CHANGES IN HEMATOLOGICAL PROFILES AND NUTRITIONAL STATUS OF HIV INFECTED CHILDREN ON PROLONGED ANTIRETROVIRAL THERAPY AT KENYATTA NATIONAL HOSPITAL

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Changes in Hematological Profiles and Nutritional Status of HIV Infected Children on Prolonged Antiretroviral Therapy at Kenyatta National Hospital

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with my approval as the University supervisor.

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DEDICATION

This is a dedication to all the upcoming research scientists who are driven by their self-ambition, determination and motivation to make health science research a one-stop shop of disease diagnosis.

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ACRONYMS AND ABBREVIATIONS

3TC	Lamivudine
ART	Antiretroviral therapy
ARV	Antiretroviral Drugs
AZT	Zidovudine
d4T	Stavudine
CD4	cluster of differentiation 4
EDTA	Ethylene diaminetetraacetic Acid
HAART	Highly Active Antiretroviral Therapy
HIV-1	Human Immunodeficiency Virus type 1
JKUAT	Jomo Kenyatta University of Agriculture and technology
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
NNRTI	Non-Nucleoside Reverse Transcriptase
RBCs	Red Blood Cells
UNAIDS	Joint United Nations Programme on AIDS
UNICEF	United Nations International Children's Emergency Fund
UNGASS	United Nations General Assembly Special Session on HIV and AIDS
WBCs	White blood Cells
ZDV	Zidovudine

ABSTRACT

Hematologic abnormalities and malnutrition are among the most common clinico-pathological manifestations in children with HIV and they are associated with increased risk of morbidity and mortality. The magnitude and severity of hematological abnormalities and malnutrition in children on prolonged ART is not well known. Short term studies have shown changes in hematological and nutritional parameters of HIV infected children but long-term hematological and nutritional evolution after prolonged ART is not completely understood. The aim of this study was to determine the changes in hematological and nutritional parameters and associated hematological abnormalities and malnutrition in the HIV-1 infected children before and after three years of ART at Kenyatta National Hospital (KNH). This study, which was nested within a preexisting prospective cohort study at KNH, extracted medical records from the parent study and evaluated the changes in hematological and nutritional parameters of 86 HIV infected children aged between 18 months to 12 years, receiving ART at KNH. The mean Hb (g/dl) increased significantly from 10.4 (±SD 2.1) to 12.5 (±SD 1.2) before and after 3 years of ART respectively (p=0.0001). Neutrophil Counts (mm3) decreased significantly from 4.2 (±SD 3.2) to 2.7 (±SD3.2) before and after 3 years of ART respectively (p=0.0001). The mean thrombocyte counts (x109/L) decreased from 297 (±SD170) to 283 (±SD84) before and after 3 years of ART respectively (p=0.4391). Anemia (Hb ≤10gm/dl) decreased significantly from 38.4% to 5.8% before and after 3 years of ART respectively (p=0.0001). Neutropenia increased from 9.3% to 22.1% before and after 3 years of ART respectively (p=0.0209). Thrombocytopenia (THR <125 x 109/L) decreased significantly from 10.6% to 1.2% before and after 3 years of ART respectively (p=0.0091). The mean anthropometric measurements, weight for age (WAZ), weight for height (WHZ), and height for age (HAZ) (z-scores) improved significantly between baseline and after 3 years of ART administration (p<0.0001). The proportion of those who were underweight dropped significantly from 58.8% to 14.1% (p=0.0001) after 3 years of ART, the proportion of those who were stunted dropped

significantly from 51.9% to 27.1% (p=0.0028) while the proportion of those who had wasting dropped significantly from 20.0% to 2.7% (p=0.0009) after 3 years of ART. This study confirms that prolonged ART in HIV-1 infected children is associated with changes in hematological and nutritional parameters and that hematological and nutritional abnormalities are common manifestations in these children. Based on these findings we recommended that physicians giving care to HIV infected children should routinely investigate and treat hematological abnormalities and malnutrition before and after ART treatment. Additionally, large scale and longitudinal studies are recommended in order to strengthen and explore in depth the problem of hematological abnormalities and malnutrition associated with HIV disease progression and prolonged ART treatment. Integration of HIV/malnutrition services and further research to determine optimal ART timing, role of supplementary feeding and antimicrobial prophylaxis are urgently required. Further, studies on a larger population of children, to ascertain the role of other factors, such as malaria and micronutrient deficiency, which may contribute to anemia, neutropenia, thrombocytopenia and malnutrition in HIV-1 infected children, are recommended.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Globally, the Human Immunodeficiency Virus (HIV) pandemic remains a serious challenge, and continues to take its toll particularly on vulnerable populations such as children. Sub-Saharan Africa is more heavily affected by HIV and Acquired Immunodeficiency syndrome (AIDS) than any other region of the world.

