ROLE OF VITAMIN D IN IRON STATUS AND THE RISK OF MALARIA INFECTION IN KENYAN CHILDREN: A CASE OF KILIFI COUNTY

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A DISSERTATION SUBMITTED TO THE UNIVERSITY OF NAIROBI IN PARTIAL FULFILMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN APPLIED HUMAN NUTRITION

2018
DECLARATION

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DEDICATION

This dissertation is dedicated to my parents, Kimita and Nyokabi. From you, I have learnt immeasurable values.
ACKNOWLEDGEMENT

I am grateful to several people who have played a role towards the success of this work.

I thank my supervisors: from the university, Prof. Wambui Kogi-Makau and Dr. John Wangoh and, from KEMRI-Wellcome Trust, Dr. Sarah Atkinson and for being there throughout this project. I would like to warmly thank Dr. Atkinson for her funding, support and mentorship during the entire research period. Her wisdom, guidance and positive criticism at every stage enabled me to complete this project. I extend my deepest appreciation to Prof. Wambui Kogi-Makau for her support, professional guidance and advice which helped me overcome various academic challenges. I am grateful to Dr. Wangoh, for his guidance and supervision.

This work would not have been possible without the supervision and guidance of Dr. Reza Morovat and his team at the Oxford University - John Radcliffe Hospital. Thank you for the technical training and advice that was crucial for this work.

I am very appreciative to John Muriuki and Reagan Moseti for, in addition to peer mentorship, assistance with statistical analyses and STATA programme. My greatest appreciation also goes to the MSc. IDEAL cohort of 2016 for the support and friendship. Your company and scientific scope of ‘all things science’ brought humour and sanity as I worked. I also wish to thank my colleagues at the University of Nairobi for their encouragement and support.

I acknowledge the support of the IDEAL Team for funding and facilitating my studentship at the KEMRI-Wellcome Trust. In particular, I am most grateful to Dr. Samson Kinyanjui, Liz Murabu, Rita Baya, Charles Kamau and Florence Kirimi. Many thanks to the KEMRI Wellcome Trust fraternity for training on research, advice and assistance throughout the project.

I would like to acknowledge the faculty and staff of the Department of Food Science, Nutrition and Technology, University of Nairobi that contributed to this study in one way or another. Special thanks to Joan and Benard for your consistent support.

Finally, I would like to thank my family for their love and encouragement. To my parents who support my academic endeavours, as well as my brothers; Gathii Kimita for mentoring me into science and, Ikambi Kimita for always cheering me on. Special thanks to Mercy Muturi, a friend who knows this journey all too well.
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OPERATIONAL DEFINITIONS

Acute phase response: An increase in serum proteins referred to as acute phase proteins in response to inflammation or tissue injury.

Asymptomatic malaria: Presence of parasite stages of *P. falciparum* in blood without causing any symptoms.

Ferritin: A protein that stores iron in tissues. It is a marker for iron stores in the body and hence iron deficiency.

Geometric means of vitamin D: Average of 25OHD

Hepcidin: Iron regulatory hormone produced by the liver and acts on ferroportin to regulate serum iron availability.

Inflammation: C Reactive Protein levels ≥5mg/l.

Iron deficiency: Plasma ferritin levels of less than 12µg/L and less than 30µg/l in the presence of inflammation indicated iron deficiency.

Iron deficient anaemia (IDA): Haemoglobin levels <11g/L in children

Kenyan children: Children from Kilifi County – Junju, Ngerenya, Chonyi locations, 6-48 months at the time of plasma sample collection.

Malaria clinical case: Fever / axillary temperature of 37.5 °C with any level of accompanying parasitaemia in children less than 1 year, and fever >37.5 °C and parasitaemia of ≥2500 parasites/µl of blood in children 1-15 years (Mwangi et al., 2005)

Risk of malaria infection: Likelihood of contracting malaria due to exposure of risk factors such as residing in a high transmission area, low immunity, young age and pregnancy.

Soluble transferrin Receptor: Blood proteins that are elevated with iron deficiency

Sub-Saharan Africa: All African countries partially or fully located south of the Sahara.
<table>
<thead>
<tr>
<th><strong>Transferrin saturation:</strong></th>
<th>Calculated by ( \frac{\text{Iron (µmol/L)}}{\text{Transferrin (g/L)}} \times 25.1 \times 100 )</th>
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</thead>
<tbody>
<tr>
<td><strong>Transferrin:</strong></td>
<td>Blood proteins that bind to iron in circulation and carry it to tissues</td>
</tr>
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<td><strong>Vitamin D deficiency:</strong></td>
<td>Blood 25OHD levels &lt; 50nmol/L (20ng/ml)</td>
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<td><strong>Vitamin D insufficiency:</strong></td>
<td>Blood 25OHD levels ≥50≤75nmol/L (≥20≤30ng/ml)</td>
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<tr>
<td><strong>Vitamin D sufficiency:</strong></td>
<td>Blood 25OHD levels ≥75nmol/L (≥30ng/ml)</td>
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<tr>
<td><strong>Vitamin D toxicity:</strong></td>
<td>Blood 25OHD levels ≥100nmol/L (≥250ng/ml)</td>
</tr>
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>1,25(OH)D</strong></td>
<td>1,25-dihydroxycholecalciferol / calcitriol</td>
</tr>
<tr>
<td><strong>25OHD</strong></td>
<td>25-hydroxycholecalciferol / calcifediol /25-vitamin D</td>
</tr>
<tr>
<td><strong>AGP</strong></td>
<td>α-acid glycoprotein</td>
</tr>
<tr>
<td><strong>CAMP</strong></td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td><strong>CGMRC</strong></td>
<td>Centre for Geographic Medicine Research Coast</td>
</tr>
<tr>
<td><strong>CRP</strong></td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td><strong>CXCL9</strong></td>
<td>Chemokine ligand 9</td>
</tr>
<tr>
<td><strong>DMT1</strong></td>
<td>Divalent Metal transporter -1</td>
</tr>
<tr>
<td><strong>EDTA</strong></td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td><strong>ESA</strong></td>
<td>Erythrocyte Stimulating Agents</td>
</tr>
<tr>
<td><strong>HAMP</strong></td>
<td>Hepcidin antimicrobial peptide</td>
</tr>
<tr>
<td><strong>Hb</strong></td>
<td>Haemoglobin</td>
</tr>
<tr>
<td><strong>HIV</strong></td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td><strong>HPLC</strong></td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td>Interferon gamma</td>
</tr>
<tr>
<td><strong>IOM</strong></td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td><strong>IRR</strong></td>
<td>Incidence Rate Ratio</td>
</tr>
<tr>
<td><strong>KEMRI</strong></td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td><strong>KHDSS</strong></td>
<td>Kilifi Demographic and Health Survey</td>
</tr>
<tr>
<td><strong>KNBS</strong></td>
<td>Kenya National Bureau of Statistics</td>
</tr>
<tr>
<td><strong>KWTRP</strong></td>
<td>KEMRI Wellcome Trust Research Programme</td>
</tr>
<tr>
<td><strong>MCV</strong></td>
<td>Mean Corpuscular Volume</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>STFR</td>
<td>Soluble Transferrin Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TS</td>
<td>Transferrin saturation</td>
</tr>
<tr>
<td>VDD</td>
<td>Vitamin D deficiency</td>
</tr>
<tr>
<td>VDI</td>
<td>Vitamin D insufficiency</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDS</td>
<td>Vitamin D sufficiency</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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ABSTRACT

Introduction: In Kilifi County, malaria infection is a major cause of mortality with children under the age of five years being the most vulnerable. Iron status influences susceptibility to malaria while Vitamin D status may also have an important influence to susceptibility and severity. Despite this, there is insufficient information on the vitamin D status of children in the coastal region where malaria infections are highly prevalent. The general objective of this study, therefore, was to find out the association between vitamin D status and iron status and how this interaction alters the risk of malaria infection in Kenyan children.

Research Methods: A cross sectional study was carried out in 1487 children in Kilifi County, Junju and Ngerenya areas. Longitudinal data on anthropometry, haemoglobin, auxiliary temperature and a malaria blood slide. Laboratory analysis was carried out for ferritin, C-reactive protein, transferrin, hepcidin and soluble transferrin receptor. Data was analysed for descriptive and inferential statistics.

Results: The overall geometric mean serum 25-hydroxycholecalciferol, 25OHD, was 81.9 nmol/L, (range 16, 215.5 nmol/L). The overall prevalence of vitamin D deficiency (25OHD <50nmol/L) was 6%, vitamin D insufficiency (25OHD >50 and ≤75nmol/L) was 34.1% and vitamin D sufficiency (25OHD <75nmol/L was 59.9%). An increase in vitamin D increased the risk of iron deficiency (OR 2.89, 95% CI; 2.02, 4.13; P=0.0005) and iron deficiency anaemia (OR, 5.70; 95%CI, 3.37, 9.64; P=0.0005) significantly in a univariate logistics model, but when adjusted for gender, study setting and age, the significance was lost. Low vitamin D levels (25OHD <75nmol/l) increased the risk of malaria incidence OR 2.52; 95% CI, 1.47; P=0.001, but when adjusted for age, gender and study setting, the effect was lost OR 1.31; 95% CI, 0.73, 2.37, P=0.36.
Vitamin D status was not associated with malaria parasitaemia. Moreover, vitamin D status had no influence on susceptibility to malaria infection.

Conclusion: This study led to the conclusion that; the prevalence of vitamin D insufficiency in children in Kilifi County is high, that low levels of vitamin D among healthy children is not associated with iron status nor does vitamin D status predict the risk of malaria infection.

Recommendations: Low vitamin D status is a public health concern among children in Kilifi County and these children should be targeted for supplementation programmes. Additionally, there should be increased sensitization by community health workers among caregivers on the health benefits of exposing children to sunshine as a source of vitamin D.
CHAPTER 1: INTRODUCTION

1.1 Background of Study

In Sub-Saharan Africa, micronutrient deficiencies and malaria infections are highly prevalent and often co-exist. The World Health Organization estimates 82 deaths per 1000 live births in children under the age of 5 years annually caused by malaria (World Health Organization, 2015). More than 70 percent of all malaria deaths occur in this age category making malaria a major cause of childhood mortality in high transmission areas (World Health Organization, 2016).

Micronutrient deficiencies are often linked to susceptibility to malaria and other infectious diseases. Vitamin D and iron status have been found to be linked to malaria susceptibility and severity in African children (Cusick et al., 2014; Sazawal et al., 2006). Findings from an observational study in Ugandan children (Cusick et al., 2014) have linked low vitamin D status to malaria susceptibility. Research on mouse models showed a positive correlation on malaria severity and low vitamin D status (Dwivedi et al., 2016; He et al., 2014).

Despite Africa having abundant sunshine throughout the year, research indicates that there may be a high prevalence of vitamin D deficiency in African children which manifests as bone mineralisation disorders such as rickets (Braithwaite et al., 2012; Edwards et al., 2014). Vitamin D is an important micronutrient for bone growth and also plays a role in immune function. Low serum vitamin D has been linked to higher risk of infections and disease severity including tuberculosis (Chan, 2000), invasive pneumococcal disease (White, 2008) HIV (Rodriguez et al., 2009) and malaria (Cusick et al., 2014).

