findings showing varying measles virus vaccine antigen specific antibody responses in vaccinated individuals (Heilmann et al., 2006). This observation could be explained by individual intrinsic characteristics including genetic makeup and maternal factors including varied natural exposure to infections and nutritional status (Kizito et al., 2013). Largely still, other factors affecting transplacental transfer such as maternal IgG levels, IgG subclasses, gestational age, placental integrity and the nature of antigen may also be playing role in these variations. To ensure vaccine efficacy, minimal levels of maternally acquired protective correlates for passive immunity are expected to avoid antibody cross reactivity. Hence, the general trend observed. Observed elevated anti-measles virus IgG levels are attributed to vaccine antigen administered at 9 months old to the children.

5.3 Varying Anti-Diphtheria Toxoid Specific Vaccine Antigen IgG Responses in Mothers and Their Children

Diphtheria toxoid vaccine, dead bacterial vaccine antigen, induced antibody responses are dependent on the host's immune status at the time of vaccination (Plotkin 2001; Savy et al., 2009; Plotkin 2010). Anti-diphtheria toxoid vaccine-induced antibody responses are affected by genetic and environmental factors including natural infections and recurrent disease outbreaks (Grassly et al., 2015). In the current study, multiple measurements of anti-diphtheria toxoid specific vaccine antigen IgG antibody responses were done in mothers at delivery and their children from delivery to 18 months old. Although mothers had slightly high anti-diphtheria IgG profiles, children were born with low anti-diphtheria toxoid specific vaccine antigen IgG profiles

which suddenly increased from birth to six months through to twelve months and declined to a constant level from twelve months to 18 months. Proportions of mothers and their children had low levels of anti-diphtheria toxoid specific vaccine antigen IgG antibody responses vital for the children's passive immunity early in life years. Earlier studies have reported similar findings showing varying anti-vaccine antibody responses in vaccinated individuals. These reduced anti diphtheria toxoid vaccine antigen IgG antibody responses to vaccination in children at birth is attributed to increased prenatal exposure to maternal antibodies (Heilmann et al., 2006). On the contrary, reduced exposure to infection antigens during the gestation period results in relatively elevated responses to the specific antigen in children, hence, low anti-diphtheria toxoid specific vaccine antigen IgG antibody responses may be due to increased exposure to the respective vaccine antigens during gestation. Low specific anti-vaccine IgG antibody responses in children may be influenced by other factors that determine transplacental transfer including maternal IgG levels, IgG subclasses, gestational age, placental integrity and the nature of antigen.

5.4 Maternal Vitamin D is Associated with Anti-Measles Virus Specific Vaccine antigen IgG Responses in Children at Delivery

Transplacental transfer of protective immune correlates for the child's passive immunity is highly dependent on the mother's nutritional status during gestation (Wu et al., 2004). Current findings further report that maternal plasma 25(OH) D concentrations are highly associated with anti-measles virus (r^2=0.71) and anti-diphtheria toxoid (r^2=0.56) specific vaccine antigen IgG antibody levels in children at delivery. This association is also depicted among vitamin D deficient children whose anti-vaccine antigen IgG responses at delivery declined by 50% up to 18 months old. Recent study findings report expression of vitamin D receptors (VDR) on human lymphocytes (Gong et al., 2017; Kempinska-Podhorodecka et al., 2017), and the modulatory effects of vitamin D on B cell function (James et al., 2016; Rolf et al., 2016; Chen et al., 2007) in

different disease status through varied mechanisms (Dankers et al., 2016; Cantorna 2010). Therefore, the current findings that vitamin D is associated with anti-vaccine antigen IgG antibody response in mothers and their children at delivery suggests its effect in the regulation of specific vaccine antigen elicited antibody profiles.

Previous study findings show that vitamin D has direct effects in both adaptive and innate arms of the immune systems (Miller and Gallo 2010; Walker and Modlin 2009). The current findings contribute to this body of knowledge indicating its association, and hence, its potential effect in modulation of optimum immune responses.

5.5 Study Limitations

Only children born to mothers with complete follow up data and samples from enrolment through the ANC visits to delivery time were considered for child follow up at 6 months interval up to 18 months old. At every follow up time point, a number of participants were lost to follow up due to variety of reasons including; migration from the study area, autonomy and respect for persons opinion. Participants' own decision to cease participation was accepted, some mothers for individual reasons not shared, preferred to give birth from different hospitals. Some had pregnancy complications attributed to still births and abortions. Lack of data on factors influencing the bioavailability of vitamin D in circulation including gastrointestinal absorption, obesity and vitamin D specific metabolism (Tsiarass et al., 2011) should be noted.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of the Findings

This study quantified vitamin D concentrations, anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody responses in pregnant mothers and their children from delivery to 18 months. Further, this study sought to establish the association between vitamin D and the specific preferred vaccine induced IgG antibody responses. Results demonstrate that vitamin D deficiency (<75nmols/L) and insufficiency (<50nmol/L) status are evident in the study population. Similarly, low anti-measles and anti-diphtheria toxoid specific vaccine antigen IgG antibody responses were evident among mothers and their children. In addition, maternal plasma 25(OH) D concentrations were associated with children's anti-measles virus and anti-diphtheria toxoid specific vaccine antigen IgG antibody levels at delivery.

6.2 Conclusion

1. Pregnant women and their children from this study area with constant sunny climatic conditions have varying vitamin D concentration status defined as; deficient (<50nmol/L), insufficient (≥50<75nmol/L) and sufficient (≥75nmol/L) status. Maternal vitamin D status influence the child's vitamin D status early in life.

2. Varying proportions of mothers and their children depict high (≥2.5), medium (≥1.1<2.5) and low (≤0.5) anti-measles virus specific vaccine antigen IgG antibody responses with respect to their respective vaccine standard references.

3. Varying proportions of mothers and their children depict high (≥3.4), medium (≥2.9<3.4) and low (≤1.6) anti-diphtheria toxoid specific vaccine antigen IgG antibody responses with respect to their respective vaccine standard references.

4. Maternal vitamin D concentration at delivery is associated with the child's specific vaccine antigen IgG antibody levels at birth, thus influencing the child's specific vaccine antigen IgG responses early in life

6.3. Recommendations from this study

1. Adequate maternal vitamin D levels should be maintained to avoid unexpected reduced plasma vitamin D concentrations associated with vitamin D deficiency in children.

2. Adequate anti-measles virus antigen specific vaccine antigen IgG levels should be maintained to ensure optimum levels in mothers and children.

3. Adequate anti-diphtheria toxoid antigen specific vaccine antigen IgG levels should be maintained to ensure optimum levels in mothers and children.

4. Adequate maternal vitamin D status should be maintained to ensure optimum anti-measles and anti-diphtheria toxoid vaccine induced IgG responses in children.

6.4. Study Recommendations for Future Work

1. More work should be carried out to explore mechanisms underlying variations in plasma vitamin D concentration in individuals from sunny climatic conditions.

2. Investigations should be carried out to understand mechanisms underlying variations in anti-measles virus antigen specific vaccine antigen IgG responses in mothers and children.

3. Investigations should be carried out to understand mechanisms underlying variations in anti-diphtheria toxoid antigen specific vaccine antigen IgG responses in mothers and children.

4. More work should be done to discover the role of vitamin D in regulation of anti-measles virus and anti-diphtheria toxoid vaccine antigen IgG responses in mothers and children.

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APPENDICES

APPENDIX 1: ENROLMENT AND MATERNAL PRENATAL FOLLOW UP FORMS

(ANC FORMS)

PARTICIPANT ID: ECH-__ | __ | __ | __ | - M
Completed By: _____Date: ____/_____/_____
(dd/mmm/yyyy)
VISIT Number: **ANC1** **ANC2** **ANC3** **ANC4**
1. Estimated gestational age (in weeks): _____
2. Fundal Height (cm): _____

Symptoms	No	Yes
3. Is the patient sick today?		
4. Does the patient have a fever today?		
5. Fever in the past two days?		
6. Cough in the past two days?		
7. Headache in the past two days?		
8. Chills in the past two days?		
9. Diarrhea in the past two days?		
10. Stomach ache in the past two days?		
11. Is a bed net being used?		
12. Other symptoms in the past two days?		