As of 2015, of the estimated nearly 37 million people living with HIV worldwide, approximately 2.6 million were children under 15 years of age, with 88 percent of these children residing in the sub-Saharan Africa. An estimated 220,000 children were newly infected with HIV in 2014; further, 600 children are newly infected with HIV every day (UNAIDS, 2015). In 2015, there were 19 million (17.7 million–20.5 million) people living with HIV in the sub-Saharan Africa with an estimated 960,000 (830,000–1.1 million) new HIV infections that year; this accounts for 46% of the global total of the new HIV infections. A total of 56,000 (40,000–76,000) of the new HIV infections in sub-Saharan Africa in 2015 were among children aged 15 years and below. In sub-Saharan Africa, 470,000 (390,000–560,000) people died of AIDS-related causes in 2015. Six out of 10 people on antiretroviral therapy globally live in the sub-Saharan Africa. In 2015, an estimated 10.3 million people were accessing antiretroviral therapy,

accounting for 54% (50–58%) of all people living with HIV in the region. (UNAIDS, 2016)

Kenya is one of the countries which have been hard hit by HIV and AIDS pandemic. An estimated 1.5 (1.3 - 1.8) million people are living with HIV with an adult prevalence of 5.9% (4.9% - 7.0%). Every year an average of 89,000 adults and about 11,000 children in Kenya are infected with HIV. Approximately 660,000 (540,000 - 810,000) children (ages 0-15) have been orphaned by AIDS (UNAIDS, 2015). There are approximately 160,000 (140,000 - 180,000) children living with HIV, with a large number still in need of ART (UNAIDS, 2014).

The discovery of drugs that slow down disease progression and improve the quality of life for those infected has greatly changed the course of the HIV pandemic. Initially only adult formulations were available but availability of pediatric formulations has resulted in a dramatic decline in those dying as a result of HIV/AIDS. The number of children (younger than 15 years) receiving ART in low- and middle-income countries more than doubled from 2009 to 2013, from 355 000 to 740 000. At the end of 2013, less than one quarter (23%, range 21– 25%) of children living with HIV were receiving ART in low- and middle-income countries compared with more than one third (37%, range 35–39%) of adults living with HIV (UNAIDS, 2014).

In 2003, only 6,000 people living with HIV were accessing ART, by 2013 this had increased to 656,000. Since 2008, the expansion of antiretroviral treatment (ART)

services throughout the healthcare system has increased the number of adults on treatment from 64 percent to 80 percent as of 2013 (UNAIDS, 2014). As in many other countries, the proportion of children receiving ART in Kenya is significantly lower. However, the scaling up of pediatric ART has increased coverage from 16 percent to 43 percent in the period 2008-13 (UNAIDS, 2014).

There is a concerted effort to increase the number of children on antiretroviral therapy through increased availability of early infant diagnosis and strengthening provider-initiated counseling and testing in health facilities. As a result of this, survival of HIV-1 infected children in Kenya and other similar settings has dramatically improved as more children access antiretroviral therapy. This has led to both decreased morbidity and mortality from HIV infection due to immune reconstitution and viral suppression.

Most clinicians agree that the benefits of ART generally outweigh the risks; however, patients who experience significant side effects sometimes disagree with this. This can lead to patient non-adherence or refusal to take any ART at all for fear of toxicity.

Antiretroviral drug toxicities may have both acute and long-term implications on hematological and nutritional indices of HIV-infected persons (Wamalwa *et al.*, 2010). Therefore, with the increased use of ART and with more and more children accessing ART, there is need to understand the long term hematological and nutritional effects of ART treatment especially among children.

1.1.2 Hematological profiles

To understand abnormal blood cell production, one must know how normal hematopoiesis occurs. To have normal hematopoiesis, the hematopoietic stem cells must be present because it is the cell from which all blood cells will be derived during a person's lifetime. Hematopoietic stem cells are situated in the bone marrow, spleen, liver, and peripheral blood. Hematopoietic stem cells in the bone marrow produce almost all blood cells, whereas the other sites assist in times of undue stress. Only about 5% of the Hematopoietic stem cells in the bone marrow are functioning at any one time, yet they can maintain the hematopoietic system for the lifetime of the person (Swartz *et al.*, 1994).