Iron deficiency anemia, on the other hand, is the leading cause of years lived with disability in Africa and its prevalence in children is high (Global Burden of Disease Pediatrics Collaboration, 2016). Iron deficiency is especially prevalent in malaria endemic areas
(World Health Organization, 2015) and, is associated with malaria infection. Studies hypothesise that poor iron status may be protective against malaria infections by starving *Plasmodium falciparum*, the malaria parasite (Ganz Tomas, 2010). In support of this, studies in coastal Kenya (Nyakeriga et al., 2004) and Tanzania (Gwamaka et al., 2012) have shown that high serum iron increases the likelihood of morbidity and mortality from malaria in children.

One of the greatest challenges in sub Saharan Africa is reducing the risk of malaria infections while countering micronutrient deficiencies. Vitamin D and iron deficiencies often co-exist, particularly in children with infections (Martineau et al., 2007). There is evidence that vitamin D plays a role in the regulation of iron status through the iron-ferroportin axis by mediating the iron regulator hormone hepcidin (Bacchetta et al., 2014; Smith et al., 2016). Vitamin D deficiency may therefore increase the risk of malaria and other infections. Interestingly, vitamin D deficiency may be more common in African children than previously thought. Studies on malaria and other infections show that poor vitamin D status is associated with the severity of disease outcomes (Yakoob et al., 2016). Knowledge about the interaction between vitamin D and iron homeostasis through hepcidin could better inform iron and vitamin D supplementation initiatives in malaria endemic areas.
1.2 Statement of problem

One of the biggest problems in Africa is controlling the rate of malaria infection. The interaction between vitamin D and iron could explain a different pathway through which both micronutrients interact to influence susceptibility to malaria. Vitamin D plays a role in iron regulation through the iron regulator hormone hepcidin (Smith et al., 2016). Though isolated studies in American (Smith et al., 2015) and Asian (Han et al., 2013) populations have shown that vitamin D status influences iron status, a thorough review of literature shows little data on African populations. Furthermore, research on vitamin D status in Africa remains an area of little coverage. A clear understanding is also lacking on the effects of low vitamin D status and susceptibility to infectious disease in the paediatric population in Africa, particularly malaria.

1.3 Justification

From the literature, little research has been done to establish the relationship between vitamin D, hepcidin concentration and other measures of iron status in malaria-endemic countries. Additionally, there appears to be no available data on the vitamin D status of children in Kilifi. Malaria infection in Kilifi County is a major cause of childhood mortality yet there is little available evidence on the relationship between vitamin D and malaria infection (Scott et al., 2012). Iron deficiency, on the other hand, affects approximately 40-50 percent of children in Kilifi and, is the leading cause of lives lived with disability in sub Saharan Africa (Atknison et al., 2014). At the same time malaria infection is common and causes childhood mortality. Though supplementation with micronutrients including iron improves general child health, it might be detrimental to children in malaria endemic areas (Sazawal et al., 2011). This is because the malaria parasite requires iron and other micronutrients for its own growth and proliferation. In such a paradoxical relationship, it is important to explore the role of vitamin D status on malaria infection. This study intends to establish whether there is an
association between vitamin D and iron status and the risk of malaria infection. Data from this study could further inform supplementation programs in Kenyan malaria endemic areas.

1.4 Aim of Study
The aim of this study was to contribute towards reduced malaria prevalence and iron deficiency among children in Kilifi County.

1.5 Purpose of the Study
This study purposed to generate data that is useful for raising awareness on the role of vitamin D and iron status in influencing malaria susceptibility, as a base for policy making on malaria and micronutrient status of children in Kilifi County.

1.6 Study Objectives
1.6.1 Overall objective
To determine the association between vitamin D status and iron status and how this interaction alters the risk to malaria infection in Kenyan children.

1.6.2 Specific objectives
1. To determine the vitamin D status of Kenyan children in a malaria endemic Kilifi County.
2. To establish the association between vitamin D and iron status in children in Kilifi County.
3. To investigate how vitamin D status alters the risk of malaria infection in Kenyan children.

1.7 Research question
How does the association between vitamin D and iron status influence the risk of malaria infection in Kenyan children?
1.8 Benefits

1. Knowledge on the prevalence of vitamin D status may inform nutritional supplementation programmes for children in Kilifi County.

2. Knowledge on the association between vitamin D status and malaria infection may lead to interventions aimed at increasing vitamin D status as well as design measures to reduce malaria infection in children below five years.

1.9 Limitation

This study is an observational study, and hence associations between vitamin D and malaria may be confounded by other environmental and physiological factors that may not be part of the study variables.
CHAPTER 2: LITERATURE REVIEW

2.1 Vitamin D: Background

Vitamin D, also known as the ‘sunshine vitamin’, is a fat soluble molecule. It was discovered in the 1920’s originally as the ‘vitamin’ that is essential for normal skeletal growth and maintenance of calcium homeostasis in the body (Holick, 2003). Further research disqualified vitamin D as a ‘vitamin’ when scientists found out that it was synthesised in the body from sunlight exposure. Instead, vitamin D is more of a steroid hormone in structure and physiology, and its hormonal action has effects on health and disease in the body (Holick, 2003).

2.1.1 Sources

Vitamin D is obtained from dietary sources or synthesized in the skin through exposure to ultra violet B rays. Dietary sources of vitamin D are in two forms; cholecalciferol (vitamin D3) from animal sources and ergocalciferol (vitamin D2) from plant sources (Holick, 2004). Food sources of vitamin D attribute to very small amount of total in the body since few foods are rich in vitamin D. These include oily fish, yeast, eggs and milk. Breast feeding infants are born with vitamin D stores and additionally obtain the nutrient from the mothers’ milk. Vitamin D is also available in fortified foods such as infant formula, milk and cheese, which may have limited access in low income countries. Supplements are of vitamin D alone or a combination with calcium and other nutrients are also sources of vitamin D (White, 2008).

2.1.2 Biosynthesis

Most of the vitamin D in circulation is obtained from cutaneous synthesis. As the skin is exposed to sunlight, ultra violet B radiation is absorbed by 7-dehydrocholesterol, a molecule found in cells of the skin, which is then converted to pre-vitamin D₃. Vitamin D₂ and vitamin
D$_3$ have no biological activity and need to be activated through the body’s metabolic pathways to exert vitamin-D effects (Holick, 2004). Vitamin D$_3$ from the diet and from the skin then enters circulation bound to carrier vitamin D binding proteins and is metabolised in the liver to 25 hydroxyvitamin D (25OHD). In the kidneys, this is then converted to the active 1,25 dihydroxy vitamin D (1,25OHD) by 1-alpha-hydroxylase enzyme. 1,25OHD acts as a hormone that regulates calcification of bones during calcium and phosphate homeostasis. It does this through regulation of parathyroid hormone levels hence reducing bone loss (Holick, 2004; White, 2008).

2.1.3 Definition and Assessment of Vitamin D Status

The definition of vitamin D status is a contentious subject. The Institute of Medicine defines deficiency as 25OHD less than 50nmol/l, insufficiency >50≤75nmol/l and sufficiency as levels >75nmol/l (IOM, 2010). However, researchers suggest the revision of these definitions since these levels are based on adequate amounts used to prevent skeletal disorders such as rickets and osteomalacia (Ross et al., 2011).

Vitamin D is quantified through the assessment of circulating 25OHD in plasma (Ross et al., 2011). High Performance Liquid Chromatography (HPLC), immunoassays and the gold standard mass spectrophotometry are used to measure 25OHD in blood. There is no consensus on a universal method of measuring 25OHD in vitamin D studies, which, complicates comparison and interpretation of individual results (Ross et al., 2011). Quantification of vitamin D is influenced by vitamin D binding protein (DBP) which influenced by disease and pregnancy.
2.2 Functions of vitamin D

2.2.1 Bone metabolism and calcium homeostasis

Vitamin D acts an endocrine hormone which regulates calcium homeostasis by maintaining plasma levels of 8.5 to 10.5 mg/Dl (Holick. 2005). When plasma concentrations fall below 8.5mg/dl, parathyroid hormone is upregulated prompting the activation of 1,25OHD. Vitamin D works to increase calcium absorption form the small intestines, renal calcium re-absorption and bone resorption through activation of osteoclasts. When on is vitamin D deficient, prolonged metabolic responses may result to increased bone resorption and bone weakening.

2.2.2 Non-skeletal Function

Recent studies have identified vitamin D receptor (VDR) in immune cells such as activated T and B cells, and cells in the colon, breast, prostate and other organs (Rodriguez et al., 2009; Bacchetta et al., 2014). Additionally, 1 α-hydroxylase enzyme has been identified in pancreatic, colon, breast, skin and immune cells, suggesting that the production of vitamin D (Holick, 2004). These discoveries evidence non-skeletal roles of vitamin D especially in immune function. Studies show vitamin D may be an important precursor in insulin response. Vitamin D deficiency has been linked to increased risk of developing type 1 diabetes (Asemi et al., 2013) while high intake of vitamin D in early childhood has been associated with decreased risk of developing type 1 diabetes mellitus (Holick, 2004).

2.2.3 Inflammation and infection

The immune system protects the body from infection. It is divided into two; innate immune system which provides non-specific first line of protection from pathogens; and adaptive immune system which is more specific and stimulated after innate response. The role of vitamin D in immune-regulation was identified decades ago when sunlight was observed to cure some infections (White, 2004) and certain illnesses were associated with low vitamin D
levels and seasons (Holick, 2003). Vitamin D is involved in innate immune response where bacteria, viruses and fungi are killed through antimicrobial peptides such as cathelicidins and defensins (Bacchetta et al., 2014). These Antimicrobial peptides are produced phagocytic cells, lymphocytes and epithelial cell lining where they defend the host against pathogens. Research has further identified the expression of 1α-hydroxylase and vitamin D receptors (VDR) in immune cells such as dendritic cells, macrophages and activated B and T lymphocytes which imply production of vitamin D in these cells (He et al., 2014; Liu, 2006). In the adaptive immune system, vitamin D function has been evidenced through the expression of vitamin D receptors in activated T and B lymphocytes (Dwivedi et al., 2016). Elevated 1,25OHD suppresses proliferation of T helper cells and modulates the production of cytokines. Associations between infections, immune function and vitamin D deficiency has been studied broadly. Acen et al. (2016) found an inverse relationship between the risk and severity of tuberculosis, acute respiratory tract infection and pneumonia and vitamin D status in Ugandan patients.