If yes, please describe: _____

Physical Exam
13. Weight (kg): _____(13)
14. Height (cm): _____(14)
15. Mid-Upper Arm Circumference [MUAC] (cm): _____(15)
16. Heartbeat Rate (beats per minute) _____(16)
17. Respiratory Rate (breaths per minute) _____(17)
18. Blood Pressure _____ / _____ (18)
Systolic **Diastolic**
19. Axillary Temperature (degrees C): _____ (19)

Medications Given at Visit	No	Yes
20. Iron Tables (N=30)		
21. Folic Acid (N=30)		
22. Fansider Tables (Malaria prophylaxis) (N=3)		
23. Tetnus booster given?		
24. Deworming (Membendazole500gms)		
25. ITN (INSECTS treated net)		
26. ARV (prophylaxis) AZT+NVP		
27. Other medications		

If yes, please specify: _____
28. Clinical Diagnosis: _____

APPENDIX 2: CHILD FOLLOW UP VISIT FORMS

Participant ID: ECH-|__|__|__|__|-|C| Date: ____/_____/_____

Scheduled Follow-up Visit Number: 1 – (~6 wks) 2 – (~10 wks) 3 – (~14 wks)
4 – (~18wks) 5 – (~6 mo) 6 – (~9 mo) 7 – (~12 mo)
8 – (~15mo) 9 – (~18mo) 10 – (~21mo) 11– (~24mo)

Vaccination History
1. 6 week DPT/HIB/AgB/Polio
2. 10 week DPT/HIB/AgB/Polio
3. 14 week DPT/HIB/AgB/Polio
4. Pneumococcal Vaccine
□ 6 week (dose 1) Date Given_____/_____/_____(dd/mmm/yyyy)
□ 10 week (dose 2) Date Given_____/_____/_____(dd/mmm/yyyy)
□ 14 week (dose 3) Date Given_____/_____/_____(dd/mmm/yyyy)
□ 1 year+ (booster) Date Given_____/_____/_____(dd/mmm/yyyy)
5. Vitamin A Oral Dose
If yes, date given (DD/MMM/YYYY): _____(5)
6. >40 week measles
If yes for measles indicate date given (DD/MMM/YYYY): _____(6)
7. Does child appear sick?
If yes, please describe briefly: _____

Symptoms
8. Does the patient have a fever today?
9. Fever in the past two days?
10.Cough in the past two days?
11. Headache in the past two days?
12. Chills in the past two days?
13. Diarrhea in the past two days?
14. Stomach ache in the past two days?
15. Is a bed net being used?
16.Other symptoms in the past two days?
If yes, please describe briefly: _____
17. Has the patient received anti-malarials?
If yes, please give the name of medication and date given: _____
18. Any other medications?
If yes, please describe: _____

Physical Examination:
19. Weight (kg): _____(19)
20. Height (cm): _____(20)
21. Temperature (°C) _____(21)
22.Mid-Upper Arm Circumference (cm) _____(22)

23.Head Circumference (cm): _____(23)
24. Is the spleen palpable?
24a: If yes, number of cm below left costophrenic margin: _____(24a)
25. Is there evidence of pallor?
26. Are any other abnormalities present?
If yes, please describe: _____
27. If child is sick, what is the diagnosis? _____
28. If child is sick, were any drugs prescribed?
If yes, please drugs/doses: _____
29. Was a finger/heel sick sample taken?
29a. If yes, please give the sample ID: ECH-|__|__|__|__|-|C|-|FP|-|____| (29a)
29b. If yes, please note time taken:
30. Was a venous blood sample taken?
30a.if yes, please give the sample ID: ECH-|__|__|__|__|-|C|-|VB|-|____| (30a)
30b. If yes, please note time taken: _____:_____ (30b)

Physical Completed By:_____ Date (DD/MMM/YYYY):____/_____/_____

Form Completed By:_____ Date (DD/MMM/YYYY):____/_____/_____

APPENDIX 3: MALARIA MICROSCOPY STANDARD OPERATING PROCEDURES

a) COLLECTION OF FINGER PRICK BLOOD AND PREPARATION OF THICK AND THIN BLOOD FILMS

1. PURPOSE AND SCOPE

To describe the procedure for collecting finger prick blood sample and preparing thick and thin blood films for malaria diagnosis by light microscopy

2. BACKGROUND

Examination of blood films by microscopy is a basic technique, which remains the gold standard for the diagnosis of malaria. Blood films for malaria diagnosis are best prepared from capillary blood obtained by a finger prick. Good-quality blood films are essential to establish accurate diagnoses.

3. SUPPLIES AND MATERIALS

- Cleaned glass slides, 25 x 75 mm, with one frosted end for labelling, preferably with ground edges, and of good quality.
- 70% ethyl alcohol or alcohol swabs
- Sterile lancets, one per patient
- Dry cotton (cotton ball, swab or gauze)
- Protective latex gloves (powder free)
- A biohazard container or any puncture-resistance sharps container
- Management of wastes generated from malaria diagnostic tests
- An infectious wastes container
- A slide tray or box and a cover to dry slides horizontally, protected from dust and flies
- A drying rack;
- Record forms (i.e. malaria register) and
- A lead pencil or permanent marker pen

4. SAFETY PRECAUTIONS

- Wear protective latex gloves before starting blood collection and when handling slides, for personal protection and to avoid leaving oil on the slide that may interfere with the smear preparation.
- Wear gloves when handling blood, and remove them before leaving the work area or when writing notes.
- Always use a new lancet for each patient. Do not recap the lancets. Never re-use lancets.
- Avoid getting blood, wet or dry, on your fingers or hands.
- Cover cuts or abrasions on your hands with a waterproof dressing.
- Avoid accidentally pricking yourself when handling sharp instruments that have been in contact with blood.
- Thoroughly wash your hands with soap and water as soon as you finish a job.

- If you get blood on your skin, quickly wipe it off with a cotton swab dampened with alcohol; then wash the affected area with soap and water as soon as possible.
- Sharps such as lancets and broken glass must be discarded in a "sharps" container for safe disposal by incineration or autoclaving.
- Materials that are not sharp but are contaminated with blood must be discarded in a covered pan or autoclave bag for safe disposal by incineration or autoclaving.
- Collect blood in an area where there is proper lighting.

5. PROCEDURE

i. Label the frosted end of the glass slide with the patient's details, and document in record form or a malaria register. See SOP 06: Labelling malaria blood films.
ii. Wearing protective latex gloves, select the third finger from the thumb of the non-dominant hand (or big toe for infants, not the heel). Do not use the thumb for either children or adults.
iii. Hold the patient's hand, palm facing upwards, and clean the selected finger with a piece of cotton soaked lightly in 70% ethanol or alcohol swab. Use firm strokes to remove dirt and oil from the ball of the finger and to stimulate blood circulation. Make sure the finger is warm by applying gentle massage if required. Let the alcohol dry from the finger.
iv. Using a new, sterile lancet and a quick rolling action, puncture the centre of the ball of the finger or toe.
v. Apply gentle pressure to the finger (or toe), and express the first drop of blood.
vi. Wipe the first drop of blood of with dry cotton, making sure that no cotton strands
vii. remain on the finger that might stick to the blood.
viii. Working quickly and handling the slides only by the edges, collect blood by applying gentle pressure to the finger and touching the slide to the blood; collect a single small drop of blood on the middle of the slide for the thin film. Apply further gentle pressure to express more blood, and collect two or three drops on the slide about 1 cm from the drop intended for the thin film. 9. Wipe the remaining blood from the finger with clean, dry cotton.
ix. Do not pause between applying and spreading the drops. Prepare the blood films with the slide lying on a flat surface.
x. To prepare the thin film, place the edge of a clean "spreader" slide at 45o in front of the blood drop intended for the thin film.
xi. Slowly pull the "spreader" back until it touches the drop of blood and the blood spreads along the edge of the "spreader".
xii. Rapidly push the "spreader" forwards (away from the centre) in a smooth, continuous motion, until the spreader leaves a "feathery" end for the thin film.
xiii. With the corner of the same "spreader" used for making the thin film, make the thick
xiv. film by swirling the three drops of blood together forming a circle of about 1 cm in diameter size.
xv. Do not stir the blood. A circular or rectangular film can be made by three to six quick strokes with the corner of the spreader.
xvi. After preparing the thin and thick blood films, allow them to dry in air in a horizontal position on a slide tray. If rapid drying is required, dry the films with low heat from a hair-dryer for 5 s, at a distance of 30 cm. Do not place the slides too close to the dryer, as the films might become heat fixed.

