IMMUNOPHENOTYPE AND HEPATO-BIOMARKER CHARACTERISTICS OF HIV INFECTED ADULTS AND HEMATOLOGICAL REFERENCE RANGES OF CHILDREN IN KISUMU WEST SUBCOUNTY, KENYA

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DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

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CERTIFICATION

The undersigned certify that they have read, and hereby recommend for acceptance of Masinde Muliro University of Science and Technology, a thesis entitled **'Immunophenotypic and hepato-biomarker characteristics of HIV infected adults and hematological reference ranges of children in Kisumu West Sub-County, Kenya.**'

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DEDICATION

I dedicate this thesis to my father, Mr. Caleb Ouma, mother, Mrs. Roseline Ouma and wife Benta Atieno. They have unrelentingly offered immense encouragement and support in my academic pursuit and endeavors. God bless you all.

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ABSTRACT

Proper diagnosis and management of clinical diseases requires succinct understanding of well-established human physiological reference values. Despite the high prevalence rates of HIV, Tuberculosis and Hepatitis infection in Kisumu area of Western Kenya, there are no locally developed hematological values that can be used as reference values of normal biological or homeostatic processes from which pathogenic, pathologic or pharmacologic responses to a therapeutic intervention can be inferred. This study enrolled 1566 study participants (1509 healthy children for hematological reference range and 57 HIV positive, anti-retroviral therapy naive adults for immunophenotypic and hepato-biomarker characterization. Statistical analysis was done by graphpad by use of percentiles, Mann-Whitney test, Spearman's correlation and Kruskall Wallis. The results of the study showed that it was possible to establish hematology reference ranges in children below two years and based on gender. It was noted that the total white blood cell counts, monocyte and granulocyte absolute counts in 1 m to 6 m age groups were statistically significantly lower than the other two age groups ($p<0.001$). In 1 m to 6 m age group, it was noted that the median values of hemoglobin, hematocrit and the RBC indices are higher compared to the age groups 6 m to 12 m and 12 m to 17 m ($p<0.0001$). For MCHC, the values were noted to be decreasing with increasing age and this was different in all the three age groups ($p<0.0001$). For platelets counts, the values were significantly different in all the three age categories with lower values in the lowest age category (1 m to 6 m) and higher values in the 6 m to 12 m age category ($p<0.001$). In regard to gender differences, hemoglobin values in females in 1m to 6 m age group were significantly higher than the male children ($p=0.0189$). Platelet counts in females in 1 m to 6 m age group were significantly higher than the male children ($p=0.0005$). The median values for RBC indices for females were higher compared to males ($p<0.0001$). In comparison to the US/European reference ranges, we observed higher ranges of WBCs, absolute lymphocyte and monocyte counts. Wider ranges were observed in platelet counts in children from Kisumu West compared to the US/Europe ranges. Compared to Harriet Lane Handbook, higher counts were observed in WBC counts, lymphocyte counts and monocyte counts in the study. Wider values were noted in RBC, platelets and RDW, while lower ranges were noted in the current study for hemoglobin, hematocrit and granulocyte counts. Median ALT values in Hepatitis C positive HIV positive ART Naive participants were higher than in the Hepatitis C negative participants ($p=0.0494$). These hematology reference ranges results in the different age categories and in relation to gender underscore the importance of using locally derived hematological reference ranges for proper inclusion and exclusions of participants into clinical trials. The study also underscores the need for monitoring liver enzymes levels in ART naive HIV positive patients as well, screening for viral hepatitis before initiation of Anti-Retro Viral therapy as these diseases pathologically alters the body's immune biomarkers.

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ABBREVIATIONS AND ACRONYMS

Ag/Ab	Antigen/Antibody
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ART	Anti-Retroviral Therapies
AST	Aspartate Transaminase
CD4	cluster of differentiation 4
CFP	Culture Filtrate Protein
DTH	Delayed Type Hypersensitivity
EDTA	Ethylene Diamine Tetra-acetic acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
FR	First Response
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
GCP	Good Clinical Practice
GSK	Glaxo SmithKline Beecham
HAART	Highly Active Anti-Retroviral Therapy
HDSS	Human Demographic Surveillance System
KEMRI	Kenya Medical Research Institute
MCH	Mean Cell Hemoglobin
MCHC	Mean Cell Hemoglobin Concentration
MCV	Mean Cell Volume
MHC	Major Histocompatibility Complex

MPV	Mean Platelet Volume
NRAMP	Natural Resistance-Associated Macrophage Protein
RDT	Rapid Diagnostic Test
RDW	Red Cell Distribution Width
RBC	Red Blood Cell
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
TMB	Tetramethylbenzidine
WBC	White Blood Cell

DEFINITIONS OF TERMS

ART: These are medications that treat HIV. The drugs do not kill or cure the virus. However, when taken in combination they can prevent the growth of the virus. When the virus is slowed down, so is HIV disease. Antiretroviral drugs are referred to as ARV.

ART-Naive: A person is considered to be treatment naive if they have never undergone treatment for a particular illness. In the context of HIV infection, ART naive is used to refer to HIV-positive patients who have never taken any antiretroviral therapy for their infection

CD4: A glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells. CD4 is a co-receptor of the T cell receptor (TCR) and assists the latter in communicating with antigen-presenting cells

Enzyme-linked immune-sorbent assay (ELISA):

This is an immunological technique of target antigen (or antibody) capture in samples using a specific antibody (or antigen), and of target molecule detection/quantitation using an enzyme reaction with its substrate. It is an immunological method used to determine if a certain substance, such as a specific cytokine or antigen, is present within a sample. It is sometimes abbreviated as "EIA."

Good Clinical Practice (GCP):

Good clinical practice is an international quality standard for conducting clinical trials that in some countries is provided by ICH, an international

body that defines a set of standards, which governments can then transpose into regulations for clinical trials involving human subjects.

Healthy children:

Children who had normal physical examination e.g. blood pressure, weight, pulse and vital signs, without acute or chronic, clinically significant pulmonary, cardiovascular, hepatobiliary, gastrointestinal, renal, neurological, mental or hematological functional abnormality or illness that required medical therapy, as determined by medical history, physical examination or clinical assessment before being enrolled into a study.

Highly active antiretroviral therapy (HAART):

Treatment with a very potent drug "cocktail" to suppress the growth of HIV

Immunophenotypic and Hepato-Biomarker Characterization:

Determining the levels of clinical characteristics of liver enzyme markers as well as immunological parameters in an HIV condition. NOTE: In this study, only CD4 immunophenotypes and two hepatic function biomarkers (AST and ALT) were evaluated

Overall age group

In this study, overall age group refers to combined age group of 1 m to 17 m taken together

Rapid diagnostic test (RDT)

Refers to medical diagnostic test that is quick and easy to perform which are suitable for preliminary or emergency medical screening and for use in medical facilities with limited resources. They provide same-day results within two hours, typically in approximately 20 minutes. They also refer to qualitative or semi-quantitative in vitro-diagnostic medical devices, used singly or in a small series, which involve non-automated procedures and have been designed to give a fast result

Reference value:

The value (test result) obtained by the observation or measurement of a particular type of quantity on a reference individual; NOTE: Reference values are obtained from a reference sample group.

Reference limit:

A value derived from the reference distribution and used for descriptive purposes; NOTE: It is common practice to define a reference limit so a stated fraction of the reference values is less than or equal, or greater than or equal, to the respective upper or lower limit; the reference limit is descriptive of the reference values and may be distinguished from various other types of decision limits.

Reference range/interval:

The interval between, and including two reference limits; NOTE: it is designated as the interval of values from the lower reference limit to the upper reference limit.

Developing hematological reference ranges:

This is the process used in creating a reference range de novo, encompassing all of the steps from selection of reference individuals, through exact details of the analytical methods, and concluding with data collection and analysis.

Tetramethylbenzidine (TMB)

This is a chromogenic substrate used in staining procedures in immunohistochemistry as well as being a visualizing reagent used in enzyme-linked immuno-sorbent assays (ELISA). It is a white solid that forms a pale blue-green liquid in solution with ethyl acetate. TMB is degraded by sunlight and by fluorescent lights.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Proper practices in regard to common public health issues more so in developing countries where medical infrastructure is under developed is urgently needed. This calls for availability and proper understanding of appropriate biomarker values that can be readily accessed for monitoring disease progression, therapeutic intervention as well as prognosis that can be applied in treatment and clinical studies for reference purposes. These values are urgently needed in sub-Saharan African that is ravaged by both parasitic diseases such as malaria and schistosomiasis, bacterial diseases as well as viral diseases such as HIV AIDS (Naylor, 2003).

It is therefore prudent to develop reference intervals in line with a more systematic process that factors the various influences on the results that have been measured. The values that are developed provide an essential tool that is really important in management of study participants and influences decision that are finally reached on subject inclusions in studies (Ngowi et al., 2009). These values are essential for appropriate screening of study subjects into clinical trials, evaluating physiological changes in disease conditions after infection or disease states, even when drugs are given to sick people as well as in clinical trials and vaccine studies. The reference values are all those ones that are obtained through observation or measurement on reference people in a defined sample group to ascertain normal physiological ranges of a given population, which have never been established in most African countries (CLSI 2008). This glaring deficiency leads to poor diagnosis and management of many clinical conditions.

Clinical trials are currently being conducted in many parts of Africa, even trials of preventive interventions for infectious diseases including HIV and *Mycobacterium tuberculosis*. Many efforts have been made towards improving the health infrastructure worldwide more so in Africa (Kibaya et al., 2008). However, the hematological reference values that are sometimes used for trial, screening and evaluating adverse events (AE) have often been derived from predominantly North American and European (largely Caucasian) populations (Gitaka et al., 2017). The use of these reference intervals may lead to unwarranted exclusion or inclusion of potential study participants that may not be appropriate (Gitaka et al., 2017).

Among the global health challenges majorly known in many parts of the world are Human Immunodeficiency Virus (HIV) infection and Acquired Immunodeficiency Syndrome (AIDS) (Beyrer and Karim, 2013). Infected subjects are usually susceptible to multiple infections. *Mycobacterium tuberculosis* and viral hepatitis are two of the commonest opportunistic co-infections seen among HIV-positive patients worldwide. The common hepatitis infection in HIV infected subjects is due to hepatitis B (HBV) and Hepatitis C (HCV) (Verucchi et al., 2004). Chronic liver diseases are impacted from the complications associated with Hepatitis B infection as well as Hepatitis C more so in the HIV infected participants as well in immunologically compromised patients. Global HIV prevalence is estimated at 40 million while, chronic Hepatitis B and Hepatitis C diseases result in approximately 370 million, and 130 million respectively. It is well known that infections with HBV and/or HCV have a severe and invasive impact on the health of millions of people around the world, as these infections are often asymptomatic (Wondimeneh et al., 2013).

Reliable information on clinical immunophenotypic biomarkers is critical in the management of these diseases. CD4 cell counts and the level of liver function bio-molecules can be monitored to assess the body status and treatment response to interventions. World Health Organization (WHO) advises that appropriate mechanisms be applied when certain virologic, immunologic, or clinical features are observed (Ekouevi et al., 2014). For instance, the WHO recommends initiation of highly active anti-retroviral therapy in HIV individuals who are also infected with Hepatitis B or Hepatitis C regardless of their levels of CD4 cells. The implication in HIV patients who have CD4 counts above 350cells/µl but who may be positive for hepatitis B and/or hepatitis C is not factored in the treatment regimen of Highly Active Anti-Retroviral Therapy (HAART) until they get to end-stage liver damage (National Institutes of Health, 1990). Therefore it is needful to develop reference intervals for liver enzymes to provide a guideline in monitoring of patients who may be prone to liver toxicity.

The WHO estimates that nearly about one third of the world's population is infected with *Mycobacterium tuberculosis* and about nine million new infections being reported annually. Globally, an estimated 13% of TB patients are co-infected with HIV (Naidoo et al., 2013).

Although data on the epidemiology of these infections in the general population in Kenya exists, there is limited information on Clinical biomarkers and immuno-phenotypic characteristics that may be putative in their management (Denue et al., 2013). There is relatively scanty information regarding liver enzyme levels (hepatic biomarker characteristics) and immuno-phenotypic characteristics among ART naive HIV positive adults with or without hepatitis B and C. Proper determination of the levels of clinical

biomarker analytes in HIV positive ART naive adults with or without Hepatitis B and C is important in clinical management.

There is limited data on what has been done to determine the level of ALT and AST clinical markers and CD4 immunophenotypes in the vulnerable population within Kisumu West sub-county. As well, hematological reference values for Kisumu West Sub-County have not been established before. The values that are currently used in the country are adopted from textbooks and some analyzer manufacture specifications (specifically the coulter ACT 5 diff analyzers). In daily practice, it is important to have hematological reference values for proper patient management. However, there are some discrepancies from one study to another, which may be related to different factors such as age, sex, geographic origin, altitude, and ethnic origin (Kibaya et al., 2008). Several multicenter trials have increased the need for regional and locally established reference values. Reference values may differ significantly by different populations, geographic regions, climate, and race and due to other factors such as gender, age, genetical factors as well as dietary patterns. For example, red blood cell indices are influenced by several factors especially the abnormalities of hemoglobin synthesis, which could be constitutional (sickle cell disease, alpha- and Beta-thalassemia) or acquired (iron deficiency) (Romeo et al., 2009, León-Velarde et al., 2000). For such reasons, using normal laboratory values that have been derived from other population can lead to selection bias causing healthy participants be excluded from clinical/vaccine trials. This also again results in other adverse events to become misclassified, also may lead to improper management of participant when they are unwell. The main objective of this study was to develop hematological reference ranges in healthy, HIV negative, children between 1 month and 17

months, and to characterize CD4 immunophenotypes and ALT and AST clinical biomarkers levels in ART Naive HIV positive adults from Kisumu West Sub-county so that overall patient care, participation of individuals in clinical trials and evaluation of adverse events can be improved.

1.2 Statement of the Problem

Appropriate management of diseases such as HIV/AIDS, tuberculosis and hepatitis requires well-delineated biomarker and immuno-phenotypic characterization of all infected subjects. The differences in regard to the CD4 immunological phenotypes have not been studied in the viral hepatitis positive and negative groups of HIV infected persons. In most rural populations living in resource-limited settings like Kisumu West Sub-County, many of the subjects are initiated on HAART therapy without any clinical background checks jeopardizing the effectiveness of the anti-retroviral therapy as well as exposing them to possible liver damage. Furthermore, clinical studies are increasingly conducted in Africa, which include trials of preventive interventions for HIV, *Mycobacterium tuberculosis* and malaria. For such reasons, the use of normal laboratory values derived from external populations could produce selection bias leading to exclusions of otherwise healthy volunteers in clinical trials, misclassification of adverse events, and a framework for allowing incorrect patient management in routine clinical care.

1.3 Objectives of the Study

1.3.1 Main Objective

To develop laboratory hematological reference values from healthy, HIV negative children below two years and to characterize CD4 immunological phenotypes and ALT and AST

liver function bio-markers in HIV positive ART naive adults from Kisumu West Sub-county.

1.3.2 Specific objectives

1. To develop laboratory hematological reference values from healthy, HIV negative children below two years from Kisumu West Sub-county.
2. To determine the levels of AST and ALT biomarkers in HIV positive ART naïve adults with or without Hepatitis B and C
3. To determine the levels of CD4 immunophenotypes in HIV positive ART naïve adults co-infected or not with hepatitis B or C
4. To examine the relationship between the immunological and liver function clinical biomarkers in HIV positive ART naive adults co-infected or not with hepatitis B or C

1.4 Research Questions

1. What are the ranges of haematology reference values from healthy, HIV negative children below two years from Kisumu West Sub-county?
2. What are the levels of AST and ALT markers in HIV positive ART naïve adults co-infected or not with Hepatitis B or C What are the levels of CD4 in HIV positive ART naïve adults co-infected or not with hepatitis B or C
3. What is the relationship between immune-phenotypic and liver function markers in HIV positive ART naive adults co-infected or not with hepatitis B or C

1.5 Justification of the study

For proper therapeutic management of patients, and due to the fact that clinical trials are increasingly performed in different parts of Africa, it is necessary to have well established local reference values that can properly be applied in inclusions and exclusions of the participants in studies as well as managing severe adverse events. Hepatitis B and C infections are among the rapidly spreading and most common infectious diseases especially in the HIV positive individuals. Studying the CD4 immunophenotypic characteristics as well as ALT and AST hepatic bio-markers in ART Naive HIV positive subjects is critical. This will help in providing valuable information to health care workers. The CD4 immunological phenotypes and liver function hepatic markers when properly monitored in the HIV infected adults will generate some relevant information that is useful on effective therapeutic strategies.

1.6 Significance of the study

Results generated from this study are useful in assisting health providers to design better strategies of HIV management in HIV ART subjects. These findings could also underscore the importance of screening all HIV positive individuals before initiating antiretroviral treatment. Determination of the CD4 immunophenotype levels in both hepatitis B and C positive and negative groups of adults is important since it leads to better management. Additionally, characterizing and understanding the phenotypic as well as the hepato-biomarker characteristics can be useful in this period of research for HIV vaccines. Reference values vary considerably in different populations, geographic regions, climate, and race and due to other factors e.g. gender, age, genetical factors as well as dietary patterns. For example, RBC parameters and probably other immunophenotypes could be

influenced by several factors especially the abnormalities of hemoglobin synthesis, which could be constitutional (sickle cell disease, alpha- and Beta-thalassemia) or acquired (iron deficiency). For such reasons, the use of normal laboratory values derived from other populations could produce selection bias leading to exclusions of otherwise healthy volunteers in clinical studies, misclassification of adverse events, and a framework for allowing incorrect patient management in routine clinical care underpinning the critical significance of this study.

1.7 Scope of the Study

This study, conducted in Kisumu West, was based on HIV positive ART naive adults and healthy children below two years whose samples were collected following informed consent. The study utilized analytical cross sectional and laboratory investigation design where laboratory assays as ELISAs for Hepatitis B, Hepatitis C, CD4 tests, hematology as well as serum ALT and AST biochemistry tests were carried out using the various laboratory tests standard operating procedures. Only the selected CD4 immunophenotype and the hepatic markers (ALT and AST) were used from a panel of many markers due to the fact that approval was only given for use of these markers and due to the fact that they are the only ones tested in larger study. The relationship between these parameters with gender and age of the study subjects were also determined.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Need for Hematological Reference Ranges from Children in Kisumu West Sub-County

Proper diagnosis and evaluation of patients and clinical study participants as well as categorization of adverse events when clinical trials are conducted calls for well-established hematological reference values. Moreover, these reference values are necessary when studies are conducted in a given area as they inform the researchers on the right study participants to be included or excluded in the clinical trials and studies based on the predetermined criterion. Hematological reference values for Kenyan pediatric populations in Kisumu West for that matter have never been established. The values that are currently used in the country are adopted from textbooks and some analyzer manufacturer specifications. These are bound to be at variations from the prevailing populations and will exhibit disparities as these values are drawn from elsewhere. These values may differ among different geographical locations due to differences in factors amongst them gender, age locations, nutritional status, genetical factors, prevailing diseases and altitude (Kibaya et al., 2008).

Kisumu West is a Sub-County in Kisumu County, Kenya, with an altitude below 1000 meters. Until now, hematological reference values for children have not been established in the area. The values usually used are those of textbooks and some analyzer manufacture specifications e.g. Act5 diff hematology analyzer ranges. There is need to have reference ranges with values as specific to different age categories in the area. This is because the types of pediatric studies mostly conducted in the area are normally varied as far as age

groups of study participants are concerned and thus an appropriate platform for different age groups is necessary. Wider reference ranges could be available in some other areas but age specific reference ranges are required in the area.

2.2 Biomarker Characterization in HIV ART Naive people

Mycobacterium tuberculosis together with Human Immuno-deficiency Virus are among the leading global infectious killers with approximated 1.4 million deaths from TB. This doesn't include those with HIV. Another about 0.4 million cases of deaths result from those who are co-infected with HIV and TB (WHO, 2016). HIV still remains the most potent risk factor for tuberculosis prevalence being one of the factors raising TB incidences especially in the Sub-Saharan Africa from late 19th century (WHO, 2016). Around 11% of the 10.4 million incident TB cases globally and around 20% of total global mortality from TB are estimated to be in people living with HIV (PLHIV) TB is implicated in approximately 36% of deaths from HIV.