2.3 Health problems related to vitamin D deficiency

Deficiencies in vitamin D lead to demineralisation of bone cells, osteoid, leading to bone mineralisation disorders (Holick, 2004). Rickets in children is characterised by soft and weak bones which result in bowed legs, curved spine, swelling of the wrist, knee or ankle, rachitic rosary and susceptibility to bone fractures (Jones et al., 2017). In adults, defective mineralisation results in osteomalacia. Vitamin D deficiency has also been linked to several conditions such as breast cancer, cardiovascular disease, autoimmune disease such as multiple sclerosis and infections (Yakoob et al., 2016; Wilkinson et al., 2000). There is evidence that vitamin D also plays a role in immune modulation (Schwartz et al., 2018).
2.4 Epidemiology of vitamin D deficiency

2.4.1 Risk factors

Processes that alter the amount of sunlight available for cutaneous synthesis of vitamin D, bioavailability and diet are factors that influence vitamin D synthesis. First, vitamin D synthesis requires Ultra violet B rays of between 290nm and 315nm wavelength (Holick, 2003). People living in high latitudes do not get enough wave length to stimulate cutaneous synthesis. Secondly, melanin acts as a barrier that prevents UVB radiation from penetrating the skin, hence decreasing the synthesis of vitamin D. Dark pigmented individuals require ten times sun exposure as light skinned persons to synthesis vitamin D. Likewise, wearing covering clothing such as in cultural dressing of the Middle East, use of sunscreen, skin injury such as in burns are often linked to low vitamin D status (Holick, 2004). Gastrointestinal disorders that alter nutrient absorption (Bikle, 2007), fat storage disorders and obesity (Rodríguez-rodríguez et al., 2009) are also associated with suboptimal levels. The risk of vitamin D deficiency also increases with age, is more prevalent in females and individuals who live a sedentary lifestyle.

2.4.2 Vitamin D deficiency in Africa

In populations living in Africa, data on vitamin D status is scant and inconsistent. However, studies identified by this literature review show much higher baseline 25OHD levels compared to other continents, and a high variation within the continent. African studies also show few nationally representative data, and most research compare unhealthy to healthy groups. Africa is a heterogeneous continent in geography, climate and topography. There also exist a great deal of variation in demography, cultural practices, religion, skin pigmentation, disease burdens, dietary intake and, water sources, all which may influence vitamin D status.
In sub-Saharan Africa where sunshine is abundant, occurrence of vitamin D insufficiency and deficiency has been reported in Ethiopia (Wakayo et al., 2015), South Africa (Poopedi et al., 2011) and Kenya (Jones et al., 2017; Toko et al., 2016). Rickets is also common in African children as evidenced by studies from Kenya (Edwards et al., 2014; Jones et al., 2017) and The Gambia (Braithwaite et al., 2012). In North African countries, studies show low 25OHD levels in children (Allali et al., 2009). Most of North Africa is located in high latitude areas and may receive little sunshine. Additionally, cultural and religious practices involving full body coverage may also hinder sun exposure and consequently cutaneous vitamin D synthesis.

2.5 Vitamin D and malaria infection

In this new area of research, few studies have examined the relationship between vitamin D and malaria through immune modulation and iron regulation.

2.5.1 Malaria

Malaria infection is a leading cause of morbidity and mortality in Sub-Saharan Africa. In 2016, sub-Saharan Africa accounted for 91 percent of all the malaria deaths (World Health Organization, 2015). Transmission in Kenya is seasonal with the highest infection cases occurring during the rainy season specifically at the coastal region and areas around Lake Victoria (Ministry of Health, 2016). In Kilifi, the highest incidence is in school going children with high levels of mortality (Mogeni et al., 2016). Malaria is mainly caused by the \textit{Plasmodium falciparum} parasite which is transmitted by the female anopheles mosquito. Clinical manifestations range from asymptomatic infection to disease and death. Populations in malaria endemic areas develop asymptomatic malaria with continued exposure, creating malaria immunity with age. Those with clinical malaria develop symptoms such as fever, headache, vomiting and nausea. In its severe form, seizures, anaemia and, respiratory distress
usually occur. In very severe complications, cerebral malaria develops where patients experience, in addition to severe malaria symptoms, delirium, febrile stupor and coma (Mwangi et al., 2005). Malaria diagnostic tests recommended by the World Health Organisation are microscopy, a technique widely used in developing countries; rapid diagnostic tests, which, are useful in remote areas; and nucleic acid amplification-based diagnostics used for epidemiological studies (http://www.who.int/malaria/areas/diagnosis/en/).

2.5.2 Evidence of vitamin D and malaria from animal studies

Animal studies indicate that vitamin D may have a beneficial anti-inflammatory role in malaria. In a study by He et al. (2014), Plasmodium berghei infected mice treated with vitamin D had significantly longer survival as opposed to the controls. The decreased time of disease progression could have been caused by the ability of vitamin D to reduce circulating cytokines, interferon gamma (IFN-γ) and tumour necrosis factor (TNF), which are elevated in severe malaria and result in hyperinflammation (He et al., 2014). In mouse studies, combined treatment of the antimalarial drug arteether with vitamin D significantly improved survival compared to mice treated with Vitamin D or arteether alone (Dwivedi et al., 2016). Yamamoto et al. (2016) also found anti-plasmodial activities where reduced parasitaemia was observed with calcitriol treatment in mice.

2.5.3 Evidence of vitamin D and malaria from human studies

Observational human studies suggest that there may be a link between 25OHD levels and malaria susceptibility and severity. A study in Ugandan children found that 25OHD concentrations were significantly lower in children with malaria, both severe and cerebral, as compared to children from the community cohort. There was also an overall high prevalence of vitamin D deficiency in all children (Cusick et al., 2014). In Tanzania, Sudfield et al.
(2015) also found an association between 25OHD levels and an increased risk of clinical malaria. On the other hand, a study in Kenya did not find any significant differences in 25OHD levels with malaria infection during pregnancy, delivery or in infant cord blood (Toko et al., 2016). Table 1 gives a summary of the human studies on vitamin D and malaria.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Study type</th>
<th>Population</th>
<th>Sample size (n)</th>
<th>25OHD nmol/l</th>
<th>Association with outcome</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cusick et al., 2014</td>
<td>Uganda</td>
<td>Cross-sectional study</td>
<td>Children 18 months to 12 years</td>
<td>60 children</td>
<td>Mean 25OHD levels</td>
<td>Yes</td>
<td>There was a 9% decrease in the odds of having severe malaria with every 1ng/ml increase in plasma 25OHD. 25OHD was significantly lower in children with SMA/CM than in children from the community cohort.</td>
</tr>
<tr>
<td>Sudfeld et al., 2015</td>
<td>Dar es Salaam, Tanzania</td>
<td>Prospective study (observational)</td>
<td>581 Infants</td>
<td>1103 HIV positive adults, ≥18 years initiating antiretrotherapy</td>
<td>Vitamin D deficiency was highly prevalent. Mean 18.5ng/ml</td>
<td>Yes</td>
<td>Children with 25OHD ≥30ng/ml had an increased risk of clinical and confirmed malaria.</td>
</tr>
<tr>
<td>Sudfeld et al., 2012</td>
<td>Dar es Salaam, Tanzania</td>
<td>Prospective study (observational)</td>
<td>1103 HIV positive adults, ≥18 years initiating antiretrotherapy</td>
<td>1103 HIV positive adults, ≥18 years initiating antiretrotherapy</td>
<td>Vitamin D insufficiency 43.6%</td>
<td>No</td>
<td>No association between vitamin D and incidence of malaria.</td>
</tr>
<tr>
<td>Toko et al., 2016</td>
<td>Western Kenya</td>
<td>Prospective study (mother infant cohort)</td>
<td>Pregnant 63 women, 43 infants women at time of enrolment 16.5 to 28.5 years, and infants from those pregnancies.</td>
<td>Low vitamin D status highly prevalent in women; 51% with VDI, 21% with VDD</td>
<td>No</td>
<td>No significant association in plasma 25OHD levels relative to malaria infection.</td>
<td></td>
</tr>
<tr>
<td>Newens et al., 2005</td>
<td>London, United Kingdom</td>
<td>Cross-sectional study</td>
<td>Adults with <em>P. falciparum</em> malaria 25 only 14 had complete follow-up data</td>
<td>Low 25OHD mean 24.3ng/ml</td>
<td>No</td>
<td>No differences in 25OHD in subjects in the course of malaria infection, and even after they got cured of malaria.</td>
<td></td>
</tr>
</tbody>
</table>

25OHD: plasma vitamin D; VDD: Vitamin D deficiency; VDI: Vitamin D insufficiency; HIV: Human Immunodeficiency virus
2.6 Mechanisms of vitamin D regulating malaria susceptibility

2.6.1 Vitamin D regulates iron

Vitamin D has been recently shown to influence iron status, and possibly influence iron availability to parasites. Iron is important in the survival of both hosts and invading microbes and, its overload has been shown to favourably promote the growth of these pathogens (Sazawal et al., 2006). Malaria infections have been shown to be less frequent and severe in patients who are iron deficient (Nyakeriga et al., 2004; Sazawal et al., 2006).

2.6.1.1 Iron status role in malaria

Studies have indicated that iron deficiency could be beneficial to hosts as it limits iron availability, diminishing the proliferation of the malaria parasite. As shown in Table 2, Nyakeriga et al (2004) found that the incidence of malaria infection was significantly lower in children who were iron deficient as compared to those who were replete.

Table 2: Incidence of *Plasmodium falciparum* malaria among iron-replete and iron-deficient children during 12 months of follow-up

<table>
<thead>
<tr>
<th>Variable</th>
<th>Iron-replete children (n = 154)</th>
<th>Iron-deficient children (n = 125)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of child weeks of observation</td>
<td>4545</td>
<td>3788</td>
</tr>
<tr>
<td><strong>Malaria definition</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of episodes</td>
<td>119</td>
<td>73</td>
</tr>
<tr>
<td>Annual incidence</td>
<td>1.36</td>
<td>1.0</td>
</tr>
<tr>
<td>Adjusted IRR (95% CI)</td>
<td>1.00</td>
<td>0.70 (0.51–0.99)</td>
</tr>
<tr>
<td><strong>Malaria definition</strong>&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of episodes</td>
<td>76</td>
<td>50</td>
</tr>
<tr>
<td>Annual incidence</td>
<td>0.87</td>
<td>0.69</td>
</tr>
<tr>
<td>Adjusted IRR (95% CI)</td>
<td>1.00</td>
<td>0.83 (0.57–1.24)</td>
</tr>
</tbody>
</table>

<sup>1</sup>fever / axillary temperature of 37.5 °C with any level of accompanying parasitaemia in children less than 1 year

<sup>2</sup>fever /37.5 °C and parasitaemia of ≥2500 parasites/µl of blood in children 1-15 years (Mwangi et al., 2005)

IRR: Incidence Rate Ratio
CI: Confidence Interval

Iron regulation is primarily controlled by the hormone hepcidin, which is produced in the liver. Hepcidin is an antimicrobial peptide and is encoded by the *HAMP* gene. It blocks export by ferroportin and the divalent metal transporter, DMT1 on macrophages and enterocytes. This control mechanism protects the body from excess iron in circulation and accumulation (Ganz and Nemeth, 2015).