6. PROCEDURE NOTES

The thick film should be dried flat and be protected from dust and flies.
The thick film may auto-fixate if exposed to extreme heat and should therefore be stained immediately.
The thick film can be dried gently with a hair-dryer set at warm or another drying method, but care must be taken to avoid heat fixation, which can occur quickly. Issue a hair-dryer only to technicians who have demonstrated competence with this method.
Do not use a ballpoint or gel pen to label slides, as the ink will spread when the film is fixed.
Correctly made slides leave little blood on the spreader slide, which can be used for making thick and thin slides from the next patient, while another, clean slide from the package is used as a fresh spreader. Do not use a slide as a spreader more than once.

b) LABELLING MALARIA BLOOD FILM

1. PURPOSE AND SCOPE

To describe the recommended procedure for labelling malaria blood films

2. BACKGROUND

Correct labelling of malaria blood films is important to ensure that the sample and the data correspond to the patient. The integrity of the diagnosis may be compromised by unlabelled or incorrectly labelled blood films. Labelling is important even if only one slide is to be prepared.
Labelling of malaria blood films also facilitates cross-checking of quality control slides from subnational levels at the national reference laboratory.

3. SUPPLIES AND MATERIALS

- a lead pencil;
- a glass slide, frosted end, 76 mm x 26 mm, 1.0–1.2 mm thick, and
- a register.

4. PROCEDURE

i. Record patient information accurately on the test request form, in the log-book , Check the patient information on the test or diagnostic request form, and record it accurately in the log-book.
ii. On the frosted end of the slide, write the laboratory code, patient's identification or code, and date of collection, before taking blood from the patient. Before taking blood from the patient, use a lead pencil to write the following information on the frosted end of the slide: laboratory code, patient identification number or code as recorded in the log-book, date of collection.

5. PROCEDURE NOTES

Labelling should be completed before taking blood from a patient. When labelling slides, avoid touching the blood film with writing instruments. Do not use a ballpoint or gel pen to label slides, as the ink will spread when the film is fixed.

- **RECOMMENDED LABELLING SCHEME**

Label the slides according to the following scheme:
Laboratory code /patient identification number or code; Date of collection (dd/mm/yyyy)

c) GIEMSA STAINING OF MALARIA BLOOD FILMS

1. PURPOSE AND SCOPE

To describe the procedure for properly staining malaria blood films with Giemsa stain.

2. BACKGROUND

A properly stained blood film is critical for malaria diagnosis, especially for precise identification of malaria species. Use of Giemsa stain is the recommended and most reliable procedure for staining thick and thin blood films. Giemsa solution is composed of eosin and methylene blue (azure). The eosin component stains the parasite nucleus red, while the methylene blue component stains the cytoplasm blue. The thin film is fixed with methanol. De-haemoglobinization of the thick film and staining take place at the same time. The ideal pH for demonstrating stippling of the parasites to allow proper species identification is 7.2.

The slow (3% stain working solution) method

The slow method is used for staining larger numbers of slides (≥ 20). It is ideal for staining blood films collected during cross-sectional or epidemiological surveys and field research and for preparing batches of slides for teaching. It is less appropriate when a quick result is needed. The slow method is less expensive than the rapid method because it requires much less stain (3% rather than 10% stain solution).

3. SUPPLIES AND MATERIALS

- Giemsa stain (3% solution) absolute methanol, acetone-free;
- A Pasteur pipette with a rubber teat;
- A small container or beaker for methanol;
- A staining troughs that can hold 20 slides placed back to back;
- A timer;
- A slide-drying rack;
- Protective latex gloves, powder-free, disposable, and
- Distilled or deionized water bufered to pH 7.2.

4. SAFETY PRECAUTIONS

1. Methanol (methyl alcohol) is inflammable and highly toxic if inhaled or swallowed; it can cause blindness and even death if swallowed in any quantity. Avoid contact and inhalation. When it is not in use, it should be stored in a locked cupboard.

2. Universal precautions – including use of relevant personal protective equipment such as gloves,safety glasses and a laboratory coat or gown – must be practised. See MM-SOP-11: General safety procedures in the malaria microscopy laboratory.

5. PROCEDURE

i. Estimate the amount of 3% Giemsa stain working solution needed for the number of slides to be stained. Prepare the stain immediately before use according to MM-SOP-04: Preparation of Giemsa working solution.
ii. Fix each thin film, preferably using a Pasteur pipette or by dipping the thin film for 2 s into a small container or beaker containing methanol. Avoid contact between the thick film and methanol, as methanol and its vapours quickly fix thick films and interfere with the haemolysis of the thick film.
iii. Place the blood film on a tray or drying rack. Allow the methanol-fixed thin smear to dry completely in air (approximately 2 min) by placing the slides on a flat surface. Never let the slide dry in a vertical position with the thin film down, as this may result in fixation of the thick film by methanol vapour.
iv. Place the slides back-to-back in a staining trough, making sure that the thick films are together at one end of the tray.
v. Pour the stain gently into the staining tray. Do not pour it directly onto the thick films, as they may float of the slides.
vi. Set the timer for 45–60 min (the exposure time should be determined previously by testing the batch of stock staining solution), and stain the blood films. Experience with the stain you are using will help indicate the time required for good staining.
vii. Gently pour buffered water into the tray to float of the iridescent “scum”. To avoid disturbing the thick films, pour the water into the thin film end. A less satisfactory way of flushing slides is to immerse the whole tray in a basin filled with clean water, making sure to avoid the iridescent scum when removing the tray from the basin.
viii. Gently pour of the remaining stain, and rinse with buffered water.
ix. Carefully remove the slides, one by one, and place them film side down in the drying rack to dry. Make sure that the thick films do not touch the edge of the rack.
x. Discard the remaining 3% Giemsa solution

6. PROCEDURE NOTES

Drying the thick blood film

Thick blood films must be completely dry before being stained. They can be dried quickly with warm air from a small hair-dryer. Avoid overheating slides, as they can “heat fix” and thus stain poorly.

Use of buffered water for rinsing slides

The pH of the water used for rinsing is important, as acidic water may decolorize the films. It is therefore recommended that slides be rinsed with the same buffered water that is used for staining and therefore has a pH of 7.2.

Care of glassware and measuring equipment

Measuring cylinders, pipettes, staining troughs and beakers must be clean and dry before use. Staining blood films with dirty utensils gives unsatisfactory results.
The equipment used for Giemsa staining should be rinsed immediately after use in clean water to remove as much of the stain as possible. It should then be soaked for a while in a detergent solution before washing. Utensils can be washed with a mild detergent, provided they are rinsed thoroughly in clean water before drying. Any detergent that is left on glass- and plastic-ware can alter the pH of the water and the stain, resulting in poor staining when the equipment is next used.

Caution

During staining with Giemsa stain (3% or 10% stain working solution), the surface becomes covered with a metallic green scum. Avoid getting it onto blood films during rinsing, as it can impair examination. Blood smears should be stained as soon as possible after they are prepared. Storage of unstained slides for a few days in hot, humid conditions before staining will result in auto-fixation, and the thick film will be rendered useless for microscopy.

Method for staining individual slides

1. Place the slides individually on the staining rack, making sure that they are not touching each other.
2. Pour the stain gently onto the slides until they are totally covered. Each slide will require approximately 3 mL of stain. Avoid pouring the stain directly onto thick films.
3. Leave the stain on the slides for 45-60 min with 3% Giemsa solution and 10–15 min with 10% Giemsa solution. Internal quality control of your stain will indicate the optimum staining time.
4. Flood the slides gently with bufered water to float of the iridescent “scum” on the surface of the stain. Water bufered to 7.2 pH should be poured onto the slides from the thin film end to avoid undue disturbance and washing-of of the thick films.
5. Remove the slides one by one and place them, thick film downwards, in a drying rack to drain and dry, making sure that the thick film does not touch the edge of the rack.

d) MICROSCOPY EXAMINATION OF THICK AND THIN BLOOD FILMS FOR IDENTIFICATION OF MALARIA PARASITES

1. PURPOSE AND SCOPE

To describe the procedure for correct detection and identification of malaria parasites in Giemsa-stained blood films by light microscopy

2. BACKGROUND

Identification of the species and stages of malaria parasites and determination of their density is crucial in clinical management of malaria patients, drug efficacy trials, malaria epidemiological surveys and control programmes. Therefore, malaria diagnoses based on examination of blood films must be correct, with an accurate parasite count.
Examination of blood films allows also detection of several blood pathogens, morphological diagnosis of anaemia and identification of several haematological disorders, which must be reported by the microscopist.