In addition to the stem cell, supportive cells, called stromal cells, must be present for normal hematopoiesis to occur. T lymphocytes, macrophages, endothelial cells, and fibroblasts help to produce the hematopoietic growth factors that are needed for production and differentiation of normal white blood cells in the bone marrow. Erythropoietin, produced in the kidney, and thrombopoietin, produced in the liver, are necessary for proliferation and production of red blood cells and platelets, respectively (Ellaurie *et al.*, 1990).

The cells of the bone marrow grow in clumps known as colonies. Cells differentiate from the earliest progenitor cells, the hematopoietic stem cells, to progenitor cells of the different cell lineages, and these colonies then further mature toward becoming red blood cells, white blood cells (neutrophils, monocytes), and platelets (Ellaurie *et al.*,

1990). The progenitors of the red blood cells are in the erythroid lineage, whereas the white blood cells are derived from the granulocytemacrophage colonies of progenitor cells, and finally the megakaryocytes of the bone marrow give rise to circulating platelets (Moses *et al.*, 1998).

Alterations in hematopoiesis can lead to abnormalities in red cell, white cell, and platelet count in the peripheral blood. A decrease in the number of white cells is called leukopenia, and a decrease in the number of platelets is called thrombocytopenia. A decrease in the white blood cells known as neutrophils is called neutropenia. Anemia is a decrease in red cell number, hemoglobin, or hematocrit and is further classified by the size of the red cells in the peripheral blood as microcytic, normocytic or macrocytic anemia (Swartz *et al.*, 1994).

1.1.3 Hematologic Abnormalities in HIV Infected Children

Clinically significant hematological abnormalities are common in children with HIV/AIDS. Impaired hematopoiesis, immune-mediated cytopenias, and altered coagulation mechanisms have all been described in HIV infected individuals and tend to be more severe in advanced stages of the disease (Forsyth *et al.*, 1996; Moses *et al.*, 1998). These abnormalities may occur as a result of HIV infection itself, as sequelae of HIV-related opportunistic infections or malignancies or as a consequence of therapies used for HIV infection and associated conditions (Swartz *et al.*, 1994). HIV infects the progenitor cells or causes them to function abnormally (Moses *et al.*, 1998). When progenitor cells, cannot produce adequate hematopoietic growth factors, decreased

production of blood cells occurs. Equally important, antiretroviral treatment for HIV infection, opportunistic infections and their treatment, and chemotherapy for treatment of HIV-associated malignancies also cause altered hematopoiesis, which can contribute to the problem (Swartz *et al.*, 1994).

1.1.4 Nutritional Profiles in HIV Infected Children

In 2012, 3.3 million children were living with Human Immunodeficiency Virus (HIV) worldwide, with more than 90 % in sub-Saharan Africa (UNAIDS, 2013). Regardless of HIV, sub-Saharan Africa is also the region of the world which is most seriously affected by malnutrition, 21 % of children under 5 years are underweight, 39 % are stunted, and 9 % are wasted (UNICEF, 2012).

Malnutrition is the underlying cause of death among 35 % of children aged <5 years (WHO, 2012) and could lead to irreversible damages such as cognitive impairment, chronic diseases and growth failure (Victora *et al.*, 2008). Therefore, malnutrition is a major problem for children and especially for HIV-infected children since it creates a vicious circle with HIV infection. Indeed, on the one hand, malnutrition worsens HIV disease as it has similar effects on the immune system as HIV infection (Jesson *et al.*, 2015). For example, among malnourished people, lymphoid tissues are damaged, and CD4 T-cell concentration is decreased (Cunningham-Rundles *et al.*, 2005).

Deficiencies in vitamins and minerals contribute to oxidative stress, which can accelerate immune cell death (Romero-Alvira *et al.*, 1998) and increase HIV replication

(Allard *et al.,* 1998). On the other hand, HIV infection increases the risk of malnutrition, because of a high pro-inflammatory cytokine activity which can cause growth impairment among children (Johann-Liang *et al.,* 2000).

HIV-related opportunistic infections such as persistent diarrhoea or oral and oesophageal candidiasis have a negative impact on nutritional status among children (Trehan *et al.,* 2012). HIV infection can also indirectly affect the child's nutritional status, when it has an impact on the child's social environment. In some contexts, when HIV concerns the most productive members of the family, the household economic capacities and the agricultural production are reduced, leading to a situation of food insecurity (Anema *et al.,* 2009). Furthermore, poor weaning practices among HIV-infected mothers can also have an impact on the child's nutritional status (Saloojee *et al.,* 2007). Thus, malnutrition is a common complication among HIV-infected children. Low weight-for-age has been reported in up to 50 % of untreated HIV-infected children in resource-limited settings (Anabwani *et al.,* 2005).