### 2.6.1.2 Role of Vitamin D in iron homeostasis

Vitamin D metabolites have recently been shown to regulate hepcidin synthesis through downregulation of the *HAMP* gene. Molecular studies have shown suppressed hepcidin mRNA expression resulting in low hepcidin concentrations (Bacchetta et al., 2014; Smith et al., 2016; Zughaier et al., 2014). In a study involving healthy volunteers, hepcidin concentrations decreased in vitamin D administered groups as compared to placebo groups (Smith et al., 2016). These finding were consistent with a study by Bacchetta et al. (2014) where individuals who received a high dose of vitamin D had decreased hepcidin levels within 24 hours of supplementation. Figure 1 shows the molecular mechanism through which vitamin D regulates iron. When vitamin D is sufficient, 250HD is activated to 1,25OHD, and binds to the vitamin D receptor (VDR). This causes the downregulation of transcription of the HAMP gene resulting in increased membrane expression of ferroportin. The resulting enhancement of iron transporters then leads to systemic increase in iron. Consequently, when vitamin D is deficient, elevated hepcidin decreases membrane expression of ferroportin decreasing systemic levels of iron. (Bacchetta et al., 2014)
Another mechanism through which vitamin D may regulate iron is through the regulation of erythropoiesis. 1,25OHD has been found to have a synergistic relationship with erythropoietin. Erythrocyte precursor cells express 1,25OHD receptors, which induce proliferation and maturation of erythroid progenitor cells. Vitamin D insufficiency may therefore result in increased resistance to erythropoietin and reduced erythropoiesis (Alon et al., 2002). These findings are supported by clinical observations, which showed that vitamin D deficiency was independently associated with low haemoglobin levels and anaemia. Supplementation with ergocalciferol has been associated with dose reduction in ESA – erythrocyte stimulating agents (ESA) in haemodialysis patients (Santoro et al., 2015).

### 2.6.2 Immune modulation properties of vitamin D in malaria

Vitamin D metabolites have been found to stimulate production of antimicrobial peptides, cathelicidin and defensin, which play a role in innate immunity. These peptides are elevated during and after malaria infection and are thought to play a multifunctional role in providing
a first line immunity against malaria parasites (Parra et al., 2013). 1,25(OH)D₃ is a direct inducer of the antimicrobial peptide gene, *hCAMP* - cathelicidin and *defensin B2* - defensin. Promoters of these genes contain vitamin D response elements which mediate 1,25(OH)D₃ dependent gene expression (Wang et al., 2017). Additionally, 1,25(OH)D₃ is also necessary for an interferon gamma mediated pathway in macrophages which propels phagosomal maturation and autophagy (He et al., 2014).

2.7 Methodological issues

The assessment of iron status in a malaria endemic area may present difficulties in determining the biochemical markers that correctly define iron status. Exposure to malaria parasites causes an acute phase response resulting in elevation of iron indicators which are also acute phase response proteins, such as ferritin and hepcidin, and in symptomatic malaria, soluble transferrin receptor (sTfR) (Silva, 2014). For this reason, plasma ferritin measurements are often accompanied by measurement of acute phase response proteins namely C-reactive protein (CRP) and α acid glycoprotein (AGP) (Silva, 2014). Using ferritin as a sole indicator of iron status may mask iron deficiency in populations with widespread infection and inflammation. Ferritin can be measured in plasma, serum or a dried serum spot, using enzyme linked immunosorbent assay (ELISA). Plasma ferritin levels of less than 12µg/L, and less than 30µg/L in the presence of inflammation indicate depleted iron stores in children less than 5 years. (Silva, 2014)

Changes in soluble transferrin receptor (sTfR) indicate changes in erythropoiesis and hence iron status. sTfR is a polypeptide derived from transferrin receptor, which is a transmembrane protein that is expressed in cells that require iron. It is a useful biomarker of iron deficiency and unlike ferritin, hepcidin and transferrin, is not strongly affected by inflammation and
infections. Levels of sTfR are elevated in iron deficiency. Levels of sTfR higher than 8.3 indicate iron deficiency (Garza, 2010).

2.8 Gap in knowledge

Generally, there is a paucity of data on the status of vitamin D levels in Africa, and particularly in Kenya. Kenya has policies on malaria control and treatment and iron supplementation programs through the Ministry of Health (Ministry of Health, 2016). However, there lacks supplementation guidelines on vitamin D status. Despite knowledge from molecular studies showing that vitamin D regulates hepcidin, few studies have been done on children population to show this association. Furthermore, vitamin D has been thought to regulate iron deficiency and that this could influence susceptibility to infection, specifically malaria. Despite this, there are few studies that have sought to establish this relationship.
CHAPTER 3: METHODOLOGY

3.1 Study context
3.1.1 Parent Study
The parent study for this present research was an ongoing study that was using genetic variation in micronutrients to determine whether blood levels of micronutrients increase the risk of infection in African children. This approach is known as Mendelian randomization. The larger study was conducted in four sites in Africa; Kilifi in Kenya, Entebbe in Uganda, Banfora in Burkina Faso and Soweto in South Africa, at different timelines.

3.1.2 Kenyan cohort
The study cohort included children from the Kilifi Health and Demographic Surveillance System (KHDSS) described by Scott et al. (2012). In summary, the KHDSS is a community-based surveillance system that recruits residents from Kilifi District. KHDSS covers approximately 891km² and currently has a population of 280, 000. Information on births, deaths, migration and pregnancies is updated every three months by enumerators who carry out household visits (Scott et al., 2012). Longitudinal data on childhood infection, genetic risk factors and prevalence of diseases is collected. All residents have a unique identity number and are investigated with standard laboratory tests. These include a malaria slide, full blood count and blood culture (Scott et al., 2012). Healthy children aged between 6 and 30 months were recruited during annual KHDSS follow-up visits, from 2000 to 2016.

3.1.3 Present study
This study used previously available data collected during surveillance, and generated data through laboratory analysis of vitamin D and iron markers. Children remained in active surveillance for malaria and other infections until their eighth birthday. Standard data on anthropometry and malaria was also collected in the annual cross-sectional survey. Additionally, 5mls of venous blood was collected from each participant. In my study, data
from the 2016 survey was included. In addition to the data from the 2016 cross-sectional bleed, this study used data from previously collected data so as to meet the objectives of this research.

3.1.4 Study area and study site
The study was conducted in Kilifi County, located 3° south of the equator in the Coastal Region of Kenya. Kilifi County has an area of 12,609.7 square kilometres and a population density of 473.6 (KNBS, 2015). Residents practice subsistence farming as their main economic activity and most of the population lives in the rural areas. The County experiences two rainy seasons, the short rains which occur between October and December and the long rains between April and July. During these seasons, malaria transmission is high (KNBS, 2015).

3.1.5 Health and nutrition
The prevalence of iron deficiency is 46.5% (Atkinson et al., 2014) and the entomological inoculation rate is 8.5-300 infective bites per person per year (Mwangi et al., 2005). A 2014 KNBS Report indicated malaria as the leading cause of mortality in Kilifi, followed by pneumonia, anaemia and tuberculosis (KNBS, 2015). The County has only one paediatric ward located in Kilifi County Hospital in Kilifi town.

This study was based at the KEMRI-Wellcome Trust Research Programme (KWTRP) – Centre for Geographical Medicine Research (CGMRC) – Coast. Children in this study were from cohorts within the Kilifi Health and Demographic Surveillance System (KHDSS) (Appendix 1) (Scott et al., 2012) which included malaria vaccine trial cohorts RTSS and ME-TRAP (SSC Protocols 1131; 3149; and 2887 Protocols 1131; 3149; and 2887) (kemri-wellcome.org). The KHDSS locations used in this study included Junju, a high malaria endemic area, and Ngerenya, a low malaria endemic area. The KHDSS spans 35kms from
Kilifi town. A census carried out in 2000 to 2001 defined a population of 198,063 (Scott et al., 2012).

3.1.6 Participant enrolment

Children residing in households within the KHDSS were recruited at birth and remained under active surveillance for malaria and other infections until their eighth birthday. Healthy children 6 to 48 months were recruited in the community cohorts and malaria vaccine studies at different timelines. Informed consent was obtained from the mothers of the child before the child was enrolled (kemri-wellcome.org). On enrolment, 5mls of venous blood was collected into heparin and EDTA tubes for each child, as well as during yearly cross-sectional bleeds. During an annual cross-sectional survey, the child was assessed and data recorded in a standard proforma per specific study protocols. These data included anthropometric information, haemoglobin, axillary temperature and a malaria blood slide if the child had a temperature above 37.5°C.

3.2 Study population

Children 6 months to 48 months at the time of plasma sample collection, living within the Kilifi Health and Demographic Surveillance at Junju, Ngengerenya locations in Kilifi County.

3.3 Study design

This study was a cross-sectional study with a retrospective aspect. Figure 2 shows the study activity flow diagram. Children were enrolled every year from 2000 to 2016. Each child was recruited at 6 to 48 months of age and followed up until their eighth birthday. At enrolment, height, weight Mid-Upper Arm Circumference (MUAC) age and, gender data was collected. Additionally, 5mls of venous blood was drawn from each child. In the present study, Vitamin
D measurement 25(OH)D, iron, sTfR, ferritin, transferrin and, C-Reactive protein (CRP) were measured from when the child was 6 to 48 months. Every year and, when a child fell sick, malaria parasitaemia data, auxiliary temperature and malaria slides were collected. Within the 8 years of follow-up, some children dropped out, others migrated, while in others, blood could not be drawn from the child due to denied consent or sickness. As this study was to determine the vitamin D status of a healthy population, all children with malaria infection at the time of vitamin D measurement were exempted.

Figure 2: Study activity flow showing points in the study for data collection for each child

3.4 Sampling
3.4.1 Sample size determination
To obtain an adequate number of participants, all healthy children registered under the KHDSS surveillance between 2000 and 2016 were included (convenient sample). A power analysis was conducted to determine an appropriate sample size that was sufficient to detect
the significance effect of vitamin D status on malaria infection. According to Cohen (1988), significance represents the likelihood of a Type I error, that is, the likelihood that the study will falsely claim a significant effect has been found where there is no effect in the population under study.

Table 3 shows the calculated power to detect the prevalence of vitamin D insufficiency of the study with a type one error rate of 0.05. A prevalence of 64.7% of vitamin D insufficiency from a study in rural community children in Ethiopia (Wakayo et al., 2015), was used to calculate power. The purpose of this study was to describe vitamin D status and how it influences malaria infection in children in Kilifi County. A sample size of 1487 gave a power >80%.

Table 3: Sample size calculation indicates sufficient power for the Kilifi samples

<table>
<thead>
<tr>
<th>Study site</th>
<th>Vitamin D insufficiency (%)</th>
<th>Sample size</th>
<th>Power (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilifi, Kenya</td>
<td>64.7%</td>
<td>1487</td>
<td>100</td>
</tr>
</tbody>
</table>

3.4.2 Sampling procedure

1. **Sampling frame**
The sampling frame included all children aged 6 to 48 months at the time of enrolment living within the KHDSS. All study participants with available stored samples, laboratory data and informed consent were included in the study.