3. SUPPLIES, MATERIALS AND EQUIPMENT

- a compound microscope, fitted with paired 10x oculars (eyepieces); 10x, 40x and 100x objectives; and a mechanical stage (An objective marker and a 60x objective may also be fitted);
- Giemsa-stained blood films to be examined; immersion oil, type A, high quality; lens paper;
- a pen and pencil and
- a malaria registry or log-book.

4. PROCEDURE

4.1 Examining the thick film

a. Place the Giemsa- stained blood film on the microscope stage with the label to the left and the thick film under the 10x objective lens. Place the Giemsa-stained blood film to be examined on the microscope stage, with the label to the left. Position the thick film in line with the 10x objective lens.
b. Switch on the microscope, and adjust the light optimally. Switch on the microscope, adjust the light source optimally and find the focus by looking through the ocular and the 10x objective.
c. Place a drop of immersion oil on the thick film. Scan the blood film for parasites and blood elements. Select part of the film that is well stained and has evenly distributed white blood cells.
d. Scan and select a well-stained, even portion of the blood film. Place a small drop of immersion oil on the thick film. To avoid cross-contamination, ensure that the immersion oil applicator never touches the slide. Do not allow the 40x objective to touch the oil.
e. Switch to the 100x oil immersion objective, and allow the lens to touch the oil. Switch the 100x oil immersion objective over the selected portion of the thick film. Use the fine focus adjustment to see the image clearly. Raise the mechanical stage to avoid damaging the slide.
f. Using the fine adjustment, focus on the blood film. Using the fine adjustment, focus on the cell elements, and confirm that the film is acceptable for routine examination: 15–20 white blood cells per thick film field will give a satisfactory film thickness. Films with fewer white blood cells per field will require more extensive examination.

g. Start with the field on the top left part of the film, and then move the slide to the right, field by field. Examine the slide in a systematic manner. Start at the top left of the film (marked with a vertical green arrow on Fig. 1) and begin at the periphery of the field, then move horizontally to the right, field by field.
h. When the other end of the film is reached, move the slide downwards, then to the left, field by field, and so forth. When the other end of the film is reached, ove the slide slightly downwards, then to the left, field by field, and so forth (see below). For efcient examination, continuously focus and refocus with the fine adjustment throughout examination of each field.

4.2. Determining whether a thick film contains malaria parasites and identifying the species

a. Examine the thick film under the oil immersion objective, field by field, horizontally or vertically.Continue to examine the slide for 100 high-power or oil immersion fields. Move the blood film by one high-power field each time, following a specific pattern using the fine adjustment to focus.
b. Read a minimum of 100 fields before declaring that no malaria parasites were seen. A minimum of 100 high-power fields must be examined before a thick film can be declared as having "no malaria parasites seen". If possible, the whole thick film should be scanned.
c. If parasites are found, scan additional 100 fields to increase the chance of identifying mixed infections. A further 100 fields must be examined before final identification of the species, ensuring that a mixed infection is not overlooked.
d. The thin blood film should always be examined to confirm the same malaria parasite. The thin blood film should always be examined to identify parasite species definitively. The thin film allows visualization of parasite and red cell morphology, unlike the thick film. Perform an examination at the feathery end or edge of the thin film.
e. Determine all species and stages observed, and record them. Identify and record all species and stages observed in the malaria microscopy blood register with reference to the WHO bench aids for the diagnosis of malaria for identification of each species.

e) RECORDING AND REPORTING MICROSCOPY RESULTS

1. PURPOSE AND SCOPE

To describe the procedure for proper recording and reporting of the results of microscopic examination of blood films for malaria diagnosis

2. BACKGROUND

Proper recording and reporting of the results of microscopy examination of blood films is very important for the clinical management of malaria patients and for the reliability of malaria surveillance data, which are the basis for monitoring, evaluating and planning programme interventions.

3. SUPPLIES, MATERIALS AND EQUIPMENT

- Laboratory register (or malaria microscopy registry),
- Patient result form,
- Pen and pencil and
- Handheld calculator (for estimating parasite density).

4. PROCEDURE

After microscopic examination, results should be recorded and reported. Record all malaria species and stages observed during microscopic examination of the blood films. When counting on the thick film is completed, if the patient's actual white cell count is not available, calculate the parasite density from an estimated white cell count of 8000/µL, as follows:

$$\text{Parasites/µL blood} = \text{Number} \frac{\text{of asexual parasites counted x 8000 white cells/µL}}{\text{No. of white cells counted}}$$

Example 1:

Plasmodium falciparum trophozoites counted = 155 White cells counted relative to parasites = 208

Parasite count: $\frac{155 \times 8000}{208}$ = 5962 parasites/µL blood

Report as: *P. falciparum* trophozoites = 5962 p/µL blood

Example 2:

P. vivax trophozoites counted = 88

White cells counted relative to parasites = 505 Actual white cell count of patient = 6500

Parasite count: $\frac{88 \times 6500}{505}$ = 1133 parasites/µL blood

Report as: *P. vivax* trophozoites = 1133 parasites/µL blood

In mixed infections or infections by more than one species, count all the species together (sexual and asexual stages), and express the results as in example 3.

Example 3:

P. falciparum gametocytes + *P. vivax* trophozoites = 360 parasites (all stages) counted in 202 white cells

Report as:

P. falciparum gametocytes + *P. vivax* trophozoites = 14 257 parasites/µL blood

Also report the presence of:

Gametocytes. Gametocytes of *P. falciparum* are counted separately, but they are still included in the general parasite count. It is rarely possible to separate the gametocytes of *P.vivax* or *P. malariae* from asexual parasites with sufficient accuracy to justify a gametocyte count.

Schizonts, as they might be an indication of disease severity.

Record in the microscopy section of the laboratory register the patient identification number, the date and time of examination and parasite species, stages and count if performed. The reporting should be uniform.

For example:

- *P. vivax* trophozoites seen.
- *P. falciparum* trophozoites seen; count, 42 000 parasites/µL.
- *P. falciparum* gametocytes seen.
- No malaria parasites seen. This phrase should be used rather than "Negative".

5. PROCEDURE NOTES

When there are approximately more than 100 parasites seen in each of the fields of the thick film, parasites are counted on the thin film, for greater accuracy. In this case, the parasite count is estimated with the formulae shown in the SOP: Counting malaria parasites below.

f) MALARIA PARASITE COUNTING

1 PURPOSE AND SCOPE

To describe the procedure for counting malaria parasites on thick and thin blood films.

2. BACKGROUND

The parasite density provides information on the severity of infection and on the response to treatment. Parasite counts are performed for *P. falciparum, P. vivax, P. malariae and P. ovale* asexual stages. Unless the protocol dictates otherwise, gametocytes are not counted, but their presence is always reported. All identified parasite species should be reported, even if they are counted together.

Most parasite counts are performed on thick blood films. If there is a no thick film or it is damaged, a thin film count is performed. A thin film count is also performed when there are > 100 parasites in each field of the thick film, which corresponds to > 8,000 parasites/µL.

