CORRELATION OF VITAMIN D LEVELS AND IMMUNE STATUS IN HIV POSITIVE CHILDREN AGED 3 TO 14 YEARS ATTENDING JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL IN KISUMU COUNTY, KENYA

BY

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DECLARATION

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

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I dedicate this thesis to my family members for their cooperation and support during the entire period.

ABSTRACT

Vitamin D deficiency is a worldwide phenomenon and it's more prevalent in Human Immunodeficiency Virus (HIV) positive individuals. This prevalence could be higher in children from sub-Saharan Africa where the HIV burden is highest. In Kenya the occurrence of Vitamin D deficiency has been reported among children, diabetics and cancer patients. Children are the worst affected by HIV because the disease progresses faster due to their naïve immune systems. Alongside other factors this is compounded by deficiency diseases, most notably Vitamin D(25OHD) which acts as an immunomodulator of the adaptive immune system where it directly affects T cell activation and antigen presenting cells. Vitamin D deficiency has been associated with poor clinical outcome among adults living with HIV. However, it is not clearly known how this would play out in children. This was a comparative cross-sectional studywhere98HIV positive children aged 3 to 14 years attending the Jaramogi Oginga Teaching and Referral Hospital and unmatched98HIV negative children in the same age visiting the outpatient clinic at the same hospital were consecutively sampled and differences in the Vitamin D levels determined using blood samples. Correlation was then done between Vitamin D,CD4 and viral load for the HIV positive group. Vitamin D was determined using the ELISA technique, while CD4 levels was determined using 3-colour flow cytometry. HIV viral load levels were determined by real-time PCR. Clinical history was collected from the participants medical records. Independent samples T-test was used to compare Vitamin D means while Pearson correlation was used to correlate Vitamin D, CD4 and viral load. The HIV uninfected group had mean Vitamin D levels of 30.88 ng/ml (30.88±6.62 ng/ml) with deficiency (<20 ng/ml) and insufficiency (21-29 ng/ml) rates at 5.1% and 37.8% respectively. The HIV infected group had mean Vitamin D levels of 28.21 ng/ml (28.21±6.39 ng/ml) with deficiency and insufficiency rates at 13.3% and 46.9% respectively. There was a significant difference between the mean Vitamin D levels of the two groups(p=0.004). There was no correlation between Vitamin D and CD4count (r=.166, N=98, p=0.101), and Vitamin D and viral load (r=-.115, N=98, p=0.776). In conclusion prevalence of Vitamin D deficiency and insufficiency is higher in HIV infected children than in uninfected children and there is no correlation between Vitamin D status and immune status in HIV infected children. These findings suggest the assessment of Vitamin Din children as the adverse health effects extend beyond bone health. Assessment of Vitamin D in this demographic could help improve the overall health status of children especially the immunosuppressed.

TABLE OF CONTENTS

LIST OFABBREVIATIONSAND ACRONYMS

25(OH)D	25 Hydroxy Vitamin D
AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
ART	Anti retroviral therapy
ART	Antiretroviral Therapy
CCR2+	Chemokine receptor type 2
CCR5	Chemokine Receptor 5
CD3	Cluster of differentiation 3
CD38	Cluster of differentiation 38
CD4+	Cluster of Differentiation 4
CD45	Cluster of differentiation 45
CD8	Cluster of differentiation 8
CDC	Center for Disease Control and Prevention
CX3CR1	Receptor for fractalkine ligand
CXCR4	Chemokine receptor type 4
CYP450	Cytochrome p450
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra Acetate
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FGF23	Fibroblast Growth Factor 23
FITC	Fluorescein isothiocyanate
Gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HLA DR	Human leukocyte antigen DR
IL-6	Interleukin 6
JOOTRH	Jaramogi Oginga Odinga Teaching and Referral Hospital
NACOSTI	National commission for science technology and innovation
NHANES	National health and nutrition examination survey

NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institute of Health
NNRTI	Non nucleoside reverse transcriptase inhibitor
PCR	Polymerase chain reaction
PE	R-phycoerythrin
perCP	Peridinin-chlorophyll-protein
PI	Protease inhibitors
PSC	Patientsupport center
PTH	Parathyroid Hormone
RNA	Ribonucleic Acid
SD	Standard deviation
SGS	School of graduate studies
SPR	Solid phase receptacle
SPSS	Statistical Packages for Social Sciences
T_H1	T Helper Subtype 1
T_H17	T Helper Subtype 17
T_H2	T Helper Subtype 2
TNFa	Tissue necrosis factor alpha
Treg	Regulatory T cell
UNAIDS	The Joint United Nations Programme on HIV and AIDS
UV	Ultraviolet B Radiation
VDR	Vitamin D Receptor
WHO	World Health Organization

DEFINITION OF TERMS

Vitamin D deficiency-this is the physiological state where the measured levels of Vitamin D are less than 20ng/ml

Vitamin D insufficiency-this is the physiological state where the measured levels of Vitamin D are between 21 and 29 ng/ml

Vitamin D sufficiency- the physiological state where Vitamin D levels are above 30 ng/ml

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Human immunodeficiency virus(HIV) defines a chronic infection in the host, characterized by progressive deterioration of the immune system through attacking the CD4+ cells(Ismail, 2015a). Globally, 37.9 million people were living with HIV at the end of the year 2018 including 1.7 million children aged 0 to 14 years. Of these 20.7 million were from eastern and southern Africa(Communications and Global Advocacy UNAIDS, 2020). Globally new infections for the 0 to 14-year age bracket have been on a steady decline from around 320,000 in the year 2010 to around 150,000 in the year 2020, mainly due to marked success in implementation of prevention of mother-to-child transmission programmes(Communications and Global Advocacy UNAIDS, 2020)In Kenya, out of the 1.5 million people living with HIV in 2017, 105213 (6%) were children aged 0-14 years(National AIDS Control Council (NACC), 2018). The county of Kisumu where the study was conducted is classified as hyper endemic for HIV, with prevalence at 16.3% against the national average of 4.8%. There were 9439 children below 15 years living with HIV, with 616 new infections and 369 AIDS related deaths as at 2018(National AIDS Control Council (NACC), 2018).

Even though the natural course of HIV is depicted by the gradual damage to the immune system from infection to clinical latency and manifestation of AIDS symptoms, (Bartlett, 2010) there are differences in disease progression in children and adults (Mothi et al., 2011). The lower efficacy of a child's immature but developing immune system results in a much more rapid disease progression of the HIV infection and a much shorter duration of each clinical stage (Centre for Disease Control and Prevention, 2015). Moreover ,the persistent chronic inflammatory state characteristic of HIV infection is more profound in children due to increased life expectancy

brought about by antiretroviral therapy (ART) drugs use, and leads to more immune dysfunction and exhaustion (Eckard et al., 2018). The 3 to 14 year age group was chosen as this is within the WHO categorization of children in relation to HIV diagnosis and management(World Health Organization, 2006) and overall health. Those below 3 years were excluded as CD4 count is not evaluated in their clinical management. The study site was selected to be JOOTRH hospital in Kisumu County as it's the largest referral facility in the region and therefore serves a wider catchment. Records verified at the study site showed that 4,396 subjects of the stated age group visited the patient support Centre in 2017 for their care (unpublished data)

Apart from advances made in anti-retroviral therapy(ART), there has been increased attention on other adjunctive interventions that slow down the infection and improve overall patient prognosis(Di Carlo et al., 2015). Key among the interventions is adequate nutrition since all cells including those of the immune system require the appropriate and adequate type of nutrition to function optimally (Solanki&Durga, 2020). Since macronutrients and micronutrients interact with the immune cells systemically in blood and lymphoid tissues, inadequate intake and lowered nutritional status of vitamins and trace elements may lead to suppressed immunity, aggravation of malnutrition and susceptibility to infections (Farhan Aslam et al., 2017), a phenomenon that is worsened in children with HIV infection(Fabusoro & Mejia, 2021).Among other vitamins, Vitamin D has been shown to exhibit extra skeletal effects with the Vitamin D receptor exhibiting pleiotropic effects on many tissues(Vojinovic & Cimaz, 2015).

Study findings indicate that Vitamin D deficiency is a worldwide phenomenon and that it's more prevalent in HIV positive subjects(Mansueto et al., 2015). Of interest is that Vitamin D is common among HIV infected youth but that HIV factors and immune restoration do not have the same relationship with Vitamin D as in adults (Ross Eckard et al., 2012) likely due to the still

developing immune systems of children and youth. Moreover, vitamin D studies done in HIV positive children are mostly in the context of skeletal diseases(Jones et al., 2018).Vitamin D concentration is affected by genetics, nutritional status, and factors that affect ultraviolet (UV) light exposure and absorption, such as season, latitude, time of day and skin pigmentation (Holick et al., 2011) which are entirely uniquely varied among different geographic regions of Africa and Kenya in particular. The few studies done in Africa on Vitamin D prevalence in healthy adult populations reveal that there is high prevalence of Vitamin D deficiency despite the prolonged sunny conditions(Mogire et al., 2020). Children are the worst affected by HIV due to their immature immune systems and thus these conclusions cannot be directly applied to children (Mothi et al., 2011).However, there are very few studies on Vitamin D status from the varied geographical, regions of Kenya. Of concern is the fact that Vitamin D status has not been determined for healthy or HIV positive populations of any age in Kisumu county, a region considered hyper endemic for HIV and with the highest number of infected children in Kenya (National AIDS Control Council (NACC), 2018). Therefore, this study examined the relationship between vitamin D levels and immune statusin 3-14 years old children.

The sunlight's ultraviolet-B (UV-B)induced vitamin D synthesis from 7-dehydrocholesterol present in the skin is its main source in the body, while dietary sources such as egg yolks, liver, red meat, and fish play a minor role (Farhan Aslam et al., 2017). Vitamin D from both the skin and diet is metabolized in the liver to calcidiol or 25- hydroxyl vitamin D (25[OH]D), which is the most important determinant of a patient's Vitamin D status. Calcidiol is then metabolized in the kidneys to calcitriol or 1, 25-dihydroxyvitamin D ($1,25(OH)_2D_3$) which is the biologically active form of vitamin D that binds stereo- specifically to the vitamin D receptors with high affinity(Wei & Christakos, 2015). The potential role of Vitamin D in many chronic illnesses and

especially its anti-microbial, anti-inflammatory, and immune modulatory roles has led to increased research on its role in HIV infection(Vojinovic & Cimaz, 2015).

All immune cells including activated $CD4^+$ T cells, B cells, neutrophils and antigen presenting cells have the Vitamin D receptor(Stagi et al., 2014).In its sufficiency Vitamin D enhances innate immune function of monocytes and macrophages through up-regulation of anti-microbial peptides production, primarily cathelicidin which has been demonstrated to inhibit HIV-replication(Wei & Christakos, 2015)In addition, vitamin D influences antigen presentation and regulates adaptive immune responses by suppressing $CD4^+$T-cells excessive proliferation and counteracting excessive immune response to infections. It promotes anti-inflammatory response by inhibiting dendritic cells(DCs) maturation, which leads to down regulation in their expression of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86), in turn decreasing their ability for antigen presentation and T cell activation(Barragan et al., 2015). Furthermore, vitamin D down-regulates IL-12 and increases IL-10 production by DCs(Wei & Christakos, 2015)It also actsdirectly on T cells to suppress their proliferation through inhibition of IL-2 production, promotion of naïve T cells differentiation into T_H2 subtypes, inhibition of inflammatory T_H1 and T_H17 subtypes development (Ismail, 2015b) and facilitation of T regulatory (Tregs) cells induction(Fisher et al., 2019). These effects results in increased production of anti-inflammatory cytokines such as IL-4 and IL-10, whereas production of pro-inflammatory cytokines such as IFN-γ, IL-2 and IL-17 is decreased(Wei & Christakos, 2015).It can thus be said that Vitamin D ultimately affects or delays disease progression through reducing $CD4^+$ activation and differentiation, and influencing cytokine expression.