2. **Sampling schema**
Figure 3 shows the sampling schema. Purposive sampling was used to select Kilifi County, Junju and Ngerenya localities. Junju and Ngerenya were selected because of their location within the KHDSS. All households in these areas were enrolled for the KDHSS as described in (Scott et al., 2012). Children were selected if they qualified the selection criteria below.
3. Selection criteria

a) Inclusion criteria
1. Availability of informed consent to participate in the study
2. Children aged 6 to 48 months at the time of sampling to be enrolled in community cohort studies
3. Children with available samples, clinical and laboratory data

b) Exclusion criteria
1. Lack of informed consent
2. Children ≥48 months or < 6 months at the time of sampling
3. Children without an available stored plasma sample and/or without follow-up data.
4. Chronically sick children or those who had sickle cell anaemia
3.5 Data acquisition methods

All children had demographic, anthropometric, malaria data and 5mls of blood collected. The baseline and follow-up data already available for each child included; anthropometric data height (cm) and weight (kg), age (months) at enrolment, gender, haemoglobin (not available for all participants), auxiliary temperature (°C), parasitaemia density (per/µL of blood) and malaria blood films for children whose temperature exceeded 37.5°C, and a child’s unique identification number. Additionally, 5ml of blood for children above 6 months were collected. Primary data was collected through laboratory assays. Plasma was kept in -80°C freezers for storage and transport. All assays were carried out by the author of this dissertation at the Department of Clinical Biochemistry, Oxford University Hospitals, Oxford; a laboratory that is well equipped with the relevant equipment.

3.5.1 Biochemical methods

1. Sample preparation
The samples were archived in KEMRI-Wellcome trust laboratories at -80 degrees, and shipped to Oxford University Hospital for laboratory tests. The samples were thawed at room temperature in batches of 81, which was the number of samples in each ELISA plate. They were then vortexed at 300s for 15seconds to mix plasma content, and later centrifuged at 10,000rpm for 10 minutes to section out any precipitate according to sample preparation guidelines by McPherson and Pincus (2016).

2. Sample analysis of analytes
The Abbot Architect cSystem (Abbott Laboratories, Abbott Park, IL) was used to assay the analytes. 250µl of sample was loaded into the automated cSystem for the analysis of ferritin, iron, transferrin, 25OHD and C-reactive protein (CRP). Table 4 shows the immunoassay used by the Abbot cSystem to assay the analytes.
Table 4: Techniques of measurement of analytes in the Abbot Architect cSystem

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique of measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>25OHD (vitamin D)</td>
<td>Chemiluminescent Microparticle Immunoassay</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Chemiluminescent Microparticle Immunoassay</td>
</tr>
<tr>
<td>Ferritin</td>
<td>Chemiluminescent Microparticle Immunoassay</td>
</tr>
<tr>
<td>CRP</td>
<td>MULTIGENT CRP Vario assay [CRPVa]</td>
</tr>
</tbody>
</table>

Source: (Abbott Laboratories, Abbott Park, IL)

a) Chemiluminescent Microparticle Immunoassay of plasma transferrin

A plasma aliquot of 250µL was loaded into the Abbot Architect cSystem (Abbott Laboratories, Abbott Park, IL) and incubated with a buffer Reagent 1 consisting of 100mmol/L TRIS, 45g/L Polyethylene Glycol and 0.1% Sodium Azide. Transferrin antibody Reagent 2: 40% Anti-human transferrin goat serum, 100mmol/L TRIS and 0.1% Sodium Azide was added to the aliquot, causing formation of insoluble immune complexes that increased turbidity. The transferrin concentration was then measured as a function of turbidity.

b) Plasma Hepcidin ELISA procedure

Plasma hepcidin was measured using competitive enzyme linked immunosorbent assay using the DRG® Hepcidin 25 (bioactive) HS ELISA Kit (DRG International, Inc. 2018). The assays are solid phase enzyme linked immunosorbent assays based on the principle of competitive binding. A sample of 20µL, standard and control were pipetted into appropriate wells and 50µL of enzyme conjugate added onto each well. The plate was then incubated at room temperature (25 degrees centigrade) for 60 minutes. After incubation, well contents were poured out and the plate was washed using a wash buffer. 100µL of the enzyme complex (streptavidin-HRP) was pipetted into the wells and the plate incubated for 30 minutes at room temperature. After incubation, a second wash step was carried out and 100µL of the substrate solution (Tetramethyl benzidine – TMB) pipetted into each well. The
plate was then incubated for 20 minutes at room temperature to allow colour development and stopped using 100µL of the stop solution (0.5M Sulfuric acid). Absorbance was taken at 450nm with a microtiter plate reader not exceeding 10 minutes after addition of the stop solution.

c) **Plasma soluble transferrin receptor ELISA**

Plasma levels of soluble transferrin receptor (sTfR) were determined using the BioVendor Human Soluble Transferrin receptor ELISA kits (biovendor.com). Plasma sample volume 20µL was diluted with a dilution buffer in the kit, dilution factor 25, and then pipetted into appropriate wells together with 100µL of standards and quality controls. The plate was then incubated for 1 hour shaking at 300 rpm on microplate shaker at room temperature. Wells were washed in the wash buffer and 100µL of conjugate solution added into each well. The plate was incubated for another hour shaking at 300 rpm then washed again. 100µL of the substrate solution was pipetted into each well in a dark room and incubated for 10 minutes to allow for colour development, which was then stopped with a 0.5M Sulfuric acid solution. Absorbance was read using a microplate reader set to 450nm the results multiplied by the dilution factor to obtain the concentration of soluble transferrin receptor in the plasma samples. The results of these ELISAs were determined from standard curves developed from standard calibrators, which were run simultaneously with study samples.

3.5.2 **Definitions**

A clinical malaria case was defined as fever / axillary temperature of 37.5 °C with any level of accompanying parasitaemia in children less than 1 year, and fever >37.5 °C and parasitaemia of ≥2500 parasites/µl of blood in children 1-15 years (Mwangi et al., 2005). A single malaria episode was defined as all cases of parasitaemia occurring within 28 days after a previous case (Gonçalves et al., 2014). Vitamin D deficiency (VDD) was defined as plasma
25(OH)D levels of <50 nmol/L, vitamin D insufficiency (VDI) as levels between 50-75nmol/L, while vitamin D sufficiency (VDS) was defined as levels >75nmol/L (IOM, 2010). Iron deficiency was defined using the plasma markers. Plasma ferritin levels of less than 12µg/L or less than 30µg/l in the presence of inflammation indicated iron deficiency (Phiri et al., 2009). Inflammation was defined as CRP levels ≥5mg/l (Phiri et al., 2009). Transferrin saturation lower than 7% was used to indicate iron deficiency, and was calculated by ((Iron (µmol/L) / Transferrin (g/L) x 25.1) x 100) (Phiri et al., 2009). Iron deficiency anaemia was defined as iron deficiency combined with a haemoglobin level of <11g/L (Cook, 2005). The nutritional status of each child was calculated based on the 2018 WHO Global Database on Child Growth and Malnutrition for Z-scores ("Weight-for-age Child growth standards", 2018). A Z-score cut-off point of <-2 standard deviation was used to classify low weight-for-age, low height-for-age and low weight-for-height as moderate and severe undernutrition, and a <-3 standard deviation Z-score to define severe undernutrition. Weight-for-age Z-scores below -2 standard deviation were grouped as underweight, height-for-age Z-score <-2 standard deviation as stunting and weight-for-height Z-score <-2 as wasting ("Weight-for-age Child growth standards", 2018).

3.6 Ethical Consideration

Ethical approval to carry out the study was granted by the Kenya Medical Research Institute’s Ethical Review Committee; Scientific and Ethics Review Unit (SERU), KEMRI/SERU/CGMR-C/046/3257, before initiation of the study. Appendix 2 shows the SERU Ethical approval.
3.7 Statistical data analysis

All the statistical analyses, data cleaning and calculations were performed using STATA version 13.1 (Stata Corporation, College Station, TX, USA, 1985-2013) statistical software package.

To explore the general characteristics of the study population, simple descriptive methods such as summary characteristics and frequency distributions were used. All variables underwent descriptive checks for missing values and outliers. The geometric mean values between groups were compared using one-way analysis of variance and independent t-test, after checking for normality and adjusting where necessary. Statistical significance was set at $P \leq 0.05$. Hepcidin, ferritin and vitamin D (25OHD) measurements were not normally distributed and were hence transformed to logarithmic scales. Data was categorised to make it easier to interpret based on previously stipulated definitions. The nutritional status of each child was calculated based on the 2006 WHO Global Database on Child Growth and Malnutrition for Z-scores (WHO, 2006). Iron deficiency anaemia, transmission area and inflammation were also grouped into categories for analysis. Categorical variables were compared using chi-square tests and analysis of variance.

Vitamin D status was modelled as a categorical variable; “low” vitamin D (25OHD<75nmol/L) and “adequate” vitamin D (25OHD≥75nmol/L). To determine the predictors of vitamin D deficiency, a univariate analysis was carried out. The predictor variables used were age, gender, study setting, inflammation and nutritional status. The variables that had $P<0.05$ were identified as significant predictors of vitamin D status, and results were reported as odds ratios and 95% confidence intervals (CI). The interaction between each predictor variable and the age of the participants was also determined.
To investigate the association between vitamin D and iron markers, univariate association between each of the iron markers (transferrin saturation, hepcidin, haemoglobin) and 25OHD was carried out. The association between vitamin D status and iron deficiency was investigated using logistic regression. The strength of association was measured by the coefficient for linear analysis and odds ratio for categorised variables. Additionally, as the age effect was a confounding factor, the model was adjusted for age, gender and the association determined by the adjusted odds ratio.

The main exposure variable was vitamin D status modelled as dichotomous variable (low, adequate) and, a categorical variable (sufficient, insufficient, deficient), depending on specific analysis. The outcome variable was the number of malaria episodes. A Poisson regression model was used to investigate the association between of sub-optimal vitamin D levels (deficiency and insufficiency) and the number of malaria episodes.
CHAPTER 4: RESULTS

4.1 Characteristics of study population

Plasma samples were available for 1487 children. Table 5 describes the characteristics of the study population. Of these, 48.9% (671) were female and 51.1% (690) were males. The minority of the children (40.2%) were in the age group 12 to 24 months. Anthropometric data was available for a total of 501 participants. The nutritional status of the study participants was determined, where 11.4% (27/237) had wasting (defined as weight-for-height z-score less than -2), 48.8% (117/240) were stunted (defined as height-for-age z-score less than -2) and 26.6% (114/429) were underweight (defined as weight-for-age z-score less than -2). Mid-Upper Arm Circumference (MUAC) data for participants below 6 months of age was available for 296 participants. The MUAC data was not normally distributed and had a geometric mean of 15.0±1.1. Only a minority of children, 0.7%, had a MUAC less than 12.5cm.