Statistics that are collected the Kenyan national HIV/AIDS body show that the prevalence of co-infections in Kisumu West Sub-County is high and likely to be underestimated. For example, tuberculosis rates in Kisumu West Sub-County are higher than the national average. Proper management of infected and affected subjects call for well-characterized immune biomarkers that improve treatment outcomes and this improves quality of life. The only biomarkers that are mostly utilized by many health personnel is the HIV viral loads as well as the CD4 T cell counts. It thus becomes paramount to establish physiological status relating to clinical characteristics and immunophenotypes in both *Mycobacterium tuberculosis* infected and non-infected individuals who are not on ART for proper patient care. This is more so important based on the fact that the area has a high prevalence rate of

HIV infection. This study in Kisumu West sought to address a group of HIV positive ART Naive individuals to determine the patterns of the immunophenotypes and clinical biomarker characteristics specifically ALT and AST liver enzyme markers in those with or without hepatitis B and hepatitis C. Many of the pharmaceutical products that are used in management of HIV have toxic adverse effects especially to the liver hence the need to understand the herpetological landscape before and during ART treatment.

2.3 The Relationship Between levels of Alanine Transaminase and Aspartate Transaminase Biomarkers and CD4 T cell counts in HIV individuals with or without Hepatitis infection

There are similarities in the transmission routes and risk factors of HIV, and the Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection (Harania et al., 2008), in spite of the fact that each has a different biology and natural history. The prevalence rates of Hepatitis B and C in HIV-infected individuals has been reported to be higher than that in the general population (Lesi et al., 2007). Hepatitis B and C infections are emerging as important causes of liver-related deaths in patients on antiretroviral therapy (ART). Therefore, with faster liver disease progression in HIV patients with these infections, declining risk of AIDS-related opportunistic infections and increased life expectancy seen in these patients, the Hepatic Biomarkers and CD4 counts should be closely monitored (Verucchi et al., 2004). Consequently, there has been increasing focus on diagnosis of HBV and HCV in the management of HIV-infected patients. Yet compliance with hepatitis screening in HIV patients prior to initiation of ART is usually poor in resource-limited settings, and the impact of HBV and HCV co-infection on ART outcomes needs to be understood (Harania et al., 2008).

Co−infection rates of HBV and HCV in HIV patients differ in different parts of the world and again depend upon the geographical location, risk groups, the type of exposure that is involved and the socio-economic condition of that particular region (Olawumi et al., 2015). In Europe and the United States of America, HIV/HBV co‑infection is around 6–14%. In India, there are only few reports of the prevalence of HBV in HIV‑infected patients. In Africa, there is lack of a well-established registry of such diseases in many countries. Hepatitis virus infection is a major cause of chronic liver diseases including cirrhosis and hepatocellular carcinoma worldwide (Wang et al., 2014). The progression of hepatic complications from Hepatitis infection is accelerated in patients co-infected with HIV. This is particularly so in HIV infected men with very low CD4 count (Wang et al., 2014, Akinbami et al., 2012). Besides, people who are infected with HIV are more likely to lose previously developed antibodies that are protective against HBs and may develop acute hepatitis B infection. This kind of risk is also associated with CD4 levels that are low counts (Olawumi et al., 2015). The risk of hepatocellular carcinoma (HCC) in HIV infected patients who are co-infected with hepatitis B virus is also elevated in individuals with lower CD4 counts (Clifford et al., 2008). Hepatic toxicity is a common complication in HIV-infected patients on Highly Active Antiretroviral Therapy (HAART). In a study done by (Olawumi et al., 2015), on the effect of Hepatitis B Virus Co-infection on CD4 Cell count and liver function of HIV infected patients, it showed that CD4 count of the HIV mono-infected patients was significantly higher than that of the patients co-infected with HBV. In that study, the mean serum level of ALT, AST and ALP of patients with CD4 count below 200/µl were significantly higher than in those with CD4 count ≥ 200/µl. In a prospective longitudinal cohort study with subjects recruited from US, Australia and

Thailand, it was found that the prevalence of hepatotoxicity at baseline was about 13% and that CD4 count < 200 cells/mm^3 and HBV DNA > 2000 IU/m (Olawumi et al., 2015). These were significantly related to increased risk of significant hepatotoxicity among HIV/HBV co-infected people on long-term HAART (Olawumi et al., 2015).

2.4 Alanine Transaminase and Aspartate Transaminase Liver biomarkers and their importance in HIV infected individuals with Hepatitis infections

Any elevation in hepatic enzyme level could be due to a liver problem, and aspartate transaminase (AST) and alanine transaminase (ALT) are two of the enzymes central to such an investigation. When used comparatively, these enzymes can help identify liver toxicity, liver disease, or liver damage. Hepatic enzyme elevations are common in HIV-infected patients, especially those treated with ART. Despite such reports, HIV-infected patients may present several risk factors for biochemical abnormalities, and a precise etiology is rarely defined clearly (Pol et al., 2004). In the longitudinal cohort study by (Olawumi et al., 2015), the serum levels of ALT and AST among co-infected patients respectively were significantly raised among male patients compared to female patients. This difference was much significant among the co-infected patients, alluding to the fact that HIV infection accelerated the progression to hepatic complications in HBV infected men (Olawumi et al., 2015). In Kisumu West Sub-County like many other regions in Kenya, information relating to clinical biomarker characteristics and immunophenotypes in study participants with HBV-HIV and/or HCV-HIV co-infected compared to the HIV ART Naive individuals without Hepatitis infection is not delineated. This study sought to characterize immunophenotypes and hepatic biomarkers on HIV infected ART Naive adults with or without Hepatitis B, C and MTB and also investigated the relationship

between the levels of the clinical biomarkers in and how this relate to disease progression and outcomes. The unique strength of the current study was the focus on HIV positive individuals category not already on ART, which is sometimes a group of HIV people normally overlooked in many studies. While most previous studies have been majorly on people on highly active ant-retroviral therapy, this uniquely study in ART Naive HIV positive adults sought to establish the patterns of the levels of the immunophenotypes and clinical biomarker characteristics in HIV positive ART naive adults in Kisumu West sub county. Despite the small number of Hepatitis B and C positive cases reported in this study which makes it inordinately impossible to draw proper conclusion partly due to the low prevalence of Hepatitis B and C, the information gathered provided valuable data in management of HIV infection.

2.5 The relationship between the Immune Phenotypic and Hepatic biomarkers in HIV Disease Status

Many HIV infected patients show elevated levels of liver enzymes such as AST and ALT (Vogel and Rockstroh, 2007). The increase in hepatic enzymes could be arising due to the fact of multiple factors such as alcoholism, lipid lowering drugs, co-infection with hepatitis viruses, or hereditary diseases. Additionally, it has been proposed that HIV causes a direct damage to hepatic cells (Vogel and Rockstroh, 2007). Several factors are associated with hepatic damage: antiretroviral treatment, co-infections with hepatitis B or C virus, opportunistic infections as *Cytomegalovirus, Mycobacterium, Leishmaniasis*, or tumors (lymphoma and Kaposi's sarcoma), cholangitis associated to parasites (Cryptosporidiosis and Microsporidiosis) and toxicity related with non-antiretroviral drugs for example trimetoprim and other antibiotics (Joy et al., 2019).

Abnormalities in liver function tests could be exercebated exclusively by direct inflammation in hepatocytes, caused by the virus. Mechanisms by which HIV causes

hepatic damage are still unknown, but the most important mechanisms could be apoptosis (induced by caspases 2, 7 and 8) and mitochondrial dysfunction with decreasing in mitochondrial DNA in several tissues; another injury mechanism is permeability alteration in mitochondrial membrane by HIV proteins which stimulate an inflammatory response (Guaraldi et al., 2008). AST and ALT are hepatic enzymes that could be used as markers of hepatocellular injury (Zechini et al., 2004).

In a study by (Mata-Marín et al., 2009) that determine the correlation between HIV viral load and serum levels of AST and ALT as markers of hepatic damage in HIV ART naive patients, it was noted that there was a mild positive correlation of ALT and AST with Viral load levels in HIV positive subjects. In the Jose study, it was observed that HIV viral loads, positively correlated with AST levels. The study also showed a significant strong, positive correlation between HIV viral load and ALT (Mata-Marín et al., 2009).

From the foregoing it is thus obvious that liver function tests should be monitored closely in HIV positive patients. It is important that ART therapy should be initiated in HIV infected patients with severe liver damage regardless of CD4+ cell count. The current study, evaluated 57 HIV positive ART Naive adults with an objective of understanding the patterns in the relationship between CD4 immunophenotypic landscapes and clinical biomarker characteristics specifically the liver enzymes.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

This study was conducted in Kisumu West Sub-County, Kisumu County of Western Kenya through a system referred to as Kombewa Health and Demographic Surveillance System (HDSS) system. The Kombewa HDSS covers an area of approximately 369km^2 area with a population of over 150,000 people and is a system that ensures that study participants are from the required area of Kisumu West Sub-County (Sifuna et al., 2014). This area is located 40 kilometers (km) west of the provincial capital of Kisumu bordering Lake Victoria. Kisumu County has an estimated HIV prevalence of 19%, notably higher than the provincial rate of 15.1% and national rate of 5.6 *(Kenya NACCo. 2014).* The majority of residents in the study area live on less than US$ 1 per day. Small scale trading, subsistence farming, animal husbandry and fishery are the leading sources of income. Farming, mostly of food crops, is done during the two rainy seasons; the "long rainy season" between March and May and the "short rainy season" between October and December. Fishing is done mostly by the Luos living along Lake Victoria. This fisher community and others on the edges and islands of Lake Victoria in Kenya, Uganda, and Tanzania have been of particular interest in the HIV field as they are very mobile, have relatively high levels of alcohol and drug abuse, high-risk sexual behaviors and consequently high HIV incidence. An entire unskilled economy is built around the fishing industry in this region. Fishermen, fish processers, fish traders, and barmaids all interact in a tightly woven, complex sexual network that bridge to the general population. HIV prevalence and incidence estimates among the fishing communities around the lake have been in excess of 30% and 5/100 person-years, respectively.

HIV prevalence in and around Kisumu, including Kombewa, is among the highest in Kenya with an estimated overall prevalence of 15.1% in adults. HIV testing surveys among specific population subsets report an HIV prevalence in the range of 30%-60% (Waruiru et al., 2014). Coupled with these factors and disease prevalence rates, and due to being a malaria holo-endemic, make it an appropriate site in conducting both clinical vaccine and drug trials.

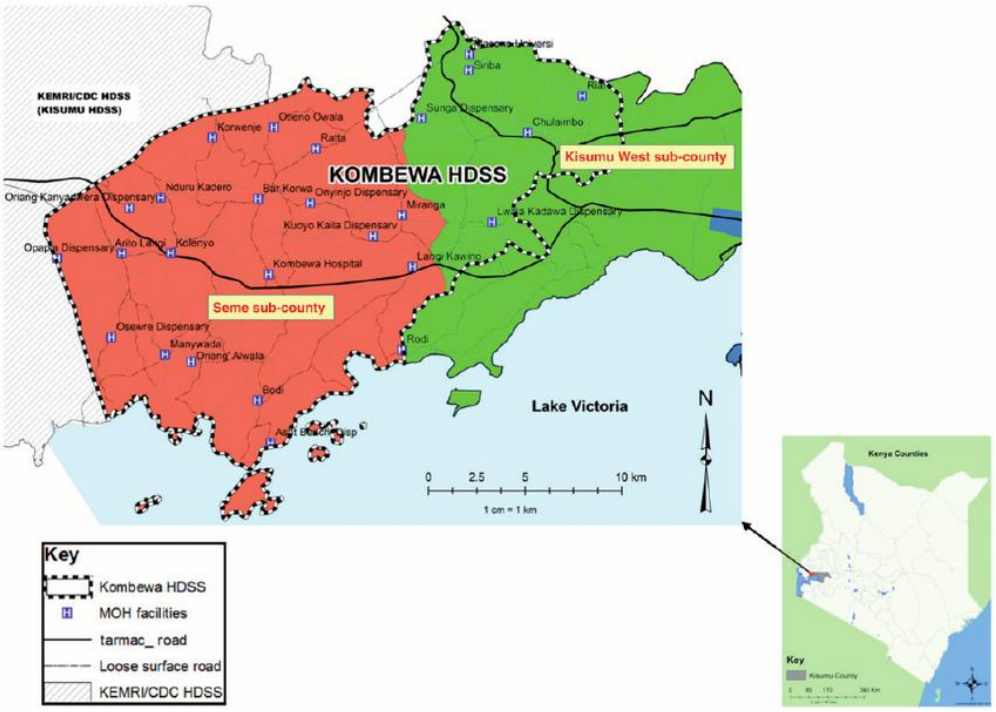


Figure 3. 1: Map of the study area, Kisumu West Sub-County

3.2. Research Design

The study was a cross sectional study with a laboratory investigation component.

3.3. Study Population

This study comprised of HIV positive ART Naive adults aged 18 years and above who were enrolled in a larger cohort study to assess the impact of clinical practices, biological factors and socio-behavioral issues on HIV infection and disease progression in an African context as well as healthy HIV negative children aged below two years of age. The

children were those who participated in a malaria vaccine clinical trial conducted under the auspices of Walter Reed Project/KEMRI. The children were enrolled between August 2009 and Jan 2014, in a phase III, Double Blind, randomized, GSK-sponsored, Vaccine Clinical Trial, while the adults were enrolled in an ongoing observational study that started in Oct 2013, and thus the data for these study participants based on the inclusion criteria was used. The actual number of children that were enrolled in this population was 1509 while the adults that were enrolled was 57.

3.4. Inclusion and Exclusion Criteria

3.4.1. Inclusion Criteria

The following inclusion criteria were applied in the current study for the immunophenotypes category of the study:

ART naive, HIV positive adults who were aged 18 years or older

Individuals who provided written informed consent for participation in the study.

For the reference ranges category, the following Inclusion criteria was used

Data from children aged 2 years and below

Baseline (screening) laboratory generated data from children whose parents/guardian signed informed consent or thumb-printed and witnessed informed consent obtained from the parent(s)/guardian(s) of the child.

Data from children who had normal physical examination e.g. blood pressure, weight, pulse and vital signs

Data generated from healthy subjects- without acute or chronic, clinically significant pulmonary, cardiovascular, hepatobiliary, gastrointestinal, renal, neurological, mental or hematological functional abnormality or illness that required medical therapy, as

determined by medical history, physical examination or clinical assessment before being enrolled into the study.

3.4.2. Exclusion Criteria

The following exclusion criteria were applied in the current study:

Adults who were HIV Negative

HIV positive adults who were on ART and individuals who did not provide written informed consent.

Data from children, who had recurrent infection, fever, including HIV and malaria before enrollment in the major study.

Data from children who had severe anemia, defined as hemoglobin level of <5.0g/dl or hemoglobin concentration of <8.0g/dl associated with clinical signs of heart failure and/or severe respiratory distress

Data from subjects who were receiving medical treatment at the time of sample collection

3.5. Sample Size Calculation

A sample size of 1566 was used in this study. As required by CLSI standard the minimum samples size required for reference range analysis is 120 for parameters not influenced by gender and 240 for parameters that are influenced by gender. The more the sample size the better the power and thus the results. The sample size for the reference range study was 1509. To obtain a sample size for data required for the immunophenotypes and clinical characteristics, Cochran's formula of sample size calculation was used as below (Ahmad and Halim, 2017).

$$n_o = \frac{Z^2 pq}{e^2}$$

Where

n_o= Sample size

Z= is the abbisca of normal distribution (Z=1.96)

P= Proportion of the population with the characteristic

In this case P (Proportion of the people with the characteristic) refers to the prevalence of the HIV positive population who are ART naive). In this case it is less than 3% for the HIV naive population.

q=1-P (Proportion without the characteristic) i.e. those individuals who are not ART naive

e= error or level of precision (e=0.05)

Inputting these figures gives a sample size **45**. For this, data for 57 HIV positive adults was used for the immunophenotypes and clinical biomarker characterization category of the study

3.6. Sampling design

The total sample size used for analysis for the study was 1566 study participants although 1688 participants had been recruited. The study was structured into two categories: one category investigated hematological values from healthy children aged 1 month to 17months, while the other category investigated immunophenotypic and clinical biomarker characterization in ART Naive HIV positive adults above 18 years of age from Kisumu West Sub-County. For the hematological reference ranges population, the study had data set of 1631 participants (n=1631) of different age groups. Data for 122 (6.95%) participants were excluded due to various reasons. Data from 2 (0.11%) participants were excluded because they were known sicklers, 10

(0.57%) participants were excluded because they were found to have fever with or without malaria during screening. 101 (5.76%) participants were excluded as they were HIV exposed and 9 (0.51%) were excluded because they had other acute illnesses during screening. Figure 3.2 shows the flow diagram on recruitment of study participants for the reference range arm of the study.

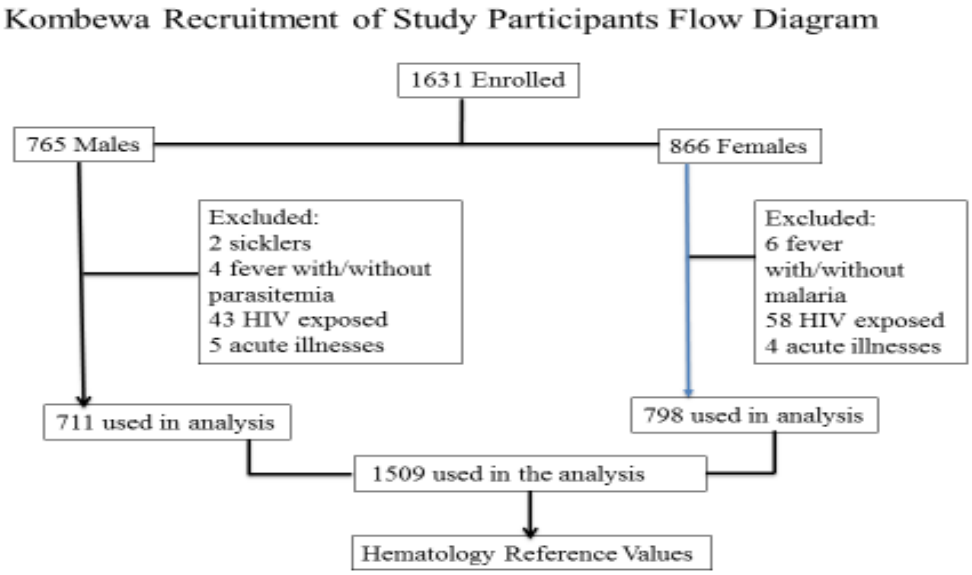


Figure 3. 2: Flow diagram on recruitment of study participants for the reference range arm of the study

The other category of the study investigated 57 (n=57) adult study participants from Kisumu West Sub-County who were ART Naive HIV positive with or without Hepatitis B and Hepatitis C.

3.7. Methods of data collection

3.7.1. Clinical information

Participants who signed informed consent had their vital signs taken that included blood pressure, pulse, heart rate and temperatures. Physical examination was also done and this was recorded down in the participant folder for records.

3.7.2. Laboratory procedures

3.7.2.1. HIV testing of the study participants

The study participants were screened for HIV infection using the Determine HIV Ag/Ab Combo RDT (Abbott Diagnostics Medical Co., Matsudo, Japan), which detects both HIV antibody and antigen following informed consent. Reactive screening results were further confirmed by First Response HIV 1-2-0 (FR) RDT, (Premier Medical Corporation Ltd., Daman, India) in accordance with the Kenyan Ministry of Health Guidelines for HIV diagnosis.

3.7.2.2. Sample collection and processing procedures

Blood samples were collected by trained phlebotomist, by use of venipuncture procedures using vacutainer® blood collection tubes (Beckton Dickinson, Plymouth, United Kingdom) from HIV positive adults who provided a written consent. Blood samples for the Hepatitis B, C and biochemical analysis were collected into 10 ml vacutainer tubes (Becton Dickson, New Jersey USA). Blood samples for CD4 analyses were collected in EDTA anticoagulated tube. Serum samples that were used for the ELISA serological assays for hepatitis B and C markers were stored at −20°C until time to analyze these. Whole blood samples from children whose parents/guardians provided informed consent

were collected in 0.5ml micro-tainer tubes containing ethylene diamine tetra acetic acid (EDTA) (Becton Dickinson, Franklin Lakes, NJ), either through capillary or venous draw.

3.7.2.3. Hematologic analysis for reference range

For the reference ranges, hematology processing and analysis were done within 24 hours of specimen collection. Prior to use, the hematology instruments were validated on site using proper validation procedures. A three-Part differential coulter counter hematology analyzer (Beckman Coulter, Paris, France) was used for the analysis. The analyzer automatically gave printout results bearing absolute numbers of leukocytes/White Blood Cells (WBC) (10^3/µl), erythrocytes/Red Blood Cells (RBC) (10^6/µl), hemoglobin (g/dL), hematocrit (%), Mean Cell Volume (MCV) (fl), Mean Cell Hemoglobin (MCH) (pg), Mean Cell Hemoglobin Concentration (MCHC) (g/dL), platelets (10^3/µl), Granulocytes (10^3/µl), lymphocytes (10^3/µl), and monocytic cells (10^3/µl), Red Cell Distribution Width (RDW) and Mean Platelet Volume (MPV).