Human immunodeficiency virus infects and destroys CD4+ T cells. In addition to CD4 receptor, attachment and fusion with the target cell also requires a co-receptor, either the cysteine-cysteine chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4)(Grande et al., 2019) Activated CD4+CCR5+ T cells are the primary target and main subset for HIV replication(Zaunders et al., 2017).Vitamin D has been demonstrated to induce antiviral gene expression, reduce the viral co-receptor CCR5 on $CD4^+$ T-cells, and promote an HIV-1-restrictive CD38+HLA-DR+ CD8+ T cell immunophenotype in *in vitro* assays, leading to HIV-1 infection inhibition in T cells (Jiménez-Sousa et al., 2018). $CD4^+$ count reflects the degree of $CD4^+$ cell destruction by HIV and also the slowed proliferation of these cells (Bartlett, 2010). It provides information on the overall immune function status of HIV infected individual and is the most significant independent marker of severity and a predictor of HIV mortality(WHO, 2017).However, the relationship between $CD4^+$ count and Vitamin D is unclear with available data giving varying results from studies done on adults. There is a dearth of information on this relationship in children in the region and in Kisumu County

An HIV Viral load is the measurement of the amount of virus in a blood sample, reported as number of HIV RNA copies per milliliter of blood(WHO, 2017).It is used as a marker of response to ART and provides prognostic information of the possibility of disease progression (WHO, 2017).Vitamin D reduces the ability of TNFα to upregulate the transcription of HIV RNA from latently infected $CD4^+$ cells. Thus, low levels of Vitamin D are related to high HIV viral load in plasma, decreased CD4+ T-cells in peripheral blood, rapid AIDS progression, and shortened survival(Yancheva et al., 2015). These effects have not been confirmed in children who normally have high CD4 count, but with an immune system that is still developing(D. E. Yin et al., 2014).Moreover, HIV Studies in adults have varying results with a no clear-cut

relationship between Vitamin D and viral load. One study on treatment naïve HIV positive adult patients found that higher viral loads may seem to induce lower levels of Vitamin D though the underlying mechanisms are unclear(Jiménez-Sousa et al., 2018). However, the different viral dynamics between treated and untreated children and in adults, calls for examination as the link between viral load and Vitamin D in children is not known.

1.2 Statement of the Problem

Human immunodeficiency virus infection continues to be a major public health problem. Globally, 37.9 million people were living with HIV at the end of the year 2018 including 1.7 million children aged 0 to 14 years. The lower efficacy of a child's immature but developing immune system results in a much more rapid disease progression of the HIV infection Therefore, in addition to advances made with ART, there is need for adjunctive interventions, including adequate nutrition, that can help slow down the infection and improve overall patient prognosis. Vitamin Dlevels has been shown to delays disease progression through reduction of $CD4^+$ activation and differentiation, as well as and influence on cytokine expression. However, Vitamin D deficiency is one of the most common comorbidities in HIV patients, worldwide. Moreover, vitamin D status has not been determined for healthy or HIV positive populations of any age in Kisumu County, a region considered hyper endemic for HIV and with the highest number of infected children in Kenya.

Human immunodeficiency virus infects and destroys CD4+ T cells. CD4 T cell count provides information on the overall immune function status of HIV infected individual while HIV viral is used as a marker of response to ART and provides prognostic information of disease progression. Vitamin D has been demonstrated to induce antiviral gene expression, reduce the viral co-receptor CCR5 on $CD4^+$ T-cells, and ability of TNFα to upregulate the transcription of

HIV RNA from latently infected $CD4^+$ cells and promote an HIV-1-restrictive CD38+HLA-DR+ CD8+ T cell immunophenotype in *in vitro* assays, leading to HIV-1 infection inhibition in T cells. However, the relationship between $CD4^+$ count and viral load with vitamin D status in children is unclear, with available data giving varying results from studies done on adults.

Therefore, this study aimed to examine the correlation between vitamin D levels and immune status in 3-14 years old children.

1.3 Objectives

1.3.1 General objective

To correlate Vitamin D levels and immune status in children aged 3 to 14 years attending JOOTRH in Kisumu County, Kenya.

1.3.2 Specific Objectives

1. To determine differences in Vitamin D levels in HIV positive and HIV negative children aged 3 to 14 years.
2. To correlate Vitamin D levels with $CD4^+$ count and viral load in HIV positive children aged 3 to 14 years.

1.4 Research hypotheses

1. There is no difference in Vitamin D levels between HIV positive and HIV negative children aged 3 to 14 years.
2. There is no correlation between Vitamin D levels and $CD4^+$ and viral load in HIV positive children aged 3 to 14 years.

1.5 Significance of the Study

The results of this study align with the findings of other Studies that HIV positive individuals have lower levels of Vitamin D compared to HIV negative individuals. Therefore, assessment of Vitamin D in this demographic will inform the need for possible supplementation. Despite the lack of a correlation between Vitamin D and immune status of the study population, there is need for assessment of Vitamin Din HIV infected children as the detrimental effects of the deficiency extend beyond the much-studied skeletal health.

CHAPTER TWO
LITERATURE REVIEW

2.1 HIV and AIDS in Children

Globally 37.9 million people were living with HIV at the end of the year 2018 including 1.7 million children aged 0 to 14 years. Of these 20.7 million were from eastern and southern Africa. In children there were 74000 new infections in the same region(Communications and Global Advocacy UNAIDS, 2020). As a result of interventions by various organizations and bodies there has been a 66% decline in new HIV infections since the year 2010 in this region. Globally new infections for the 0 to 14-year age bracket have been on a steady decline from around 310,000 in the year 2010 to around 150,000 in the year 2018. Among this age group in Kenya there were 105213 children living with HIV, with 8000 new infections and 4312 deaths. (Kenya AIDS Progress Report, 2017). Treatment coverage was at 77%. The county of Kisumu where the study was conducted is classified as hyper endemic for HIV prevalence at 16.3% against the national average of 4.8%. There were 9439 children below 15 years living with HIV, with 616 new infections and 369 AIDS related deaths as at 2018. Mother to child transmission rate was at 20%. By the end of 2017 a total of 144,303 people were living with HIV in the county with 6% (8600) being children aged below 15 years. Within the same period there were 909 new infections in this age group(National AIDS Control Council (NACC), 2018).The study site was selected to be JOOTRH hospital in Kisumu County as it's the largest referral facility in the region and therefore serves a wider catchment. Records verified at the study site showed that 4,396 subjects of the stated age group visited the patient support Centre in 2017 for their care.

Human immunodeficiency virus refers to the virus that attacks immune cells and damages them. AIDS refers to the advanced stage of HIV infection characterized by a severely weakened immune system (WHO 2006). The primary receptor for HIV is the CD4+ molecule on T-helper

cells of humans. In addition to CD4 receptor, attachment and fusion with the target cell also requires a co-receptor, either the cysteine-cysteine chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) (Grande et al., 2019) Activated CD4+CCR5+ T cells are the primary target and main subset for HIV replication (Zaunders et al., 2017).Even though the natural course of HIV is depicted by the gradual damage to the immune system from infection to clinical latency and manifestation of AIDS symptoms, (Bartlett, 2010) there are critical differences in disease progression in children and adults(Mothi et al., 2011). This stems largely from the lower efficacy of a child's immature but developing immune system. This phenomenon results in a much more rapid disease progression and a much shorter duration of each clinical stage(Centre for Disease Control and Prevention, 2015). In perinatal acquired infection viral load is usually very low at birth but slowly rises in the first two months to over 100,000 copies/ml(Obimbo et al., 2009). This slowly declines over time up to the age of 4 to 5 years (D. E. Yin et al., 2014). The high viral load is associated with the somatic growth of the lymphatic system and the inability of the immune system to launch an HIV specific response (Krogstad et al., 2015). This explains why it is important to measure the plasma viral load together with $CD4^+$ counts as both are independent predictors of disease progression and mortality risk, which increases prognostic accuracy (Ndegwa L, Mathenge A, 2014).

Viral load (HIV RNA) levels indicate the amount or number of HIV viruses in the circulation. The higher the viral load the faster the disease progression towards AIDS (Giusti et al., 2011) due to increased $CD4^+$ depletion. $CD4^+$ count as a biomarker reflects the degree of $CD4^+$ cells destruction by HIV and also the slowed proliferation of these cells(Bartlett & Hirsch, 2010). Viral load is used as an adjunctive measure to $CD4^+$ to evaluate the degree of HIV suppression; both markers gauge disease severity and progression, and predict mortality and survival. In

addition, both $CD4^+$ count and HIV RNA levels are considered upon treatment initiation and are closely monitored to determine the effectiveness of HIV medical management using ART(WHO, 2017). Clinically, it is used to test the effectiveness of ART therapy in reducing the viral load to a non-detectable level. The child's $CD4^+$ T cell count should always be compared with the age-appropriate values (Boyd et al., 2010) as Lymphocyte counts are very high in infancy and decline to adult levels at around 5 years of age. Studies have shown a high mortality by 1 year and up to 89% by 3 years(Slogrove et al., 2020). With treatment and effective control of the viral load, the immune system of HIV infected children is capable of efficient CD4+ recovery through naive and memory T-cell population and expansion from the thymic reservoir (Hazenberg et al., 2004).However, the persistent chronic inflammatory state characteristic of HIV infection is more profound in children due to this increased life expectancy, and leads to more immune dysfunction and exhaustion, similar to adults(Eckard et al., 2018).The 3 to 14 year age group was chosen as this is within the WHO categorization of children in relation to HIV diagnosis and management(World Health Organization, 2006) and overall health. Younger children outside this age group were not used as CD4 count values are not measured and used for management and monitoring of the HIV infection for that age bracket.

2.2 Vitamin D and HIV

2.2.1 The Role of Vitamin D in Immunity

That nutrition plays a vital role in the immune system is without doubt and has been studied extensively. All cells including those of the immune system require the appropriate and adequate type of nutrition to function optimally(Solanki & Durga, 2020).Macronutrients (carbohydrates, proteins and fats) and micronutrients (trace elements, vitamins) interact with the immune cells systemically in blood and lymphoid tissues. Inadequate intake and lowered nutritional status of vitamins and trace elements such as iron, zinc, selenium and copper, and vitamins A, B6, B12,

folic acid, C, D and E may lead to suppressed immunity, aggravation of malnutrition and susceptibility to infections (Farhan Aslam et al., 2017).Of the vitamins that have been explored, Vitamin D has been shown to exhibit extra skeletal effects, through the Vitamin D receptor and exhibiting pleiotropic effects on many tissues(Vojinovic & Cimaz, 2015). The potential role of Vitamin D in many chronic illnesses and especially its role as an immunomodulator of the adaptive and innate immune system has led to increased research on its role in HIV infection.

The primary source of Vitamin D is irradiation of 7-dehydrocholesterol in the skin. Solar Ultraviolet-B(UVB) of wavelength 290 to 315 nm penetrates the skin and converts 7-dehydrocholesterol to pre Vitamin D, which forms vitamin D by spontaneous isomerization (Baeke et al., 2010)Dietary sources include the flesh of fatty fish and fish liver oils, beef, liver, egg yolks and cheese(Hossein-Nezhad & Holick, 2013). Vitamin D from both the skin and diet is metabolized in the liver tocalcidiol or 25(OH)D, which is the most important determinant of a patient's Vitamin D status as it correlates well with end organ effects. Calcidiol is metabolized in the kidneys to calcitriol or $1,25(OH)_2D_3$.While 25(OH)D reflects the total amount of Vitamin D available to the body, $1,25(OH)_2D_3$ is considered the biologically active compound accounting for its immune modulator effects on both the innate and the adaptive immune system(Wejse & Patsche, 2018).The mechanism of action of $1,25(OH)_2D_3$ is similar to that of other steroid hormones and is mediated by the Vitamin D receptor(VDR) which is found in over 35 body tissues that are not involved in bone metabolism(Jiménez-Sousa et al., 2018). $1,25(OH)_2D_3$ binds stereo-specifically to VDR with high affinity. Classic actions of $1,25(OH)_2D_3$ are its regulation of calcium and phosphate movement across the gut, bone and kidney (Hossein-Nezhad & Holick, 2013). Plasma levels of $1,25(OH)_2D_3$ demonstrate a positive association with intestinal

absorption and renal reabsorption of Calcium. In this way it helps build bones and helps keep them healthy(Battault et al., 2013). It also acts together with two peptide hormones parathyroid hormone(PTH) and fibroblast growth factor 23(FGF23). PTH is the major stimulator of $1,25(OH)_2D_3$ in the kidney which in turn suppresses PTH production by feedback mechanisms and increase of serum calcium levels(M. Yin, 2012)The end result is the down regulation of PTH directly and indirectly by $1,25(OH)_2D_3$. Studies have shown that PTH begins to plateau at Vitamin D levels of between 16 and 40 ng/ml(Kagotho et al., 2018).