Among children with severe malnutrition, mortality risk is three times higher in HIV-infected children than in non-HIV-infected children (Chinkhumba *et al.,* 2008). Thus, nutritional care is fully part of the pediatric HIV healthcare package. The World Health Organization recommends that an asymptomatic HIV- infected child should increase his energy requirements by 10 %, compared to a non-infected child; this is extended to 20 to 30 % during symptomatic HIV infection or episodes of opportunistic infections, and up to 50 to 100 % when a severe malnutrition episode occurs (WHO, 2008). However, the

burden of malnutrition remains difficult to quantify in HIV-infected people, most of all in children. A better understanding of the changes in hematological and nutritional profiles and associated factors in HIV-1 infected children is therefore necessary to improve HIV pediatric healthcare, especially in sub-Saharan Africa (Jesson *et al.*, 2015).

1.2 Statement of the Problem

HIV/AIDs pandemic remains a serious challenge, and continues to take its toll particularly on vulnerable populations including children. Sub-Saharan Africa is the most heavily affected by HIV/AIDS than any other region of the world leading to increased risk of morbidity and mortality. Globally, nearly 37 million people are currently living with HIV/AIDS. Every day more than 5,600 people contract HIV- nearly 230 every hour. More than two-thirds (70%) of all people living with HIV, 25.8 million, live in sub-Saharan Africa- including 88% of the world's HIV-positive children. An estimated 220,000 children were newly infected with HIV in 2014 with statistics showing that 600 children are newly infected with HIV every day (UNAIDS, 2015). Kenya is one of the countries which are hard hit by the HIV/AIDS pandemic where an estimated 1.5 million people are living with HIV. There are approximately 170,000 children living with HIV, with a large number of them still in need of ART (UNAIDS, 2015).

The discovery of antiretroviral drugs that slow down disease progression and improve the quality of life for those infected has greatly changed the course of the HIV pandemic. This has led to both decreased morbidity and mortality from HIV infection

due to immune reconstitution and viral suppression. However, short term studies show that antiretroviral drug toxicities may have both acute and long-term implications to the health of HIV-infected persons. Therefore, with the increased use of ART and with more and more children accessing ART, there is need to understand the long term effects of ART treatment especially among children.

The long-term hematological and nutritional evolution after prolonged ART is not completely understood. Short term studies have shown that the main laboratory abnormalities associated with ART are hematological changes that may lead to increased prevalence of anemina, neutropenia and thrombocytopenia in HIV-1 infected children (Sperling *et al.,* 1998). These adverse events are most common with long term therapy. Information on the effect of long term use of ARV drugs on hematological and nutritional parameters in HIV infected children is limited. Data on the effect of prolonged ART on hematological and nutritional parameters in HIV-1 infected children is lacking in the Kenyan setting and the sub-Saharan African region at large.

1.3 Justification

There is yet no available published work on hematological abnormalities and malnutrition in HIV positive children in Kenya. This study seeks to identify, measure, and explain the changes in hematological and nutritional parameters in children who were on ART for a period of three years. Further, the study examines the patterns and severity of hematological abnormalities and malnutrition among these children before

ART initiation and after three years of ART. The study provides information on alterations of total white blood cells; specifically neutrophils and T-helper cells, hemoglobin levels and nutritional status among HIV infected children on prolonged ART.

The information gathered through this study will serve as supportive evidence of the effectiveness of antiretroviral drug therapy among HIV positive children on long term ART and will help inform clinicians on the management of the various hematological manifestations as observed in HIV-1 infected children such as neutropenia, anemia and thrombocytopenia.

1. 4 Research Hypothesis

1. 4.1 Null Hypothesis

This study hypothesis that there are no significant changes in the mean hematological profiles and nutritional status in HIV-1 infected children who have been on ART for a period of three years.

1.5 Research Questions

1. What are the changes in the mean hemoglobin levels, neutrophils, and platelets in HIV-1 infected children between baseline and after three years of ART?

2. What are the changes in mean anthropometric measurements; weight-for-age (WAZ), weight-for-height (WHZ), and height-for-age (HAZ) (z-scores) in HIV-1 infected children between baseline and after three years of ART at Kenyatta National Hospital?

3. What is the prevalence of hematological abnormalities and malnutrition before and after three years of ART in HIV-1 infected children at Kenyatta National Hospital?

1.6 Objectives

1.6.1 Broad objective

To determine the changes in hematological profiles and nutritional status and the related hematological and nutritional abnormalities in HIV-1 infected children on prolonged ART at the Kenyatta National Hospital.