Inflammation, which was defined as CRP≥5mg/L, had a prevalence of 26.4% (393/1440). Malaria parasitaemia was also present in 21.9% (262/1191) of malaria blood smears were positive for *P. falciparum*. The overall geometric mean parasite density was 2317 parasites/µl (with a range of 40 – 380000 parasites/µl).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Frequency, N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n=1361)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>671</td>
<td>49.0</td>
</tr>
<tr>
<td>Male</td>
<td>690</td>
<td>51.0</td>
</tr>
<tr>
<td>Age groups (n=1361)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12 months</td>
<td>300</td>
<td>21.6</td>
</tr>
<tr>
<td>12 to ≤24 months</td>
<td>555</td>
<td>40.2</td>
</tr>
<tr>
<td>24 to ≤36 months</td>
<td>153</td>
<td>11.5</td>
</tr>
<tr>
<td>36 to ≤48 months</td>
<td>146</td>
<td>10.7</td>
</tr>
<tr>
<td>&gt;48 months</td>
<td>207</td>
<td>16.0</td>
</tr>
<tr>
<td>Study setting (n=1273) &lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ngerenya</td>
<td>275</td>
<td>21.6</td>
</tr>
<tr>
<td>Junju</td>
<td>998</td>
<td>78.4</td>
</tr>
<tr>
<td>Inflammation (n=1347)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP&lt;5mg/ml</td>
<td>983</td>
<td>73.0</td>
</tr>
<tr>
<td>CRP≥5mg/ml</td>
<td>364</td>
<td>27.0</td>
</tr>
<tr>
<td>Parasitaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With parasitaemia</td>
<td>227</td>
<td>21.0</td>
</tr>
<tr>
<td>Without parasitaemia</td>
<td>855</td>
<td>79.0</td>
</tr>
<tr>
<td>Nutritional status &lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stunted (n=240)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height-for-Age</td>
<td>117</td>
<td>48.8%</td>
</tr>
<tr>
<td>Wasted (n=237)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight-for-Height</td>
<td>27</td>
<td>11.4%</td>
</tr>
<tr>
<td>Underweight (n=429)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight-for-Age</td>
<td>114</td>
<td>26.6%</td>
</tr>
<tr>
<td>Mid Upper Arm Circumference (MUAC) &lt;sup&gt;c&lt;/sup&gt;</td>
<td>2/294</td>
<td>0.7%</td>
</tr>
<tr>
<td>&lt;12.5cm</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Study setting: Ngerenya is a low malaria transmission area; Junju is a high malaria transmission area

<sup>b</sup> Nutritional status: Stunting defined as height-for-age z-scores less than -2; Wasting defined as weight-for-height z-scores less than -2; Underweight defined as weight-for-age z-scores less than -2.

<sup>c</sup> MUAC: Mid Upper Arm Circumference for children less than 6 months

CRP: C reactive protein
4.2 Vitamin D status of children in Kilifi County

A total of 1364 (92%) children had vitamin D measurements. The geometric mean of serum 25-hydroxycholecalciferol (25OHD) was 81.9 nmol/L, (range 16, 215.5 nmol/L). The overall prevalence of vitamin D deficiency (25OHD <50nmol/L) was 6% (82/1364), vitamin D insufficiency (25OHD >50 and ≤75nmol/L) was 34.1% (465/1364) and, vitamin D sufficiency (25OHD <75nmol/L) was 59.9% (817/1364). (Figure 4)

![Pie chart showing Vitamin D status](chart.png)

**Figure 4: Vitamin D status in study population**

Children with inflammation (CRP≥5mg/L) had a significantly lower 25OHD level compared to the non-inflamed (CRP<5mg/L). Children with malaria parasitaemia had a 16% lower 25OHD level compared to those without parasitaemia (95% CI 0.21,0.11; P=0.000). Moreover, there was no statistically significant difference in 25OHD geometric means between males and females as well as no difference in study setting and, in nutritional status indices as shown in the Table 6.
<table>
<thead>
<tr>
<th>Study variables</th>
<th>Frequency</th>
<th>Plasma 25OHD nmol/l mean± SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>671</td>
<td>81.2±1.4</td>
<td>0.416</td>
</tr>
<tr>
<td>Male</td>
<td>690</td>
<td>82.4±1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Study setting</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ngerenya</td>
<td>275</td>
<td>82.2±1.3</td>
<td>0.138</td>
</tr>
<tr>
<td>Junju</td>
<td>998</td>
<td>79.7±1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Nutritional status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stunted</td>
<td>99</td>
<td>70.1±1.4</td>
<td>0.997</td>
</tr>
<tr>
<td>Not Stunted</td>
<td>109</td>
<td>70.1±1.4</td>
<td></td>
</tr>
<tr>
<td>Wasted</td>
<td>24</td>
<td>67.7±1.4</td>
<td>0.519</td>
</tr>
<tr>
<td>Not Wasted</td>
<td>181</td>
<td>70.1±1.4</td>
<td></td>
</tr>
<tr>
<td>Underweight</td>
<td>102</td>
<td>75.6±1.4</td>
<td>0.196</td>
</tr>
<tr>
<td>Not Underweight</td>
<td>287</td>
<td>79.7±1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Parasitaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With parasitaemia</td>
<td>227</td>
<td>68.4±1.3</td>
<td>0.0005</td>
</tr>
<tr>
<td>Without parasitaemia</td>
<td>855</td>
<td>80.3±1.4</td>
<td></td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP&lt;5mg/L</td>
<td>983</td>
<td>81.0±1.4</td>
<td>0.042</td>
</tr>
<tr>
<td>CRP≥5mg/L</td>
<td>364</td>
<td>84.1±1.4</td>
<td></td>
</tr>
</tbody>
</table>

*Study setting: Ngerenya is a low malaria transmission area; Junju is a high malaria transmission area.*

*Nutritional status: Stunting defined as height-for-age z-scores less than -2; Wasting defined as weight-for-height z-scores less than -2; Underweight defined as weight-for-age z-scores less than -2.*

CRP: C-Reactive protein

4.2.1 Distribution of Vitamin D by Age

The geometric mean of 25OHD level was lower in older compared to younger children (P<0.0001). Children less than 12 months had the highest mean 25OHD of 105 nmol/L. Figure 5, shows a box plot of 25OHD levels by age categories. Vitamin D levels reduced in
the first three years of life (6 months to 36 months), and then stabilised to the same level with from 48 months onwards.

![Graph showing distribution of Vitamin D (25OHD) by age in months]

**Figure 5: Distribution of Vitamin D (25OHD) by Age in months**

### 4.2.2 Predictors of a Low Vitamin D status (25OHD <75nmol/L)

The study participants were dichotomised using serum 25OHD of less than 75nmol/L as “Low vitamin D status” and levels greater than 75nmol/L as “Normal vitamin D status”. The predictors of low vitamin D status were determined using logistic regression analysis as shown in Table 7. The risk of having a low vitamin D status increased with age from children less than 12 months and this was statistically significant. Children living in Junju, a high malaria transmission area, had a 50% increased risk of having a low vitamin D status (OR, 1.50; 95% CI, 1.11, 2.01; P=0.008). Males seemed to have a trend towards a lower risk of having a low vitamin D status (OR, 0.80; 95% CI, 0.63, 1.02; P=0.072) whereas inflammation was not associated with low vitamin D status (OR, 0.81; 95% CI, 0.62, 1.07; P=0.146).
Table 7: Predictors of a low vitamin D status (25OHD <75nmol/L) for children in Kilifi County

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D status</th>
<th>OR (95% CI)</th>
<th>P value</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal n (%)</td>
<td>Low n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>386 (47.36)</td>
<td>285 (52.2)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>429 (52.64)</td>
<td>261 (47.8)</td>
<td>0.82 (0.66,1.02)</td>
<td>0.08</td>
<td>0.80 (0.63,1.02)</td>
</tr>
<tr>
<td>Age-groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;12months</td>
<td>246 (30.18)</td>
<td>54 (9.89)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>12 ≤ 24months</td>
<td>392 (48.1)</td>
<td>163 (29.85)</td>
<td>1.89 (1.34,2.68)</td>
<td>0.000</td>
<td>1.62 (1.10,2.37)</td>
</tr>
<tr>
<td>24 ≤ 36months</td>
<td>65 (7.98)</td>
<td>88 (16.12)</td>
<td>6.17 (3.99,9.53)</td>
<td>0.000</td>
<td>5.26 (3.30,8.39)</td>
</tr>
<tr>
<td>36 ≤ 48months</td>
<td>48 (5.89)</td>
<td>98 (17.95)</td>
<td>9.30 (5.91,14.64)</td>
<td>0.000</td>
<td>8.22 (5.06,13.36)</td>
</tr>
<tr>
<td>&gt;48months</td>
<td>64 (7.85)</td>
<td>143 (26.19)</td>
<td>10.18 (6.71,15.44)</td>
<td>0.000</td>
<td>8.81 (5.62,13.82)</td>
</tr>
<tr>
<td>Study setting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ngerenya</td>
<td>178 (24.22)</td>
<td>97 (18.03)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Junju</td>
<td>557 (75.78)</td>
<td>441 (81.97)</td>
<td>1.45 (1.10,1.92)</td>
<td>0.008</td>
<td>1.50 (1.11,2.01)</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP&lt;5mg/L</td>
<td>582 (72.03)</td>
<td>401 (74.4)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>CRP≥5mg/L</td>
<td>226 (27.97)</td>
<td>138 (25.6)</td>
<td>0.89 (0.69,1.13)</td>
<td>0.338</td>
<td>0.81 (0.62,1.07)</td>
</tr>
</tbody>
</table>

Multivariable estimates (Odds ratio) adjusted for all other variables in the model. OR, Odds Ratio; CI, confidence interval; MUAC, Mid-Upper Arm Circumference. Low vitamin D status 25OHD<75nmol/L; Normal Vitamin D status 25OHD≥75nmol/L. P value indicates logistics regression for differences in predictor variables.
4.2.3 Vitamin D status of children in Malaria transmission areas

Vitamin D status; normal vitamin D and low vitamin D, were mapped according to the study settings. Location coordinates of study participants were used to map children in Junju, where malaria transmission was high and, Ngerenya, where malaria transmission was low. Figure 6 shows that Junju had a higher proportion of children with malaria (27 episodes in Ngerenya verses 225 episodes in Junju). Moreover, Ngerenya had more children with vitamin D sufficiency (178 (24.22%) compared to Junju, 557 (75.8%). To further explore this observation, logistic regression analysis showed that study participants in Junju had a 50% higher chance of having vitamin D deficiency than Ngerenya (OR 1.50; 95% CI, 1.11,2.01; P=0.008).
Figure 6: Map of KHDSS showing Malaria Transmission area and coordinates for study participants
4.3 Association of vitamin D status and iron status

4.3.3 Mean differences of vitamin D by iron markers
Table 8 summarizes the geometric mean differences in iron markers. Soluble transferrin receptor levels were significantly higher in children with normal vitamin D status than those with low vitamin D status (19.0mg/L versus 16.7mg/L). Hepcidin levels were higher in the low vitamin D group compared to the normal Vitamin D group (6.5µg/L versus 5.1µg/L; P=0.0005). Iron deficient children had significantly higher levels of vitamin D (70.0% versus 30.0%) in normal and low vitamin D status.