Liu *et al* demonstrated that macrophages but not dendritic cells after in vitro activation showed increased expression of VDR gene and gene products and induction of the antimicrobial cathelicidin gene(Liu et al., 2006). This led to mycobacterial killing as a response to Vitamin D and $1,25(OH)_2D_3$. Microbial killing was more efficient with 25OHD than with $1,25(OH)_2D_3$ implying that the human innate response to microbial change is dependent on the 25OHD status of the host (Campbell & Spector, 2012). In vitro studies have also demonstrated that Vitamin D induces innate antimicrobial effector responses through induction of reactive oxygen intermediates and activation of antibacterial autophagy (Baeke et al., 2010).In vivo studies need to be done to shed more light on the role of Vitamin D in innate host defenses against infection. Vitamin D regulates the adaptive immune responses by intercepting $CD4^+$ T-cells excessive proliferation and counteracting excessive immune response to infections. It promotes anti-inflammatory response by inhibiting dendritic cells (DCs) maturation, which leads to down regulation in their expression of MHC class II and co-stimulatory molecules (CD40, CD80, and CD86), in turn decreasing their ability for antigen presentation and T cell activation(Barragan et al., 2015).Furthermore, vitamin D down-regulates IL-12 and increases IL-10 production by DCs (Wei & Christakos, 2015) It also acts directly on T cells to suppress their proliferation through

inhibition of IL-2 production, promotion of naïve T cells differentiation into T_H2 subtypes, inhibition of inflammatory T_H1 and T_H17 subtypes development and facilitation of T regulatory (Tregs) cells induction (Fisher et al., 2019). T_H1 and T_H17 promote HIV infection while Vitamin D diminishes the inflammation environment and stops tissue destruction through promoting of T_{reg}, a subtype of regulatory T-cells (Ismail, 2015a). These effects results in increased production of anti-inflammatory cytokines such as IL-4 and IL-10, whereas production of pro-inflammatory cytokines such as IFN-γ, IL-2 and IL-17 is decreased (Wei & Christakos, 2015).In its sufficiency Vitamin D enhances normal immune function through production of peptides to combat pathogens and control autophagy in $CD4^+$ cells, macrophages and other immune cells (Campbell & Spector, 2012). For instance, in a systematic review and meta-analysis of 11 randomized controlled trials, Vitamin D showed a protective effect against respiratory tract infections in a paediatric population. The protective effect was larger in studies using once-daily dosing compared with bolus doses (OR = 0.51 vs OR = 0.86, p = 0.01) (Mohd et al., 2014). It can thus be said that Vitamin D ultimately affects or delays disease progression through reducing $CD4^+$ activation and differentiation, and influencing cytokine expression. In insufficiency it is postulated that immune function is disrupted and slowed, coupled with increased $CD4^+$ destruction and disease progression (Allison C. Ross et al., 2011). With reduced $CD4^+$ reserves and decreased $CD4^+$ production in HIV, Vitamin D deficiency in this state causes further reduction in immune function, with lower $CD4^+$ counts and reduced response to intracellular pathogens such as *Mycobacterium tuberculosis*(Martineau et al., 2011).

2.2.2Vitamin D in HIV positive and HIV negative children

There is an increase in recognition of the role of Vitamin D in the progression and course of the HIV disease in adults. Though it has been estimated that over 1 billion people worldwide suffer from Vitamin D deficiency or insufficiency(Hossein-Nezhad & Holick, 2013), it is not alarming

to find high rates of Vitamin D deficiency in HIV infected individuals especially children. The overall estimated prevalence of Vitamin D deficiency among people living with HIV is in the range of 70.3% to 83.7 % (Mansueto et al., 2015). Gichuhi *et al* found a 39 % prevalence in a study of HIV positive adults in a Kenyan study(Gichuhi et al., 2014). Dao *et al* found that Vitamin D deficiency was not different between HIV positive and HIV negative subjects(Dao et al., 2011). In another study Ross et *al* found no difference in Vitamin D among HIV positive and HIV negative cohorts with 77% of HIV positive and 74% of HIV negative subjects having Vitamin D of less than 20 ng/ml(Allison C. Ross et al., 2011). Rutstein et al., found that Vitamin D deficiency and insufficiency (36% and 89%) was higher in HIV positive individuals than the uninfected (15% and 84%) in unmatched HIV uninfected children or young adults (mean age 13.8±4.1) years) from a pediatric clinic (Rutstein et al,2011).

In these studies, black skin type is a likely contributory factor for the low Vitamin D levels regardless of the geographical location as higher prevalence has been observed in African American population and are thus considered high risk for Vitamin D deficiency and insufficiency (Kwon et al., 2016). In uninfected children, a Ugandan study found that 80%(n=16) of children had Vitamin D insufficiency (<30 ng/ml)(Cusick et al., 2014). In Ethiopia 42% of studied participants (age 11-18 years) had deficient Vitamin D (Wakayo et al., 2015).There is no similar data available on HIV infected children from the two countries, limiting comparisons with the healthy group. A Johannesburg study reported insufficiency in 19% of 10-year-old children and deficiency in 7%, with black children having more deficiency(Poopedi et al., 2011). This is in agreement with multiracial studies that also found black subjects to have higher prevalence of Vitamin D deficiencies than nonblacks (Dao et al., 2011)(Taksler et al., 2015). However, seasonality is more pronounced in south Africa compared to the western Kenya region

where this study was done, which could have an effect on the findings that were obtained. There is scarcity of data on this comparison in the African context and specifically the Kenyan context, where Vitamin D studies have mainly focused on the non-immune aspectin children. This could be due to lack of screening in low-income settings characteristic of most African countries, Kenya included. The data from adults cannot be directly extrapolated to children bearing in mind the way in which in which the HIV infection progresses in children.

Several studies have examined the potential effects of low Vitamin D and immune function of HIV infected individuals. A deficiency has been shown to affect disease progression and mortality with patients with higher Vitamin D levels having decreased disease progression(Sudfeld et al., 2012). Low Vitamin D levels have been associated with cerebrovascular diseases, type 2 diabetes mellitus, bacterial vaginosis and oral candidiasis (Mara Pinto, 2014). Several mechanisms have been put forward to explain the association between Vitamin D deficiency and high severity of HIV disease. Vitamin D by itself maybe a contributory causal agent in HIV infection as Tissue necrosis factor alpha (TNF-α) overproduction in chronic HIV infection may impair 1-αhydroxylase production. This alterations in 25OHD metabolism during HIV-1 infection is associated with a rise in proinflammatory cytokines which block the effect of the parathyroid hormone (PTH) and the hydroxylation of calcidiol in the kidney, preventing the synthesis of calcitriol(Alvarez et al., 2019). Moreover, opportunistic infections often require hospitalization which reduces patient exposure to sunlight. Both opportunistic infections and hospitalization may lead to malnutrition and reduced oral intake of Vitamin D containing food (Mansueto et al., 2015).These factors may be true in the Kenyan context but is yet to be demonstrated as a majority of these patients are on ART which delays the onset of opportunistic infection and maintains the $CD4^+$ count within normal limits.

However, ART by itself has been cited as a contributor to the reduction of Vitamin D, as certain non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors(PIs) negatively affect the function of hydroxylase enzymes in the kidneys, inducing a decrease in calcitriol (Piloya et al., 2021). The effect of this confounder has not been explored in the current context as this would be an important factor in the Vitamin D levels of the HIV negative and HIV positive paediatric populations.

Of the studies done on healthy subjects in Africa, results show a higher baseline compared to other regions of the world but with a significant continental variation(Mogire et al., 2020). This could be attributed to climatic conditions in Africa as a result of overhead or near overhead sunlight with no pronounced seasons. Consequently, most people spend time outdoors increasing their exposure to UVB rays. On the other hand, studies performed in the United States on black people for instance indicate that they have the highest prevalence for Vitamin D deficiency. In one study consisting of 72 % black youth the prevalence of Vitamin D insufficiency was 87 % at an insufficiency cut off of 37.5 ng/ml (Stephensen et al., 2006). This cut off is however higher if compared to the insufficiency cut off of 21-29 ng/ml used by most studies and this study. The rate could therefore be lower. Another study found a 63 % rate of deficiency in women, with higher rates in African-American women(Adeyemi et al., 2011). In this study black race was a significant predictor of Vitamin D deficiency. This could be attributed to the negative correlation between Vitamin D and increasing distance from the equator(Durazo-Arvizu et al., 2014) especially for the black skin type. As can be seen, the number of studies from Africa on Vitamin D and HIV is scarce and the existing few have been done on adults, with conflicting results. Data on children from this region is conspicuously absent, with no data from Kenya, a country

that straddles the equator and has adequate sunshine for most of the year. Moreover, the data cannot be generalized to children, whether HIV infected or not.

2.2.3 Vitamin D levels, CD4count and viral load in HIV positive children

The relationship between Vitamin D levels, viral load and $CD4^+$ count is not clearly defined. Vitamin D has been shown to reduce the ability of TNFα to upregulate the transcription of HIV RNA from latently infected CD4+ cells (Nunnari et al., 2016). This therefore could explain why low levels of Vitamin D are related to high HIV viral load in plasma, decreasedCD4+ T-cells in peripheral blood, rapid AIDS progression, and lower survival in HIV- infected patients(Jiménez-Sousa et al., 2018). Some studies have described a positive correlation while others have found no correlation or association. In a study of 160 HIV positive youth severe Vitamin D of less than 10 ng/ml was related to lower $CD4^+$ count and $CD4^+$ % but not viral load. However, $CD4^+$count did not increase under Vitamin D supplementation especially in children with relatively preserved immunologic function (Kakalia et al., 2011). In Botswana however, Vitamin D supplementation improved the overall immunological outcome of children and adults with HIV(Steenhoff et al., 2015) , with age (3 to 13 years) being a significant factor in the outcome. Bang et al., found no correlation between Vitamin D, age, HIV infection or $CD4^+$ count (Bang et al., 2010). Bearden et al., found a nonlinear association between viral load and $1,25(OH)_2D_3$ but found no association between Vitamin D and $CD4^+$(Bearden et al., 2013). Havers et al., found that low Vitamin D was associated with lower viral load and also with increased risk of HIV disease progression and death (Havers et al., 2014) in their study low Vitamin D was identified as an independent risk factor for clinical and virologic failure. Another study found an association between increased Vitamin D levels and elevated viral loads. They found no correlation between Vitamin D and CD4 count/CD4 %(Abedi & Rosenthal, 2016). Eckard et al., on the other found significant correlations between low Vitamin D and longer duration of HIV

disease and use of ART(Eckard, Leong, et al., 2013).Their findings showed no correlation between Vitamin D and CD4 count. A Ugandan study found an association between severe Vitamin D deficiency and severe immunosuppression at ART initiation(Piloya et al., 2021) but failed to find an association between Vitamin D and viral load. It remains to be seen if these findings can be replicated as HIV patients are put on treatment immediately upon diagnosis, including children of all ages. The study was done in Kampala which has almost the same climatic features as Kisumu. These factors have been marginally investigated in the paediatric demographic in Africa or Kenya and it will be interesting to find out if the same factors from the other studies extrapolate to the current study context, where currently there is a dearth of information on the Vitamin D status of the paediatricdemographic for healthy as well as the HIV infected.