3.7.2.4. Diagnosis of Hepatitis B Surface Antigen using ELISA

Hepatitis B Surface Antigen (HBsAg) was tested using HBsAg 3.0 ELISA kit (Bio-Rad, Johannesburg, South Africa). The procedure for Hepatitis B Surface antigen ELISA summarily involved addition of 100µl of the kit controls and/or patient specimens to the appropriate wells of the microwell plate. Two Positive Controls, two Low Positive Controls, and three Negative Controls were assayed on each plate. The plate was then covered with a plate sealer to minimize evaporation and then incubated for 60 to 65 minutes at 37±1°C using a dry-heat static incubator. At the end of the incubation period, the plate cover was removed and the fluid aspirated from each well into a biohazard

container. The plate was then washed a minimum of five times with a Wash Solution (at least 400 µl/well/wash), with a soaking duration of 30 to 60 seconds between each wash. The wash solution was aspirated after each wash and after the last wash, the inverted plate was blotted on clean, absorbent paper towels. 100µl of Working Conjugate Solution was then added to each well containing a specimen or control. The plate was then covered with a plate sealer to minimize evaporation and then incubated for 60 to 65 minutes at 37±1°C using a dry-heat static incubator. At the end of the incubation period, the plate was then washed as in the steps above. 100µl of the Working Tetramethylbenzidine (TMB) Solution was then added to each well containing a specimen or control and incubated for 30 to 33 minutes at room temperature (15-30°C) in the dark. 100µl of Stopping Solution was then added to each well to terminate the reaction and then the results of the absorbance read within 30 minutes after adding the Stopping Solution, using the 450 nm filter with 615 nm to 630 nm as the reference and results evaluated for acceptability (Appendix 5 shows a full procedure for the ELISA test for Hepatitis B Surface Antigen).

3.7.2.5. Diagnosis of anti-hepatitis C antibody using ELISA

Anti-hepatitis C antibodies were tested using Ortho® HCV3.0 ELISA test kit (Ortho-Clinical Diagnostics Inc, Raritan, NJ) in accordance with manufacturer's recommendations. ELISA procedure for determination of anti-HCV antibodies summarily involved addition of 200ul of specimen diluents to all wells. 20ul of the kit controls, kit calibrators and/or patient specimens were then added to the relevant wells. The microwell strip holder was then covered with a plate sealer and incubated at 37 degrees C±1C for 60min±5min. The sample solutions were then aspirated from the microwells then filled completely with wash buffer and washed for 5 times. The plate was inverted and firmly

tapped on a clean paper towel to remove excess wash buffer, if necessary. 200ul of conjugate was then added to all wells and then the micro-well strip holder was covered with a new plate sealer and then incubated at 37 degrees C for 60min±5min. The plate was then washed as above. The substrate solution, that contains OPD tablets, was then added to the plate and then incubated at room temperature in the dark for 30min±1min. 50ul of 4N sulfuric acid was then added to all wells and the plate was read at a wavelength of 490nm with a reference wavelength of 620nm or 630nm within 60min following the addition of sulfuric acid. The results were then evaluated for acceptability (Appendix 6 shows a full procedure for the ELISA test for anti-Hepatitis C antibody).

3.7.2.6. Evaluation of serum ALT and AST Liver Function markers

Serum ALT and AST biochemistry levels were assessed the same day of blood collection by use of cobas® C311 biochemistry analyzer (Roche, Mannheim, Germany). The serum biochemistry tests were evaluated as per the instrument manufacturer instructions to determine the serum levels for the liver Biomarker characteristics.

3.7.2.7. Evaluation of serum CD4 immunophenotypes

CD4 determinations were done by use of FacsCount flow cytometer analyzer. These samples were analyzed using whole blood within 24 hours of blood collection as per the manufacture instructions.

The HBV results, HCV results, CD4, ALT, AST results as well as age and gender were recorded in a data collection sheet, (Appendix 7).

3.8. Statistical Data Analysis

For the reference ranges, the Clinical Laboratory Standards Institute (CLSI) recommends a sample size of at least 120 values for non-parametric reference intervals and for parameters not influenced by gender and 240 for parameters that are influenced by gender (CLSI, 2008). This study had data for sample size of 1509 children for the reference range category that were used to derive the ranges for the different age categories stated. The extracted data was entered and organized into an excel spreadsheet and verified by the principal investigator as per the inclusion and exclusion criteria and kept for analysis. Graphpad V5 software was used to analyze the data using non-parametric tests and both descriptive and inferential statistics were used in the analysis. Non-parametric methods were used as the data was verified to be not normally distributed. Shapiro-Wilk and Kolmogorov Smirnov test of normality were used in evaluating the data distribution and verified as not normally distributed thus non-parametric tests were used.

Rationale for using non-parametric tests

Besides carrying out normality test, as per CLSI guideline in establishing this, the following is a direct quotation:

> ‘’*The working group recognizes the reality that, in practice, very few laboratories perform their own reference interval studies. As indicated in this document, the working group endorses its previous recommendation that the* ***best*** *means to* ***establish*** *a reference interval is to collect samples from a sufficient number of qualified reference individuals to yield a minimum of 120 samples for analysis, by nonparametric means, for each partition (e.g. sex, age range)’’ (*CLSI, 2008). *In cases where parametric methods have to be used, outlying values should*

be removed. However this often leads to biasness and thus nonparametric tests would be highly preferable. (CLSI, 2008).

The nonparametric method of estimation of the reference interval is again strongly recommended as the preferred method for analysis because of its simplicity and reliability. More importantly, this method requires no specific assumption about the mathematical form of the probability distribution of reference values. For the nonparametric method, a minimum of 120 reference values is recommended for each reference population or sub-class. This is the smallest number of samples that allows the determination of a 90%CI around the reference limits (e.g. the 2.5th and 97.5th percentiles). Greater confidence or improved precision in an estimated 95% reference interval can be accomplished using a larger samples of reference individuals (CLSI, 2008).

From other published work on this topic non-parametric methods have been used.

To establish hematological reference ranges in children, data from a total of 1509 healthy children were used in the study. The children were stratified according to gender and age groups with a class interval of 6 months. Spearman's rank correlations were used in bi-variate analysis Mann Whitney test was used in comparison of medians in two groups. To establish the differences in the median values for various hematological indices due to gender, median values were compared across the study groups using Mann-Whitney U tests. To establish the differences in the median values of hematological parameters across different age groups, median values were compared among the different age categories using Kruskall Wallis test. A two-sided p value of <0.05 was considered significant. Reference intervals were considered the central 95% interval of 2.5th and 97.5th percentile as per the CLSI guidelines (CLSI, 2008).

For the immunophenotype and bio-marker characteristics, to investigate if hepatitis B and C infections affected ALT and AST liver markers in adults from Kisumu West, the levels of AST and ALT were compared in the HIV positive ART Naive adults. To investigate the relationship between CD4 levels and serum levels of ALT and AST spearman's correlation analysis was used.

3.9. Ethical Considerations

Ethical issues regarding the researcher, the participants and the research process were taken into consideration to protect the integrity of the researcher and to ensure honest results. Data was actually collected to avoid fraudulence. Privacy and confidentiality of the information and data obtained from the participants were maintained by concealing their identity. The researcher requested an approval for the use of the data for this sub-study from the sponsor's protocol chair and the study Principal Investigator. A sub study template for this was documented showing this permission. The larger study had been approved by the appropriate regulatory authorities as the KEMRI and WRAIR IRBs. (Appendices 8 and 9). For the reference ranges, approval from the study sponsor, the Malaria Vaccine Initiative, was sought and granted before the secondary study was carried out (Appendix 3). The protocol for reference ranges study was written and successfully defended at the KEMRI Scientific and Ethics Review Unit (SERU) before execution of the study.

CHAPTER FOUR

RESULTS

4.1. Socio-demographic characteristics of the study population

Table 4. 1: Demographic Characteristics of study participants in the reference ranges arm of the study.

Age groups	Males (%)	Females (%)	Total (%)
1 m to 6 m	362 (24)	394 (26)	756 (50.1)
6 m to 12 m	200 (13)	187 (12)	387 (25.6)
12 m to 17 m	149 (10)	217 (14)	366 (24.3)
Total	711 (47.1)	798 (52.9)	1509 (100)

Reference range data presented as summary for the different age categories based on gender. Total number represented as raw scores with percentages in parenthesis.

Table 4. 2: Socio-demographic characteristics of the study participants in the immunophenotypic arm of the study

Characteristics	Gender		Total (%)
Age bracket (years)			
	Males n (%)	**Females n (%)**	
21 to 30	7 (26.9)	19 (73.1)	26 (45.6)
31 to 40	12 (54.5)	10 (45.5)	22 (38.6)
41 to 50	2 (33.3)	4 (67.7)	6 (10.5)
51 to 60	1 (33.3)	2 (67.7)	3 (5.3)
Marital status			
Single	8 (32)	17 (68)	25 (43.9)
Married	9 (64.3)	5 (35.7)	14 (24.6)
Divorced	2 (67.7)	1 (33.3)	3 (5.3)
Widowed	3 (60)	2 (40)	5 (8.8)
Religion			
Christian	22 (39.3)	34 (60.7)	56 (98.2)
Muslims	0 (0.0)	1 (100.0)	1 (1.8)
Tribe/ethnicity			
Luo	21 (38.2)	34 (61.8)	55 (96.5)
Luhya	1 (50.0)	1 (50.0)	2 (3.5)
Other	0 (0.0)	0 (0.0)	0 (0.0)
Education			
College and above	5 (45.5)	6 (54.5)	11 (19.3)
High school	6 (33.3)	12 (67.7)	18 (31.6)
Primary school	7 (43.8)	9 (56.2)	16 (28.1)
Illiterate	4 (33.3)	8 (67.7)	12 (21.0)

Socio-geodemographic characteristics of participants in immunophenotype arm of the study showing age, marital status, religion and tribe. The percentages are shown in the parenthesis.

The age and the gender of the participants included were recorded in an excel sheet. Of the 1509 children that were included for the reference range category, 756 (50.1%) were from the age bracket 1m to 6m, 387 (25.6%) were from 6m to 12m and 366 (24.3%) were from

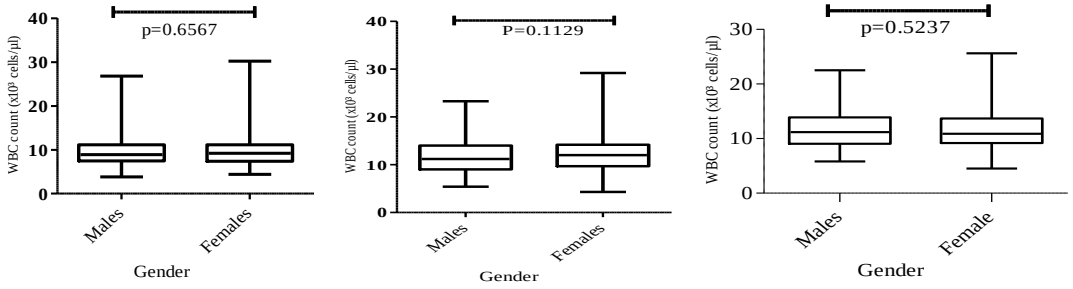
the age bracket 12m to 17months. 711 (47.1%) of the participants were males while 798 of the participants were females. The median age was 6 (IQR 1.25-12) months as shown in table 4.1. For the immunological phenotypic characteristics, the study had data set of 57 ART Naive HIV positive adults (n=57. Majority (45.6%) of the study participants were from the age group 21 to 30 years, while 21.0% of the study participants were illiterate. More than 96% of the population was from the Luo ethnic group (see Table 4.2). 35 (61.4%) of these were females.

4.2 Establishment of Hematological References ranges in children (1 month to 17months of age)

4.2.1. Hematological indices vary according to Gender in Children in Kisumu West Sub-county

4.2.1.1 White blood cell counts do not differ according to gender in all the 3 age groups

There were no significant differences in regard to gender in the three different age groups (See figure 4.1 below). Table 4.3 (a) shows the median values of WBC in males compared to females in different age groups



WBC levels in 1 to 6 m group WBC levels in 6 to 12 m group WBC levels in 12 to 17m group

Figure 4. 1: WBC counts in males and females across different age categories

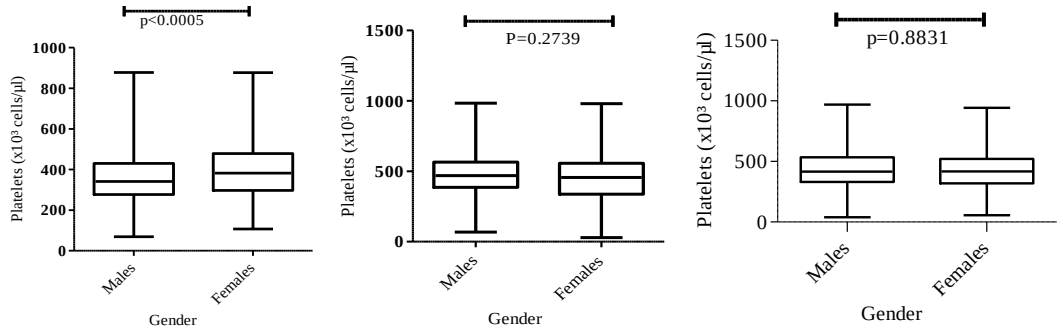
Table 4. 3: (a)-95% Hematology reference ranges for white blood cells in children aged 1-17months from Kisumu

Parameter/Age group		**N**	**Median (95% reference values)**	**P values**
White Blood Cells ($\times 10^3$ cells/μl)	Male	362	8.9 (5.2-19.5)	0.6567
1 to 6 months	Female	394	9.2 (5.0-16.9)	
6 to 12 months	Male	200	11.2 (6.2-19.8)	0.1129
	Female	187	12.0 (6.5-22.1)	
12 to 17 months	Male	149	11.2 (6.5-21.3)	0.5237
	Female	217	10.9 (6.3-19.5)	
Overall	Male	711	10.0 (5.8-20.0)	0.3421
	Female	798	10.1 (5.6-19.3)	
Overall	All gender	1509	10.1 (5.7-19.8)	

This table shows data for 95% reference values for white blood cells in different age categories stratified by gender.

4.2.1.2 Higher platelet counts in females than males in 1 m to 6 m age group

In 1m to 6m age group, females were noted to have higher median value (382×10^3 cells/μl) compared to median value in male children (341×10^3 cells/μl)**,** $p<0.0005$. There were no significant differences in the median platelet values in 6 to 12 month age group and 12 to 17 month age groups (p=0.2739 and 0.8831 respectively). (See figure 4.2 below). Table 4.4 shows the median values of platelets in males compared to females in different age groups.



Plt levels in 1 m to 6 m group Plt levels in 6 m to 12 m group Plt levels in 12 m to 17 m group

Figure 4. 2: Box and whisker plots showing the platelet values of children of different age categories from Kisumu West in regard to gender

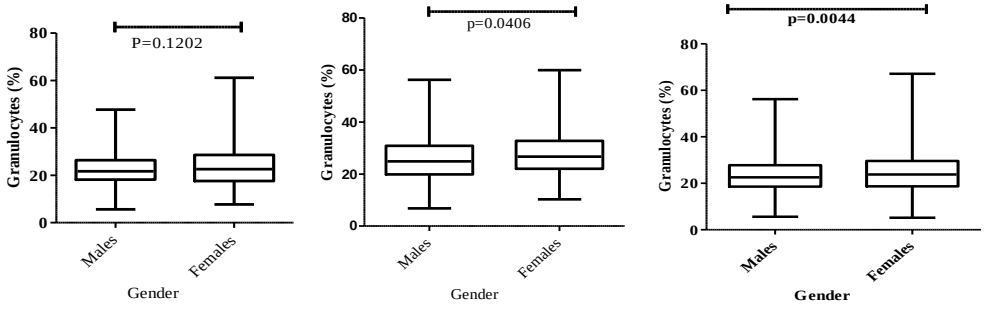
Table 4. 4: 95% Hematology reference ranges for platelets in children of different age categories from Kisumu.

Parameter/Age group		**N**	**Median (95% reference values)**	**P values**
Platelets ($\times 10^3$ cells/μl)				
1 to 6 months	Male	362	341 (136-704)	
	Female	394	382 (160-695)	**0.0005***
6 to 12 months	Male	200	469 (119-771)	
	Female	187	456 (98-816)	0.2739
12 to 17 months	Male	149	415 (93-808)	
	Female	217	418 (112-776)	0.8831
Overall	Male	711	393 (120-755)	
	Female	798	408 (139-759)	0.1654
Overall	All gender	1509	400 (129-757)	

The results in this table show 95% reference values for platelets in different age categories stratified by gender. ***-**values in **bold** with asterisk shows statistically significant result. In only 1 to 6m age group, female had higher median values compared to males.

4.2.1.3 Higher granulocyte counts in females than males in 12 to 17m age group

It was noted that percent granulocyte counts were higher in females compared to the males. In 12 to 17m age group, the granulocyte median count in female was 26.7%, while the median count in male was 24.9% (p=0.0406). Also in the overall age group (i.e. 1 m to 17 m group), the female median count, was 23.8%, compared to the male median count which was 22.6%, p=0.0044 (see figure 4.3). Table 4.5 shows the median values of granulocyte percent in males compared to females in different age groups.



1 m to 6 m group

12 m to 17 m

Overall age group
(1 m to 17 m age group

Figure 4. 3: Box and whisker plots showing the percent granulocyte counts in children of different age categories from Kisumu West in regard to gender.

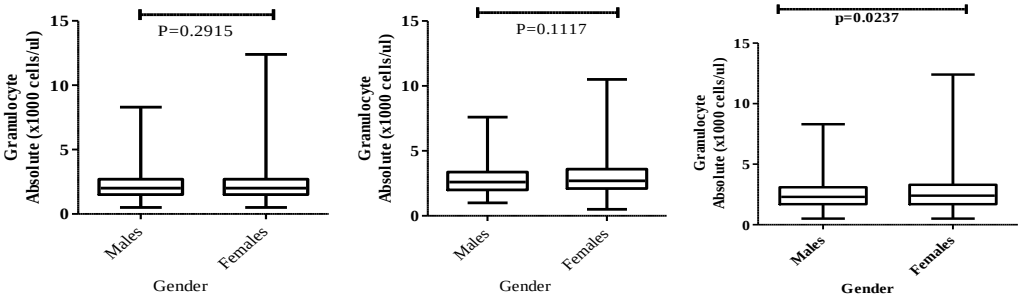
Table 4. 5: 95% Hematology reference ranges for percent granulocyte counts in children of different age categories from Kisumu

Parameter/Age group		N	Median (95% reference values)	P values
Percent granulocyte counts (%)				
1 to 6 months	Male	362	21.7 9.0-39.0)	0.1202
	Female	394	22.6 (11.3-41.8)	
6 to 12 months	Male	200	22.7 (12.5-41.1)	0.6237
	Female	187	23.9 (12.1-41.1)	
12 to 17 months	Male	149	24.9 (9.7-44.3)	**0.0456***
	Female	217	26.7 (14.0-49.4)	
Overall	Male	711	22.6 (9.7-41.8)	**0.0044**
	Female	798	23.8 (11.6-44.7)	
Overall	All gender	1509	23.2 (10.9-43.0)	

The results in this table show 95% reference values for granulocyte (%) in different age categories stratified by gender.

***-values in **bold** with asterisk shows statistically significant result. Females had higher median values for percent granulocytes in 12 m to 17 m and in the 1 m to 17 m age group)

It was noted for absolute granulocyte count in 1 m to 17 m age category only that female had higher median values than males (p=0.0237). (Figure 4.4). Table 4.6 shows the median values of absolute granulocyte counts in males compared to females in different age groups.



1 m to 6 m

6 m to 12 m

Overall age group
1 m to 17 m age group

Figure 4. 4: Box and whisker plots showing the absolute granulocyte counts in children of different age categories from Kisumu West in regard to gender

Table 4. 6: 95% Hematology reference ranges for granulocyte absolute counts in children of different age categories from Kisumu

Parameter/Age group		N	Median (95% reference values)	P values
Granulocytes Absolute counts ($\times 10^3$ cells/μl)				
1 to 6 months	Male	362	2.0 (0.7-4.5)	0.2915
	Female	394	2.0 (0.8-5.0)	
6 to 12 months	Male	200	2.6 (1.2-5.7)	0.1117
	Female	187	2.7 (1.1-6.9)	
12 to 17 months	Male	149	2.7 (1.0-6.7)	0.2400
	Female	217	2.9 (1.2-7.2)	
Overall	Male	711	2.3 (0.8-5.6)	**0.0237***
	Female	798	2.4 (0.9-6.3)	
Overall	All gender	1509	2.4 (0.9-6.0)	

This table shows data for 95% reference values for granulocyte absolute counts in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. Females had higher granulocyte absolute counts only in 1 m to 17 m age group

4.2.1.4 Lymphocyte counts are not statistically significantly different in females as in males in Kisumu West Sub county

It was observed that there were no significant differences in the median values between males and females in different age categories (figure 4.5). Table 4.7 shows the median values of lymphocyte absolute counts in males compared to females in different age groups.