3. SUPPLIES, MATERIALS AND EQUIPMENT

- A compound microscope fitted with paired 10x oculars (eyepieces), 10x, 40x and 100x objectives and a mechanical stage (an objective marker and a 60x objective may also be fitted);
- A multiple tally counter or two-key tally counters, one to count malaria parasites and one to count white blood cells;
- Giemsa-stained blood slides to be examined;
- Immersion oil, type A, high quality;
- Lens paper;
- A pen and pencil and
- A handheld calculator**.**

4. PROCEDURE

Performing a parasite count on a thick film and calculating parasite density
Note: Before starting the counting, examine 100 fields of the thick film to detect the presence of malaria parasites at 100x oil immersion.

i. Place the glass slide on the microscope stage with the label to the left. This allows a standardized approach for the start point for counting and also to record parasite locations using the marked divisions on the slide holder.
ii. If malaria parasites are present, count asexual forms (in either single or mixed species infections) without sexual (gametocyte) forms, which are not counted but just reported. In mixed infections, all asexual parasites are counted together and the presence of multiple species is reported
iii. Starting at the top most part of the film, look for a field where a good number of white blood cells are observed together and start counting.
iv. Using a multiple type tally counter, count parasites and white blood cells simultaneously by clicking on the assigned key as parasites or white blood cells are observed. If two tally counters are being used use one for the WBCs and the other for parasites.
v. After counting all the parasites and white blood cells in one field, move to the next field following the pattern of movement shown in Figure 1 and repeat the same counting procedure and so on. Be careful not to overlap fields.
vi. Depending on the number of parasites observed, stop counting after you have examined 200 or 500 white cells.
    a. If you have counted ≥ 100 parasites in 200 white cells, stop counting, and record the results as the number of parasites per 200 white cells.
    b. If you have counted ≤99 parasites in 500 white cells, stop counting, and record the results as the number of parasites per 500 white cells.
vii. Count all parasites and white blood cells in the final field, even if the white cell count exceeds 200 or 500.

viii. Record the actual numbers of parasites and white blood cells counted on an appropriate worksheet.

ix. Calculate the parasite density from:

ii. Parasites / µL blood = $\frac{\text{Number of parasites counted x 8,000 white cells/µL}}{\text{No. of white cells counted}}$

When counting is completed, calculate the parasite density on the basis of the patient's actual white cell count. If this is not available, use an estimated average white cell count of 8,000/µL.

Performing a parasite count on the thin film and calculating parasite density

Note: If ≥ 100 parasites are present in each field of a thick film under the 100x objective, calculate the parasite count on the thin film.

i. If infected red blood cells are present, count all parasitized red blood cells. If sexual forms
   - (gametocyte) are seen, do not count them, but report them. In mixed infections, all parasitized
   - red blood cells are counted together, and the presence of multiple parasite species is reported.

ii. In the top section of the thin film, locate a field with about 250 red blood cells. Count the total number of red blood cells in that field and the number of parasitized red blood cells. A typical

iii. field (at 100x magnification) should contain approximately 250 red blood cells.

iv. Using a multiple type tally counter, count parasitized and other red blood cells by clicking the assigned keys for parasitized and non- parasitized red blood cells. If you have two tally counters, use one for parasitized red blood cells and the other for non-parasitized red blood cells.

v. After counting all the parasites and white blood cells in one field, move to the next field, following the pattern of movement shown in Fig. 1, and repeat the counting procedure in each field. Be careful not to overlap fields. Continue in a longitudinal manner, moving stepwise across the film as required. Count all parasitized and other red cell in each field, even if the total red cell count per field exceeds 250.

vi. Stop counting when about 20 fields with about 250 red blood cells (about 5000 red blood cells) have been counted. Record the actual numbers of parasitized and other red blood cells counted on an appropriate worksheet. Use these figures to calculate the total parasite count per µL of blood.

vii. When counting is completed, calculate the parasite density from the patient's actual red cell cell count. If this is not available, use an estimated average red cell count of 5 000 000/µL and the following formula. Note that the final result is rounded to the nearest whole number.

viii. Number of parasites per µL blood:

Parasites / µL = $\frac{\text{No. of parasitized red blood cells x 5 000 000}}{\text{No. of red blood cells counted}}$

APPENDIX 4: Malaria Quantitative Real Time Polymerase Chain Reaction

This protocol describes the detailed experimental procedure for real-time RT-PCR using SYBR Green I as mentioned in Xiaowei Wang and Brian Seed (2003) A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Research 31(24): e154; pp.1-8. The procedure begins with reverse transcription of total RNA. The cDNA is then used as template for real-time PCR with gene specific primers.

Time required

cDNA synthesis: 2 hours.
real-time PCR: 2 hours.
Dissociation curve analysis: 0.5 hour.

Reagents and Equipments

- Oligonucleotide Primers. Gene specific primers are retrieved from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). These primers are ordered from the MGH DNA Core facility (https://dnacore.mgh.harvard.edu/synthesis/index.shtml). All the primers are desalted and both UV absorbance and capillary electrophoresis are used to assess the quality of primer synthesis.
- Whole blood DNA
- Human blood total DNA master panel (BD Biosciences / Clontech).
- SYBR Green PCR master mix, 200 reactions (Applied Biosystems).
- Optical tube and cap strips (Applied Biosystems).
- SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).
- 25 bp DNA ladder (Invitrogen).
- ABI Prism 7000 Sequence Detection System (Applied Biosystems).
- ABI Prism 7000 SDS software (Applied Biosystems).
- 3% ReadyAgarose Precast Gel (Bio-Rad).
- Agarose gel electrophoresis apparatus (Bio-Rad).

Detailed procedure

Reverse Transcription

Reverse Transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol.

1. Prepare the following RNA/primer mixture in each tube:

Total RNA	5 μg
random hexamers (50 ng/μl)	3 μl
10 mM dNTP mix	1 μl
DEPC H_2O	to 10 μl

2. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min.
3. Prepare reaction master mixture. For each reaction:

10x RT buffer	2 μl
25 mM $MgCl_2$	4 μl
0.1 M DTT	2 μl
RNAaseOUT	1 μl

4. Add the reaction mixture to the RNA/primer mixture, mix briefly, and then place at room temperature for 2 min.
5. Add 1 μl (50 units) of SuperScript II RT to each tube, mix and incubate at 25°C for 10 min.
6. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice.
7. Add 1 μl RNase H and incubate at 37°C for 20 min.
8. Store the 1st strand cDNA at -20°C until use for real-time PCR.

Real-time PCR

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5 pmol/μl.
2. Set up the experiment and the following PCR program on ABI Prism SDS 7000. Do not click on the dissociation protocol if you want to check the PCR result by agarose gel. Save a copy of the setup file and delete all PCR cycles (used for later dissociation curve analysis).
    1. 50°C 2 min, 1 cycle
    2. 95°C 10 min, 1 cycle
    3. 95 °C 15 s -> 60 °C 30 s -> 72 °C 30 s, 40 cycles
    4. 72°C 10 min, 1 cycle
3. A real-time PCR reaction mixture can be either 50 μl or 25 μl. Prepare the following mixture in each optical tube.

25 μl SYBR Green Mix (2x)		12.5 μl SYBR Green Mix (2x)
0.5 μl liver cDNA		0.2 μl liver cDNA
2 μl primer pair mix (5 pmol/μl each primer)	OR	1 μl primer pair mix (5 pmol/μl each primer)
22.5 μl H_2O		11.3 μl H_2O

4.After PCR is finished, remove the tubes from the machine. The PCR specificity is examined by 3% agarose gel using 5 μl from each reaction.
5.Put the tubes back in SDS 7000 and perform dissociation curve analysis with the saved copy of the setup file.
6.Analyze the real-time PCR result with the SDS 7000 software. Check to see if there is any bimodal dissociation curve or abnormal amplification plot.

Troubleshooting

Here I listed a few major causes for real-time PCR failures. Please read the Primer Bank Help page for more details.
Little or no PCR product. Poor quality of PCR templates, primers, or reagents may lead to PCR failures. First, please include appropriate PCR controls to eliminate these possibilities. Some genes are expressed transiently or only in certain tissues. In our experience, this is the most likely cause for

negative PCR results. Please read literature for the gene expression patterns. One caveat is that microarrays are not always reliable at measuring gene expressions. After switching to the appropriate templates, we obtained positive PCR results in contrast to the otherwise negative PCRs (see our paper for more details).

Poor PCR amplification efficiency. The accuracy of real-time PCR is highly dependent on PCR efficiency. A reasonable efficiency should be at least 80%. Poor primer quality is the leading cause for poor PCR efficiency. In this case, the PCR amplification curve usually reaches plateau early and the final fluorescence intensity is significantly lower than that of most other PCRs. This problem may be solved with re-synthesized primers.

Primer dimer. Primer dimer may be occasionally observed if the gene expression level is very low. If this is the case, increasing the template amount may help eliminate the primer dimer formation.