1.6.2 Specific objectives

1. To determine the changes in hemoglobin levels, neutrophils, and platelets in HIV-1 infected children on ART for a period of three years at Kenyatta National Hospital.
2. To determine the changes in weight-for-age (WAZ), weight-for-height (WHZ), and height-for-age (HAZ) (z-scores) in HIV-1 infected children on ART for a period of three years at Kenyatta National Hospital.
3. To determine the prevalence of hematological abnormalities (anemia, neutropenia and Thrombocytopenia) and malnutrition in HIV-1 infected children on ART for a period of three years at Kenyatta National Hospital.

CHAPTER TWO

LITERATURE REVIEW

2.1 HIV Disease Progression in Children

HIV disease is a continuum of progressive damage to the immune system from the time of infection to the manifestation of severe immunologic damage by opportunistic infections (OI), neoplasms, wasting, or low CD4 lymphocyte count that define AIDS (Longini *et al.*, 1991).

HIV infection in the infant or young child, whether acquired perinatally or via breast milk, occurs at a time when the immune system is developing and the infant is simultaneously exposed to a multitude of new infections as well as frequent immunizations. This repeated antigenic stimulation undoubtedly causes immune activation, immune dysfunction and thereby facilitating HIV viral replication. Some of these new infections are opportunistic infections that cause significant immune dysfunction. Moreover, in HIV infected children, altered blood cell production is common resulting in hematological abnormalities such as neutropenia, anemia, and thrombocytopenia (Consolini *et al.*, 2007).

2.2 Antiretroviral Therapy and Associated Hematological Abnormalities in Children

Antiretroviral treatment reduces illness and mortality among children living with HIV in much the same way that it does among adults. In one study in Brazil, three-quarters of

HIV-positive children receiving ART were alive after a four-year follow-up period (Ryavanki *et al.,* 2013). Positive outcomes have also been seen in pediatric ART programmes in Thailand, Kenya, and Ukraine (Aurpibul *et al.,* 2008; Wamalwa *et al.,* 2007; Mahdavi *et al.,* 2010). A study released in 2007, which monitored 586 HIV positive children receiving antiretroviral treatment in 14 countries in Africa and Asia, found that 82 percent were still alive after two years (O'Brien *et al.,* 2007). Advances in the treatment of HIV infected children have increased patient survival rates and decreased morbidity and mortality (Cohen *et al.,* 2011). With the availability of more effective antiretroviral regimens, researchers and clinicians have prolonged the time interval during which viral replication is controlled and therefore effective immune function is maintained, thus preventing or greatly delaying onset of opportunistic infections (OIs) and other complications of HIV infection. As a result, these patients undergo long-term exposure to a large number of drugs including those that comprise antiretroviral therapy (ART) and antibiotic therapy for the treatment of opportunistic infections (like co-trimoxazole) (Cohen *et al.,* 2011).

However, while these drug regimens have been shown to be extremely effective in treatment of HIV infected children, decreasing child morbidity and mortality, certain agents have adverse consequences on immune function, with many causing toxicity to the bone marrow and negatively affecting bone marrow cell survival and replication, thus resulting in anemia, neutropenia, thrombocytopenia and lymphocytopenia in children (Aurpibul *et al.,* 2008). The impact of long term therapy and the degree to which this has an impact on the child's immune system has not been investigated

especially in sub-Saharan Africa which is home to 9 out of 10 children living with HIV/AIDs.

2.3 Hematological Abnormalities in HIV Infected Children on ART

Altered hematopoiesis occurs in patients with HIV infection. This change affects all three cell lines (red blood cells, white blood cells, and platelets) that come from stem cells in the marrow. Consequently, HIV-infected children may suffer from hematological abnormalities which are among the most common manifestations of HIV infection in children.

The causes of these conditions are varied and are not fully understood. Evidence shows that HIV infects the progenitor cells in the bone marrow, the hematopoietic stem cells, and causes abnormal function. When hematopoietic stem cells cannot produce adequate hematopoietic growth factors (the substances that stimulate the production of blood cells in the bone marrow), decreased production of blood cells occurs (Ellaurie *et al.*, 1990). Moreover, antiretroviral treatment for HIV infection, opportunistic infections and their treatment, and chemotherapy for treatment of HIV-associated malignancies also cause altered hematopoiesis, which can result in hematological abnormalities including anemia, neutropenia and thrombocytopenia or any combination of these three.

2.3.1 Anemia

Anemia is the most common abnormality seen in people with HIV and occurs in 16% to 94% of HIV-infected children (Ellaurie *et al.*, 1990). In patients with HIV, anemia is a

statistically significant predictor of progression to AIDS, and is independently associated with an increased risk of death (Ellaurie *et al.,* 1990).