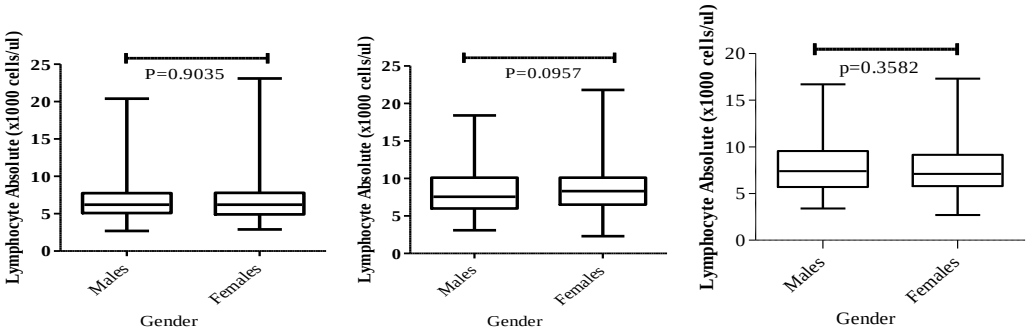


Figure 4. 5: Box and whisker plots showing the lymphocyte absolute counts in children of different age categories from Kisumu West in regard to gender

Table 4. 7: 95% Hematology reference ranges for lymphocyte absolute counts in children of different age categories from Kisumu

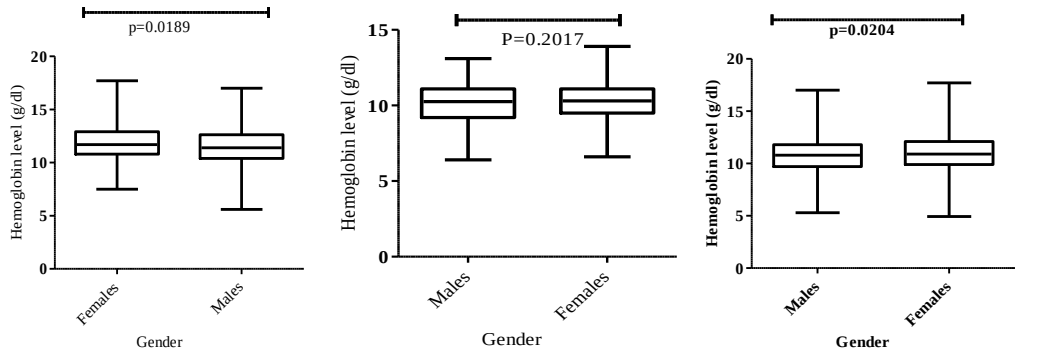
Parameter/Age group		N	Median (95% reference values)	P values
Lymphocyte absolute counts ($\times 10^3$ cells/μl)				
1 to 6 months	Male	362	6.2 (3.4-14.3)	0.9035
	Female	394	6.2 (3.4-12.5)	
6 to 12 months	Male	200	7.6 (3.7-14.5)	0.0957
	Female	187	8.3 (4.5-14.8)	
12 to 17 months	Male	149	7.4 (4.2-15.9)	0.3582
	Female	217	7.1 (3.5-12.9)	
Overall	Male	711	6.7 (3.6-14.5)	0.7527
	Female	798	6.8 (3.4-13.2)	
Overall	All gender	1509	6.8 (3.5-13.6)	

The results in this table show 95% reference values for lymphocyte absolute counts in different age categories stratified by gender

4.2.2. Erythrocyte Indices vary according to Gender in Children in Kisumu West Sub-county

4.2.1.5 Hemoglobin values vary according to gender in 1m to 6m age group

It was noted that in 1m to 6m age groups, males had significantly lower values than females (median values in males was 11.4g/dl compared to median values in females at 11.7g/dl, p=0.0189). Also when hemoglobin median values were also compared in the overall age group (1m to 17m age group), the median value in males was 10.8g/dl compared to median values in females at 10.9g/dl (p=0.0204). Figure 4.6 shows the box and whisker plots for hemoglobin levels in children in the study. Table 4.8 shows the median values of hemoglobin in males compared to females in different age groups.



1 m to 6 m

6 m to 12 m

Overall age group
1 m to 17 m age group

Figure 4. 6: Box and whisker plots showing the hemoglobin levels in children in different age categories from Kisumu West in regard to gender

Table 4. 8: 95% Hematology reference ranges for hemoglobin in children of different age categories from Kisumu

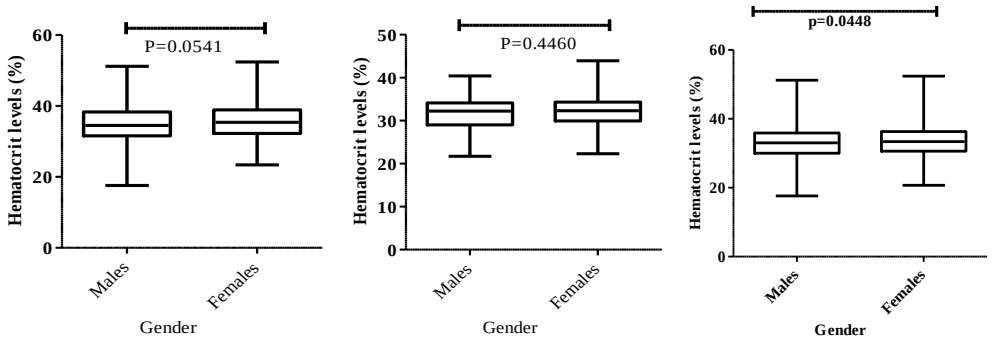
Parameter/Age group		N	Median (95% reference values)	P values
Hemoglobin (g/dL)				
1 to 6 months	Male	362	11.4 (8.5-15.0)	**0.0189***
	Female	394	11.7 (8.6-15.4)	
6 to 12 months	Male	200	10.3 (7.5-12.4)	0.2017
	Female	187	10.3 (7.9-13.0)	
12 to 17 months	Male	149	10.0 (6.6-12.6)	0.1387
	Female	217	10.2 (6.9-12.6)	
Overall	Male	711	10.8 (7.4-14.5)	**0.0204***
	Female	798	10.9 (7.7-14.7)	
Overall	All gender	1509	10.9 (7.6-14.6)	

The results in this table show 95% reference values for hemoglobin in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. Females have higher median values of hemoglobin in 1 m to 6 m age group and in the overall age group (1 m to 17 m) as compared to males

4.2.1.6 Hematocrit values vary according to gender in the overall age group

To establish the differences in the median values for hematocrit, in the overall group, the median value in females was 33.4% compared to males who had median value of 33.0%. (p=0.0448). There were no significant differences between the male and female median values in the other age groups (see figure 4.7). Table 4.9 shows the median values of hematocrit in males compared to females in different age group



1 m to 6 m | 6 m to 12 m | Overall age group 1 m to 17 m age group

Figure 4. 7: Box and whisker plots showing the hematocrit values of children of different age categories from Kisumu West in regard to gender

Table 4. 9: 95% Hematology reference ranges for hematocrit in children of different age categories from Kisumu.

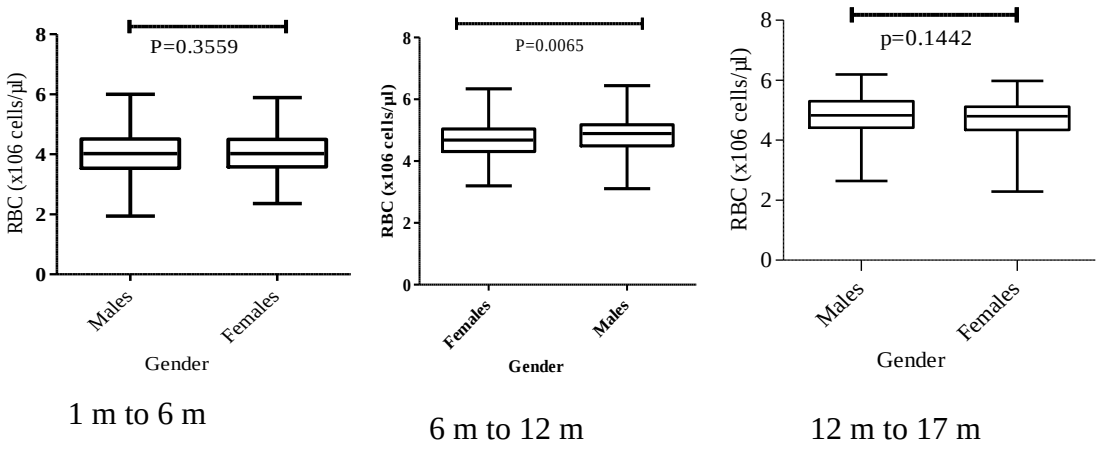
Parameter/Age group		N	Median (95% reference values)	P values
Hematocrit (%)				
1 to 6 months	Male	362	34.5 (26.4-45.8)	0.0541
	Female	394	35.4 (26.6-46.9)	
6 to 12 months	Male	200	32.2 (24.0-38.2)	0.4460
	Female	187	32.3 (25.5-38.8)	
12 to 17 months	Male	149	31.4 (21.7-38.7)	0.1400
	Female	217	32.1 (23.3-38.9)	
Overall	Male	711	33.0 (23.9-43.8)	**0.0448***
	Female	798	33.4 (24.7-43.9)	
Overall	All gender	1509	33.2 (24.4-43.8)	

The results in this table show 95% reference values for hematocrit in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result.

4.2.1.7 Males had higher median red blood cell count compared to females in 6m to 12m age category in Kisumu West Sub-county

To establish the differences in the median values of RBC counts, it was noted that in 6m to 12m age group, there were differences in RBCs in median levels in females compared to males. Males had higher median RBC value in this group ($4.89x10^6$ cells/μl) compared to female children who had median value of 4.68×10^6 cells/μl (p=0.0065) (figure 4.8) Table 4.10 shows the median values of RBC counts in males compared to females in different age group



1 m to 6 m 6 m to 12 m 12 m to 17 m

Figure 4. 8: Box and whisker plots showing the RBC counts in children of different age categories from Kisumu West in regard to gender.

Males had higher median RBC value in 6 m to 12 m group compared to female children (p=0.0065).

Table 4. 10: 95% Hematology reference ranges for red blood cells in children of different age categories from Kisumu

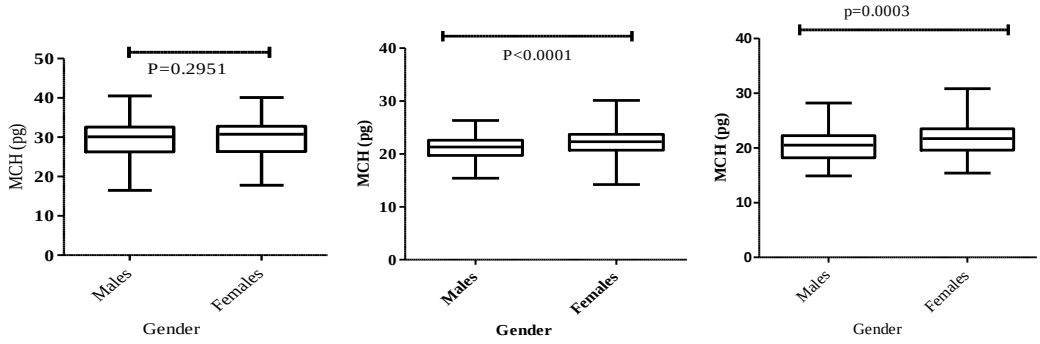
Parameter/Age group		**N**	**Median (95% reference values)**	**P values**
Red Blood Cells ($\times 10^6$ cells/μl)				
1 to 6 months	Male	362	4.02 (2.73-5.37)	0.3559
	Female	394	4.02 (2.87-5.40)	
6 to 12 months	Male	200	4.89 (3.43-5.91)	**0.0065***
	Female	187	4.68 (3.65-5.86)	
12 to 17 months	Male	149	4.83 (3.29-6.08)	0.1442
	Female	217	4.80 (3.23-5.86)	
Overall	Male	711	4.47 (2.90-5.86)	0.4362
	Female	798	4.40 (2.99-5.66)	
Overall	All gender	1509	4.44 (2.94-5.78)	

The results in this table show 95% reference values for red blood cells in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result.

4.2.1.8 Females have higher Mean Cell Hemoglobin values compared to males in 6 m to 12 m age and 12 m to 17 m age groups in Kisumu West Sub-county.

MCH Female children had median values of 22.3pg for MCH in 6 m to 12 m group, compared to male children who had median value of 21.3pg ($p<0.0001$). In 12 m to 17 m age group same pattern was depicted where females had higher median values at 21.7pg compared to males with median value of 20.5pg ($p=0.0003$). Figure 4.9 below shows the MCH values in children in regard to gender. Table 4.11 shows the median values of MCH in males compared to females in different age group.



1 m to 6 m 6 m to 12 m 12 m to 17 m

Figure 4. 9: Box and whisker plots showing the MCH values of children of different age categories from Kisumu West in regard to gender

Table 4. 11: 95% Hematology reference ranges for Mean Cell Hemoglobin in children of different age categories from Kisumu

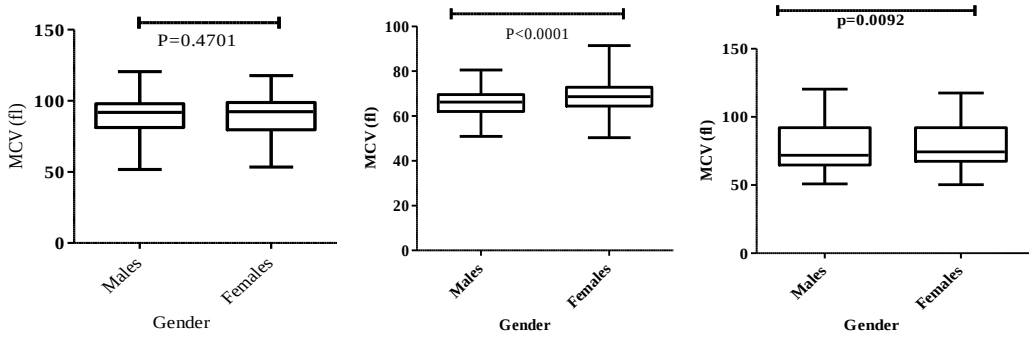
Parameter/Age group		N	Median (95% reference values)	P values
Mean Cell Hemoglobin (pg)				
1 to 6 months	Male	362	30.1 (19.6-36.7)	0.2951
	Female	394	30.8 (20.4-36.2)	
6 to 12 months	Male	200	21.3 (16.2-25.9)	**<0.0001***
	Female	187	22.3 (16.6-27.6)	
12 to 17 months	Male	149	20.5 (15.4-26.3)	**0.0003***
	Female	217	21.7 (16.2-26.8)	
Overall	Male	711	23.4 (16.8-35.5)	**0.0089***
	Female	798	24.3 (17.5-35.5)	
Overall	All gender	1509	24.0 (16.9-35.5)	

The results in this table show 95% reference values for MCH in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. In 6 m to 12 m and 12 m to 17 age groups, females had higher median values for MCH compared to males.

4.2.1.9 Females have higher Mean Cell Volume values compared to males in 6 m to 12 m and 12 m to 17 m age groups in Kisumu West Sub-county

For MCV, females were noted to have higher median values (68.6fl) compared to males in 6 m to 12 m age group (66.2fl), ($p<0.0001$). Similarly in the overall age group, females had higher median MCV value (74.4fl) compared to the male children (71.9fl), ($p=0.0092$). Figure 4.10 below shows the MCV levels in children in regard to gender. Table 4.12 shows the median values of MCV in males compared to females in different age group.



1 m to 6 m | 6 m to 12 m | Overall age group 1 m to 17 m

Figure 4. 10: Box and whisker plots showing the MCV levels in children from Kisumu West in regard to gender

Table 4. 12: 95% hematology reference ranges for Mean Cell Volume in children of different age categories from Kisumu

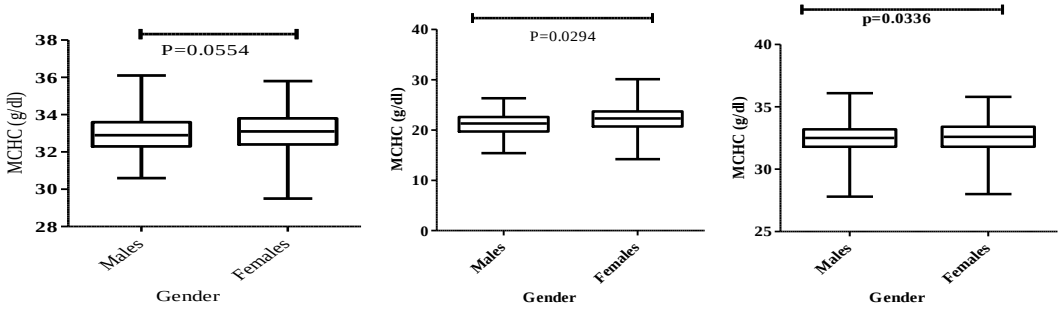
Parameter/Age group		N	Median (95% reference values)	P values
Mean Cell Volume (fl)				
1 to 6 months	Male	362	91.8 (60.6-110.4)	0.4701
	Female	394	92.3 (63.7-109.7)	
6 to 12 months	Male	200	66.2 (53.6-77.5)	**<0.0001***
	Female	187	68.6 (52.34-82.16)	
12 to 17 months	Male	149	64.6 (52.8-79.93)	**<0.0001***
	Female	217	67.9 (53.76-80.76)	
Overall	Male	711	71.9 (54.7-106.34)	**0.0092***
	Female	798	74.4 (55.9-105.4)	
Overall	All gender	1509	73.4 (54.9-105.6)	

The results in this table show 95% reference values for MCV in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. In 6 m to 12 m and 12 m to 17 m age groups females had higher mean cell volume median values compared to males.

4.2.1.10 Females have higher Mean Cell Hemoglobin Concentration values compared to males in 6 m to 12 m age group in Kisumu West.

For MCHC, females were noted to have higher median values compared to males in 6 m to 12 m age group (p=0.0294). Similarly in the overall age group, females had higher median MCHC value compared to the male children (p=0.0336). Figure 4.11 below shows the MCHC values in children in regard to gender. Table 4.13 shows the median values of MCHC in males compared to females in different age group.



1 m to 6 m

6 m to 12 m

Overall age group
1 m to 17 m

Figure 4. 11: Box and whisker plots showing the MCHC levels in children from Kisumu West in regard to gender

Table 4. 13: 95% Hematology reference ranges for Mean Cell Hemoglobin Concentration in children of different age categories from Kisumu

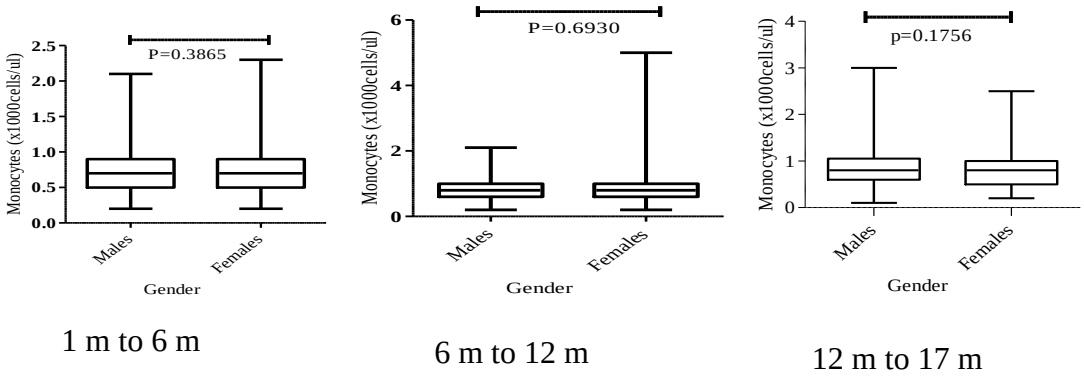
Parameter/Age group		N	Median (95% reference values)	P values
Mean Cell Hemoglobin Concentration (g/dl)				
1 to 6 months	Male	362	32.9 (31.1-34.9)	0.0554
	Female	394	33.1 (31.2-35.0)	
6 to 12 months	Male	200	32.2 (29.6-33.7)	**0.0294***
	Female	187	32.3 (30.4-34.1)	
12 to 17 months	Male	149	31.9 (28.9-34.0)	**0.0003***
	Female	217	31.9 (29.4-34.1)	
Overall	Male	711	32.5 (29.8-34.7)	**0.0336***
	Female	798	32.6 (30.0-34.7)	
Overall	All gender	1509	32.6 (30.0-34.7)	

The results in this table show 95% reference values for MCHC in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. Females had higher median values compared to males in 6 m to 12 m age group.