2.3.4 Vitamin D ranges

Even though there isno agreement among experts on the optimal levels of Vitamin D, the Institute of Medicine (IOM) has defined a serum 25-OH Vitamin D level of >20 ng/mL as sufficient, based on integrated measures of calcium absorption, bone mineral density, osteomalacia and rickets(A. Catharine Ross et al., 2011).the Endocrine Society on the other hand has defined >30 ng/mL as sufficient(Holick et al., 2011). The Pediatric Endocrine Society (PES) guidelines published in 2008 defined deficiency as level <15 ng/mL and > 20 ng/mL as optimal(Misra et al., 2008). The findings are derived from data in adults. Limited studies have evaluated the effect of Vitamin D on bone mass at doses that raise serum 25-OH Vitamin D concentrations above 30 ng/mL in pediatric patients. The results are so far inconsistent. Therefore, the higher Vitamin D cutoffs of 30 ng/mL have not been the official recommendation of the American Academy of Pediatrics, PES, or their European counterparts. However, the cut offs established by the endocrine society have generally been accepted worldwide as the cut offs

for all ages and sex (Holick et al., 2011). This cutoff was used for this study (appendix XI). Deficiency is defined as levels of less than 20ng/ml, insufficiency as values between 21 to 29 ng/ml and sufficiency as values equal to or more than 30 ng/ml, and toxicity as values above 150 ng/ml (Mohd et al., 2014). These cut offs have been adopted globally even though there is no data for the African or Kenyan populations or children for that matter.

CHAPTER THREE
METHODOLOGY

3.1 Study Setting

The study was conducted at the Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH), Kisumu County from November 2019 to March 2020. The hospital is located in the city of Kisumu which is located at the Equator on co-ordinates 0°6' S 34°45' E with an altitude of 1131 meters above sea level (appendix I).This region receives overhead or near overhead sunshine throughout the year. The hospital has a Patient Support Centre (PSC) for the HIV infected that serves the whole county and also acts as a referral point for other support centers in the western region. The hospitals catchment area of Kisumu County where the study was conducted is classified as hyper endemic for HIV prevalence at 16.3% against the national average of 4.8%. There were 9439 children below 15 years living with HIV, with 616 new infections and 369 AIDS related deaths as at 2017(National AIDS Control Council, 2018). Samples were obtained from children attending the HIV patient support Centre clinic on Thursdays and from HIV negative children visiting the outpatient clinic. JOOTRH is the largest referral hospital in the western region.

3.2 Study Population

The study population was HIV infected children visiting the PSC at the JOOTRH and HIV uninfected children visiting the outpatient clinic at the same site. According to records verified at the study site (unpublished data) 4,396 subjects of the stated age group visited the patient support Centre in 2017 for their care.

3.3 Study Design

This was a comparative cross-sectional study of unmatched children aged 3 to 14 years visiting the JOOTRH, where children who are HIV positive and those who are HIV negative were

compared on the basis of their Vitamin D levels. Vitamin Dmetabolism and its resultant effects is a process that is not affected by sex of the host (Mohd et al., 2014) though older age has been associated with low Vitamin D levels (Giusti et al., 2011)and therefore it was not necessary to age and sex match the study participants. The HIV positive children were further analyzed to correlate their immunological status ($CD4^+$ count and viral load) against their Vitamin D levels.CD4 count was not measured in HIV negative children as the levels of CD4 in this group are not directly affected by HIV but by other factors that are beyond the scope of this study.

3.4 Participants' Recruitment and Consent Process

Permission to conduct the study was sought and given by the JOOTRH administration (appendix VI). Participants were recruited from the children visiting the HIV patient support Centre and the outpatient clinic. A brief explanation about the study was given to the parent or guardian about the study and the consent process explained. For those who were willing to participate informed written consent was sought and filled by the parents or guardians accompanying them to the clinics (appendix II). younger children gave verbal assent while those older and who could write signed the assent forms. These were then recruited and blood samples taken from them.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Criteria

HIV positive and HIV negative children aged 3 to 14 years whose parents or guardians consented by signing the consent form, and those who assented.

3.5.2 Exclusion Criteria

All eligible children who did not assent and whose parents or guardians declined consent to the study. Children on supplements for Vitamin D, recent blood transfusions, chronic diseases were also excluded from the study. All children outside the stated age limit of <3 years and >14 years.

All participants who had been recruited, but whose blood samples were insufficient/difficult draw.

3.6 Sample Size Determination and Sampling Techniques

3.6.1 Sample Size Determination

Sample size was determined according to formula(Sharma et al., 2019) used for comparison between two groups when endpoint is quantitative data;

Sample required (n)= $(r+1)/r * \sigma^2 (Z_{1-\beta} + Z_{1-\alpha/2})^2/d^2$

Where:

n = Number of samples required

r = ratio of control to cases; 1 for equal number of case and control

$Z_{1-\beta}$= Standard normal variate for desired power; It is 0.84 for 80% power.

$Z_{1-\alpha/2}$= Critical value and a standard value for the corresponding level of confidence.

(At 95% CI it is 1.96)

σ = Standard deviation (SD) which is based on a previous published study; SD= 35ng/ml according to Eckard et al., 2013.

d = Effect size (Expected mean difference between case and control based on previous studies= 14ng/ml according to Eckard et al., 2013(Eckard, Tangpricha, et al., 2013).

$N = (r+1)/r * \sigma^2 (Z_{1-\beta} + Z_{1-\alpha/2})^2/d^2$

$= (1+1)/1 * 35^2 (0.84 + 1.96)^2/14^2$

$= (2) * 1225(2.8)^2/14^2$

$= (2) * 1225(7.8)/14^2 = 97.5$

= 98 for infected group and 98 for uninfected group

98HIVinfected children were selected for the study. 98unmatchedchildren were selected from the HIV uninfected pool.

3.6.2 Sampling Technique

Due to the small number of children (5 to 10) within the desired age group who visited the PSC clinic every Thursday of the week, Consecutive sampling was used on all eligible participants for

both groups until the required sample size was attained. Choice of this sampling method was also informed by resource constraints notably time and finances required to reach the sample size.

3.7 Laboratory Procedures

3.7.1 Blood Collection

venous blood(2 ml)was collected into serum separator vacutainers(Becton Dickinson, USA)tubes form the antecubital area of the arm or any other appropriate site. The serum tube was allowed to clot and centrifuged at 3000 revolutions per minute for 5 minutes to obtain serum. This was then stored at -20^0 Celsius awaiting analysis. A further 2ml each was collected into 2 separate EDTA tubes (Becton Dickinson, USA)for$CD4^+$enumeration and HIV viral load estimation respectively. The viral load tube was centrifuged at 1500g for 10 minutes to obtain plasma. This plasma was frozen at -20^0Celsius awaiting analysis. The tube for CD4 enumeration was stored at room temperature awaiting analysis.

3.7.2 Determination of Vitamin D

Vitamin Dwas determined using the Minividas immunoassay analyzer (Biomereaux, France) using the ELISA principle according to the manufacturer's instructions. Briefly, the frozen samples(sera)were thawed at room temperature and centrifuged if necessary, to clarify them. Vortexing was then done and100 microliters (uL) of samples pipetted into the VIT D reagent strips. Reagent strips and solid phase receptacles (SPRs)was loaded into the equipment and the assay initiated from the equipment screen. Results were expressed in nanograms per milliliter (ng/ml)

3.7.3 Determination of HIV Viral Load

The HIV-1 RNA in plasma was quantitated using COBAS AmpliPrep/COBAS TaqMan HIV-1 real time PCR test (Roche diagnostics, Germany) version 2. Briefly 850 microliters (µL) of

plasma to be tested was lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer to release nucleic acids and protect the released HIV-1 RNA from RNases in plasma. Protease and a known number of HIV-1 QS (quantitation standard construct containing fragments of HIV-1 sequences with primer binding regions identical to those of the HIV-1 gag target sequence,) Armored RNA molecules were then introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture was then incubated so that the HIV-1 RNA and HIV-1 QS RNA bind to the surface of the magnetic glass particles. Unbound substances were removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the adsorbed nucleic acids were eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA and HIV-1 QS RNA, was then added to the amplification mixture and transferred to the COBAS®TaqMan® Analyzer for reverse transcription, amplification and simultaneously detection by cleavage of two target-specific and one QS-specific dual-labeled oligonucleotide probe. To achieve this the reaction mixture was heated to allow the downstream primers (primers to the gag and the long terminal repeat[LTR] region of HIV-1) to anneal specifically to the HIV-1 target RNA and to the HIV-1 QS RNA. In the presence of Manganeseions (Mn^{2+}) and excess deoxynucleotide triphosphates (dNTPs), including deoxyadenosine, deoxyguanosine, deoxycytidine, deoxyuridine and deoxythymidine triphosphates, DNA polymerization occurred, extending the annealed primers and forming DNA strands complementary to the RNA target. Analyzer automatically repeated this process for a preprogrammed number of cycles, with each cycle doubling the amount of amplicon DNA. Amplification occurred only in the two regions of the HIV-1 genome between the upstream and downstream primers, so that the entire HIV-1 genome was not amplified. The

DNA amplicons were then quantitated by the analyzer and expressed as HIV RNA copies per milliliter (ml) of plasma.

3.7.4 Determination of $CD4^+$ Count

The $CD4^+$ cell count was done using fluorescence-activated cell sorter (FACS) system (Becton Dickinson, USA) that uses flow cytometry principle, using manufacturer's instructions. Briefly 20 uLof antibody containing 3colour panel MultiTEST CD3-FITC, CD45-PerCP and CD4-APC fluorochromes (Cat no.340491, Becton Dickinson, USA)was pipetted into labelled tubes to which 50 microliters of whole blood was added. This was vortexed briefly and then incubated in the dark for 15 minutes. FACS lysing solution of 450 uL (Becton Dickinson, USA) was added and further incubated in the dark for 15 minutes at room temperature. The samples were analyzed within two hours of setup. During analysis appropriate gating was applied to achieve the right cell populations. Control beads, low and normal were included in the run once a day to check for integrity of the system. The $CD4^+$ count was expressed in cells per millimeter^3 (cell/mm^3).

3.8 Data Management and Statistical Analysis

Age and sex were obtained from the patients' medical records at the facility. Data for each participant was entered into excel spreadsheets form denoting the participant's age, viral load, $CD4^+$ count, 25OHD levels and date sample was collected. The data was verified and checked for consistency and analyzed using IBM SPSS version 20.The Pearson correlation coefficient was used to examine correlation between 25OHD, CD4 and viral load of children infected with HIV. Differences in means for each Vitamin D category for both HIV positive and negative groups were compared using independent samples T-test, since the data was found to have a

normal distribution. All tests were two-tailed and a p value ≤ 0.05 considered statistically significant.

3.9 Ethical Considerations

Initial approval to conduct the study was provided by School of Graduate Studies (SGS) of Maseno University (appendix III). Ethical approval of the study protocol was obtained from Ethical Review Committee (ERC) of Maseno University number MSU/DRPI/MUERC/00629/18(appendix IV) while institutional approval for the study was obtained from Institutional Review Board of Jaramogi Oginga Odinga Teaching and Referral Hospital number ERC.IB/VOL.3/3(appendix V).Permission to collect data was also granted from the institution (appendix VI). Additional approval was obtained from the NACOSTI permit number NACOSTI/P/19/46935/28130(appendix VII). Written informed consent and assent was obtained from each participant's guardian by taking them through the consent form. After they confirmed that the study details were well explained and that they understood them, they then filled the consent form (Appendix II).Children asented verbally and by signing the assent form, for those who could write.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of HIV Uninfected and HIV Infected Participants

The study involved 98 participants from the HIV infected group and 98 participants from the HIV uninfected group between the ages of 3 and 14 years. Of the HIV infected group, the age range was 5 to 14 years with a mean age of 10.65years. Males were 39(39.8%) and females were 59(60.2%). From the HIV uninfected the age range was 3 to 13 years with a mean age of 6.62 years. Males were 53(53.1%) while females were 46(46.9%) from this group (Table 4.1).