Causes of HIV-related anemia are multifactorial and include direct and indirect effects of HIV infection. HIV-related anemia is generally due to reduced red blood cell (RBC) production secondary to bone marrow suppression by HIV, as a result of cytokine production and alteration in the bone marrow microenvironment. Anemia may also occur due to nutritional deficiencies, increased RBC destruction, or a combination of these problems. Evaluation of hemoglobin level, reticulocyte count, bilirubin, and mean corpuscular volume and review of the peripheral blood smear are necessary for diagnosis (Coyle, 1997).

HIV infection alone, without other complicating illness, may produce anemia in some patients. A study of serum immuno-reactive erythropoietin, in HIV-infected patients, in various stages of illness, showed that levels of the hormone failed to rise commensurately with increasing anemia, suggesting that insufficient amounts of erythropoietin may be one cause of anemia in this setting (Spivak, 1989). Other studies have suggested that soluble factors in the serum of HIV-infected patients may inhibit hematopoiesis, or that direct HIV infection of marrow progenitor cells may play a role in producing anemia and other hematological abnormalities associated with HIV infection (Folks *et al.,* 1988).

A cross-sectional study conducted on hematological abnormalities associated with pediatric HIV/AIDS among 68 HIV infected children in Lagos, Nigeria found anemia (Hb < 10 g/dL) was present in 77.9%, with severe anemia (Hb < 6 g/dL) present in 5.9%, moderate anemia (Hb: 6-7g/dL) in 32.3% and mild anemia (Hb: 8-9.9 g/dL) in 39.7% of the children (Adetifa, *et al.,* 2006). Other studies conducted in HIV infected African children have found the prevalence of anemia to be 73%, to 94% (Eley *et al.,* 2002; Erhabor, *et al.,* 2005; Ellaurie, *et al.,* 1994).

Antiretroviral therapy is the only regimen potent enough to drastically reduce viral replication, prevent the emergence of resistance, restore immune status, slow HIV disease progression, exert durable therapeutic responses, improve quality of life and promote normal growth and development in children (Moses *et al.,* 1998). Zidovudine or stavudine is commonly paired with lamivudine or abacavir as the nucleoside components of ART regimen. Zidovudine is well known for its bone marrow suppressive effect; the suppression being related to bone marrow reserve, dosage of the drug, duration of treatment and the stage of HIV disease (Aurpibul *et al.,* 2008). Anaemia is one of the major bone marrow suppressive effects of ZDV and is commonly seen 4 to 6 weeks after commencement of use. The Zidovudine induced anemia has been found in 18-20% of children receiving ZDV and the degree and severity were found to be dose dependent (Ellaurie *et al.,* 1990)

Mathews *et al.,* 1991, studied the hematological manifestations in 187 HIV positive children. One hundred and one of them were on ART (Group A) and remaining 86 were

not on ART (Group B). The study found anemia in 36% of patients in Group A and 45% in Group B. In a study done to investigate the hematological changes after switching from stavudine to zidovudine in 78 HIV-infected children on ART, a statistically significant decrease in haemoglobin level occurred, but the magnitude of the decrease was small and not clinically significant (Aurpibul *et al.*, 2008).

2.3.2 Neutropenia

Neutropenia is a problem commonly encountered in patients with HIV infection. Although it usually reflects the toxicity of therapies for HIV infection or associated conditions, studies of untreated patients have also shown a high incidence of neutropenia, particularly in patients with more profound immunodeficiency. This may be an isolated event or may occur simultaneously with anemia and thrombocytopenia. Approximately 41% of HIV-infected pediatric patients will develop neutropenia, defined as an absolute neutrophil count <500 μL (Consolini *et al.*, 2007).

Neutropenia can result from the HIV infection or related malignancies, drug therapies, or opportunistic infections. HIV can cause neutropenia by directly or indirectly impairing hematopoiesis (Ellaurie *et al.,* 1990). Similarly, microorganisms that cause opportunistic infections, such as cytomegalovirus and Mycobacterium avium complex, can infiltrate the bone marrow and cause myelosuppression (Ellaurie *et al.*, 1990).

Studies have shown that neutropenia is common in children with HIV; in a study of 100 children infected with HIV, Ellaurie *et al.,* 1994 observed neutropenia in 34% of

children without an opportunistic infection and 65% in those with opportunistic infection. In a study of 68 HIV positive children (Adetifa *et al.,* 2006) found that 17.5% had neutropenia. A prevalence of 24% of neutropenia was noted in a study conducted by (Erhabor, *et al.,* 2005) among 100 cases of HIV positive children.