4.2.1.11 No differences in monocyte counts between males and female children in the different age groups in Kisumu West.

It was observed that there were no significant differences in the median values for monocyte counts between males and females in different age categories (figure 4.12). Table 4.14 shows the median values of monocyte absolute counts in males compared to females in different age groups



1 m to 6 m 6 m to 12 m 12 m to 17 m

Figure 4. 12: Box and whisker plots showing monocyte counts in children from Kisumu West in regard to gender

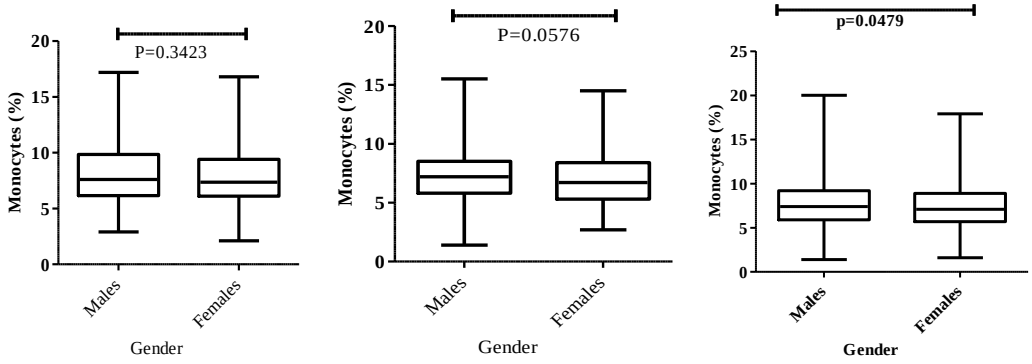
Table 4. 14: 95% Hematology reference ranges for monocyte absolute counts in children of different age categories from Kisumu West

Parameter/Age group		N	Median (95% reference values)	P values
Monocyte absolute counts ($\times 10^3$ cells/μl)				
1 to 6 months	Male	362	0.7 (0.3-1.6)	0.3865
	Female	394	0.7 (0.3-1.6)	
6 to 12 months	Male	200	0.8 (0.3-1.6)	0.6930
	Female	187	0.8 (0.4-1.7)	
12 to 17 months	Male	149	0.8 (0.3-2.1)	0.1756
	Female	217	0.8 (0.3-1.7)	
Overall	Male	711	0.8 (0.3-1.6)	0.1400
	Female	798	0.7 (0.3-1.6)	
Overall	All gender	1509	0.7 (0.3-1.6)	

The results in this table show 95% reference values for monocyte absolute counts in different age categories stratified by gender.

4.2.1.12 No differences in percent monocyte counts between males and females in Kisumu West

It was observed that there were no significant differences in the median values in percent monocyte counts between males and females in other age categories apart from the overall age category (figure 4.13). Table 4.15 shows the median values of percent monocytes in males compared to females in different age group



1 m to 6 m

6 m to 12 m

Overall age group
1 m to 17 m age group

Figure 4. 13: Box and whisker plots showing the percent monocyte counts in children from Kisumu West in regard to gender

Table 4. 15: 95% Hematology reference ranges for percent monocyte counts in children of different age categories from Kisumu West

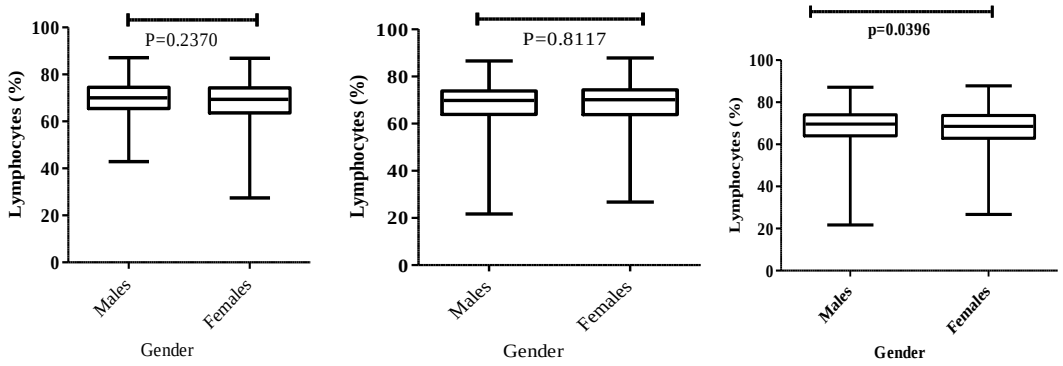
Parameter/age group		N	Median (95% reference values)	P values
Percent Monocytes (%)				
1 to 6 months	Male	362	7.6 (3.9-13.29)	
	Female	394	7.4 (3.38-13.52)	0.3423
6 to 12 months	Male	200	7.2 (3.0-12.8)	
	Female	187	6.7 (3.3-11.8)	0.0576
12 to 17	Male	149	7.0 (3.0-15.7)	
months	Female	217	6.8 (2.8-12.2)	0.4721
Overall	Male	711	7.4 (3.6-13.8)	
	Female	798	7.1 (3.2-12.8)	**0.0479***
Overall	All gender	1509	7.2 (3.4-13.3)	

The results in this table show 95% reference values for monocyte (%) in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result.

4.2.1.13 No differences in percent lymphocyte counts between males and females in other age groups except in the overall age group in children from Kisumu West

It was observed that there were no significant differences in the median values for percent lymphocyte counts between males and females in other age categories apart from the overall age group (1 m to 17 m age group) (fig 4.14). Table 4.16 shows the median values of percent lymphocyte counts in males compared to females in different age groups.



1 m to 6 m

6 m to 12 m

Overall age group
1 m to 17 m age group

Figure 4. 14: Box and whisker plots showing the percent lymphocyte levels in children from Kisumu West in regard to gender

Table 4. 16: 95% Hematology reference ranges for lymphocytes % in children aged 1-17months from Kisumu West

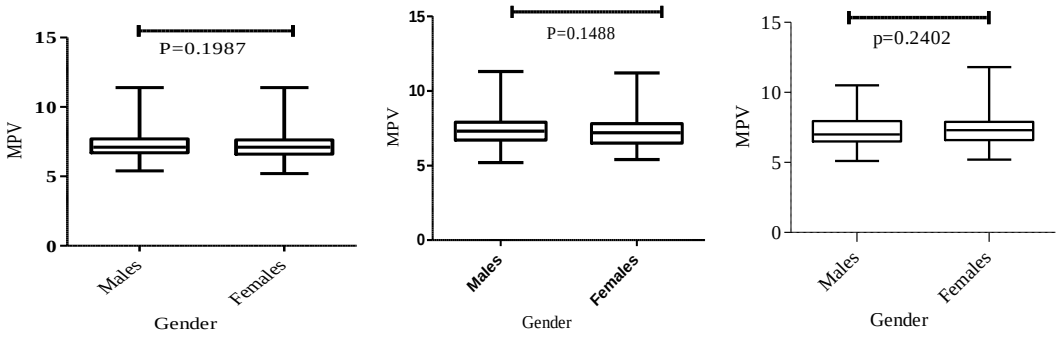
Age group		N	Median (95% reference values)	P values
Percent Lymphocytes (%)				
1 to 6 months	Male	362	70.0 (52.5-83.6)	0.2370
	Female	394	69.4 (49.1-81.8)	
6 to 12 months	Male	200	69.8 (50.7-82.3)	0.8117
	Female	187	70.1 (48.5-82.5)	
12 to 17 months	Male	149	67.7 (48.0-81.8)	0.070
	Female	217	66.4 (44.8-78.5)	
Overall	Male	711	69.6 (50.7-82.6)	**0.0396***
	Female	798	68.5 (46.3-81.4)	
Overall	All gender	1509	69.1 (48.6-82.2)	

This table shows data for 95% reference values for lymphocyte percent in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result. Males had higher percent lymphocyte counts compared to females in the overall age group (1 m to 17 m age group).

4.2.1.14 Mean Platelet Volume values do not differ between males and females in Kisumu West

It was observed that there were no significant differences in the median values for MPV values between males and females in all the age categories (fig 4.15). Table 4.17 shows the median values of MPV in males compared to females in different age groups.



1 m to 6 m 6 m to 12 m 12 m to 17 m

Figure 4. 15: Box and whisker plots showing the MPV values in children from Kisumu West in regard to gender

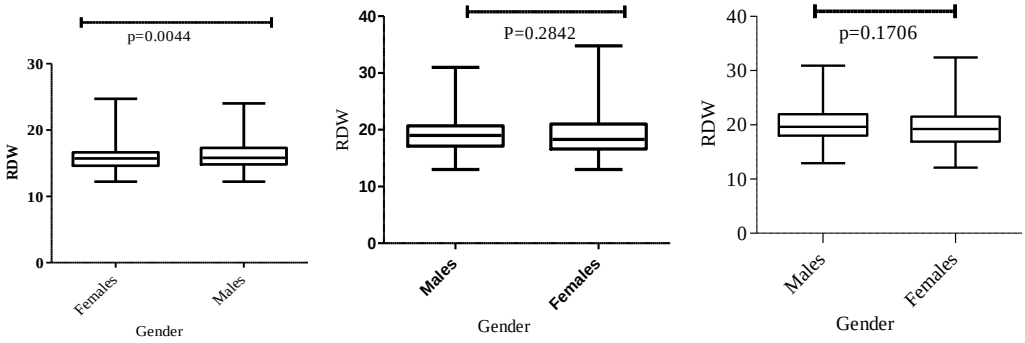
Table 4. 17: 95% Hematology reference ranges for Mean Platelet Volume in children of different age categories from Kisumu

Age group		N	Median (95% reference values)	P values
1 to 6 months	Male	362	7.1 (5.7-9.3)	0.1987
	Female	394	7.1 (5.4-8.9)	
6 to 12 months	Male	200	7.3 (5.7-9.7)	0.1488
	Female	187	7.2 (5.6-9.7)	
12 to 17 months	Male	149	7.0 (5.6-9.5)	0.2402
	Female	217	7.3 (5.6-9.5)	
Overall	Male	711	7.1 (5.7-9.5)	0.1930
	Female	798	7.1 (5.6-9.4)	
Overall	All gender	1509	7.1 (5.6-9.4)	

The results in this table show 95% reference values for MPV in different age categories stratified by gender.

4.2.1.15 Red Cell Distribution Width differ between gender in the age category 1m to 6m

It was observed that in 1m to 6m age category, RDW in males were higher compared to females. Males had RDW values of 15.8 compared to the females who had RDW values of 15.7 (p=0.0044). There were no significant differences in the median values between males and females in the other age categories (fig 4.16). Table 4.18 shows the median values of RDW in males compared to females in different age groups.



1 m to 6 m 6 m to 12 m 12 m to 17 m

Figure 4. 16: Box and whisker plots showing the RDW values in children from Kisumu West in regard to gender

Table 4. 18: 95% Hematology reference ranges for Red Cell Distribution Width in children of different age categories from Kisumu

Age group		N	Median (95% reference values)	P values
1 to 6 months	Male	362	15.8 (13.4-21.5)	**0.0444***
	Female	394	15.7 (13.2-20.3)	
6 to 12 months	Male	200	19.0 (14.9-28.7)	0.2842
	Female	187	18.3 (13.5-25.6)	
12 to 17 months	Male	149	19.6 (14.5-26.6)	0.1706
	Female	217	19.2 (13.9-27.3)	
Overall	Male	711	17.3 (13.7-25.3)	0.3360
	Female	798	16.7 (13.3-25.1)	
Overall	All gender	1509	17.0 (13.4-25.1)	

The results in this table show 95% reference values for RDW in different age categories stratified by gender.

***-**values in **bold** with asterisk shows statistically significant result.

Table 4.19 below shows a summary of the median values for the hematological and erythrocyte indices in the different age groups in relation to gender

Table 4. 19: Summary of the median values for hematological and erythrocyte Indices in the different age groups in relation to gender

Analyte	Age group 1 m to 6 m			Age group 6 m to 12 m			Age group 12 m to 17 m		
	Median in males	Median in females	P value	Median in males	Median in females	P value	Median in males	Median in females	P value
White blood cells	8.9	9.2	0.6567	11.2	12	0.1129	11.2	10.9	0.5237
Lymphocytes %	70	69.4	0.2370	69.8	70.1	0.8117	67.7	66.4	0.070
Monocytes %	7.6	7.4	0.3423	7.2	6.7	0.0576	6.8	7.4	0.4721
Granulocytes %	21.7	22.6	0.1202	22.7	23.9	0.6237	24.9	26.7	**0.0406***
Lymphocytes Abs	6.2	6.2	0.9035	7.6	8.3	0.0957	7.4	7.1	0.3582
Monocytes Abs	0.7	0.7	0.3865	0.8	0.8	0.6930	0.8	0.8	0.1756
Granulocytes Abs	2.0	2.0	0.2915	2.6	2.7	0.1117	2.7	2.9	0.2400
Red blood cells	4.02	4.02	0.3559	4.89	4.68	**0.0065***	4.83	4.80	0.1442
Hemoglobin	11.4	11.7	**0.0189***	10.3	10.3	0.2017	10.0	10.2	0.1387
Hematocrit	34.5	35.4	0.0541	32.2	32.3	0.4460	31.4	32.1	0.1400
Mean cell volume	91.8	92.3	0.4701	66.2	68.6	**<0.0001***	64.6	67.9	**<0.0001***
Mean cell hemoglobin	30.1	30.8	0.2951	21.3	22.3	**<0.0001***	20.5	21.7	**0.0003***
Mean cell hemoglobin concentration	32.9	33.1	0.0554	32.2	32.3	**0.0294***	31.9	31.9	
Red cell distribution width	15.8	15.7	0.0444	19.0	18.3	0.2842	19.6	19.2	0.1706
Mean platelet volume	7.1	7.1	0.1987	7.3	7.2	0.1488	7.0	7.3	0.2402
Platelets	341	382	**<0.0005***	469	456	0.2739	415	418	0.8831

This table shows a summary of the median values in different hematological and erythrocyte indices in different age groups relation to gender. *-values in **bold** with asterisk shows statistically significant result.

4.2.3. Median values of hematological indices vary across children of different age groups in Kisumu West Sub-County

4.2.3.1. Hematological Indices Differ according to Age

Median values and ranges for total White Blood Cell counts, Monocyte absolute counts and Granulocyte Absolute counts

For white blood cells, children aged between 1-6 months had the lowest median for WBCs (9.1×10^3 cells/µl), followed by those aged between 12-17months (11.1×10^3cells/µl) and lastly those aged between 6-12months (11.6×10^3 cells/µl) ($p<0.0001$). For absolute monocyte counts the median value in 1m to 6m age group was 0.7, both the median value in 6m to 12m and 12m to 17m was 0.8 ($p=0.0011$). Figure 4.17 below shows the box and whisker plots for WBC and absolute monocyte counts in the different age categories.

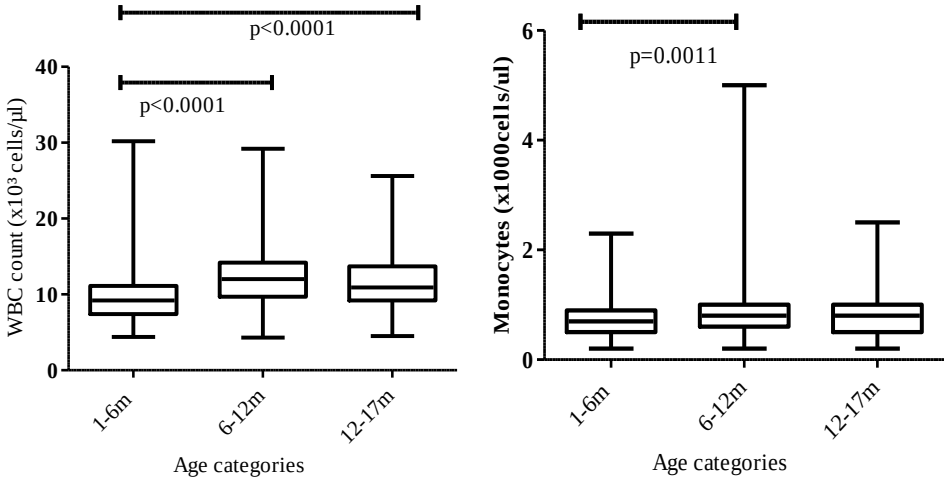


Figure 4. 17: Box and whisker plots showing the levels of WBC and monocytes across different age categories

For granulocyte absolute counts the median value in 1m to 6m age group was 2.0, the median value in 6m to 12m age group was 2.6 while the median value in 12m to 17m

age group was 2.85. (p<0.0001). Figure 4.18 below shows the box and whisker plots for absolute granulocyte counts in children in different age categories.

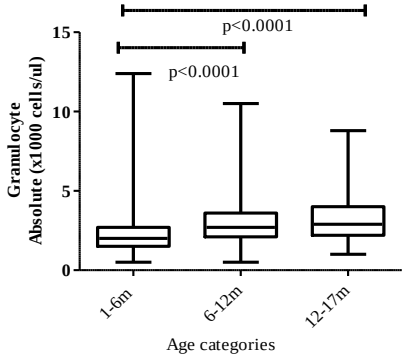


Figure 4.18: Box and whisker plots showing the levels of granulocyte absolute across different age categories

It was noted that median granulocyte percentiles increased with increasing age ((*p<0.0001*). Children aged 1-6m had the lowest percentage granulocyte (22.1%) followed by 6-12months age group (23.1%) and lastly 12m to 17m (26.3%).

Median Percent lymphocytes were noted to be lower in the upper age group (12-17months) compared to the two lower age categories Children aged 1 to 6m had median lymphocyte percent of 69.8%, those aged 6-12months had median lymphocyte counts of 69.9% while those aged 12m to 17m had lymphocyte counts of 66.8% (p<0.0001). Figure 4.19 below shows the box and whisker plots in percent granulocyte and lymphocyte in children across different age categories.

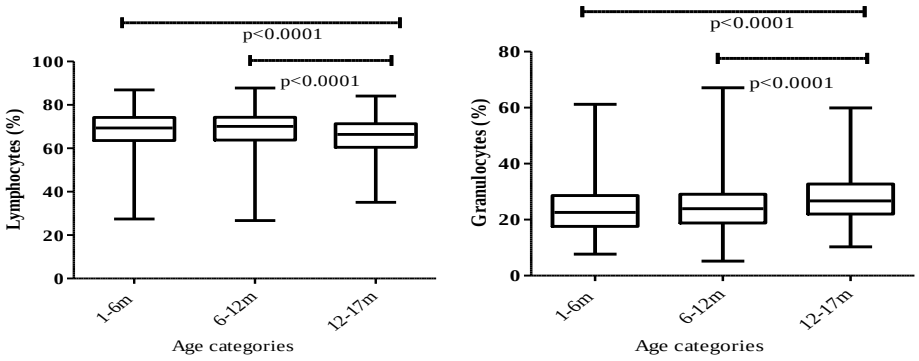


Figure 4. 18: : Box and whisker plots showing the levels of lymphocyte and granulocyte percent across different age categories

Median values and ranges of absolute lymphocytes counts in children of different age categories

Median absolute lymphocyte counts were noted to be significantly different from all the three age categories with lower values (6.2×10^3 cells/μl) in the lowest age category (1m to 6m) and higher values in the 6-12m age category (8×10^3 cells/μl) ($p<0.0001$). Children aged 12m to 17m had median value of $7.2x10^3$ cells/μl. Figure 4.20 below shows the box and whisker plots for absolute lymphocyte across different age categories.

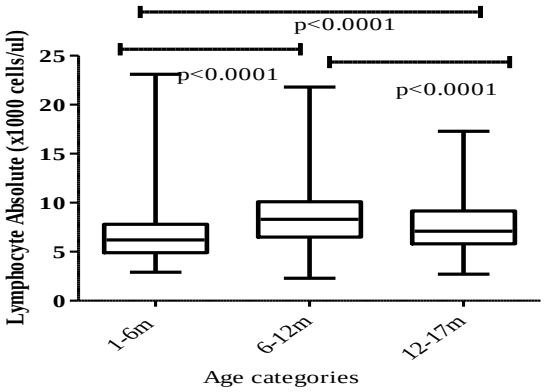


Figure 4. 19: Box and whisker plots showing the levels of lymphocyte absolute across different age categories

4.2.3.2. Median values and ranges of percent monocytes vary in children of different age categories

It was noted in 1m to 6m age group, the median values of the percent monocytes are statistically significantly higher compared to the age groups 6m to 12m and 12m to 17m ($p<0.0001$). Median values in 1m to 6m age group was 7.4%, children aged 6m to 12m and 12m to 17m each had median values of 6.9%. Figure 4.21 shows the box and whisker plots for percent monocytes in children across different age categories.