Multiple bands on gel or multiple peaks in the melting curve. Agarose gel electrophoresis or melting curve analysis may not always reliably measure PCR specificity. From our experience, bimodal melting curves are sometimes observed for long amplicons (> 200 bp) even when the PCRs are specific. The observed heterogeneity in melting temperature is due to internal sequence inhomogeneity (e.g. independently melting blocks of high and low GC content) rather than non-specific amplicon. On the other hand, for short amplicons (< 150 bp) very weak (and fussy) bands migrating ahead of the major specific bands are sometimes observed on agarose gel. These weak bands are super-structured or single-stranded version of the specific amplicons in equilibrium state and therefore should be considered specific. Although gel electrophoresis or melting curve analysis alone may not be 100% reliable, the combination of both can always reveal PCR specificity in our experience.

Non-specific amplicons. Non-specific amplicons, identified by both gel electrophoresis and melting curve analysis, give misleading real-time PCR result. To avoid this problem, please make sure to perform hot-start PCR and use at least 60°C annealing temperature. We noticed not all hot-start Taq polymerases are equally efficient at suppressing polymerase activity during sample setup. The SYBR Green PCR master mix described here always gives us satisfactory results. If the non-specific amplicon is persistent, you have to choose a different primer pair for the gene of interest. You are also encouraged to report bad primers to Xiaowei Wang.

APPENDIX 5: 25 Hydroxy-Vitamin D Enzyme-Immunoassay (EIA)

Purpose
For quantitative determination of 25-hydroxyvitamin D {25(OH)D} and other hydroxylated metabolites in plasma samples collected from expectant mothers and their children up to 24 months old.

Reagents
Calibrators (Standards)-(CAL)
Antibody coated plates (MICROPLATE)
25-D biotin concentrate (25-D BIOTIN 50x)
Buffer (BUF)
Enzyme conjugate (ENZYMCONJ)
Controls (CTRL)
TMB substrate (SUBS)
Stop solution (HCL)
Wash concentrate (WASHBUF 20x)
Adhesive plate sealer

Materials Required:
Disposable 12 x 75 mm Borosilicate glass (*polypropylene tubes not suitable*)
Pipettes (25and 200ul; 1ml)
Multichannel pippetes (100ul)
Votex machine
Automated microplate washer
Photometric microplate reader and data analysis equipment

Assay Summary Description
The IDS 25-hydroxy vitamin D EIA kit is an enzyme-immunoassay for the quantification of 25(OH) D and other hydro-xylated metabolites in serum or plasma. Calibrators, controls and samples are diluted with biotin labeled 25-(OH) D. The diluted samples are incubated in micro plate wells which are coated with a highly specific sheep 25-OH D antibody for 2 hours at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labeled avidin, is added and binds selectively to complexed biotin and, following a further wash step, color is developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures are read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 25-OH D.

Reagent preparation

25-D Biotin Solution (25-D BIOTIN SOLN)
Add 3 ml of buffer (BUF) to the bottle of lyophilized 25-D Biotin concentrate (25-D Biotin; 50x (blue in colour). Replace the stopper and let it stand for 10-15 minutes at room temperature. Invert several times to ensure complete reconstitution. Add the reconstituted 25-D biotin concentrate (25.D Biotin, 50x -3ml) back in to the bottle containing the remaining buffer (BUF). Mix well by inversion. The 25-D biotin solution (50ml) is green in colour. Mark the bottle "25-D Biotin Solution". ***Store at 2-8^{o}C in the dark ALWAYS- stable for 8 weeks.***

Wash Solution (WASHBUF SOLN)

Add the contents of each bottle of wash concentrate (WASHBUF 20x) to 950ml 0f distilled or deionized water and mix. Store at room temperature.

All other reagents are supplied ready for use.

N/B
-Allow all reagents to come to room temperature before use.
-Mix all reagents by repeated inversion before use.
-Unused antibody coated plate (MICRPLATE) strips must be returned to the foil pouch with the desiccant sachet. Fold over the end of the foil pouch and seal in one of the plastic self-seal bags provided. Store at 2-8^{o}C for up to 8 weeks after opening.
- Controls (CTRL) can be stored at 2-8^{o}C for up to 8 weeks after opening
- Wash Solution (WASHBUF SOLN) can be stored at room temperature for up to 8 weeks.

Specimen Handling
Collect blood specimens in to EDTA or sodium heparin anticoagulant. Separate plasma soon after collection and store immediately at -20^{o}C. Avoid repeated freeze and thawing of samples multiple times

Assay procedure

1. Prepare labeled borosilicate glass or polypropylene tubes, one for each calibrator (CAL, control (CTRL) and sample (SPE).

    i. Tube 1; CAL
    ii. Tube 2; CTRL
    iii. Tube 3- 96: SPE
2. Add 25µl of each Calibrator (CAL, control (CTRL) or sample to the appropriately labeled tubes.

3. Add 1000µl of 25-D Biotin Solution (25-D BIOTIN SOLN) to all tubes. Vortex thoroughly for 10 seconds.
    From _____ to _____

4. Add 200ul of each diluted Calibrator, control or sample to the appropriate wells of the antibody coated plate (MICRPLATE) in duplicate. Cover the plate with an adhesive plate sealer. Incubate at 18-^{24o}C for 2 hours.

    Plate No. _____ Date _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

From _____ to _____

5. Wash all wells three times with wash solution (WASHBUF SOLN). *(Automatic plate wash; set plate washer to dispense at least 300ul of wash solution per well. Fill and aspirate for 3 cycles).* Tap the inverted plate firmly on absorbent tissue to remove excess wash solution before proceeding to the next step.

6. Add 200ul of Enzyme Conjugate (ENZYMCONJ) to all wells using a multichannel pipette. Cover the plate with an adhesive plate sealer. Incubate at 18-25^{o}C for 30 minutes.
   From_____ to_____

7. Repeat wash step 5.

8. Add 200ul of TMB substrate (SUBS) to all wells using a multichannel pipette. Cover the plate with an adhesive plate sealer. Incubate at 18-25^{o}C for30 minutes. **N/B**: TMB substrate is easily contaminated. Only remove the required amount for the assay from the bottle. Dispose of unused TMB substrate. Do not return the bottle.
   From _____to_____

9. Add 100ul of stop solution (HCL) to all wells using a multichannel pipette.

10. Measure the absorbance of each well at 450nm using a micro-plate reader within 30miutes of adding the stop solution.

From_____to_____

Performer's Name _____Signature _____

Assay Date of Performance: _____

APPENDIX 6: Anti-Measles Antigen Total IgG ELISA Protocol

Materials:

*__Immulon-4 HBX__ 96 well microtiter plate (Dynatech)

*__Coating buffer__ - 1 X PBS

*__Blocking buffer__ - 5% milk/PBST (5 % dry milk/ 1 x PBS/ 0.05% Tween 20)

* __Wash buffer__- 1 x PBS, 0.05% Tween 20

* __ELISA diluent buffer -1% BSA PBST__ (1 x PBS/ 1 % Bovine Fraction V serum Albumin/0.05% Tween 20)

* __Goat anti-human IgG Fc □ □ fragment□ specific□ /alkaline phosphatase conjugate__ (Jackson Immuno Labs Cat #109-055-098 lot#105207)

* __Alkaline phosphatase substrate tablets__ (Sigma, Cat # S0942, 5 mg tablets)

* __Alkaline phosphatase substrate buffer =Diethanolamine Buffer:__
118mL Diethanolamine (Fisher # BP348-500) in 882 ml dH_2O. pH to 9.6, wrap in foil and store @ 4^oC

*__Measles__ (Rubeola)(Genway, catalog # GWB-9A35FE, San Diego, CA, 92121, USA)

*__2nd International Standard for anti-Measles immunoglobulin, human (NIBSC code: 97/648) 3 IU/ml__

Procedure:

1. Coat each well with 100 µl coating buffer containing 8 µg/ml Measles. Incubate overnight at 4 C

2. Wash plates x 3 with wash buffer

3. Block plates with 150 µl 5% milk blocking buffer per well. Incubate at 37 °C for 1 hour, or 4 °C overnight

4. Wash plates x 3 with wash buffer

5. Prepare 1:100 plasma sample dilutions in 5% milk ELISA diluent buffer. Add 100 ul of sample to the appropriate well

6. Add standards and controls. NIBSC standard is 3 IU/ml. First dilution is 1:200, and then twofold dilutions x 10 or if optimized use three dilutions, high, medium and low ie. 1:200, 1:800, 1:3200; for NAM use 1:100 dilutions.