Table 4.1: demographic characteristics of the study participants

		HIV infected(n=98)	HIV uninfected(n=98)	p value
Age(years)		10.65±2.17	6.68±2.81	<0.001
sex	male	39(39.8%)	52(53.1%)	
	female	59(60.2%)	46(46.9%)	

Data shows numbers(n) and proportions (%) for each group**.**

Table 4.2: 25OHD characteristics of the study participants

25OHD (ng/ml)	HIV infected(n=98)	HIV uninfected(n=98)	p value
mean	28.21±6.39	30.88±6.62	0.004[a]
Deficient (≤20)	13(13.3%)	5(5.1%)	
mean	18.1	18.4	0.733[a]
Insufficient (21-30)	46(46.9%)	37(37.8%)	
mean	25.9	25.5	0.575[a]
Sufficient (≥30)	39(39.8%)	56(57.1%)	
mean	34.3	35.7	0.075[a]

Data shows numbers(n) and proportions (%) for each group**.** a= independent samples T test

4.2 25OHD Status in HIV Infected and Uninfected Patients

The HIV uninfected group had mean25OHDlevels of 30.88 ng/ml (30.88±6.62 ng/ml) with a range between 16.1 to 46.5 ng/ml. Of these,5(5.1%) participants had deficient (<20 ng/ml) 25OHDlevels with a mean of 18.4ng/ml, 39(39.8%) had insufficient (21-30 ng/ml) levels with mean 25.5 ng/ml while 56(57.1%) had sufficient (≥30 ng/ml) 25OHDlevels with mean 35.7 ng/ml. The HIV infected group had mean25OHD levels of 28.21 ng/ml (28.21±6.39 ng/ml) with a range between 12.5 to 45.9 ng/ml. For this group 13(13.3%) participants had deficient (<20 ng/ml)25OHD levels with a mean of 18.1 ng/ml,46(46.9%) had insufficient (21-30 ng/ml) levels with a mean of 26.0 ng/ml while 39(39.8%) had sufficient (≥30 ng/ml) 25OHDlevels with a mean of 34.3 ng/ml (Table 4.2, figure 4.1).

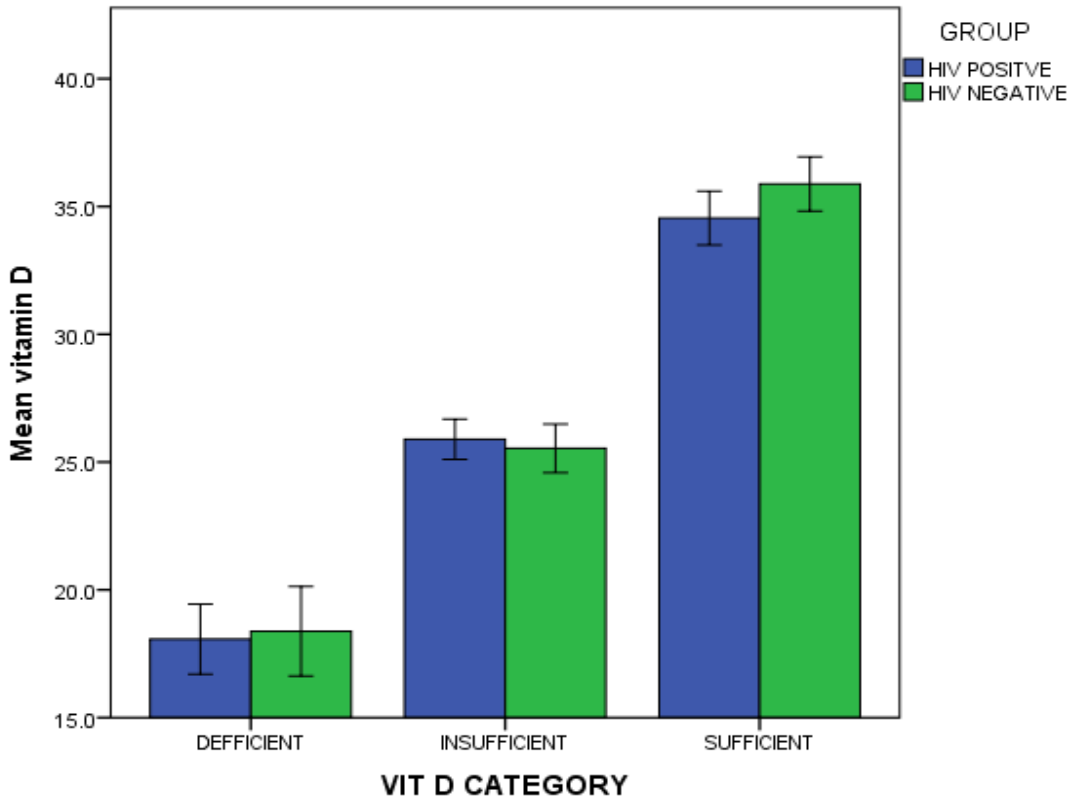

Figure 4.1: mean 25OHD levels in HIV infected and HIV uninfected patients

4.3 Differences in Vitamin D in HIV Infected and HIV Uninfected

There was a significant difference between the mean Vitamin D levels of the two groups(p=0.004). The mean for the HIV negative group was 30.88 ng/ml while for the HIV positive group it was 28.21 ng/ml.

4.4 CD4 Levels in HIV Positive Patients

The mean CD4 level was 931.57±318.3 cells/mm3, with lowest and highest values as 42 and 1804 cells/uL respectively;7(7.0%) subjects had <500 cells/mm^3(Table 4.3)**.**

4.5 Viral load levels in HIV Positive Patients

Subjects with low viral load (<10000 copies/ml) were 95, of which 33 had levels of <20 copies/ml;1 had moderate viral load (10000 -100000 copies/ml) while 2 had high viral load (>100000 copies/ml). Median (IQR) viral load was 70.5(232) copies/ml (Table 4.3)**.**

Table 4.3: Vitamin D, CD4 and viral load results for the HIV infected group.

		VIT D CATEGORY N (%) =98		
		DEFFICIENT	**INSUFFICIENT**	**SUFFICIENT**
VIRAL LOAD	<10000	13(13.27%)	43(43.88%)	39(39.80%)
(copies/mL)	10000 -100000	0(0%)	1(1.02%)	0(0%)
	>100000	0(0%)	2(2.04%)	0(0%)
CD4(cells/mm3)	<500	1(1.02%)	4(4.08%)	1(1.02%)
	>500	12(12.24%)	42(42.86%)	38(38.78%)

Table shows numbers(n) and proportions (%) Viral Load low(<1000),moderate(10000-100000),high(>100000) CD4<500(significant immunosuppression),>500(no significant immunosuppression)

4.6 correlation between Vitamin D, CD4, and Viral load

There was no correlation between Vitamin D and CD4count.There was also no correlation between Vitamin D and viral load. (Table 4.4).

Table 4.4: correlations between Vitamin D, CD4 and viral load (Pearson correlation)

		CD4	**VIRAL LOAD**
VITAMIN D	Correlation coefficient	0.166	-0.115
N=98	significance 0.01 (2 tailed)	0.101	0.26

Significance is at 0.01 level

CHAPTER FIVE

DISCUSSION

5.1 Vitamin D Levels in HIV Positive and HIV Negative Children

From the study the results established that the HIV negative subjects had significantly higher levels of Vitamin D compared to the HIV positive group and thus lower prevalence of Vitamin D deficiency and insufficiency than the HIV positive group (table4.2).Similar findings have been demonstrated in previous studies. Rutstein et al found that Vitamin D deficiency and insufficiency (36% and 89%) was higher in HIV positive individuals than the uninfected (15% and 84%)in unmatched HIV uninfected children or young adults (mean age 13.8±4.1) years) from a pediatric clinic (Rutstein et al,2011).In the mentioned study Vitamin D deficiency and insufficiency were defined as 25-OHD <11 ng/mL and 25-OHD <30 ng/mL, respectively. Compared to this study that used deficiency as <20 ng/ml and insufficiency as 20-30 ng/ml the matched and adjusted categorization from their study could be different. It was not clearly explained why the study authors went with this categorization. Gordon et al found that Vitamin D deficiency was highest in African American teenagers during winter, although the problem seemed to be common across sex, season, and ethnicity(Gordon et al., 2004).

In these studies, black skin type is a likely contributory factor for the low Vitamin D levels regardless of the geographical location as the high melanin content in black skin reduces the efficiency of Vitamin D synthesis,(Adeyemi et al., 2011). All the participants in this study were black. In uninfected children, a Ugandan study found that 80%(n=16) of children had Vitamin D insufficiency (<30 ng/ml) (Cusick et al., 2014) compared to 39% (39) for this study, likely due to the small sample size in the Ugandan study. Similar Vitamin D cut offs were used same as for this study. In Ethiopia 42% of healthy studied participants (age 11-18 years) had deficient Vitamin D compared to 5% for this study. In the age category of 11-14 years the deficiency rate

was 21.2%.(Wakayo et al., 2015). The Ethiopian study also explored factors that could affect the Vitamin D levels, something that was not done for this study. However, the amount of sunshine received by the two countries is roughly similar as they both lie in the tropics with Ethiopia having sunshine all year round. The Ethiopian study had a bigger sample size and therefore probably more power. There is no similar data available on HIV infected children from the two countries, therefore comparisons cannot be done with the same group from this study. In Tanzania 39% of pregnant women had low Vitamin D levels of less than 32ng/ml(Mehta et al., 2010). The Tanzanian study used a higher cutoff for Vitamin D which could explain their higher percentage than that obtained for this study (37.8%). In a south African study 74% of patients were found to have Vitamin D deficiency using similar cutoffs for this study. This could be explained by seasonality experienced in South Africa compared to the region of this study. Gichuhiet alfound a higher prevalence(49%) in a study of HIV positive adults on ART(Gichuhi et al., 2014) in a Kenyan study. In the same city Wambui and colleagues found that 6.3%and 29.7% of HIV infected adult patients had Vitamin D deficiency and insufficiency respectively (Wambui *et al*,2018). This was lower than the prevalence obtained by Gichuhi et al (49%)and this study. Chokephaibulkit et al found similar results in Thai adolescents with median age14.3 (IQR 13.0–15.7) years where 46.5%adolescents had Vitamin D insufficiency, and 24.7% had Vitamin D deficiency using similar cutoffs from the endocrine society that were used for the this study(Chokephaibulkit et al., 2013). For healthy adults in Kenya Kagotho et al found a 17.4% deficiency in HIV negative adults, compared to 5% HIV negative children obtained for this group(Kagotho et al., 2018).

Even though the factors that affect Vitamin D levels generally cut across the general population with older age cited as a factor(Giusti et al., 2011),better conclusions and generalizations on this difference can be done if more studies are done on the same, focusing on children. A Johannesburg study reported insufficiency in 19% of 10-year-old children and deficiency in 7%, with black children having more deficiency(Poopedi et al., 2011).The results of this study are therefore in agreement with the generally accepted findings that HIV infected people have higher prevalence of Vitamin D deficiency and insufficiency than healthy/uninfected people. Black ethnicity which is predominant in Africa and the study region is a contributory factor in deficiency as dark skin is a factor affecting Vitamin D levels synthesized from the sun (Richard et al., 2017). It would be expected that this would be balanced out by the longer sun hours and stronger solar rays in this region, but which is unlikely from the results of this study and others done in Africa(Mogire et al., 2020), meaning that other factors are at play. To the best of my knowledge this study is the first at attempting to characterize the Vitamin D status in a HIV infected and uninfected pediatric demographic in Kenya and the study region. More studies would be needed to shed more light on this characterization in this demographic, including analyzing other factors like serum phosphorus, parathyroid hormone and calcium for both the HIV infected and the uninfected. Explanations suggested to explain the hypovitaminosis that is more frequent in HIV individuals than the uninfected include use of HAART, chronicity of the HIV infection and opportunistic infections which impair sun exposure and oral nutrition(Mansueto et al., 2015).More studies are needed to shed light on other factors that cause hypovitaminosis D in the study region.