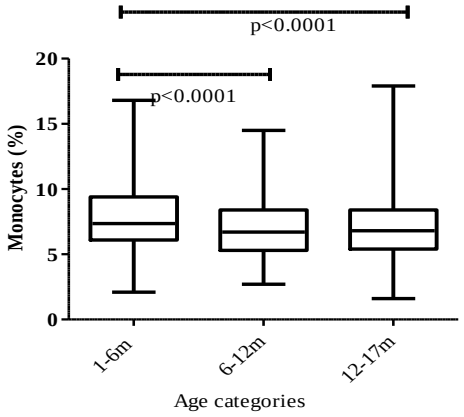


Figure 4. 20: Box and whisker plots showing the levels of monocytes percent across different age categories

4.2.3.3. Median values of erythrocyte indices vary across children of different age groups in Kisumu

It was noted in 1m to 6m age group, the median values of the hemoglobin and red cell indices are statistically significantly higher compared to the age groups 6m to 12m and 12m to 17m ($p<0.0001$). The median values for hemoglobin in children aged 1m to 6m was 11.6g/dl compared to median values in children aged 6m to 12m and 12m to 17m who had median values of 10.3 g/dl and 10.2 g/dl respectively. Children aged 1m to 6m had hematocrit median values of 35.1% compared to children aged 6m to 12m and 12m

to 17m who had median values of 32.2% and 31.8% respectively. Figure 4.22 shows the levels of hemoglobin and hematocrit in children in different age categories.

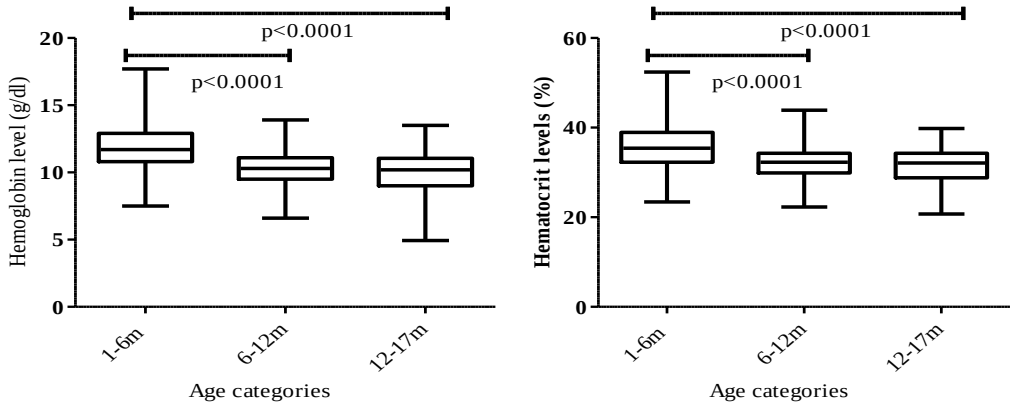


Figure 4. 21: Box and whisker plots showing the levels of hemoglobin and hematocrit across different age categories

Children aged 1m to 6m had MCV median values of 92.1fl compared to children aged 6m to 12m and 12m to 17m who had median values of 67.4fl and 66.1fl respectively. For MCHC, the values were noted to be decreasing as per increasing age and this was different in all the three age groups ($p<0.0001$). Children aged 1m to 6m had MCHC median values of 33g/dl, while children aged 6m to 12m had MCHC median values of 32.3 g/dl. Children aged 12m to 17m had the least MCHC median values of 31.9g/dl. Figure 4.23 shows the MCV, MCHC and MCH levels in children across different age categories.

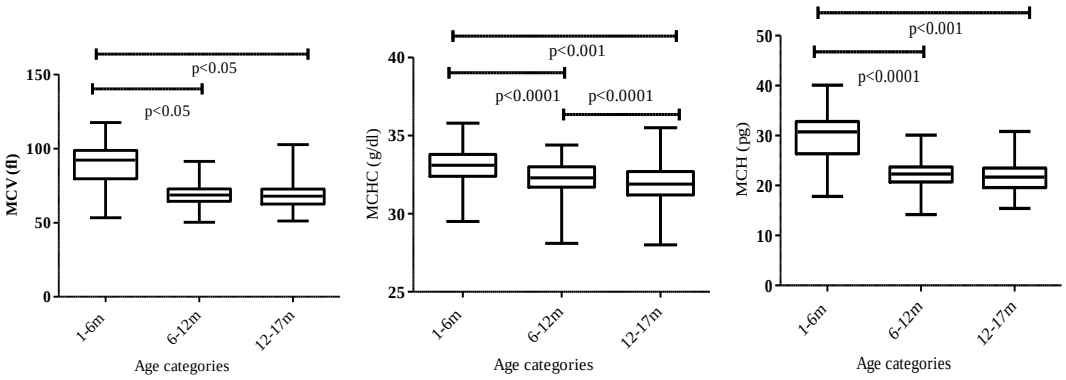


Figure 4. 22: Box and whisker plots showing the levels of values of red blood cell indices across different age categories

It was observed that red blood cells and RDW in 1 m to 6 m age groups were statistically significantly lower than other two age groups (*p<0.001*). No differences were observed in the other age categories 6 to12 m and 12 to 17 m for these analytes. Children aged 1 m to 6 m had RBC median values of 4.02×10^6cells/µl, followed by children aged 12 m to 17 m who had median values of 4.81×10^6cells/µl and lastly children aged 6 m to 12 m who had median values of 4.83×10^6cells/µl. Figure 4.24 shows the levels of RBC and RDW in children across different age categories.

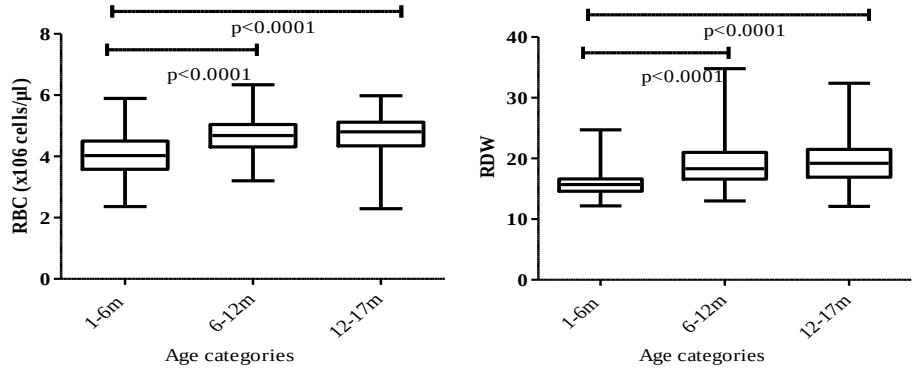


Figure 4. 23: Box and whisker plots showing the levels of values of RBC and RDW across different age categories.

It was noted that for platelets counts, the values were significantly different in all the three age categories with lower values in the lowest age category (1 m to 6 m) and

higher values in the 6 to12 m age category (*p<0.001*). Children aged 1 m to 6 m had median platelets counts of 357×10^3 cells/µl followed by children aged 12 m to 17 m who had median platelet counts of 416×10^3 cells/µl and lastly children aged 6 m to 12 m had median platelet counts of 465×10^3 cells/µl. Figure 4.25 shows the levels of platelet in children across different age categories.

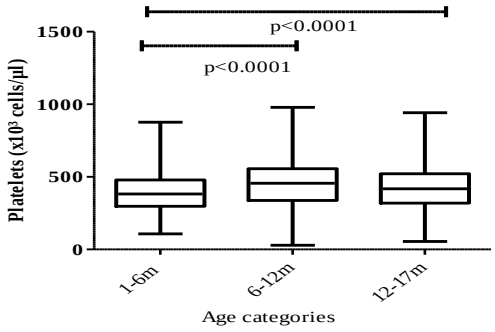


Figure 4. 24: Box and whisker plots showing the levels of values for platelets across different age categories.

Table 4. 20: Summary of the median values for the various parameters in the different age groups

Table 4.5-Summary of the median values for the various hematology parameters in the different age groups

	Age groups			
Parameter	**1 m to 6m**	**6 m to 12 m**	**12m to 17m**	**p value (groups with differences)**
White blood cells	9.1	11.6	11.1	<0.0001 (G1 and G2; G1 and G3)
Lymphocytes %	69.8	69.9	66.8	<0.0001 (G1 and G3; G2 and G3)
Monocytes %	7.4	6.9	6.9	<0.0001 (G1 and G2; G1 and G3)
Granulocytes %	22.1	23.1	26.3	<0.0001 (G1 and G3; G2 and G3)
Lymphocyte absolute	6.2	8	7.2	<0.0001 (All groups)
Monocyte absolute	0.7	0.8	0.8	0.0011 (G1 and G2; G1 and G3)
Granulocyte absolute	2	2.6	2.85	<0.0001 (G1 and G2; G1 and G3)
Red blood cells	4.02	4.83	4.81	<0.001 (G1 and G2; G1 and G3)
Hemoglobin	11.6	10.3	10.2	<0.0001 (G1 and G2; G1 and G3)
Hematocrit	35.1	32.2	31.8	<0.0001 (G1 and G2; G1 and G3)
Mean cell volume	92.1	67.4	66.1	<0.0001 (G1 and G2; G1 and G3)
Mean cell hemoglobin	30.5	21.7	21.1	<0.0001 (G1 and G2; G1 and G3)
Mean cell hemoglobin concentration	33	32.3	31.9	<0.0001 (G1 and G2; G1 and G3)
Red cell distribution width	15.7	18.8	19.5	<0.001 (G1 and G2; G1 and G3)
Platelets	357	465	416	<0.001 (All groups)

Summary table of results showing median values of hematological and erythrocyte indices across different age groups. Table indicates p values in groups that have statistical differences.

G1- 1 m to 6m, G2- 6 m to 12 m, G3- 12 m to 17 m

4.2.4. Current reference ranges for this study compared to other available reference values

Table 4.21 shows 95% reference intervals for hematological parameters for Kisumu children compared to published data from Kilifi for children aged 1 m to 6 m. Despite Kilifi and Kisumu being in the same country, some differences were noted in 95% reference intervals compared to the reference intervals in the children of the similar age category in Kilifi. In 1 m to 6 m age group, reference intervals for WBCs, hemoglobin, hematocrit, MCV and lymphocytes absolute count values for Kisumu were noted to be higher with higher median values than the Kilifi values ($p<0.0001$). The median values for Absolute monocyte counts in Kisumu children were lower compared to the Kilifi children ($p<0.0001$). Platelets ranges for Kisumu children were narrower with lower median values as compared to Kilifi values ($p<0.0001$). In 1 m to 17 m age group, the same pattern as for the 1 m to 6 m age groups was depicted whereby Kisumu reference intervals and median values for WBCs, hemoglobin, hematocrit, MCV and lymphocytes absolute count values were higher compared to Kilifi values ($p<0.0001$). Similarly, the platelet ranges were narrower with lower median values for Kisumu children as compared to Kilifi values ($p<0.0001$).

Table 4. 21: 95% reference intervals for hematological parameters for Kisumu children aged 1 m to 6 m compared to published data for Kilifi for children aged 1 m to 6 m

	95% reference intervals in males			95% reference intervals in females		
Parameter	Kisumu (current)	Kilifi	P value	Kisumu (current)	Kilifi	P value
White Blood Cells ($\times 10^3$ cells/μl)	5.2-19.5	4.60-13.71	**0.0001***	5.0-16.9	5.01-4-15.93	0.1877
Lymphocytes (%)	52.5-83.6	-	-	49.1-81.8	-	-
Monocytes (%)	3.9-13.3	-	-	3.4-13.5	-	-
Granulocytes (%)	9.0-39.0	-	-	11.3-41.8	-	-
Red Blood Cells ($\times 10^6$ cells/μl)	2.73-5.37	-	-	2.87-5.40	-	-
Hemoglobin (g/dl)	8.5-15.0	8.0-14.0	**0.0001***	8.6-15.4	8.3-13.8	**0.0001***
Hematocrit (%)	26.4-45.8	24.6-41.9	**0.0001***	26.6-46.9	25.2-42.5	**0.0001***
Platelets ($\times 10^3$ cells/μl)	136-704	93-746	**0.0001***	160-695	22-833	**0.0001***
Mean cell volume (fl)	60.6-110.4	58-98	**0.0001***	63.7-109.7	55-102	**0.0001***
Mean cell hemoglobin (pg)	19.6-36.7	-		20.4-36.2	-	-
Mean cell hemoglobin concentration (g/dl)	31.1-34.9	31.2-34.7	0.2358	31.2-35.0	31.0-35.1	0.1343
Mean platelet volume	5.7-9.3	-	-	5.4-8.9	-	-
Red cell distribution width	13.4-21.5	-	-	13.2-20.3	-	-
Lymphocyte absolute counts ($\times 10^3$ cells/μl)	3.4-14.4	2.25-8.99	**0.0001***	3.4-12.5	3.39-9.20	**0.0001***
Monocyte absolute counts ($\times 10^3$ cells/μl)	0.3-1.6	0.37-1.88	**0.0001***	0.3-1.6	0.35-1.91	**0.0001***
Granulocyte absolute count ($\times 10^3$ cells/μL)	0.7-4.5	-	-	0.9-6.0	-	-
Reference					**(Gitaka et al., 2017).**	

Table showing comparison of Kisumu ranges against Kilifi ranges for 1 m to 6 m age group

-Some data were missing from published data for Kilifi values to compare with Kisumu reference ranges

*- values in **bold** with asterisk shows statistically significant results

Table 4. 22: 95% reference intervals for hematological parameters for Kisumu children aged 1 m to less than 12 m and 1 m to 17 m, compared to published data for Kilifi

Table showing comparison of Kisumu and Kilifi hematological median values.

Parameter	**95% reference intervals in children aged 1m to less than 12months**			**95% reference intervals in children aged 1m to 17months**		
	Kisumu (current)	Kilifi	P value	Kisumu (current)	Kilifi	P value
White blood cells ($\times 10^3$ cells/μl)	9.7 (5.4-19.2	9.7 (5.6-16.6)	**0.0023***	10.1 (5.7-19.8)	10.0 (5.71-16.72)	**0.0001***
Lymphocytes (%)	69.9 (51.1-82.6)	-	-	69.1 (48.6-82.2)	-	-
Monocytes (%)	7.3 (3.7-13.2)	-	-	7.2 (3.4-13.3)	-	-
Granulocytes (%)	22.3 (10.3-40.7)	-	-	23.2 (10.9-43.0)	-	-
Red blood cells ($\times 10^6$ cells/μl)	4.26 (2.90-5.58)	-	-	4.44 (2.94-5.78)	-	-
Hemoglobin (g/dl)	11.1 (8.1-14.9)	9.8 (7.3-13.2)	**0.0001***	10.9 (7.6-14.6)	9.8 (7.2-12.7)	**0.0001***
Hematocrit (%)	34 (25.5-44.7)	30.6 (23.5-39.2)	**0.0001***	33.2 (24.4-43.8)	30.6 (23.9-38.3)	**0.0001***
Platelets ($\times 10^3$ cells/μl)	393 (147-734)	451 (73-770)	**0.0001***	400 (129-757)	462 (84-773)	**0.0001***
Mean cell volume (fl)	86.3 (57.1-108.1)	72 (53.4-98.6)	**0.0001***	73.4 (54.9-105.6)	69 (52-97)	**0.0001***
Mean cell hemoglobin (pg)	28.1 (18.1-36.1)	-	-	24 (16.9-35.5)	-	-
Mean cell hemoglobin concentration (g/dl)	32.8 (30.7-34.8)	-	-	32.6 (30.0-34.7)	32.1 (29.4-34.4)	**0.0001***
Mean platelet volume	7.1 (5.6-9.4)	-	-	7.1 (5.6-9.4)	-	-
Red cell distribution width	16.2 (13.3-23.0)	-	-	17 (13.4-25.1)	-	-
Lymphocyte absolute counts ($\times 10^3$ cells/μl)	6.6 (3.4-13.5)	5.96 (3.3-10.2)	**0.0001**	3.5-13.6	6.0 (3.13-10.2)	**0.0001***
Monocytes absolute counts ($\times 10^3$ cells/μl)	0.7 (0.3-1.6)	1.02 (0.5-2.0)	**0.0001**	0.3-1.6	1.02 (0.48-1.93)	**0.0001***
Granulocyte absolute counts ($\times 10^3$ cells/μl)	2.2 (0.8-5.3)	-	-	0.9-6.0	-	-
Reference					**(Gitaka et al., 2017)**	

-Some parameters were not available in Kilifi data for comparison with Kisumu results.

*- values in **bold** with asterisk show statistically significant results

Table 4. 23: 95% reference intervals for hematological parameters for Kisumu children aged 1 m to less than 12 m, compared to published data for Kilifi, Tanzania and United States/Europe.

Parameter	**Kisumu (current)**	**Kilifi**	**Tanzania**	**USA/Europe**
White blood cells ($\times 10^3$ cells/μl)	9.7 (5.4-19.2)	5.6-16.6	2.0-17.3	5.0-17.0
Lymphocytes (%)	69.9 (51.1-82.6)	-	-	-
Monocytes (%)	7.3 (3.7-13.2)	-	-	-
Granulocytes (%)	22.3 (10.3-40.7)	-	-	-
Red blood cells ($\times 10^6$ cells/μl)	4.26 (2.91-5.58)	-	-	-
Hemoglobin (g/dl)	11.1 (8.1-14.9)	7.3-13.2	8.1-13.2	9.4-13.0
Hematocrit (%)	34 (25.5-44.7)	23.5-39.2	25.1-38.6	28-42
Platelets ($\times 10^3$ cells/μl)	393 (147-734)	72.7-769.2	25-708	150-400
Mean cell volume (fl)	86.3 (57.1-108.1)	53.4-98.6	53.3-96.6	70-98
Mean cell hemoglobin (pg)	28.1 (18.1-38.1)	-	-	-
Mean cell hemoglobin concentration (g/dl)	32.8 (30.7-34.8)	-	-	-
Mean platelet volume	7.1 (5.6-9.4)	-	-	-
Red cell distribution width	16.2 (13.3-23.0)	-	-	-
Lymphocyte absolute ($\times 10^3$ cells/μl)	6.6 (3.4-13.5)	3.3-10.2	3.3-11.8	3.3-11.5
Monocyte absolute ($\times 10^3$ cells/μl)	0.7 (0.3-1.6)	0.5-2.0	0.2-1.5	0.2-1.3
Granulocyte absolute ($\times 10^3$ cells/μl)	2.2 (0.8-5.3)	-	-	-
Reference		**(Gitaka et al., 2017).**	**Buchan *et al.*, 2010**	**Simpkin &Hinchliffe, 2006)**

Table showing comparison of Kisumu hematological reference ranges with some published data

-Some data were missing from other published data to compare with Kisumu reference ranges

4.3. Elevated ALT and AST Liver bio-markers in HIV positive ART naive adults Co-infected with Hepatitis

Initiation of anti-retroviral therapy requires a proper understanding of the health status of HIV infected individuals as these drugs have been known to lead to liver toxicity and pathology. The study examined the levels of liver enzymes (ALT and AST) in HIV positive adults who were either infected or not with hepatitis B or hepatitis C but who were not yet on ART.

4.3.1. Effect of Hepatitis B on ALT and AST Liver biomarkers

It was observed that there were no significant differences in the median levels of ALT and AST between hepatitis B positive and negative groups. The median ALT value in hepatitis B positive individuals was 20.35 IU/L compared to the median value in hepatitis B negative individuals which was 20.3 IU/L (p=0.6282). For AST values, the median AST value in hepatitis B positive individuals was 30.0 IU/L compared to the median values in hepatitis B negative individuals which was 26.2 IU/L (p=0.7786). Figure 4.26 show the levels of clinical biomarkers in hepatitis B positive adults compared to hepatitis B negative adults.