7. Incubate at 37 C for 1 hour

8. Wash plates x 3 with wash buffer

9. Add 100 ul of 1:1000 dilution of goat anti-human IgG / alkaline phosphatase conjugate antibody to each well (dilute in 5% milk ELISA diluent buffer)

10. Incubate at 37 C for 1 hour

11. Wash plates x 3 with wash buffer

12. Dissolve 2 alkaline phosphatase substrate tablets per 10 ml of alkaline phosphatase substrate buffer (10 ml per plate). Ensure tablet is well-dissolved. Add 100 ul of alkaline phosphatase substrate solution to each well.

13. Wait for color to develop. Read OD using ELISA reader set to 405 nm. Stop reaction with 50 ul of 5% EDTA when highest standard reaches OD of approximately 2.5-3.0. Usually 10-15 minutes.

APPENDIX 7: Diphtheria Toxoid Total IgG ELISA Protocol

Materials:

* **Immulon-4 HBX** 96 well microtiter plate (Dynatech)

***Coating buffer** – Carbonate buffer (pH 9.6)= 1M = 4.53 ml 1 M $NaHCO_3$ solution + 1.82 ml 1 M Na_2 CO_3 solution , pH , bring up to 100 ml with Deionized water

***Blocking buffer – 3% BSA PBST** (1 x PBS/ 3 % Bovine Fraction V serum Albumin/ 0.05% Tween 20)

***Wash buffer**- 1 x PBS, 0.05% Tween 20

***ELISA diluent buffer -1% BSA PBST** (1 x PBS/ 1 % Bovine Fraction V serum Albumin/0.05% Tween 20)

* **Goat anti-human IgG Fc □ □ fragment□ specific** /alkaline phosphatase conjugate(Jackson Immuno Labs Cat #109-055-098 lot#105207)

***Alkaline phosphatase substrate tablets** (Sigma, Cat # S0942, 5 mg tablets)

***Alkaline phosphatase substrate buffer** =**Diethanolamine Buffer:**

118mL Diethanolamine (Fisher # BP348-500) in 882 ml dH_2O. pH to 9.6, wrap in foil and store @ 4^oC

***#149 Diphtheria Toxin CRM mutant** (List Biological Lab, Inc., lot# 14934A1, 2 mg/ml)

***Diphtheria Antitoxin Human Serum** (NIBSC code: 10/262, 2 IU/ml)

Procedure:

1. Coat each well with 100 µl coating buffer containing 0.5 g/ml Diphtheria toxin. Incubate overnight at 4 C

2. Wash plates x 3 with wash buffer

3. Block plates with 150 µl 3% BSA PBST blocking buffer per well. Incubate at 37 °C for 1 hour, or 4 °C overnight

4. Wash plates x 3 with wash buffer

5. Prepare 1:100 plasma sample dilutions in 1% BSA PBST ELISA diluent buffer. Add 100 µl of sample to the appropriate well.

6. Add standards and controls. NIBSC standard is 2 IU/ml. First dilution is 1:100, and then twofold dilutions x 10 or High, Medium and Low standards ie: 1: 100, 1:200, 1:400.

7. Incubate at 37 C for 2 hours or 4 C overnight.

8. Wash plates x 3 with wash buffer

9. Add 100 µl of 1:1000 dilution of goat anti-human IgG / alkaline phosphatase conjugate antibody to each well (dilute in 1% BSA PBST ELISA diluent buffer)

10. Incubate at 37 C for 1 hour

11. Wash plates x 3 with wash buffer

12. Dissolve 2 alkaline phosphatase substrate tablets per 10 ml of alkaline phosphatase substrate buffer (10 ml per plate). Ensure tablet is well-dissolved. Add 100 µl of alkaline phosphatase substrate solution to each well.

13. Wait for color to develop. Read OD using ELISA reader set to 405 nm. Stop reaction with 50 µl of 5% EDTA when highest standard reaches OD of approximately 2.5-3.0. Usually 15-20 minutes.

APPENDIX 8: School of Graduate Studies Proposal Approval Letter

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PG/PHD/00120/2011

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 11th June, 2013

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR NAMUYENGA TOKO EUNICE— PG/PHD/00120/2011

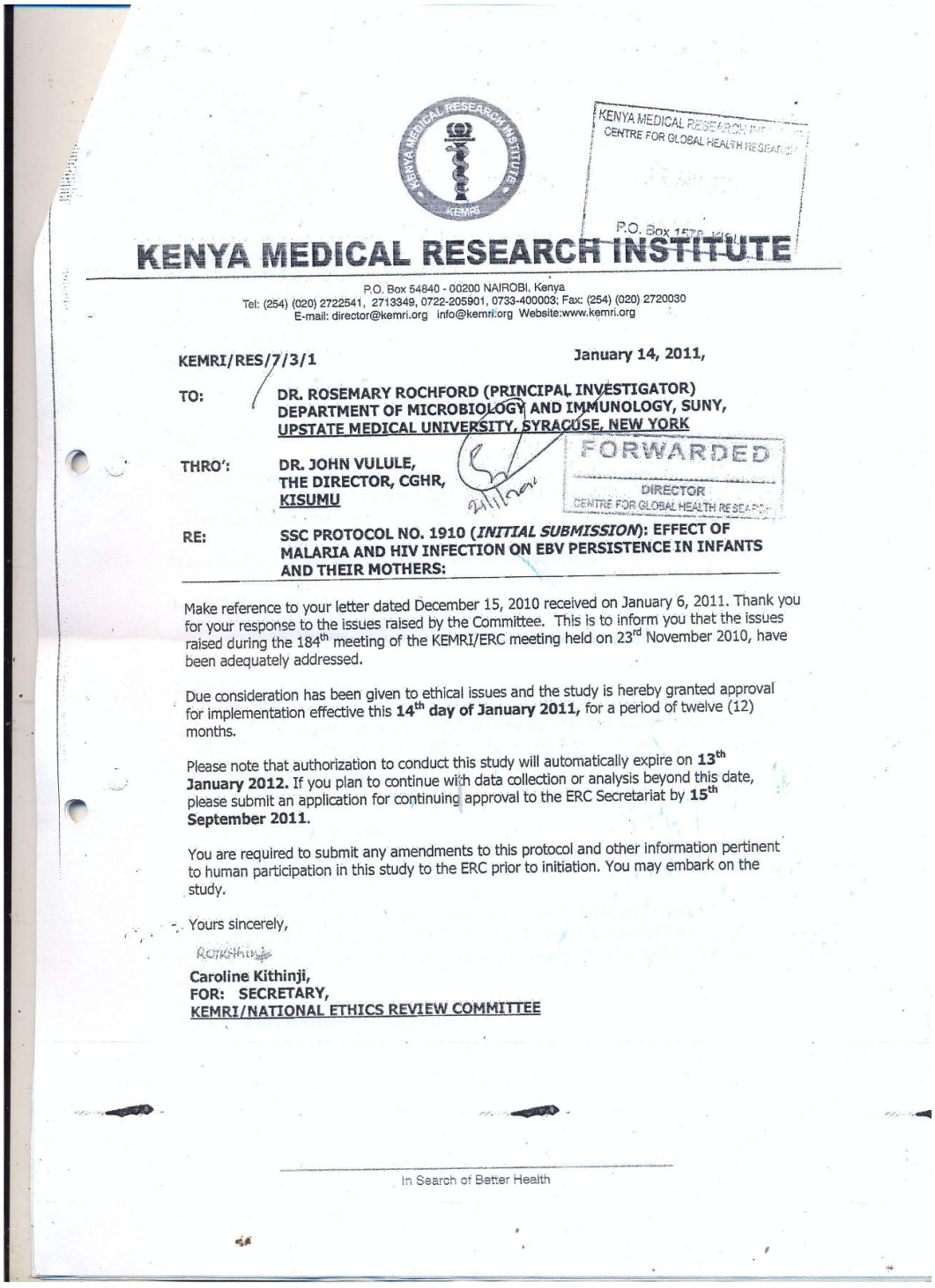
The above named is registered in the Doctor of Philosophy in Biomedical Sciences of the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled "Maternal Selenium Status, Inflammation and Associated Birth Outcomes in Pregnant Women Exposed to Holoendemic Malaria in Western Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. P.O. Owuor
DEAN, SCHOOL OF GRADUATE STUDIES