5.2 Correlation between Vitamin D,CD4 and Viral Load

This study found no correlation between the Vitamin D levels and immune status using CD4countand viral load (table 4.4)as the immune markers. In a similar study of 160 HIV

positive youth (2 -26 years) severe Vitamin D (<10 ng/ml) was related to lower $CD4^+$ count and $CD4^+$ % but not viral load(Poowuttikul et al., 2014). Chokephaibulkitet al also found no correlation between Vitamin D,CD4 or viral load (Chokephaibulkit et al., 2013) in a Thaiadolescent's study. Bearden et al, found a nonlinear association between viral load and $1,25(OH)_2D_3$(calcitriol)but found no association between 25OHD (calcidiol) and$CD4^+$in adults(Bearden et al., 2013). Kakalia et al found that $CD4^+$count did not increase under Vitamin D supplementation for 6 months, especially in children (mean age 10.3 years) with relatively preserved immunologic function (Kakalia et al., 2011). Other studies identified a correlation between CD4 levels and Vitamin D. Adeyemi et al found that lower CD4 count was associated with higher odds of Vitamin D deficiency in black women(Adeyemi et al., 2011).Ross *et al* found that in adults on ART there was an increase in CD4 count in subjects with higher Vitamin D levels(Allison C. Ross et al., 2011). However, they did not find this association in HIV infected youth (17.4±4.6 years)(Ross Eckard et al., 2012).Welz et al also found that low CD4levelswereassociated with severe Vitamin D deficiency(Welz et al., 2010). Havers et al found that low Vitamin D was associated with lower viral load in adults and (Havers et al., 2014). Abedi and rosenthal found a correlation between increased Vitamin D levels and elevated viral loads. However, their population was older as it encompassed 13- to 25-year-old population(Abedi & Rosenthal, 2016). They found no correlation between Vitamin D and CD4 count/CD4 % in this population from a retrospective review of data.

The reason for these different results might be due to relatively higher Vitamin D levels observed in the subjects under this study compared to those in other regions of the world that are temperate and experience seasons. The study location being close to the equator with mostly overhead sunlight with no prominent seasonality means that exposure to sunlight is maximum.

The current guidelines outline the use of ART regardless of the CD4 or viral load levels. Confirmed HIV positive individuals are immediately put on HAART at diagnosis and CD4 and viral load used to monitor the response to the HAART regimen. This leads to better prognosis of the patient as regimen switching is done to offer maximum benefit. This would explain why 33(33.04%) of the subjects in this study had undetectable (<20 copies) levels of viral load and 92(93.9%) subjects had >500 cells/ul. Of these 42.9% had insufficient Vitamin D levels while 38.8% had insufficient levels. Only 12.2% had deficient Vitamin D levels in this category as has been summarized in table 4.3.

The normal CD4 count in majority of the subjects could possibly be due to the fact immune system of HIV-infected children appears to be capable of efficient CD4 recovery through naïve T-cell population and expansion. This potential progressively decreases with duration of infection and with age(Lewis et al ,2012). Use of HAART in itself has been found to be associated with low Vitamin D levels in HIV infected individuals.25OHD deficiency has been observed to be more prevalent in HIV infected individuals on HAART than those HAART-naïve or those who are HIV negative. It would be worthy to explore the outcome of Vitamin D supplementation in HAART use in this region for this demographic so that the relationship between HAART use and Vitamin D can be explained. Specifically, non-nucleoside reverse transcriptase inhibitors (NNRTIs) and efavirenz in particular has been demonstrated to have a marked effect on Vitamin D due to impairment of theCYP450Vitamin D metabolic pathways in the kidneys(Mara Pinto, 2014).

5.3 Study Limitations

This study had several limitations. The Cross-sectional design used in the study limits the derivation of cause-effect relationships from the variables of the study. The study was conducted in a single site serving an urban and rural population, which may limit the generalization of the

finding to other similar populations. Other physiological markers of Vitamin D levels like Parathyroid hormones and calcium levels were not determined, all which affect the Vitamin D status. The dietary profile of the participants, which could have an effect on the Vitamin D levels, was not factored into the analysis. Even though the study participants between the two groups were not age and sex matched this has been shown in studies to have no major significance on the outcome. However, the study has been effective at obtaining new knowledge on Vitamin D in a never before studied demographic in Kenya.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION

6.1 Summary of the Findings

In this study HIV infected children had lower Vitamin D levels compared to the HIV uninfected children. In the HIV infected children there was no correlation between Vitamin D and immune status using CD4 count and viral load as the immune markers.

6.2 Conclusions

1. From the study HIV infected children have lower Vitamin D levels than the uninfected children.
2. There is no correlation between Vitamin D status and immune status in HIV infected children.

6.3 Recommendations from the Study

Assessment of Vitamin D for children especially the HIV infected would help and inform the management and improvement of their health as the benefits of Vitamin D extend beyond skeletal health.

6.4 Recommendations for Future Studies

The data suggests the need for large properly designed prospective studies that will investigate the prevalence of Vitamin D in healthy as well as HIV positive populations in Kenyan children and the risk factors associated with this deficiency in both groups.

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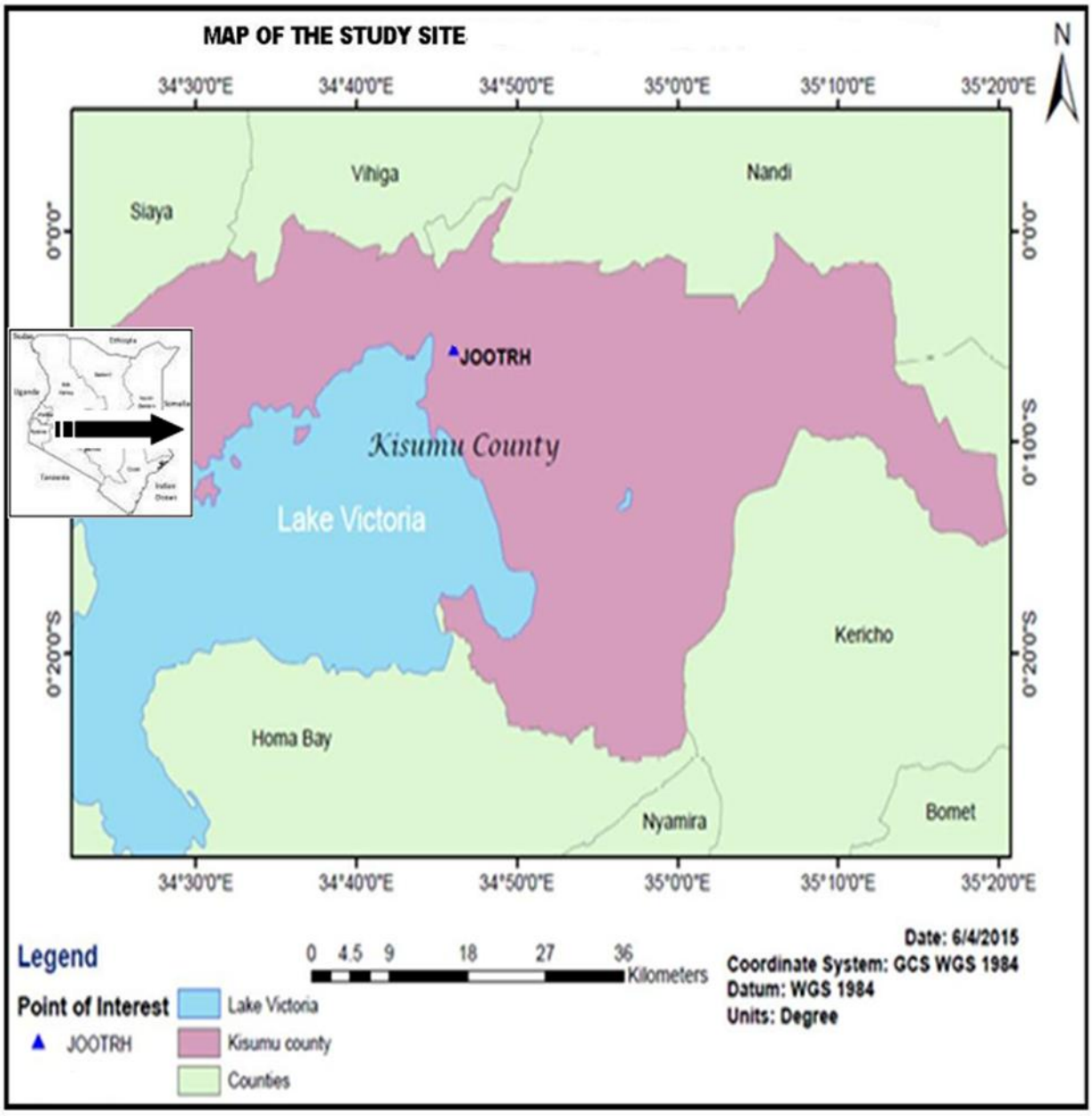
APPENDICES

Appendix I: Map of Kisumu County



Appendix II: Letter of consent

CORRELATION OF VITAMIN D LEVELS AND IMMUNE STATUS IN HIV POSITIVE CHILDREN AGED 3 TO 14 YEARS ATTENDING JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL IN KISUMU COUNTY, KENYA

Introduction: My name is Maurice Songoreh Asamuka from Maseno university. You are being requested to allow your child participate in this study. This form tells you why this research study is being done. Please read and decide whether to join the study or not.

Investigators: The principle investigator in this study is Maurice Songoreh Asamuka from Maseno University under the supervisor-ship of Dr Bernard Guya and Dr Lillian Ogonda.

Objective of the study: The purpose of this study is to determine the correlation of Vitamin D levels and immune status of children aged between 3 and 14 years. This will be achieved by determining the levels of Vitamin D in HIV positive and negative children and then correlating Vitamin D levels with CD4 count and viral load in HIV positive children

Study location: The study will be done at JOOTRH in Kisumu county. However, the study samples will be analyzed at various labs notably JOOTRH lab (CD4 count), Aga khan hospital Kisumu lab (Vitamin D) and the KEMRI/CDC labs (HIV viral load).

Anticipated benefit: You will be able to know the Vitamin D levels of your child as part of his/her overall health. Relevant findings from this exercise will be provided to your health care provider to facilitate the child's healthcare management.

Risk and discomfort: Blood will be collected by venipuncture from the child's arm at the elbow or at any other appropriate site by qualified phlebotomists. Therefore, there will be a slight pain and discomfort which will immediately wear off after the procedure is complete. Any bleeding will be handled and resolved before the child leaves the phlebotomy area. The amount of blood drawn will be small (6 ml) and won't affect the child's health adversely. Please note that there is a very minimal chance of infection arising from this procedure.

Protection against risk: JOOTRH Institutional Review Board and Maseno University Ethical review Committee have reviewed this protocol. In addition, I have ensured that all procedures conform to MUERC, Kenyan and international ethical standards regarding research involving human subjects. Guidance from the study supervisors and JOOTRH clinical research committee will be sought in assuring that the protocol meets strict the mentioned guidelines for protection of children as research subjects and that all processes and procedures are done according to laid down standard operation procedures and policies.

Confidentiality: All paper and electronic medical records for potential participants in this study will be coded, secured and maintained in compliance with Maseno university Ethical Review Committee policy. The child's identification will be anonymized throughout the study and results won't be disclosed to any other individuals outside the study setup. Data obtained from the child's file will be similarly treated with utmost confidentiality.

Volunteerism: participation in this study is entirely voluntary. It is upon your will to allow your child to participate. There is no renumeration or compensation financial or otherwise for your

child's participation. However, you or the child are free to decline your participation or even withdraw at any time and this won't affect the child's care in any way.

Results dissemination: results of this study will be disseminated through presentations and reports.

I consent by signing my name here below and confirm that;

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I have been explained the purpose of this study and I do understand the risk and benefits of this study.

I hereby grant consent voluntarily for the subject to participate as a participant in this study.

Participant's Name..... Signature.....Date.....

Principal Investigator..... Signature.....Date.....

For any question or concern about the research study or study related injuries, please contact Maurice Songoreh Asamuka 0727902603

For any questions pertaining to the child's rights as a research participant, contact person is: The Secretary, Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address**:** muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com.

Appendix III: Initial approval of study protocol by SGS Maseno university

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/PH/000100/14

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

Date: 2nd August, 2018

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR MAURICE SONGOREH — MSC/PH/000100/2014

The above named is registered in the Master of Science in Medical Immunology in the School of Public Health and Community Development, Maseno University. This is to confirm that His research proposal titled "*Correlation of Vitamin D Levels and Immune Status in HIV Positive Children Aged 3 to 14 Years Attending Jaramogi Oginga Odinga Teaching and Referral Hospital in Kisumu County, Kenya.*" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Appendix IV: MUERC approval

MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221
Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 25th January, 2019

TO: Maurice Songoreh Asamuka
PG/MSc/00100/2014
Department of Biomedical Sciences
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00629/18

RE: Correlation of Vitamin D levels and Immune Status in HIV Positive Children Aged 3 to 14 years attending JOOTRH in Kisumu County, Kenya. Proposal Reference Number MSU/DRPI/MUERC/00629/18

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 25th day of January, 2019 for a period of one (1) year. This is subject to getting approvals from NACOSTI and other relevant authorities.