Table 8: Mean differences of iron markers between low vitamin D and high vitamin D status groups

<table>
<thead>
<tr>
<th>Iron markers</th>
<th>Frequency</th>
<th>Low Vitamin D status</th>
<th>Normal Vitamin D status</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTfR (mg/L)</td>
<td>1346</td>
<td>16.7±1.5</td>
<td>19.0±1.5</td>
<td>0.000</td>
</tr>
<tr>
<td>Hepcidin (µg/L)</td>
<td>1257</td>
<td>6.5±3.4</td>
<td>5.1±3.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Ferritin index</td>
<td>1308</td>
<td>5.5±1.9</td>
<td>7.0±2.0</td>
<td>0.003</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>1300</td>
<td>10.5±2.1</td>
<td>8.3±2.1</td>
<td>0.000</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>793</td>
<td>10.4±1.2</td>
<td>9.9±1.2</td>
<td>0.000</td>
</tr>
<tr>
<td>Iron deficient, no (%)</td>
<td>477</td>
<td>143 (30.0%)</td>
<td>334 (70.0%)</td>
<td>0.000a</td>
</tr>
<tr>
<td>Iron deficiency anemia, no (%)</td>
<td>199</td>
<td>57 (28.6%)</td>
<td>142 (71.4%)</td>
<td>0.000b</td>
</tr>
</tbody>
</table>

Low Vitamin D is 25OHD <75nmol/L; Normal Vitamin D is 25OHD≥75nmol/L. Geometric mean values are presented for continuous variables and categorical variables are represented by proportions (%). Iron deficiency was defined as ferritin<12µg/L or ferritin <30 µg/L with inflammation CRP≥5mg/L. Iron deficiency anaemia was defined as iron deficiency with Hb<11g/dl.
SD, standard deviation
P values for continuous variables are Chi-square differences in mean.
*P values for categorical variables
4.3.4 Effect of vitamin D status on iron markers

The correlations between 25OHD and iron markers are demonstrated in Figure 7. All the variables were log transformed as they were not normally distributed. There was a significant negative correlation between log vitamin D and; log ferritin, log hepcidin and log TS, and a positive correlation with log sTfR.

Two-way scatterplots of vitamin D status shows:

a) log ferritin Coeff. -0.04 (95% CI -0.05, -0.02; P=0.000)
b) log hepcidin Coeff. -0.02 (95% CI -0.04, -0.01; P=0.004)
c) log sTfR Coeff. 0.13 (95% CI 0.09, 0.17; P=0.000)
d) log transferrin saturation (TS) Coeff. -0.09 (95% CI -0.12, -0.07; P=0.000)

Figure 7: The correlation between Vitamin D and Iron markers among children in Kilifi County, Kenya
A linear relationship. Univariate regression analyses of the effect of vitamin D levels on iron markers showed a 21% increase in sTfR, (95% CI; 0.15, 0.29; P=0.000); 31% decrease in hepcidin -0.31 (95% CI; -0.52, -0.10; P=0.004); 34% increase in ferritin index (95% CI; 0.23, 0.45; P=0.000) and 89% decrease in haemoglobin (95% CI; -1.19, -0.60; P=0.000) with increasing vitamin D levels. When adjusted for gender, age and study setting, the model showed significant association with increasing vitamin D levels where; 18% increase in sTfR, 71% increase in ferritin index and a 32% decrease in transferrin saturation. The effect on all the other variables was not significant. Increase in vitamin D levels increased iron deficiency (OR 2.89, 95% CI; 2.02, 4.13; P=0.0005) and iron deficiency anaemia (OR, 5.70; 95%CI, 3.37, 9.64; P=0.0005) significantly in a univariate logistics model, but when adjusted for gender, study setting and age, the significance was lost, as shown in Table 9.
Table 9: Effect of Vitamin D level on iron status markers

<table>
<thead>
<tr>
<th>Variables</th>
<th>N</th>
<th>Univariate estimates β (95% CI)</th>
<th>P value</th>
<th>Multivariable estimates β (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sTfR (mg/L)</td>
<td>1346</td>
<td>0.21 (0.15, 0.29)</td>
<td>0.000</td>
<td>0.18 (0.10, 0.26)</td>
<td>0.000</td>
</tr>
<tr>
<td>Hepcidin (µg/L)</td>
<td>1257</td>
<td>-0.31 (-0.52, -0.10)</td>
<td>0.004</td>
<td>-0.19 (-0.43, 0.04)</td>
<td>0.271</td>
</tr>
<tr>
<td>Ferritin index</td>
<td>1308</td>
<td>0.34 (0.23, 0.45)</td>
<td>0.000</td>
<td>0.71 (-2.20, 3.61)</td>
<td>0.023</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>1300</td>
<td>-0.46 (-0.58, -0.34)</td>
<td>0.000</td>
<td>-0.32 (-0.45, -0.19)</td>
<td>0.000</td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>793</td>
<td>-0.89 (-1.19, -0.60)</td>
<td>0.000</td>
<td>-0.31 (-0.67, 0.05)</td>
<td>0.094</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate estimates OR (95% CI)</th>
<th>P value</th>
<th>Multivariable estimates OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency</td>
<td>1325</td>
<td>2.89 (2.02, 4.13)</td>
<td>0.000</td>
<td>1.21 (0.77, 1.90)</td>
</tr>
<tr>
<td>Iron deficiency anaemia</td>
<td>771</td>
<td>5.70 (3.37, 9.64)</td>
<td>0.000</td>
<td>1.39 (0.68, 2.84)</td>
</tr>
</tbody>
</table>

OR and β coefficients adjusted for the confounding variables age (months), gender, study site and, inflammation.

sTfR: soluble Transferrin receptor; CI: confidence intervals; OR: odds ratio.

Iron deficiency defined as plasma ferritin levels of less than 12µg/L and less than 30µg/l in presence of inflammation.
Iron deficiency anemia defined as haemoglobin <11g/L in children.
The prevalence of iron deficiency was 36.0% (477/1325). Interestingly, the age distribution of vitamin D status versus that of iron status in children showed an inverse trend; Vitamin D insufficiency increased with age (Figure 8) whereas iron deficiency decreased with age (Figure 9).

Figure 8: Distribution of children’s Vitamin D status by age (months)
4.4 Vitamin D status and the risk of malaria infection

a) Association of asymptomatic parasitaemia and vitamin D status

Asymptomatic parasitaemia was defined as presence of any parasitaemia in blood without causing any symptoms. About 21.9% (262/1191) of the children had malaria parasitaemia. Asymptomatic parasitaemia at the time of vitamin D measurement was determined using regression model with vitamin D as continuous variable and in a logistic regression model as a categorical variable. The outcome variable was with or without asymptomatic parasitaemia. Low vitamin D levels (25OHD <75nmol/l) increased the risk of malaria incidence OR 2.52; 95% CI, 1.47, 4.33, P=0.001, but when adjusted for age, gender and study setting, the effect was lost OR 1.31; 95% CI, 0.73, 2.37, P=0.362. Table 10 shows the estimates of association of vitamin D and malaria at the time of vitamin D measurement.
Table 10: Association of vitamin D status of study children and malaria infection

<table>
<thead>
<tr>
<th>Vitamin D (nmol/L)</th>
<th>N</th>
<th>Unadjusted Estimate (coefficient) (95% CI)</th>
<th>P value</th>
<th>Adjusted Estimate&lt;sup&gt;a&lt;/sup&gt; (coefficient) (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Vitamin D (continuous)</td>
<td>1126</td>
<td>β -0.09 (-0.14, -0.04)</td>
<td>0.000</td>
<td>β -0.02 (-0.07, 0.03)</td>
<td>0.427</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td></td>
<td>OR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Vitamin D status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal vitamin D (≥75)</td>
<td>551</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Low vitamin D (&lt;75)</td>
<td>462</td>
<td>2.52 (1.47, 4.33)</td>
<td>0.001</td>
<td>1.31 (0.73, 2.37)</td>
<td>0.362</td>
</tr>
<tr>
<td>Vitamin D categories</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D sufficient (≥75L)</td>
<td>551</td>
<td>Reference</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Vitamin D insufficient (&lt;75≤50)</td>
<td>389</td>
<td>2.41 (1.38,4.23)</td>
<td>0.002</td>
<td>1.34 (0.73,2.46)</td>
<td>0.345</td>
</tr>
<tr>
<td>Vitamin D deficient (&lt;50)</td>
<td>73</td>
<td>3.11 (1.32, 7.30)</td>
<td>0.009</td>
<td>1.21 (0.48, 3.02)</td>
<td>0.688</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimate (coefficient and odds ratio), adjusted for age (months), gender and study setting. OR, odds ratio; CI, confidence interval
b) Vitamin D status and the occurrence of future malaria episodes

A Poisson regression model was used to investigate the effect of vitamin D on future malaria episodes. To help assess whether the Poisson model fit the data, a goodness-of-fit chi-squared test was run. The Poisson model was found to fit reasonably well as the goodness-of-fit chi-squared test was not statistically significant. The Poisson regression results were presented as incidence rate ratios (IRR). There was a significant interaction between vitamin D status and future malaria episodes in unadjusted models. However, there was no significant association between vitamin D status and prospective malaria episodes in adjusted models, and age was a significant confounder (Table 11).

Table 11: Susceptibility to malaria infection by vitamin D status of study children

<table>
<thead>
<tr>
<th>Vitamin D (nmol/L)</th>
<th>Unadjusted IRR (95% CI)</th>
<th>P value</th>
<th>Adjusted IRR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Vitamin D (continuous)</td>
<td>0.51 (0.39, 0.66)</td>
<td>0.000</td>
<td>0.87 (0.65, 1.17)</td>
<td>0.375</td>
</tr>
</tbody>
</table>

Vitamin D status

<table>
<thead>
<tr>
<th>Vitamin D status</th>
<th>Reference</th>
<th>P value</th>
<th>Reference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal vitamin D status (≥75)</td>
<td>Reference</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low vitamin D status (&lt;75)</td>
<td>1.50 (1.26, 1.78)</td>
<td>0.000</td>
<td>1.06 (0.87, 1.28)</td>
<td>0.572</td>
</tr>
</tbody>
</table>

Vitamin D categories

<table>
<thead>
<tr>
<th>Vitamin D sufficiency (≥75)</th>
<th>Reference</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D insufficiency (&lt;75≤50)</td>
<td>1.51 (1.26, 1.81)</td>
<td>0.000</td>
</tr>
<tr>
<td>Vitamin D deficiency (&lt;50)</td>
<td>1.42 (1.01, 2.00)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

NOTE: The analysis represents 1191 children with vitamin D measurement during 6 months of follow-up. Incidence rate ratios (IRRs) were calculated by Poisson regression analysis that included malaria episodes as the dependent variable and the explanatory variable vitamin D status, age (months), gender and study setting.