If a child with HIV develops neutropenia, drug-related toxicity is the most likely cause. Zidovudine and other nucleoside analogues can cause a dose-dependent neutropenia. In these situations, when the ARV therapy is discontinued, the neutrophil count will often increase, but the platelet count may fall.

In a study conducted in Ethiopia to investigate hematological changes in children taking ART, neutropenia was seen both before and after initiation of treatment. The prevalence of neutropenia before treatment (7.8%) was higher than after treatment (4.7%). In this study, all cases of neutropenia were patients who were taking AZT based ART. These findings are lower as compared to a meta-analysis which reported 26-46% neutropenia in AZT based ART recipients (Moyle *et al.,* 2004) and the prospective randomized comparative trial of d4T and AZT in children which found neutropenia of 20% among AZT recipients who had been on treatment for more than a year (Mark *et al.,* 1998).

2.3.3 Thrombocytopenia

Thrombocytopenia occurs often in patients with HIV infection and is the second most common hematological abnormality found in children with HIV infection. The association of thrombocytopenia with HIV infection was first established in 1982 and

has since been observed in children as, either an initial manifestation of AIDS or during the course of the infection (Labrune *et al.,* 1989). Although there is no clear correlation between the severity of thrombocytopenia and the rate of progression to AIDS, the incidence of thrombocytopenia is higher in more advanced stages of HIV infection (Sullivan *et al.,* 1997). Thrombocytopenia can occur in 20% to 33% of pediatric patients with HIV at some time during the course of their disease (Ellaurie *et al.,* 1986). Possible etiologies of thrombocytopenia in patients with HIV infection include immune-mediated destruction, thrombotic thrombocytopenic purpura, impaired hematopoiesis, and toxic effects of medications (Najean *et al.,* 1994).

In many instances, however, thrombocytopenia is a relatively isolated hematological abnormality associated with a normal or increased number of megakaryocytes in the bone marrow and elevated levels of platelet-associated immunoglobulin. These patients have the clinical syndrome commonly referred to as immune thrombocytopenic purpura (ITP). A patient with thrombocytopenia has true HIV related ITP if there is no other condition or treatment that could cause thrombocytopenia (Jost *et al.,* 1988).

There are other causes of thrombocytopenia in HIV infection including any of the infectious or neoplastic conditions that involve the bone marrow and any of the medications that cause generalized myelosuppression in patients with HIV infection can produce thrombocytopenia.

The prevalence of thrombocytopenia in HIV infected children has been described in various studies. A cross-sectional study of baseline hematological parameters was undertaken in 68 HIV infected children and in this study prevalence of thrombocytopenia was found to be 12.5% (Adetifa *et al.,* 2006). Other studies conducted to investigate the prevalence of hematological abnormalities in HIV-1 infected children, have found the prevalence of thrombocytopenia to be 10%, to 33% (Erhabor *et al.*, 2005, Ellaurie *et al.,* 1988, Suarez *et al*.., 1994).

Medications used in the management of HIV that are most commonly associated with thrombocytopenia include ganciclovir (48%), pentamidine (18%), and trimethoprim-sulfamethoxazole (3%) (Ellaulie *et al.,* 1990). However, some studies have shown that HIV-related thrombocytopenia responds well to antiretroviral therapy. Some studies evaluating the use of Zidovudine (AZT) have shown increased platelet production in the HIV infected individuals. In other studies however, thrombocytosis and thrombocytopenia were found before and after treatment with ART. A moderately low prevalence of thrombocytopenia was found in a study conducted to investigate the hematological changes in children with HIV who were on ART treatment (Muluneh *et al.,* 2007). In this study, prevalence of thrombocytopenia after ART treatment was 7.8%; which was similar with findings of a randomized comparative trial of AZT and d4T in HIV infected children which showed a prevalence of 7% (Mark *et al.,* 1998).

2.4 Nutritional Indices in HIV Infected Children

Nutrition and HIV are strongly related and complement each other. HIV causes immune impairment leading to malnutrition which leads to further immune deficiency, and contributes to rapid progression of HIV infection to AIDS (Smith *et al.*, 1992). A malnourished child after acquiring HIV is likely to progress faster to AIDS, because his body is weak to fight infection whereas a well-nourished child can fight the illness better. It has been proved that good nutrition increases resistance to infection and disease, improves energy, and thus makes the child stronger and more productive. Wasting syndrome is defined by loss of more than 10% of the usual body weight with a lack of other detectable cause of wasting other than the HIV infection itself. Nutritional improvement measures must be initiated before a patient reaches this stage (Smith *et al.*, 1992).