MASENO UNIVERSITY DIRECTOR 13 JUN 2013 SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

APPENDIX 9: KEMRI Institutional Review Board (IRB) Approval Letter



KENYA MEDICAL RESEARCH INSTITUTE

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Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 January 14, 2011,

TO: DR. ROSEMARY ROCHFORD (PRINCIPAL INVESTIGATOR)
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY, SUNY,
UPSTATE MEDICAL UNIVERSITY, SYRACUSE, NEW YORK

THRO': DR. JOHN VULULE,
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

RE: SSC PROTOCOL NO. 1910 (*INITIAL SUBMISSION*): EFFECT OF MALARIA AND HIV INFECTION ON EBV PERSISTENCE IN INFANTS AND THEIR MOTHERS:

Make reference to your letter dated December 15, 2010 received on January 6, 2011. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 184th meeting of the KEMRI/ERC meeting held on 23rd November 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **14th day of January 2011,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **13th January 2012.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **15th September 2011**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

In Search of Better Health

APPENDIX 10: INFORMED CONSENT FORM

Namuyenga Toko Eunice MSN/KEMRI Consent for Human Investigational Studies
Study Title: The association of Vitamin D with Measles Virus and Diphtheria Toxoid Vaccine Specific Immunoglobulin G Antibody Responses in Mother-Child pairs at Chulaimbo Sub-County Hospital in western Kenya

Consent form: Maternal nutrition and immunity prospective study
Purpose: Vitamin D concentration in circulation correlates with immune responses hence controls the body's general physiological responses to infections. Adequate Vitamin D status is associated with improved pregnancy outcomes and reduced disease progression. Inadequate Vitamin D levels are associated with pre-eclampsia, a condition characterized with strong inflammatory responses that serve as an important cause of preterm birth, high blood pressure and significant protein levels in urine during pregnancy. There is no information about the prevalence and risk factors associated with pregnancy outcomes in malaria holoendemic areas. This study therefore hypothesizes that, maternal plasma Vitamin D deficiency is associated with increased pro-inflammatory responses linked to adverse birth outcomes and reduced antibody responses to vaccine antigens among expectant women exposed to holoendemic malaria. Namuyenga Toko Eunice of Maseno University in collaboration with her colleagues at Kenya Medical Research Institute (KEMRI) and Suny Upstate Medical School, USA, are inviting you to enroll and participate in this research study. We are doing this study because we want to understand the association of micronutrient variation in the control of the body's inflammatory reactions during pregnancy and the associated immune response birth outcomes among women exposed to holoendemic malaria transmission. Micronutrients through reduction of inflammation can be used in the control and prevention of adverse impacts of maternal malaria infections and consequently, improved pregnancy outcomes. This initiative will investigate plasma Vitamin D concentrations associated with specific vaccine IgG antibody responses in mother-child pairs in a malaria holoendemic area. Data showing maternal malaria infection, Vitamin D inadequacy and inadequate specific vaccine IgG antibody responses will require public health policy on maintenance of adequate vitamin D levels besides the recommended malaria treatment during pregnancy.

Procedure: In order to understand the functional role of micronutrients on the control of immune reactions and the associated birth outcomes, we need to know about your immune responses and nutritional status by measuring your inflammatory cytokine responses, getting information about your Vitamin D micronutrient levels, dietary habits, specific vaccine antigen antibody responses and some cultural practices. We will request for a small blood sample (4mls) at the time of enrolment, and 2mLs once every 3 months for 9 months. It is important if you agree to be in this study that you will not migrate from the area for the next three years. Blood will be drawn from a vein in your arm using a needle attached to a blood collection syringe or tube. On subsequent visits, I finger prick will be performed to collect the 2mL blood sample. Blood will be transported to the KEMRI/SUNY laboratory at Kisian in Kisumu city. Tests done in the laboratory will tell us your nutritional status, inflammatory cytokine responses and malaria infection status. To assess your new born baby's condition, we will also measure their height and weight.

The blood sample will be destroyed after we have tested it for nutritional status inflammatory responses and malaria parasite infections. If, at any time you wish to withdraw your agreement, please contact Namuyenga Toko Eunice at KEMRI Kisumu Tel.254-715-458878 or 254-736-614248 and we will destroy the samples. If you do not wish to have the blood stored, you may still participate in this study. You will still be examined for nutritional deficiency and participate in environmental questionnaire. In case of any clinical disorders like fever or anemia (lack of blood) and is infected with malaria, the clinical officer or nurse assisting the investigators in this project will provide you with free medicine to treat malaria infection as recommended by ministry of health malaria treatment policy.

Long-term storage and future studies: I agree for KEMRI to store my blood for futures studies of etiological factors that may influence adverse birth outcomes.. I understand that if any tests results are found that are important for my health, KEMRI will try to report this to me, if possible. I understand that I have the right to withdraw my agreement for future research anytime and for any reason. I may also ask that my blood not be used for certain types of testing. To do this, I may tell Namuyenga Toko Eunice the study principal investigator, of my request and he will inform the other investigators at KEMRI and SUNY Upstate Medical School. I understand that the KEMRI SSC and ERC will approve any future testing not described here.

If you agree, circle "YES". If you do not agree, circle "NO" YES NO

Signature*_____ Date _____

Witnessed by _____Date_____
*A person can sign, or verbally state his/her consent in the presence of a witness who will then sign or put a thumb impression

Risks and Benefits: There are minimal risks in donating blood. The blood drawings include a little bleeding, pain, bruising and rarely infection. All of these are uncommon events that may occur in very few people. The benefit of having your ecological environment tested is to establish the essential background information likely to be in the food chain. The benefits of your blood donation are free testing and treatment for malaria illness and establishing the micronutrient and inflammatory responses. Blood will be checked for malaria by blood smear. If the blood smear is positive and your child has malaria symptoms, such as fever and headache, the Clinical Officer assisting the principal investigator will offer you the drugs currently recommended by the Kenya Ministry of Health for the treatment of uncomplicated malaria.
The current medication recommended by the Kenya National Health Guidelines for the treatment of uncomplicated malaria is Artemether-Lumefantrine (CoArtem®). Artemether-Lumefantrine has been shown to be very effective for treating uncomplicated malaria even if the parasites are resistant to other anti-malarial drugs. The side effects of this drug may include dizziness and fatigue, loss of appetite, nausea, vomiting, abdominal pain, myalgia, racing heartbeat, trouble sleeping headache, rash and aching joints. The benefits of taking the medication are the treatment of symptomatic malaria, which can cause severe sickness and even death.

Confidentiality: The results of the studies using blood will be assigned a study number to preserve confidentiality. A database linking personal identifiers to the study number will be kept by the principal investigator and relevant key personnel. The other people assisting the investigators will be blinded to the true identity of the study participants.

Summary of your rights as a participant in a research study: Your participation in this research study is voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits to which you are otherwise entitled. If you decide to join the study, you may withdraw at any time and for any reason. If information generated from this study is published or presented, your identity will not be revealed. If you experiences physical injury or illness as a result of participating in this research study, contact The Director of the Center for Global Health and Research CGHR) at KEMRI in Kisumu at PO Box 1578, Kisumu 40100 Tel. 254-57-2022924 or Peter Odada Sumba at 254-57-2022989/ 254-733746854/254-720-766550

Contact Information: Further information with respect to illness or injury resulting from a research procedure as well as a research subjects' rights is available from KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi at 254- 2-02722541 or The Director of KEMRI, PO Box 54840, Nairobi at 254- 2-02722541. If you have any questions about this study, you may also speak to The Director of CGHR, KEMRI in Kisumu at 254-57-2022924. Peter Odada Sumba can be contacted at CGHR, KEMRI, P.O Box 1578, Kisumu at 254-57-2022989 / 254-733-746854 / 254-720-766550

Signature: Signing below indicates that you have been informed about the research study in which you voluntarily agree to participate; that you have asked any questions about the study; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study. By signing this consent form, you do not waive any legal rights, and the investigators are not relieved of any liability they may have. You can withdraw from this study at any time. A copy of this consent form will be provided to you.

Name ---------- Date ----------