* Adjusted for age (months), gender and study setting.
CHAPTER 5: DISCUSSION

5.1 Vitamin D status of children in Kilifi

This study established prevalence of vitamin D deficiency (6%; 25OHD <50nmol/L) and of vitamin D of insufficiency (34%; prevalence 25OHD <75nmol/L) among otherwise healthy children in Kilifi County. The study site is located at 3ºS latitude along Indian Ocean the Coast of Kenya; where sunlight is strong. The study participants were from rural communities where environmental air pollution was not evident. Considering these conditions, the hypothesis that vitamin D deficiency is almost non-existent in the coastal region was nullified. Despite this, comparison with data from other African studies shows significant variation. For instance, a study in South African children found a 36% prevalence (Poopedi et al., 2011) while another in Ethiopian school going children found a 42% prevalence of vitamin D deficiency (Wakayo et al., 2015), compared to 6% in this study population. The reason for these disparities across the continent could be because Africa is not homogenous in geography, climate, cultural practices, skin pigmentation and disease burden, and these differences could have led to the variability in vitamin D status. Additionally, these results show higher vitamin D levels in African children in Kilifi compared to other continents such as America, where one study found an 18% prevalence of vitamin D deficiency in children (Mansbach et al., 2009) while another in Asian children showed a prevalence of 90% deficiency in children one to six months of age (Choi et al., 2013). Both America and South Korea are in higher latitudes and do not receive ample sunlight throughout the year for optimal vitamin D synthesis.

This study showed that low vitamin D status decreases with age. This is in agreement with a study by Mansbach and colleagues (2009) where, vitamin D levels among children 0 to 48 months decreased with increasing age. Infants less than six months had the highest mean levels of vitamin D. This may due to of exclusive breastfeeding practice where children are
exclusively breast fed from birth until six months, and up to two years with complementary feeding (World Health Organization, 2009). According to these recommendations, many of the health care providers and community health workers within the KHDSS advise caregivers on exclusive breastfeeding (Scott et al., 2012). Other studies (Mansbach et al., 2009; Zhu et al., 2012) attribute this trend to decreased intake of vitamin D fortified infant food, which is not comparable to this population in Kilifi. Age differences in vitamin D in these children could be explained by an increased need for vitamin D due to rapid skeletal growth (Holick, 2004). Additionally, it could also be due to transplacental transfer of vitamin D where children are born with high vitamin D stores. This could be depleted as the child grows or, due to the cultural practice of covering infants and keeping them indoors which denies them exposure to sunlight.

This study did not find a significant difference in vitamin D levels by gender. Similar results were found for Chinese children where no significant difference was found between the vitamin D levels of boys and girls at any stage (Zhu et al., 2012). This could be because children in this study population, 3 months to 30 months at the time of vitamin D measurement, had not reached puberty where girls tend to have a higher fat storage than boys, and hence lower vitamin D levels in circulation (Poopedi et al., 2011). Vitamin D is a fat soluble vitamin which has stores in the adipose tissue (Martinaityte et al., 2017)

This study found no correlation between serum vitamin D levels and inflammation in all age groups. While some observational studies have shown an inverse association between vitamin D and inflammation in newborns (Tao et al., 2015), others have shown no relationship in women (Azizieh et al., 2016) and in elderly subjects (Shea et al., 2008). Prior randomized control studies on participants with autoimmune and chronic diseases with baseline CRP of <5nmol/L showed increased CRP with vitamin D supplementation (Asemi et al., 2013). It appears that the beneficial effects of high vitamin D levels in reduction of CRP and other
inflammatory cytokines are found in individuals diagnosed with autoimmune and chronic diseases as in the study by (Asemi et al., 2013).

There was no statistically significant difference in geometric means of vitamin D in children according to their nutritional status. However, malnourished children had a lower vitamin D geometric mean as compared to those who were not malnourished. Previous studies have shown an association between nutritional status and low vitamin D levels. In hospitalized Ugandan children, 6 to 24 months of age subjects with severe acute malnutrition and moderate acute malnutrition had significantly lower vitamin D levels compared to those who were non-malnourished (Nabeta and Kasolo, 2017). Also, very few children in this cohort must have had severe malnutrition. Malnourished children are more likely to have accompanying infections, poor absorption and utilization of food, which may explain the differences in vitamin D levels. Additionally, malnourished children may feel weak, and may spend fewer hours in the sun as compared to healthy children, resulting in a low vitamin D status. The assumption in this study was that the children were healthy community subjects who were exposed to the sun daily, whereas those from the Ugandan study were hospitalized and sickly and hence did not get sufficient sunshine for vitamin D synthesis.

5.2 The association between vitamin D status and iron status

In this study, no significant association of iron deficiency and vitamin D status was observed. Moreover, haemoglobin, ferritin index and transferrin saturation were not significantly associated with vitamin D status. Study participants with low vitamin D had lower soluble transferrin receptor (sTfR) compared to those with normal vitamin D status. Soluble transferrin receptor is used as a measure of iron status especially during inflammation, when ferritin, which is a routine investigation for iron deficiency is confounded by inflammation.
Contrary to these results, previous cross-sectional studies have shown an association between low vitamin D and iron deficiency (Lee et al., 2009; Sim et al., 2010). Some studies show that supplementation with vitamin D does not increase ferritin, haemoglobin and iron.

Hepcidin concentrations were lower in children with higher vitamin D levels in an unadjusted model though this was not significant when adjusted for age, study site and gender. Elevated levels of vitamin D resulted in decreased hepcidin levels, an observation agreeing with a study in pregnant adolescent girls adolescents (Thomas et al., 2015). Previous studies also demonstrate that supplementing with vitamin D decreases hepcidin concentrations in adults (Bacchetta et al., 2014; Smith et al., 2016). Studies in mice show that vitamin D is capable of suppressing the expression of the *HAMP* gene which codes for hepcidin (Bacchetta et al., 2014). This association was however not significant in this study population and may have been confounded by factors not measured in our study. The study population was highly inflamed (27% prevalence) and this could have highly influenced hepcidin concentrations. The precise mechanism through which vitamin D influences iron status is not well understood.

There are few plausible reasons for the negative findings in this study. One possibility is that the study participants were inflamed, underestimating iron deficiency anaemia. Iron deficiency anaemia is defined using the iron marker ferritin, an acute-phase protein that is elevated during inflammation. As this study population has a 27% prevalence of inflammation, this could have resulted in non-identification of iron deficient children. A second reason could be because of age-specific differences in iron deficiency. Iron deficiency in our study population was highest in infancy 0 to 6 months, and decreased gradually with age, making age a strong confounder. This finding may be explained by the age variability in hepcidin concentrations which has been reported in malaria endemic regions (Atkinson et al., 2015). Other potential confounders influencing the relationship between vitamin D and iron
markers, which were not considered in this study, include dietary calcium, fat intake, body mass index, folate deficiency, vitamin B12 deficiency, sickle cell trait, iron metabolism disorders and medication (Holick, 2003).

5.3 Vitamin D status alters the risk of malaria infection

In this study, vitamin D status was not associated with malaria parasitaemia at the time of vitamin D measurement in a cross-sectional model. Moreover, vitamin D status had no influence on susceptibility to malaria infection. Findings from previous studies are contradictory. A previous prospective study of adults receiving antiretroviral therapy in Tanzania found no association between vitamin D deficiency and malaria incidence (Sudfeld et al., 2013), and another in western Kenya, a malaria endemic area, found no significant difference in vitamin D levels and malaria during pregnancy, delivery, or in infant cord blood (Toko et al., 2016). Contrary to these findings, a study in Ugandan children found low vitamin D levels in children with severe malaria compared to community cohorts, and the odds of having severe malaria decreased with increase in plasma vitamin D levels (Cusick et al., 2014). However, one observational study among HIV-exposed uninfected infants at 6 weeks of age with 25OHD ≥50nmol/L were found to have an increased risk of clinical and confirmed malaria (Sudfeld et al., 2015).

Despite these findings, there are multiple direct immune mechanisms through which vitamin D deficiency may impair malaria immunity and increase the risk of asymptomatic and symptomatic malaria infection. Malaria infection is characterised by an overwhelming inflammatory response that leads to clinical symptoms typical of a systemic infectious disease. Patients with malaria experience tiredness, aching joints and muscles, fever, and anorexia, which are characterised by release of inflammatory cytokines (Mecheri, 2012). Animal studies have established that treatment with vitamin D reduces circulating cytokines
interferon gamma (IFN-γ) and tumour necrosis factor (TNF) hence decreasing time of malaria progression and minimising susceptibility (He et al., 2014). Moreover, in one study, mice infected with Plasmodium treated with the antimalarial drug arteether¹, and vitamin D combined showed improvement in disease pathogenesis compared to mice treated with vitamin D or arteether alone (Dwivedi et al., 2016). Interestingly, the chemokines CXCL9 and CXCL10, which play a role in the pathogenesis of cerebral malaria by attracting T-cells into the central nervous system (Campanella et al., 2008), were found to be suppressed in vitamin D treated mice (Dwivedi et al., 2016).

In addition to suppressing pro-inflammatory cytokines, vitamin D metabolites have been found to stimulate production of antimicrobial peptides, cathelicidin and defensin, which play a role in innate immunity (Bacchetta, et al., 2014). These peptides are elevated during and after malaria infection and are thought to play a multifunctional role in providing a first line immunity against malaria parasites (Parra et al., 2013). 1,25(OH)D₃ is a direct inducer of the antimicrobial peptide gene, hCAMP - cathelicidin and defensin B2 - defensin. Promoters of these genes contain vitamin D response elements which mediate 1,25(OH)D₃ dependent gene expression (Wang et al., 2017). This could mean that an elevated level of vitamin D could potentially stimulate immune mechanisms that cause decrease malaria infection.

¹ Arteether is an antimalarial drug used to treat malaria.
5.4 Strengths and Limitations of the Study

The main strength of this study is the large sample size (n=1487) which gave a wide representation of the population under research. Secondly, the research involved a relatively homogenous cohort of children 6 to 48 months which helped reduce confounding.

However, there are general limitations in this study. First, it is an observational study and does not prove causality. Secondly, information on iron, vitamin B12, folate and sun exposure was not collected. These are important determinants of vitamin D synthesis that influence vitamin D status, therefore they could confound these results.

5.5 Conclusion

This study led to a conclusion that; the prevalence of vitamin D insufficiency in children in Kilifi County is high, that vitamin D deficiency and insufficiency are not associated with iron status nor is vitamin D status associated with future malaria episodes. Hence, vitamin D and does not alter the risk of malaria infection among children in Kilifi County.
5.6 Recommendations

1. Vitamin D insufficiency and deficiency are a public health concern among children in Kilifi County and these children should be targeted for supplementation programmes. Additionally, there should be increased sensitization by community health workers among caregivers on the health benefits of exposing children to sunshine as a source of vitamin D.

2. Similar studies should be carried out in other counties in Kenya to determine the vitamin D status of children and other age groups. There is scarcity of data on this area.

3. Randomised control trials investigating the effect of vitamin D supplementation on iron status of children should be carried out to further explore the effect of improving the vitamin D status on iron deficiency and iron deficiency anaemia.

4. Vitamin D level was not associated with future malaria episodes in this study. However, malaria data may have limited the ability to detect a significance level. Further studies are needed to assess the relationship between vitamin D levels and malaria immune response proteins to assess the molecular relationship of vitamin D and malaria.
REFERENCES


APPENDIX 1: Map of Kenya showing Kilifi County and Kilifi Demographic and Health Survey (KHDSS)
APPENDIX 2: KEMRI/SERU Ethical Approval