Please note that authorization to conduct this study will automatically expire on 24th January, 2020. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 15th December, 2019.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 15th December, 2019.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

Dr. Bernard Guyah
Ag. Secretary,
Maseno University Ethics Review Committee

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix V: JOOTRH ERC approval

Telegrams: "MEDICAL", Kisumu
Telephone: 057-2020801/2020803/2020321
Fax: 057-2024337
E-mail: ercjootrh@gmail.com
When replying please quote
ERC.IB/VOL.3/3
Ref: .....

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849
KISUMU

Date: 27th March, 2019

Maurice Songoreh Asamuka

Dear Maurice,

RE: FORMAL APPROVAL OF THE PROTOCOL STUDY ENTITILED:-
"CORRELATION OF VITAMIN D LEVELS AND IMMUNE STATUS IN HIV POSITIVE CHILDREN AGED 3 TO 14 YEARS ATTENDING JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL INKISUMU COUNTY. KENYA"

The JOOTRH ERC reviewed your protocol and found it ethically satisfactory. You are therefore permitted to commence your study immediately. Note that this approval is granted for a period of one year (w.e.f. 27th March, 2019 to 27th March, 2020). If it is necessary to proceed with this research beyond approved period, you will be required to apply for further extension to the committee.

Also note that you will be required to notify the committee of any protocol amendment(s), serious or unexpected outcomes related to the conduct of the study or termination for any reason.

In case the study site is JOOTRH, kindly report to the Chief Executive Officer before commencement of data collection.

Finally, note that you will also be required to share the findings of the study in both hard and soft copies upon completion.

The JOOTRH – IERC takes this opportunity to thank you for choosing the Institution and wishes you the best in your future endeavours.

Yours sincerely,

WILBRODA N. MAKUNDA
SECRETARY- IERC
JOOTRH - KISUMU

Appendix VI: JOOTRH permission to collect data

COUNTY GOVERNMENT OF KISUMU
DEPARTMENT OF HEALTH

Telephone: 057-2020801/2020803/2020321
Fax: 057-2024337
E-mail: medsuptnpgh@yahoo.com
ceo@jaramogireferral.go.ke
Website: www.jaramogireferral.go.ke
When replying please quote
GEN/21A/V
Ref: .....

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849-40100
KISUMU

4TH APRIL, 2019
Date .....

Maurice Songoreh Asamuka
KISUMU

RE: PERMISSION TO COLLECT DATA

Following approval of protocol titled "Correlation of vitamin D levels and immune status in HIV positive children aged 3 to 14 years attending Jaramogi Oginga Odinga Teaching and Referral Hospital", you are hereby permitted to proceed with the activity.

Thank you.

CHIEF EXECUTIVE OFFICER
JARAMOGI OGINGA ODINGA TEACHING &
REFERRAL HOSPITAL/JOOTRH
P.O. BOX 849-40100, KISUMU
DATE:.....

DR. OKOTH PETER.,
CHIEF EXECUTIVE OFFICER,
JOOTRH – KISUMU.

Appendix VII: NACOSTI certification

THIS IS TO CERTIFY THAT:
MR. MAURICE SONGOREH ASAMUKA
of MASENO UNIVERSITY, 0-40100
KISUMU,has been permitted to conduct
research in *Kisumu County*

on the topic: *CORRELATION OF VITAMIN D LEVELS AND IMMUNE STATUS IN HIV POSITIVE CHILDREN AGED 3 TO 14 YEARS ATTENDING JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL IN KISUMU COUNTY, KENYA*

for the period ending:
11th March,2020

.....
Applicant's Signature

Permit No : NACOSTI/P/19/46935/28130
Date Of Issue : 11th March,2019
Fee Recieved :Ksh 1000

.....
Director General
National Commission for Science, Technology & Innovation

Appendix VIII:PROCEDURE FOR DETERMINATION OF VITAMIN D USING MINIVIDAS ANALYZER

Only remove the required reagents from the refrigerator. They can be used immediately.

Use one "VITD" strip and one "VITD" SPR® from the kit for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after the required SPRs have been removed.

The test is identified by the "VITD" code on the instrument. The calibrator must be identified by "S1",and tested induplicate. If the control is to be tested, it should be identifiedby"C1".

If necessary, clarify the samples by centrifugation.

Mix the calibrator, control and samples using a vortex-type mixer (for serum or plasma separated from the pellet).

Before pipetting ensure that samples, calibrators, controls and diluent are free of bubbles. For this test, the calibrator, control, and sample test portion is 100 µL.

Insert the "VITD" SPRs and "VITD" strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the Reagent Strips match.

Initiate the assay as directed in the User's Manual. All the assay steps are performed automatically by the instrument.

Reclose the vials and return them to 2–8°Cafter pipetting. The assay will be completed within approximately 40minutes.Aftertheassayiscompleted,remove the SPRs and strips from the instrument. Dispose of the used SPRs and strips into an appropriate waste container.

Once the assay is completed, results are analyzed automatically by the computer. Fluorescence is measured twice in the Reagent Strip’s reading cuvette for each sample tested.

The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme

remaining on the interior of the SPR. The RFV (Relative Fluorescence Value)is calculated by subtracting the background reading from the final result. This calculation appears on the result sheet.

The results are automatically calculated using calibration curves which are stored by the instrument (4-parameter logistics model) and are expressed in ng/mL ornmol/L.

Appendix IX: PROCEDURE FOR DETERMINATION OF CD4+ COUNT USING FACSCALIBUR

Calibration and Calibration Verification Procedures

Preparation of the Cali BRITE Beads

BDC at a log number 310486-CAliBRITE3 and number 340487-APCbeads. Pipette FACSF low sheath reagent intotwo12×75mm plastic tubes, 0.5ml for the unlabeled+activated protein C (APC) bead sand1ml for the mixture of unlabeled fluoresceinisothiocyanate(FITC), peridinin chlorophyll-α protein (PerCP), and APC beads. Gently mix the unlabeled + APC beads by inversionandad done drop to the 0.5ml tube and the 1ml tube. Gently mix the FITC, Per CP and APC beads and done drop of each to the 1ml tube. Cap the tubes and mix by gently inverting several times. Harsh mixing can cause high background noise. Make fresh daily.

Select the FACS Comp software.

On the first screen, enter your initials under Operator ID. Make sure the lot identification for the current batch of Cali BRITE Beads is correct in the software (matches the bead lot number son the insert from the Cali BRITE box). The suffix letter is important because it tells the software what the minimum signal-to- noise separation is for that lot. Acquire the unlabeled + APC bead mixture; if "PMT's" set successfully, acquire the mixed beads after gently inverting the tube several times to mix. If all tests passed successfully, print the results. See BD's FACS comp manual for detailed instructions for performing FACS comp on the three different flowcytometers

Operating Procedures; Calculations; Interpretation of Results

Lyse/No Wash Procedure (LNW) (for single-plat form lymphocyte sub set determin ationpanel)

1. Remove the cryovials from the–70°C free

2. Zerinbatches of no more than 10. Make sure to compare the specimen number or patient ID on the specimen vial to the paper work (transmittal form, etc.) Thaw quickly in a 37°C water bath.
3. Log in specimen under appropriate project spread sheet.
4. CD-chex will be used as QC control and frozen whole blood pool as normal control.
5. Label Tru COUNT tubes as follows:
    a) Write the panel and antibody added on the top part of the tube
    b) Write the specimen's assigned lab ID on the lower part of the tube
6. Pipette 20µl of the appropriate antibody into the corresponding labeled tube
7. Make sure specimens are well mixed before adding 50µl (using BD pipette) of frozen whole blood to the corresponding tube.
8. Vortex briefly at vortex setting 4 to 6.
9. Incubate at room temperature in the dark for15minutes
10. Add450µl (0.45ml) of FACSlysing solution and vortex well.
11. Incubate at room temperature in the dark for10minutes
12. Add 50µl of Tru COUNT control beads to the CD-Chex tube.
13. Incubate at room temperature in the dark for at least 15 minutes before acquiring the sample. Acquire within 2 hours of setup.
14. Multi SET Acquisition and analysis:
    a. TruCOUNT3Color-4tube and 4Color-2 tube LNW panel scan be performed using BD Multi set software.

b. Open Multi SET software from the Apple menu and then open appropriate schedule (XXX.sch) from the CLIA Lab folder on the Desktop. Follow the prompting messages from the programme to proceed.

c. Sign in with your initials

d. Make sure to select the appropriate locations for data files, reports, and export document storage.

e. Multi SET data files are saved.

f. Make sure the appropriate absolute bead count, reagents, and control bead lot ID's are filled and saved.

g. Fill out the schedule with sample name, sample id, case number and panel name.

h. Open Cytometer from the Apple menu and make sure the LNW instrument settings file from the current day's FACS comp results is selected before clicking the Run Test command. Append statistics to the same file created for the project

15. Run tests and use Manual Gate for analysis; print report be foregoing to the next sample.

16. Save schedule before quitting the program when finished.

Calculations

Calculations for the proportion of lymphocytes that are of particular cell type are determined in the Multi SET software. This software calculates the proportion of cells that are positive with a particular monoclonal

Antibody and reports them in whole numbers. Additionally, the values are automatically corrected for the purity of lymphocytes inside the gate (%of bright CD4+ 5+,CD 14-cells of all cells in the gate). All results reported are corrected for lymphocyte purity. Additional calculations are meant for data quality control. These include adding the CD3+CD4+ and CD3+CD8+ results to equal the CD3 results (+10%) and accounting for all of the lymphocytes in the specimen by adding the T,B, and NK cells together to total 90% to 110%.

The absolute counts of each subset are calculated automatically by the following formula: absolute CD4+ or CD8orCD3Count=(#of target population events /# of bead events collected)× (# of beads per test from the package insert/ test volume of 50µl).

APPENDIX X: PROCEDURE FOR DERTEMINATION OF HIV VIRAL LOAD USING COBAS TAQMAN/COBAS AMPLIPREP

Note: For detailed operating instructions, a detailed description of the possible configurations, printing results and interpreting flags, comments and error messages, refer to the COBAS TaqMan 48 analyzer Instrument Manual for use the AMPLILINK software version 3.3 Series Application Manual, or the AMPLILINK software Version 3.3 Series Application Manual for use with COBAS AmpliPrep instrument, COBAS TaqMan analyzer, COBAS TaqMan 48 analyzer,

Batch Size: Each kit contains reagents sufficient for 48 tests, which may be performed in batches of 12 to 24 tests. At least one of each control (CTM (-) C, HIV-1 L (+) C and HIV-1 H (+) C, v2.0) must be included in each batch (see "Quality Control" section).

Workflow: The COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation.

Note: Do not freeze or store processed specimens and controls at 2-8ºC.

Specimen and Control Preparation

Note: If using frozen specimens, place the specimens at room temperature (15-30ºC) until completely thawed and vortex for10-15 seconds before use. Controls should be removed from 2-8ºC storage and equilibrated to room temperature before use.

COBAS AmpliPrep Instrument Set-up

Part A. Maintenance and Priming

A1. The COBAS AmpliPrep Instrument is ready for operation in stand-by mode.

A2. Turn the Data Station for the AMPLILINK software ON. Prepare the data Station as follows:

a. Log onto the Windows XP operating system
b. Double click the AMPLILINK software icon
c. Log onto AMPLILINK software by entering the assigned User ID and password

A3. Check the supply of PG WR using the Status Screen and replace if necessary.

A4. Perform all Maintenance that is listed in the Due Tab on the Maintenance section of the Status Screen. The COBAS AmpliPrep Instrument will automatically prime the system.