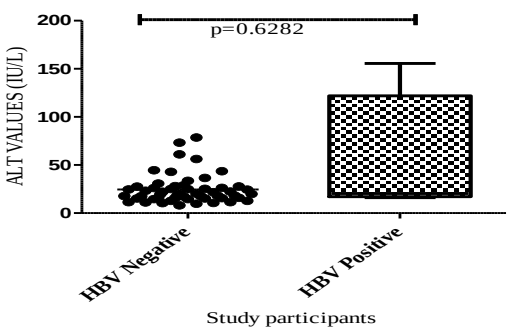


Fig 4.26 (a)

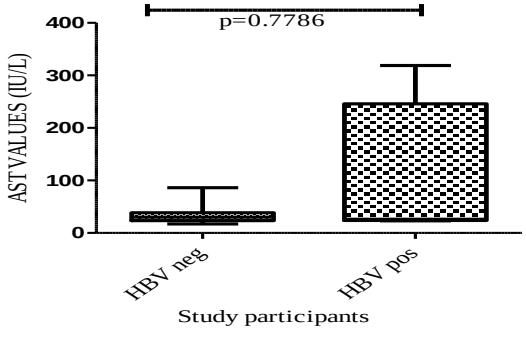


Fig 4.26 (b)

Figure 4. 25: : The levels of ALT and AST Liver bio-markers in HIV positive ART naïve adults with and without Hepatitis B

4.3.2. Effect of Hepatitis C on ALT and AST Liver biomarkers

The study found that there were significantly high differences in the levels of liver enzymes in the HIV positive participants who were hepatitis C positive compared with those who were negative. The median ALT value in hepatitis C positive individuals was 30.8 IU/L compared to the median values in Hepatitis C negative individuals which was 19.8 IU/L (p=0.0494). For AST values, the median AST value in hepatitis C positive individuals was 42.4 IU/L compared to the median values in Hepatitis C negative individuals which was 29.4 IU/L (p=0.3630). Fig 4.27)

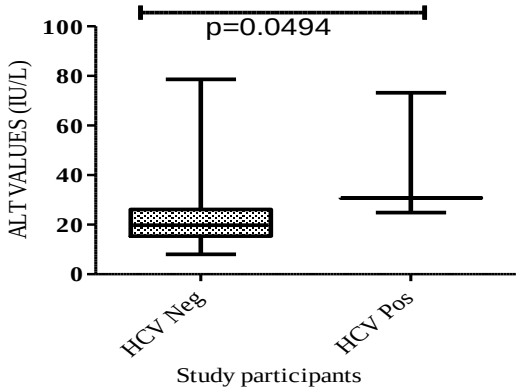


Fig 4.27 (a)

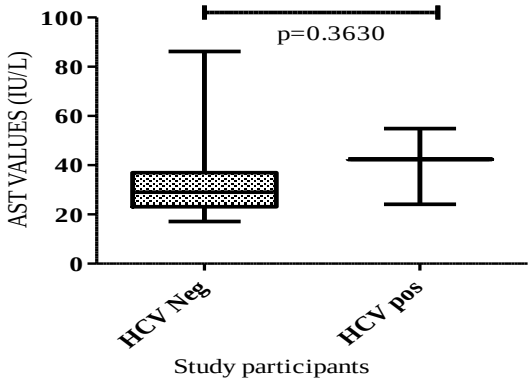


Fig 4.27 (b)

Figure 4. 26: Box and whisker plots showing the levels of ALT and AST liver biomarker characteristics in HIV positive ART naïve adults with and without Hepatitis C from Kisumu West

Table 4. 24: Summary of the median levels of the ALT and AST Biomarker characteristics in HIV positive ART Naive adults in Kisumu West

	Median Levels of ALT and AST Biomarker Characteristics (IU/L)					
	Hepatitis B status			Hepatitis C status		
	HBV Neg	HBV Pos	P values	HCV Neg	HCV Pos	P values
ALT	20.3	20.35	0.6282	19.8	30.8	0.0494
AST	26.2	30	0.7786	29.4	42.4	0.363

This table shows the summary of median levels of clinical biomarker characteristics in HIV positive ART Naive individuals from Kisumu West

4.4. CD4 Immunophenotypic characterization of HIV infected ART naive adults with hepatitis B and hepatitis C

The study also examined the levels of CD4 immunophenotypes in HIV positive ART naive adults with and without hepatitis B and hepatitis C co-infections.

4.4.1. Hepatitis B and C infection and CD4 Immunophenotypes levels

The study revealed that there were no significant differences in the median levels of CD4 in the HIV positive ART naïve adults who had hepatitis B positive against those who didn't have hepatitis B. The median CD4 value in hepatitis B positive individuals was 519.5 cells/ul compared to the median values in Hepatitis B negative individuals which was 378 cells/ul (p=0.6732). Similarly for hepatitis C, the study revealed that there were no significant differences in the median levels of CD4 in hepatitis C positive individuals compared to the median levels in hepatitis C negative individuals (p=0.2918). (Figure 4.28)

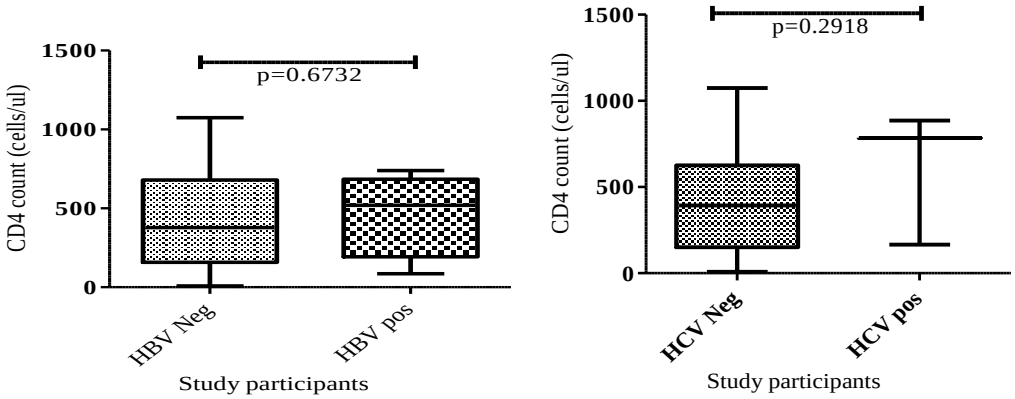


Fig 4.28 (a) Fig 4.28 (b)

Figure 4. 27: : Box and whisker plots for the CD4 immunophenotype levels in Hepatitis B and C negative and positive adults

4.5. The relationship between the CD4 immunophenotypes and ALT and AST biomarkers in adults in Kisumu West

It was observed that there was a positive correlation, where a rise in ALT was correlated with a rise in AST. Figure 4.29 shows the correlation of the clinical biomarkers and the immunophenotypes.

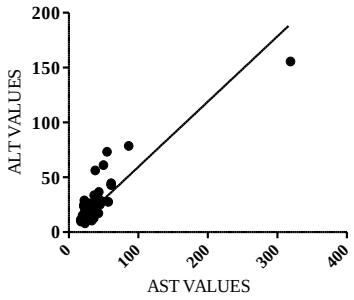


Figure 4. 28: Spearman's correlation showing the relationship between the clinical biomarker analytes in HIV positive ART naive adults

CHAPTER FIVE

DISCUSSION

The main objective of the study was to develop hematological reference ranges in HIV negative, healthy children between 1 month and 17months, and to characterize CD4 immunophenotypes and ALT and AST liver biomarker levels in ART Naive HIV positive adults from Kisumu West Sub-County. Reference ranges have a significant effect on interpretation of test results, and there is always a need to compare results obtained with these values (Choong ML and (1995)). Health of an individual can be assessed by the data provided from the laboratory values and these include hematological values. These values are frequently used in research studies and clinical trials in monitoring health status of study participants. The reference values are such values that are derived from observation or measurement on reference individuals in the reference sample group to ascertain the normal physiological ranges of a given population, which have never been established in most African countries (CLSI, 2008). In settings where locally established reference values aren't available research and clinical care providers usually resort to already established American and European reference ranges. There could be many variations in such values as pertains age, nutrition, genetic differences, exposure to infectious diseases, ethnic origins, socio-demographic characteristics and other factors based on the environment. (Kibaya et al., 2008). It is thus quite beneficial to develop region and age specific reference values that can well be applied for efficient patient management and generally for proper conduct of clinical research especially the research studies geared towards reducing the disease burden for most African infectious diseases.

For the immunophenotypic characteristics, the study looked at the CD4 immunophenotypic and ALT and AST hepato-biomarker characteristics of HIV positive ART naive adults who were infected or not with hepatitis B and hepatitis C from Kisumu West Sub-County. Most studies have focused on different aspects of HIV positive group on ART since they are the majority and they are mostly accessible. As some modes of exposure/transmission to HIV virus are almost the same as in viral hepatitis relating blood infections, this necessitates studying the immune-phenotypic and hepato-biomarker characteristics in this group of ART Naive individuals. The study also assessed the relationship between the immunophenotypic and hepato-biomarker characteristics in HIV ART Naive adults in Kisumu West Sub-county.

5.1. Hematological Reference ranges in children between 1 month and 17 months from Kisumu West Sub-county

This study was carried out in Kisumu West Sub-County in Kisumu County, Western Kenya, a region in Kenya where many clinical research studies are being carried out. One of the objectives of this study was to develop hematological reference ranges in healthy, HIV negative children between 1 month to 17 months from Kisumu West Sub county, which may serve as standards for interpretation of laboratory results. The age groups were based on the previous GSK sponsored vaccine study that was done and for which data was plenty and available to develop the hematological reference ranges. The values currently mostly used in Kisumu West Sub-County for the children are from Harriet Lane Handbook (Gunn et al.) and analyzers. From the study, it was possible to

develop hematological reference ranges that are more applicable in different and specific age brackets especially for studies that enroll children in different age categories. The results describe what is typical in a population whose participants' data were evaluated as being normal through predetermined criteria including physical examination.

In comparison to Tanzanian values, Kisumu hematology reference ranges were observed to be higher than the ranges of Tanzanian children for the WBC, Absolute lymphocyte and monocyte counts, hemoglobin, hematocrit and MCV. In this study, higher ranges of WBCs, absolute lymphocyte and monocyte counts were observed compared to the values in US/Europe. This implies that cases that would otherwise be considered as leukocytosis when American/European values are used would actually be normal when the values that were developed in Kisumu West are used. Several African studies have shown lower red blood cell indices in children and adults. In this study, wider ranges were observed in hemoglobin, hematocrit, and MCV as indicated in table 4.3. This could be attributed to factors such as malaria, other parasitic infections, haemoglobinopathies and iron deficiency anemia. Thus, if American or European hematological ranges are used in Kisumu County, participants who would otherwise have been enrolled into clinical trials/studies as healthy would be classified as unhealthy or excluded in the trials. Wider ranges were observed in platelet counts in Kisumu children compared to the American/European ranges. Thus, if American /European values are used in determining eligibility for participants into study, some healthy participants would otherwise be excluded on the basis of being considered as having

thrombocytopenia or thrombocytosis, confirming the necessity to apply the right ranges for a particular population. The analyzer that was used in performing the hematology analysis wasn't able to extrapolate basophiles, eosinophils, and neutrophils separately rather grouping these as granulocytes. There were no values for RBC and other RBC indices from these populations that could be used to compare the values that were obtained in Kisumu West.

In comparison to reference ranges that were obtained in Kilifi, there were noted some differences in the reference values and ranges from Kisumu West despite being in the same country. In 1 month to 6 month age group, reference intervals for WBCs, hemoglobin, hematocrit, MCV and Lymphocytes absolute count values for Kisumu were noted to be higher with higher median values than the Kilifi values. The median values for Absolute monocyte counts in Kisumu children were lower compared to the Kilifi children. There were narrower platelets ranges for Kisumu children compared to the ones for Kilifi. This same pattern was depicted in the 1 month to 17 months age group. The researcher isn't pointing out exactly the reason for such differences, but some suspected possibilities could be due to differences in altitude, differences in regard to exposure to infectious diseases, ethnic constitution of the study population, differences in nutritional deficiencies or other environmental factors.

Compared to Harriet Lane Handbook, higher counts were observed in WBC counts, both absolute and percent lymphocyte counts, as well as monocyte counts for current study. Wider ranges were observed in RBC, platelets and RDW, while lower ranges noted in the current study for hemoglobin, hematocrit and granulocyte counts.

5.2. Alanine transaminase and Aspartate Transaminase liver biomarker characteristics in HIV positive adults with or without hepatitis B and C in Kisumu West

In this study, regarding liver enzymes, there was a significant increase in the serum liver enzymes ALT in HIV positive HCV positive co-infected people as compared to HIV positive HCV negative individuals. Although the study participants who were co-infected with the viral hepatitis and HIV were few, the study supports other studies conducted by Zhou *et al*., (2007) who reported that many people with chronic hepatitis have elevated liver enzymes levels.

ALT is produced in liver cells, the major cell type in the liver. ALT is often inaccurately referred to as a liver function test. The level of ALT in the blood may be elevated in situations the liver cells are destroyed. As these are destroyed, the enzyme leaks out into the blood. All types of hepatitis whether viral, alcoholic or drug-induced, can cause liver cell damages which can result in increase of serum ALT activity (Schiff et al., 2017). The ALT level may also be rise in situations where there occur liver cell death that could occur due to other causes, for example, drug toxicity and even shock. The levels of the ALT enzyme may correlate with the extent to which cell death or inflammation occurs, although may not always be the case. An accurate estimate of inflammatory activity or the amount cell death can only be made by liver biopsy (Schiff et al., 2017).

AST is an enzyme similar to ALT but less specific for liver disease as it is also produced in muscle and can be elevated in other conditions (for example, early in the course of a heart attack). AST is also inaccurately referred to as a liver function test by

many physicians. In many cases of liver inflammation, the ALT and AST activities are elevated roughly in a 1:1 ratio. In some conditions, such as alcoholic hepatitis or shock liver, the elevation in the serum AST level may higher than the elevation in the serum ALT level (Schiff et al., 2017). In a similar research in Northern Nigeria, Adewole et al., (2009) reported a high mean level of ALT among HIV patients co-infected. Increased levels of liver enzymes observed among HIV patients co-infected with HCV in this present research may be because of the asymptomatic nature of HCV that leads to late diagnosis. At this stage, the patient might have developed most symptoms and thus, in chronic stage of the infection since 70-80% of people infected with HCV develop chronic infections Also, Boyer, (2001) reported that HCV is the leading cause of chronic liver disease in the United States. Even though the normal levels of AST and ALT vary depending on the individual, the range for normal AST is reported between 10.4-40.5 U/L and ALT between 7.1 – 56.4 U/L. More specifically, the recommended AST values are 14.0-20.4 U/L for males, and 10.3-36.7 U/L for females (Schiff et al., 2017). 44 (77.19%) participants had AST values within the recommended range while 52 (91.23%) of the participants had ALT values within the recommended range.

Liver enzyme elevations are a frequent finding in HIV-infected patients as a consequence of several risk factors. However the analysis of these events is limited as precise etiology is rarely clearly defined. Abnormalities in liver function tests could be produced exclusively by direct inflammation in hepatocytes, caused by the HIV virus. Although the mechanisms by which HIV causes hepatic damage are still unknown, studies have shown that it may be as result of apoptosis (induced by caspases 2, 7 and 8) and mitochondrial dysfunction with decreasing mitochondrial DNA in several tissues.

Another injury mechanism is permeability alteration in mitochondrial membrane by HIV proteins which stimulate an inflammatory response (Pol et al., 2004). Alanine aminotransferase (ALT) is a hepatic enzyme that may be used as a marker of hepatocellular injury. (Zechini et al., 2004). However, the impact of viral hepatitis on the immune system and liver enzymes needs further studies in both on HAART and ART naive HIV positive patients.

5.3. CD4 immunophenotypes in HIV positive adults with or without hepatitis B, hepatitis C in Kisumu West

The results for this study showed an overall median CD4 count of 433 cells/mm3 and median of 407cells/mm3 for the 57 adult participants studied. CD4 levels were studied in those who were Hepatitis B and C positive adults against those who were negative for Hepatitis B and C. In this study there were no significant differences in the median CD4 levels in the HIV positive ART Naive adults from Kisumu West who are either co-infected or not with Hepatitis disease. This was inconsistent with a study done by Sajadi that reported that within a NVS cohort, individuals without chronic HCV had a statistically significant elevation in mean CD4 count compared to those NVS with chronic HCV. The correlation of CD4 count with HCV status in the study was statistically significant for earliest recorded CD4 count and there was a trend toward significance with the most recent CD4 count as well (Sajadi et al., 2010). In this study there was a similarly statistically significant elevation in the mean CD4% and mean CD4/CD8 ratio in those without chronic HCV compared to the chronic HCV group. (Sajadi et al., 2010)

However in the current study from Kisumu West, the researcher acknowledges a limitation of low numbers of Hepatitis positive cases partly due to low prevalence of Hepatitis in the area thus limiting adequate conclusions to be drawn from this.

In this study of 22 males and 35 females, there was also difference on the mean CD4 values in relation to gender. The median CD4 value in the females was slightly higher than males (437 cells/mm3 in females compared to 427 cells/mm3 in males). Similar findings as this were observed in studies which have been conducted in. Kenya (Tugume et al., 1995). The higher CD4 values in females compared to males could probably due to biological factors. It has been speculated that gender and age-related variations within the immune system parameters may contribute to the pathogenesis of several gender and age-related diseases such as autoimmune disorders in female patients (Schiff et al., 2017). Since the number of subjects with Hepatitis B and C were few, comparison of the positive Hepatitis B and C individuals in terms of gender could not be done to effectively yield statistically significant conclusions

Globally, the recommended CD4 range is 400 – 1600 cells/mm^3. Some people would be initiated to medication when their CD4 counts reaches 350 cells/mm^3. On the other hand, according to AIDS.gov, one of the qualifications for an AIDS diagnosis is CD4 count less than 200 cells/mm^3. Almost half of the patients (49.1%) had CD4 count less than 400 cells/mm^3. Those who required to be initiated on medication were 42.10%, while 28.07% had CD4 counts less than 200 cells/mm^3.

In this study, again it was not possible to characterize the immune and biomarker characteristics in the hepatitis B and C positive individuals. This is because even in the general population prevalence of the hepatitis B and C infections is low and so getting

higher numbers to be studied sometimes is hard. This study only had four individuals who were positive for hepatitis C and four who were positive for Hepatitis B, one of whom had Hepatitis B and C co-infection. Characterizing these on their own would not provide statistical significant conclusions and thus causality could not be inferred.

5.4. The relationship between the CD4 immunophenotypes and ALT and AST liver biomarkers in HIV positive ART naive adults with or without hepatitis B and hepatitis C

To investigate the relationship between CD4 levels and serum levels of ALT and AST among other variables, spearman's correlation analysis was used to do this. Rationale for studying this is that for example, liver dysfunction is a real challenge in the management of HIV infected patients, thus evaluation of the liver enzymes and CD4 levels in relation to viral load levels is necessary. Especially a proper understanding on how this is depicted in HIV positive ART Naïve patients is required. This is due to the fact that the use of ART has completely modified the pattern of hepatic events in HIV infection resulting in significant decrease in morbidity and mortality among HIV infected patients (Guaraldi et al., 2011). It is important to monitor trend of these liver functions especially before introduction of anti-retrovirals in order to evaluate effects that could result. The results of the study shows that a positive correlation between HIV viral load and ALT was observed. This is consistent with findings for another study (Denue et al., 2013). As seen above, ALT could be used as markers of hepatocellular injury. Liver enzyme elevations are frequent in human immune deficiency virus (HIV)-infected patients which may be caused by the HIV virus in those without other risk factors for liver damage. It is thus recommended evaluating patients with high ALT for

early anti-retroviral therapy (ART) in those without risk factor for liver damage regardless of the CD4+ cell count, especially where facility for estimating viral load is not available. ALT should be monitored given the observed positive correlation between the HIV viral load and serum levels of ALT enzyme in HIV infected ART naive patients.

CHAPTER SIX:

6.0. Summary of study findings, conclusions and recommendations

6.1. Summary of study findings

The following is the summary of the findings of this study:

1. This study was able to establish gender and age specific hematological reference ranges that can be used in Kisumu West Sub-county, Kisumu County for clinical vaccine and drug trials, clinical management of children as well as evaluation of adverse events. It was observed that the total white blood cell counts, monocyte and granulocyte absolute counts in 1 m to 6 m age groups are lower than the other two age groups. In 1 m to 6 m age group, it was noted that the median values of hemoglobin, hematocrit and the RBC indices are higher compared to the age groups 6 m to 12 m and 12 m to 17 m. In regard to gender differences, hemoglobin values in females in 1 m to 6 m age group were significantly higher than the male children. Platelet counts in females in 1 m to 6 m age group were significantly higher than the male children. The median values for RBC indices for females were higher compared to males. In comparison to the American /European reference ranges, we observed higher ranges of WBCs, absolute lymphocyte and monocyte counts. Wider ranges were observed in platelet counts in children from Kisumu West compared to the American /Europe ranges. Compared to Harrriet Lane Handbook, higher counts were observed in WBC counts, lymphocyte counts and monocyte counts in the study. Wider values were noted in RBC, platelets and RDW, while lower ranges were noted in the current study for hemoglobin, hematocrit and granulocyte counts.

2. This study shows that median ALT in HIV positive individuals was elevated by Hepatitis infection. It was observed that hepatitis C positive HIV positive adults had higher values than the Hepatitis C negative participants.