Part B. Loading of Reagent Cassettes

Note: All reagent cassettes should be removed from 2-8ºC storage, immediately loaded onto the COBAS AmpliPrep Instrument and allowed to equilibrate to ambient temperature on the instrument for at least 30 minutes before the first specimen is to be processed. Do not let reagent cassettes come to ambient temperature outside the instrument as condensation may form on the barcode labels. Do not wipe off condensation if it appears on the barcode label.

B1. Place HIV-1 CS1 onto a reagent rack. Place HIV-1 CS2, HIV-1 CS3 and HIV-1 CS4 onto a separate reagent rack

B2 Load the reagent rack containing HIV-1 CS1 onto rack position A of the COBAS AmpliPrep Instrument

B3. Load the reagent rack containing HIV-1 CS2, HIV-1 CS3 and HIV-1 CS4 onto rack position B, C, D or E of the COBAS AmpliPrep Instrument.

Part C. Loading of Disposables

Note: Determine the number of COBAS AmpliPrep reagent cassettes, Sample Processing Units (SPUs), Input Sample tubes (s-tubes), K-tips and K-tubes needed. One SPU, one Input S-tube, one K-tip and one K-tube are needed for each specimen or control.

Multiple workflows for use of the COBAS AmpliPrep Instrument with the COBAS TaqMan Analyzer or COBAS TaqMan 48 Analyzer are possible. Depending on the workflow used, load the appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, K-tube racks and K-carriers on K-carrier racks onto the respective rack positions of the COBAS AmpliPrep Instrument.

C1. Place the SPUs in the SPU rack(s) and load the rack(s) onto rack position J, K, or L of the COBAS AmpliPrep Instrument.

C2. Depending on the workflow used, load full K-tube rack(s) onto rack position M, N, O or P of the COBAS AmpliPrep Instrument.

C3. Load full K-tip rack(s) onto rack position M, N, O or P of the COBAS AmpliPrep Instrument.

C4. For workflow 3 using the COBAS TaqMan 48 Analyzer, load K-carriers on K-carrier rack(s) onto rack position M & N, or O & P of the COBAS AmpliPrep Instrument.

Part D. Ordering and Loading of Specimens

D1. Prepare sample racks as follows: Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed. Attach one of the specific barcode label clips for the controls [CTM (-) C, HIV-1 L (+) C, v2.0 and HIV-1 H (+) C, v2.0] to each sample rack position where the controls (S-tube) are to be placed. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit. Take care in assigning the right control to the position with the appropriate control barcode clip. Place one Input S-tube into each position containing a barcode label clip.

D2. Write the sample rack order on the HIV-1 viral load bench form.

D3. Using the AMPLILINK software, create specimen orders for each specimen and control in the orders window sample folder. Select the appropriate test file and complete by saving.

D4. Assign specimen and control orders to sample rack positions in the Orders window

Sample Rack folder. The sample rack number must be for the rack prepared in step D1.

D5. Print the Sample Rack Order report to use as a worksheet.

D6. Prepare specimen and control racks in the designated area for specimen and control

addition as follows: Vortex each specimen and control [CTM (-) C, HIV-1 L (+) C, v2.0

and HIV-1 H (+) C, v2.0] for 10-15 seconds. Avoid contaminating gloves when

manipulating the specimens and controls.

D7. Transfer 1000µL of each specimen and control [CTM (-) C, HIV-1 L (+) C, v2.0 and

HIV-1 H (+) C, v2.0 to the appropriate barcode labeled Input S-tube using a micropipettor

with an aerosol barrier or R Nase-free tip. Avoid transferring particulates and/or fibrin clots from the original specimen to the Input S-tube. Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet in step D2. The barcode label clips for controls should have the same control lot number as the lot number on the control vials in the kit. Assign the right control to the position with the appropriate control barcode clip. Avoid contaminating the upper part of the S-tubes with specimens or controls.

D8. For workflow 3 using the COBAS TaqMan 48 Analyzer, load sample rack(s) with

Input S-tubes and K-tubes (one for each Input S-tube, loaded in the right position adjacent to Input S-tubes) onto rack position F, G or H of the COBAS Ampli Prep Instrument.

Part E. Start of COBAS Ampli Prep Instrument Run

E1. Start the COBAS Ampli Prep Instrument using the AMPLILINK software.

Part F. End of COBAS Ampli Prep Instrument Run and Transfer to COBAS TaqMan

48 Analyzer

F1. Check for flags or error messages.

F2. Remove processed specimens and controls from the COBAS Ampli Prep Instrument on K-carrier racks (for COBAS TaqMan 48 Analyzer), depending on the workflow.

F3. Remove waste from the COBAS Ampli Prep Instrument

Note: All processed specimens and controls should not be exposed to light after completion of specimen and control preparation.

Amplification and Detection

COBAS TaqMan 48 Analyzer Set-up

The COBAS TaqMan 48 Analyzer run must be started within 120 minutes following completion of specimen and control preparation.

Note: Do not freeze or store processed specimens and controls at 2-8^{0}C

Part G. Loading Processed Specimens

G1. Depending on the workflow, perform the appropriate steps to transfer the K-tubes to

the COBAS TaqMan 48 Analyzer.

Workflow 3: Manual transfer of K-carrier on K-carrier rack(s) to the COBAS TaqMan 48 Analyzer. Manual transfer of K-carriers into COBAS TaqMan 48 Analyzer using the K-carrier Transporter.

Part H. Start of COBAS TaqMan 48 Analyzer Run

H1. Start the COBAS TaqMan 48 Analyzer by one of the options below depending on

the workflow used.

Workflow 3: Fill K-carrier with empty K-tubes if there are fewer than 6 K-tubes on the K-carrier. Filling is guided by the AMPLILINK software. Open thermal cycler cover, load K carrier into thermal cycler and close lid. Start the COBAS TaqMan 48 Analyzer run.

Part I. End of COBAS TaqMan 48 Analyzer Run

I1. At the completion of the COBAS TaqMan 48 Analyzer run, print Results Report.

Check for flags or error messages in the Result report. Specimens with flags and

comments are interpreted as described in the Results section. After acceptance, store

data in arcHIVe.

I2. Remove used K-tubes from the COBAS Taqman 48 Analyzer.

1. Results

The COBAS TaqMan 48 Analyzer automatically determines the HIV-1 RNA concentration for the specimens and controls. The HIV-1 RNA concentration is expressed in copies/mL. The conversion factor between HIV-1 RNA copies/mL and HIV-1 RNA IU/mL is 0.6 copies/IU, using the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid Technology (NAT) Assays Testing (NIBSC 97/746).

AMPLILINK Software

- Determines the Cycle Threshold value (Ct) for the HIV-1 RNA and the HIV QS RNA
- Determines the HIV-1 RNA concentration based upon the Ct values for the HIV-1 RNA and the HIV-IQS RNA and the lot-specific calibration coefficients provided on the cassette barcodes.
- Determines that the calculated CP/mL for HIV-1 L(+)C, v2.0 and HIV-1 H(+)C, v2.0 fall within he fixed ranges

Batch Validation – AMPLILINK Version 3.3 Series

Check AMPLILINK software results window or printout for flags and comments to ensure that the batch is valid. For control orders, a check is made to determine the CP/mL value for the control is within its specified range. If the CP/mL value for the control lies outside of its range, a FLAG is generated to show the control has failed.

The batch is valid if no flags appear for any of the controls [HIV-1 L (+) C, v2.0 and CTM (-) C].

The batch is not valid if any of the following flags appear for the HIV-1 Controls:

Negative Control:

Flag	**Result**	**Interpretation**
NC_INVALID	Invalid	An invalid result or a "valid" result that was not negative for HBV target

HIV-1 Low Positive Control, v2.0:

Flag	Result	Interpretation
LPCINVALID	Invalid	An invalid result or a control out of range

HIV-1 High Positive Control, v2.0:

Flag	Result	Interpretation
HPCINVALID	Invalid	An invalid result or a control out of range

If the batch is invalid, repeat the entire batch including specimen and control preparation, amplification and detection.

Interpretation of Results

For a valid batch, check each individual specimen for flags or comments on the results printout. Interpret the results as follows:

- A valid batch may include both valid and invalid specimen results depending on whether flags and/or comments are obtained for the individual specimens.

Specimen results are interpreted as follows:

Titer Result	Interpretation
Target Not Detected	Ct value for HIV-1 above the limit for the assay or no Ct value for HIV-1 obtained. Report results as "HIV-1 RNA not detected".
<2.00E+01 cp/mL	Calculated CP/mL are below the Limit of Detection of the assay. Report results as "HIV-1 RNA detected, less than 20 HIV-1 RNA cp/mL''.
≥2.00E+01 cp/mL	Calculated results greater than or equal to 20 CP/mL and less than or equal to 1.00E+07CP/mL are within the Linear Range of the assay.

and≤1.00E+07 cp/Ml	
>1.00E+07 cp/mL	Calculated CP/mL are above the range of the assay. Report results as "greater than 1.00E+07 HIV-1 RNA cp/mL". If quantitative results are desired, the original specimen should be diluted 1:100 with HIV-1-negative human EDTA plasma and the test repeated. Multiply the reported result by the dilution factor.

- Note: The analytical measurement range of analyte values that can be directly measured on a specimen without any dilution using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 is 20 to 1.0E+07 cp/mL.
- Note: Specimens above the range of the assay that produce an Invalid result with a flag "QS_INVALID" should not be reported as >1.00E+07 cp/mL. The original specimen should be diluted with HIV-1-negative human EDTA-plasma and the test repeated. Multiply the reported result by the dilution factor.
- Note: Titer Result "Failed". Interpretation: Specimen is not correctly processed during specimen preparation on the COBAS AmpliPrep Instrument.
- Note: Titer Result "Invalid". Interpretation: An Invalid Result.

APPENDIX XI: VITAMIN D RANGES

These are the ranges that were used in this study and are the recommendations from the endocrine society.

Vitamin D status	**serum level(ng/ml)**
Deficiency	<20
Insufficiency	21-30
Sufficiency	>30
Toxicity	>150

Appendix XII: Assent form

CORRELATION OF VITAMIN D LEVELS AND IMMUNE STATUS IN HIV POSITIVE CHILDREN AGED 3 TO 14 YEARS ATTENDING JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL IN KISUMU COUNTY, KENYA

Introduction: My name is Maurice Songoreh Asamuka from Maseno university. You are being requested to participate in this study. This form tells you why this research study is being done. Please read and decide whether to join the study or not.

Objective of the study: The purpose of this study is to determine the correlation of Vitamin D levels and immune status of children aged between 3 and 14 years. This will be achieved by determining the levels of Vitamin D in HIV positive and negative children and then correlating Vitamin D levels with CD4 count and viral load in HIV positive children

Study location: The study will be done at JOOTRH in Kisumu county.

Anticipated benefit: You will be able to know your Vitamin D levels as part of your overall health. Relevant findings from this exercise will be provided to your doctor to facilitate healthcare management.

Risk and discomfort: Blood will be collected by venipuncture from your arm at the elbow or at any other appropriate site by qualified phlebotomists. Therefore, there will be a slight pain and discomfort which will immediately wear off after the procedure is complete. Any bleeding will be handled and resolved before you leave the phlebotomy area. The amount of blood drawn will be small (6 ml) and won't affect your health adversely. Please note that there is a very minimal chance of infection arising from this procedure.

Volunteerism: participation in this study is entirely voluntary. Your parent will be requested for permission for your participation It is upon your will and that of your parent to participate. You will be required to discuss with your parent/guardian first before agreeing to participate. There is no renumeration or compensation financial or otherwise for you or your parent for participation. However, you are free to decline your participation or even withdraw at any time and this won't your care in any way. Also note that withdrawal or decline can be decided by your parent, even if you agree.

I assent by signing my name here below and confirm that;
I hereby grant assent voluntarily to participate in this study.
Participant's Name..... Signature.....Date.....
Principal Investigator..... Signature.....Date.....
For any question or concern about the research study or study related injuries, please contact Maurice Songoreh Asamuka 0727902603

For any questions pertaining to the child's rights as a research participant, contact person is: The Secretary, Maseno University Ethics Review Committee, Private Bag, Maseno; Telephone numbers: 057-51622, 0722203411, 0721543976, 0733230878; Email address**:** muerc-secretariate@maseno.ac.ke; muerc-secretariate@gmail.com