6.2. Conclusions

1. For reference ranges, the current study provides the first locally established clinical hematological laboratory reference ranges in children below two years in Western Kenya, Kisumu County. The findings of the study show that most of the ranges and median values across different age groups and gender are different and thus need to use ranges for specific age categories and gender as developed in table 4.3. In spite of the factors influencing hematological values, it was possible from this study to develop the hematological reference in children in Kisumu West Sub-County, Kenya.
2. For the immunophenotypic characterization, there were significant differences in regard to the median levels of the ALT in hepatitis C infected group compared to hepatitis C non infected participants. For the other CD4 immunophenotypes and clinical biomarker analytes (ALT and AST enzyme levels) there were no significant differences in both the HIV positive ART Naive adults with or without hepatitis B and hepatitis C. However, before arriving at a final conclusion, a study with more respondent who tested positive for Hepatitis B or C would give a correct picture. Screening for viral hepatitis should also be performed alongside HIV testing in order to detect it early for effective therapy and good prognosis.

6.3. Recommendations

6.3.1. Recommendations from the current study

1. In evaluating and establishing reference ranges completely healthy children would be required in line with assessing other co-infections for example the hepatitis B, Hepatitis C, and syphilis given availability of resources to screen for these diseases.

2. Further, similar study of clinical characterization and immunophenotypes that would utilize higher number of Hepatitis B and C positive individuals and with higher number of individuals in general could be warranted for proper characterization of the immune-phenotypic and hepato-biomarker characteristics. The sample size could have been small for this study due to the criteria of limited data that was only accessible in ART Naive individuals.

6.3.2. Recommendation for future studies

For more characterization, future studies could utilize more of these immune-phenotypes and clinical biomarker analytes

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APPENDICES

APPENDIX 1- APPROVAL DOCUMENT REQUEST FOR DATA USE

DOCUMENTED SUB-STUDY TEMPLATE TO REQUEST FOR USE OF DATA FOR MSc THESIS

AFRICOS Repository Request

Internal document requesting de-identified AFRICOS samples/data under existing approved protocol language, completed for study activity tracking purposes.

Topic/Title:
A study to determine the description of clinical characteristics, liver enzymes and CD4 changes in Hepatitis B and Hepatitis C HIV infected ART naïve subjects enrolled in a large cohort study in Western Kenya

Requesting AFRICOS Investigator:
Principal investigator
Name: DR MILTON OMONDI

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Sub-study Investigator(s):

Name: JEW OCHOLA
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Other participating AFRICOS team members:

Applicable AFRICOS Objective(s): **Bullet list applicable protocol objectives**

- To investigate the prevalence and incidence of HBsAg, HBeAg, HBcAb and HBV DNA viremia in HIV infected and uninfected subjects

Objectives of Current Study:

Primary Objective

To study immunological phenotypic and hepato-biochemical analytes characteristics in HIV positive ART naive adults from Kisumu West Sub-county

Secondary Objectives

1. To assess the levels of clinical biomarker analytes in HIV positive ART naive adults with or without Hepatitis B and C.
2. To determine immuno-phenotypic biomarker in HIV positive ART naive adults with or without Hepatitis B and C
3. To correlate phenotypic and clinical biomarkers in HIV positive ART naïve adults with or without Hepatitis B and C

Significance of Study: **Briefly summarize any background information and the significance of this project.**

The use of ELISA as an immunological diagnostic method is important in assessment of negativity and positivity status of some diseases. Some of these include Hepatitis B, C and *Mycobacterium Tuberculosis*. Immunological pheno and biomarker characterization in ART NAÏVE HIV positive individuals with or without Hepatitis B, C and Mycobacterium tuberculosis is important since it could lead to a deeper understanding of why the differences could occur. This could also help to establish if there could exist as a result of these in regard to age and gender i.e. whether exposure rates are higher in a particular age group or gender compared to another and if this could be of any significance especially in regard to intervention. The generated information will help health providers to come up with better strategies of HIV management in HIV ART subjects.

Project description: **Briefly summarize planned work**

Sites from which samples/data requested:

- Data is requested from Kericho and Kisumu West District

Description of requested data:

The study will utilize data from the initial visit.

	HBV Pos	HBV Neg
Sex		
Age		
Education		
Risk Factors: Blood Transfusion Intravenous Drug Use Casual Sex Work MSM Alcohol Use		
HIV status		
Hepatitis C Status		
ALT Grade		

Data Management:

No samples are requested. Data from the repository will be used for the indicated analysis. The work will be done in fulfillment of the requirements for an MSc in Immunology.

Data Analysis assistance requested: ❒no

Responsible laboratory personnel to receive samples: No samples

Unused samples will be: No samples

Special instructions/requests:

Statistical Considerations: **If analysis is exploratory, can state N/A**

APPENDIX 2-SIGNED APPROVAL FOR USE OF DATA FOR Msc thesis

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20910

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www.hivresearch.org

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22 August 2014

Mr. Ouma 'Jew' Ochola
P.O. Box 54
Kisumu, Kenya

To whom it may concern:

As the Protocol Chair for RV 329: African Cohort Study (AFRICOS), I hereby authorize Mr. Jew Ochola to evaluate de-identified AFRICOS data from our study sites in the South Rift Valley and Kisumu West. The data is authorized for use regarding the proposed investigation of the prevalence of Hepatitis B and/or C in both HIV positive and negative participants, as well as description of clinical characteristics, liver enzyme and CD4 changes in subjects with and without Hepatitis B and/or C from the HIV positive subject population.

Sincerely,

Julie Ake M.D., MSc., F.A.C.P.
MAJ(P), MC
AFRICOS Protocol Chair

The U.S. Military HIV Research Program at the Walter Reed Army Institute of Research is supported by a cooperative agreement between the United States Army Medical Research and Materiel Command and the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.

APPENDIX 3- GSK approval for reference range data

APPENDIX 4- **Blood sample collection Procedures:**

Collection tubes

- **Clot Tube/Red top tube** – tube does not contain anticoagulant. Used to obtain clotted blood or serum specimen. Serum is required for serological tests, most chemistries and blood typing.
- **Serum Separation Tube (SST**) – tube contains "Thixotropic Gel" in the bottom. During centrifugation, the gel temporarily becomes fluid and moves to the dividing point between the serum and cells. These tubes are known as "Quick Clot" tubes and are for STAT chemistry testing.
- **ACD-Yellow top tubes—**tubes contain anticoagulant ACD for PBMC isolation
- **Purple Top** – Tubes contain the anticoagulant ethylene-diamine tetra- acetic acid (EDTA). Used to obtain whole blood or plasma. Most common tests drawn are CBC, differential, sedimentation rate, reticulocyte count, red blood cell cholinesterase, and lead.

Order of Draw

i. First draw **– T**ubes with no additives (i.e. red).
ii. Second Draw – purple top tubes
iii. Third draw-ACD, Yellow-top tubes.
iv. Last draw – Tubes with additives, Quantiferon tubes.

Preparation

- Assemble all materials to ensure easy access.
- Properly identify the subject. Ensure laboratory request form and labels are for subject.
- Check tests requested and select the appropriate tube. If test is unknown, ask laboratory supervisor.
- Tubes that contain additives should be gently tapped to dislodge any additive that may be trapped around the stopper.
- Sit the subject comfortably in the chair, and if necessary, have the subject roll up sleeve.
- Place the tourniquet above elbow and tighten enough to find the appropriate vein.
- Palpate the ante cubit al fossa area and locate the desired vein. Loosen tourniquet.
- Starting from the center and moving outward clean area with 70% isopropyl alcohol in a circular motion. Do not re-palpate disinfected site.
- Choose the proper needle depending on the size of the selected vein.

Vacutainer Technique

- Open needle package but do not remove the needle shield. Thread the needle into the holder until secured. Apply tourniquet. Do not re-palpate disinfected site.
- Have the subject make a fist and straighten the arm.

- Remove the needle cover and inspect the needle to ensure that it is not damaged.
- Position needle with bevel up, parallel to, and over the top of the vein. Insert the needle quickly under the skin and then into the vein.
- After entry into the vein, push the tube all the way into the holder and allow the blood to fill the tube.
- When blood starts to flow into the tube, release tourniquet and have subject release fist. If multiple specimens are needed, release the tourniquet after the first tube is collected. To fill other tubes, remove the full tube and insert new tubes until all required tubes are filled. Follow the order of draw described above.

➢ If no blood flows into the tube or blood ceases to flow before an adequate specimen is collected, the following steps are suggested to complete a satisfactory collection.
➢ Push tube forward until tube stopper is penetrated. If necessary, hold in place to ensure complete vacuum draw.
➢ Confirm correct position of needle in vein.
➢ Tubes may have bad vacuum. Remove tube and replace with new tube.
➢ If second tube does not draw, remove needle and discard and repeat procedure.
➢ Upon completion of the venipuncture, remove the needle from the subject's arm and apply pressure to the site, using a sterile piece of gauze. Instruct the subject to elevate the arm slightly and continue to applying pressure for 2-3 minutes.

b. Immediately dispose of needle into sharps container. If an accidental needle stick occurs, contact the laboratory supervisor immediately. Wash the area with soap and water for follow up with treatment.
    i. Place labels on tubes and place tubes in appropriate rack/rocker for testing/transporting to testing laboratory. Labels should include study subject's, identification number, date and time of draw, and any additional information as required.
    ii. When the venipuncture site has stopped bleeding, place a bandage over the site and escort the subject out of the phlebotomy area.
    iii. The only areas that are authorized for the phlebotomist to draw blood are the arms and the hands. Arterial sticks are strictly prohibited. In the event that an accidental arterial stick occurs, immediately remove the needle and place direct pressure on the site.
    iv. Maintain pressure on the site for minimum of five minutes. After bleeding has clearly stopped, place a pressure dressing on the site and have the subject apply more direct pressure to the site for another 15 minutes.
c. Place labels on tubes and place tubes in appropriate rack/rocker for laboratory testing/transportation to testing lab. Labels should include study subject's identification number, date and time of draw, and any additional information as required.

Appendix 5-HEPATITIS B ELISA PROCEDURE

a. Perform equipment maintenance and calibration when necessary as required by the manufacturer
b. Bring all of the reagents, except Conjugate Concentrate, to room temperature before beginning the assay procedure.
c. Prepare working concentrations of Wash Solution, Conjugate Solution, and TMB Solution. Mix gently, by inversion. **Be sure that Conjugate Solution is completely mixed.** Mix again just before use.
d. Remove strips not needed for the assay and replace them with labelled Null Strips, if necessary. Micro-well strips not needed for the assay may be returned to the plate pouch and sealed, and then used at a later time. Strips from different plates can only be mixed to assemble full or partial plates if they are from the same plate lot and have come from plates that have previously been tested with kit controls and yielded valid runs. When assembling a plate that contains strips from a newly opened, previously untested plate, one of these strips should be placed at the beginning of the plate and tested with the kit controls. If sample identity is not maintained by an automatic procedure, identify the individual wells for each specimen or control on a data sheet.
e. **Add 100 µl of the controls or specimens to the appropriate wells** of the microwell plate. In addition to patient specimens, one reagent blank (Blank on Air), two Positive Controls, two Low Positive Controls, and three Negative Controls should be assayed on each plate or partial plate of specimens.
f. Cover the microwell plate with a plate sealer or use other means to minimize evaporation.
g. Incubate the plate for 60 to 65 minutes at 37±1°C using a dry-heat static incubator. At the end of the incubation period, carefully remove the plate cover and aspirate the fluid from each well into a biohazard container. **Wash the microwell plate or strip a minimum of five times** with the Wash Solution (at least 400 µl/well/wash), or as otherwise validated. **Soak for 30 to 60 seconds between each wash.** Aspirate the Wash Solution after each wash. After the last wash, if excess liquid remains, blot the inverted plate on clean, absorbent paper towels.

   NOTE: Grasp the plate holder firmly at the center of the long sides before inverting to blot.
h. **Add 100 µl of Working Conjugate Solution to each well** containing a specimen or control except H1. Cover the microwell plate with a plate sealer or use other means to minimize evaporation. Incubate the plate for 60 to 65 minutes at 37±1°C using a dry-heat static incubator.At the end of the incubation period, carefully remove the plate cover and aspirate the fluid in each well into a biohazard container. **Wash the plates a minimum of five times** with Wash Solution (at least 400 µl/well/wash), or as otherwise validated. **Soak for 30 to 60 seconds between each wash.** Aspirate the Wash Solution after each wash. After the last wash, if excess liquid remains, blot the inverted plate on a clean, absorbent paper towel. NOTE: Grasp the plate holder firmly at the center of the long sides before inverting to blot.

i. **Add 100 µl of the Working TMB Solution to each well containing a specimen or control** except H1**.** Cover the microwell plate with a fresh plate sealer or use other means to minimize evaporation. Incubate plates in the dark for 30 to 33 minutes at room temperature (15-30°C**).** (For example, cover the plates with black plastic or place in a drawer.)

j. Carefully remove the plate cover and **add 100 µl of Stopping Solution to each well except H1** to terminate the reaction. Tap the plate gently, or use other means to ensure complete mixing. Complete mixing is required for acceptable results**.**

k. **Read absorbance within 30 minutes** after adding the Stopping Solution, using the 450 nm filter with 615 nm to 630 nm as the reference. (Blank on air.) Ensure that all strips have been pressed firmly into place before reading.

i. Cut-off Value

ii. Determine the cut-off value by adding the NCX to 0.070

Validation

iii. A run is valid if the following criteria are met:

- The absorbance values of the Negative Controls are greater than 0.000 AU and less than or equal to 0.100 AU. One Negative Control value may be discarded. If two or more Negative Controls are out of limit, the run must be repeated.
- The average of the absorbance values of the Positive Control must be greater than or equal to 1.000 and the individual absorbance values must be within range of 0.65 to 1.35 times the PCX. No Positive Control values may be discarded.

APPENDIX 6-HEPATITIS C ELISA PROCEDURE

a. Approximately 30min prior to the beginning of procedure, bring kit reagents to RT. Invert liquid reagents gently several times but avoid foaming. Check incubator temp; between 36-38C
b. Determine the total number of wells needed for the assay. In addition to specimens, one reagent blank, 3 negative calibrators and 2 positive controls must be included on each plate. Store unused wells at 2-8C in the supplied foil pouch with dessicant, tightly sealed and used within 42 days of opening the foil pouch. Record the date the pouch is opened and the expiry date of the unused

    NB; Handle microwell strips with care. Do not touch the bottom exterior surface of the wells
c. Assemble the microwell strips in the microwell strip holder, if necessary. Microwell strips must be level in the strip holder. For incomplete plates add uncoated microwell strips
d. Prepare a record (plate map) identifying the placement of the controls, calibrators and specimens in the microwells. Arrange the assay control/calibrator wells so that well 1A is the reagent blank. From well 1A, arrange all the controls and calibrators in a row (horizontal) or column (vertical) configuration as follows; B1, C1, D1 (Neg calibrators), E1, F1 (positive control) Configuration is dependant upon software
e. Visually inspect the microwells upon addition of specimens, calibrators and controls. A color change from green to blue indicates that the specimen, calibrator, or control has been added to the well. Add specimens, calibrators or controls to the wells as follows;
f. Add 200ul of specimen diluents to all wells, including 1A
g. Add 20ul of the controls, calibrators or specimens to the appropriate wells
h. If the controls, calibrators or specimens have been manually delivered, ensure that the contents of the wells are thoroughly mixed. Use microwell shaker, manual mixing with pipette is acceptable. Avoid splashing contents to test wells.
i. Cover the microwell strip holder with a plate sealer
j. Incubate at 37C±1C for 60min±5min
k. Level the strips in the microwell if necessary. With an aspirator washer device, aspirate and wash all wells 5 times with wash buffer IX
l. Aspirate the sample solutions from the microwells then fill completely with wash buffer. Do not let over-flow. Allow 20 soak seconds
m. Complete the aspirate/fill sequence 4 additional times. Completely aspirate wells. Invert the plate and firmly tap on a clean paper towel to remove excess wash buffer, if necessary
n. Add 200ul of conjugate to all wells, including 1A
o. Cover the micro-well strip holder with a new plate sealer. Incubate at 37C for 60min±5min
p. Prepare sufficient substrate solution to be used to allow time for OPD tablets to dissolve completely. Do not use more than a single preparation of substrate solution on a plate.
q. After the second incubation, wash the wells as above, 6.1.12
r. Add 200ul of substrate solution to all wells including 1A

s. Incubate at RT in the dark for 30min±1min
t. Add 50ul of 4N sulfuric acid to all wells, including 1A. To ensure proper mixing, acid should be added forcibly in a steady stream
u. Read the micro-well strip plate at a wavelength of 490nm with a reference wavelength of 620nm or 630nm. Blank the reader on well 1A. The blank value well 1A should be substracted from all control, calibrator and specimen well values prior to applying the QC criteria. Microwell plates must be read within 60min following the addition of sulfuric acid. Plates must be stored in the dark until read.

Quality control procedures/Validation

Reagent Blank Acceptance Criteria

A plate is considered valid with respect to the reagent blank if the absorbance value of the reagent blank well 1A is greater than or equal to -0.020 and less than or equal to 0.200. The plate is invalid if the substrate blank well is invalid.

Negative Calibrator acceptance criteria

The individual negative calibrator values must be less than or equal to 0.120 and greater than or equal to -0.005. if one of the 3 calibrator values is outside either of these limits, recalculate the negative calibrator mean (NCalx) based upon the 2 acceptable calibrator values. The plate is invalid and the test must be repeated if 2 or more of the 3 calibrator values are outside either of the limits

Determine the mean of the negative calibrator values (NCalx)

Positive Control Acceptance Criteria

A plate is considered valid with respect to the positive control if both positive control values are greater than or equal to 0.800, within the linear range of the reader and do not differ by more than 0.600

Calculation of the Cut-off Value

Cut-off value=NCalx + 0.600

APPENDIX 7-DATA COLLECTION SHEET

SID anonymized	CD4 levels	AGE	GENDER	HEPATITIS B STATUS	HEPATITIS C STATUS	ALT	AST

APPENDIX 8 SSC CRR APPROVAL DOCUMENT

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **March 12, 2015**

TO: **DR. MILTON OMONDI,**
USA MEDICAL RESEARCH UNIT,
PRINCIPAL INVESTIGATOR

THROUGH: **AG DIRECTOR, CCR,**
NAIROBI

18/3/15

Dear Sir,

RE: SSC PROTOCOL NO. 2396 *(RESUBMISSION-REQUEST FOR ANNUAL RENEWAL):* AFRICAN COHORT STUDY (AFRICOS)

Reference is made to your letter dated 26th February 2015. The ERC Secretariat acknowledges receipt of the revised document on 2nd March 2015.

This is to inform you that the Scientific and Ethics Review Unit (SERU) reviewed the document submitted, and determined that the issue raised at the 236th B meeting, has been adequately addressed.

This study is granted approval for continuation effective this **March 12, 2015.** Please note that authorization to conduct this study will automatically expire on **March 11, 2016**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by **January 29, 2015.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

APPENDIX 9-SERU APPROVAL-AFRICOS STUDY

KENYA MEDICAL RESEARCH INSTITUTE

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Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
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KEMRI/RES/7/3/1 **February 28, 2013**

TO: **DR MILTON OMONDI (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. JUMA RASHID,**
THE DIRECTOR, CCR,
NAIROBI

Forwarded 7/3/2013

Dear Sir,

RE: SSC PROTOCOL No. 2396 - REVISED (*RESUBMISSION):* AFRICAN COHORT STUDY (AFRICOS) PROTOCOL RV 329
(*MAIN STUDY PROTOCOL - VERSION 1.1 DATED 01 AUGUST 2012*)

Reference is made to your letter dated January 18, 2013. The ERC Secretariat acknowledges receipt of the revised proposal on 04 February, 2013.

The Committee noted that:

(a) The aim of the proposed descriptive study is assess the contribution of clinical practices, biological factors and socio-economic circumstances on HIV infection and disease progression in Africa.
(b) The proposed open-ended cohort study will involve both retrospective and prospective collection of data as well as routine blood collections for the duration of the study with study visits occurring every six (6) months.
(c) The blood samples will be separated into plasma, serum and peripheral blood mononuclear cells (PBMCs) and archived in the AFRICOS Repository (AFRICOSR).
(d) The use of AFRICOS specimens will be regulated by WRAIR IRB and the Ethics Committee for the country of specimen origin.
(e) AFRICOS sites in Kenya will be in South Rift Valley Province and Kisumu West in Nyanza Province enrolling a total of 1,200 and 600 participants respectively.

This is to inform you that at the Committee determines that the issues raised at the 209th ERC meeting of 30th October 2012 and consequently reviewed at the **212th ERC meeting** of the **26th February 2013** are adequately addressed.
Consequently, the study is granted approval for implementation effective this **26th February 2013** for a period of one year. Please note that authorization to conduct this study will automatically expire on **February 25, 2014**.