HIV accounts for significant immunosuppression in an infected individual. If the corroboratory indices of good health are satisfactory, the suppression of immune defences can be mitigated. One such index is nutrition. HIV, immune expression, and nutrition interactions are complex and related to each other (Shalini *et al.,* 2012). Malnutrition adds fuel to the fire by accelerating the progress of HIV infection to AIDS. HIV/AIDS is associated with biological and social factors that affect the individual's ability to consume, utilize, and acquire food. Once there is an infection with HIV, the patient's nutritional status declines further leading to immune depletion and HIV progression (Shalini *et al.,* 2012).

2.4.1 Effect of HIV and Malnutrition on Hematological Parameters

One of the factors responsible for malnutrition in an HIV-infected child is reduced appetite, which could be due to difficulty in ingesting food as a result of infections like oral thrush or oesophagitis caused by Candida, a common opportunistic infection in HIV-infected children and fever, side effects of medicines, or depression. Poor absorption of nutrients may be due to accompanying diarrhea which may be because of bacterial infections like Salmonella or Mycobacterium avium intercellular; viral like cytomegalo virus or parasitic infections like Giardia, C. parvum, and E. Bieneusi; due to nausea/vomiting as a side effect of medications used to treat HIV or opportunistic infections. 30–50% of HIV patients in developed and nearly 90% in developing countries complain of diarrhoea and malabsorption (Smith *et al.*, 1992). Gastrointestinal tract is the largest lymphoid organ in the body and is directly affected by HIV infection. HIV causes damage to the intestinal cells by causing villus flattening and decreased D-xylose absorption. This leads to carbohydrate and fat malabsorption thereby affecting fat soluble vitamins like vitamins A and E, which are important for proper functioning of immune system.

Whereas larger amounts of nutrients are required during fever and infections that accompany an HIV infection, they are utilized poorly by the body. This leads to loss of weight and lean muscle tissue, further causing damage to the immune system. Lack of iron in the diet and infections such as malaria and hookworm lead to anaemia. Anaemia causes lethargy, further reduces food intake and nutrient absorption, and also causes

disruption of metabolism, chronic infections, muscle wasting, or loss in lean body tissue (Smith *et al.,* 1992). AIDS-related dementia or neuropsychiatric impairment may make the patients unable to care for themselves, forget to eat, or unable to prepare balanced meals. Even in households with HIV-infected members, nutritional impacts can be seen if the infected adult becomes too sick to work and provide food for themselves and their families (Shalini *et al.*, 2012). Dietary intake also varies inversely with level of virus, suggesting that viral replication directly or indirectly suppresses appetite. Malnutrition is frequent and is considered a marker for poor prognosis among HIV-infected subjects (Shalini *et al.*, 2012).

Malnutrition and HIV form a vicious cycle and ultimately aim at reducing the immunity of the patient. In both malnutrition and HIV there is reduced total lymphocyte as well as CD4 and CD8 T-lymphocyte numbers. According to a study, (Yolken *et al.*, 1991), approximately 30–60% of asymptomatic children infected with HIV malabsorb carbohydrates, 30% malabsorb fat, and 32% malabsorb proteins. Whereas micronutrient deficiencies may affect replication of the invading virus, they also induce several metabolic alterations in the body. This includes changes in whole-body protein turnover, increased urinary nitrogen loss, and elevated hepatic protein synthesis as well as increased skeletal muscle breakdown providing for proliferation of neutrophils, lymphocytes, and fibroblasts, and for synthesis of immunoglobulins and hepatic acute phase proteins, manifesting clinically as fever (Yolken *et al.*, 1991).

2.4.2 Malnutrition in HIV Infected Children

Malnutrition affects over one-quarter of under-fives in the developing world and is thought to contribute to one-third of deaths in this age group- estimated at 1–2 million deaths per year (Rose *et al.,* 2014). HIV infection and malnutrition often coexist, and the two conditions overlap and interact. Prevalence of concurrent HIV infection in children presenting with malnutrition is variable, with figures of up to 71.8% reported (Rose *et al.,* 2014). Meta-analysis of 17 large studies in sub-Saharan Africa suggested that 29.2% of children presenting with malnutrition were HIV-positive, although given the higher prevalence of HIV in this region, this might not be globally representative. Importantly, mortality from malnutrition is more than three times higher in HIV-positive children than their HIV-negative peers (Geissler *et al.,* 2006).

Severe acute malnutrition is defined by the WHO as a weight-for-height z-score of less than –3, or a midupper arm circumference of less than 11.5 cm in children aged 6 months to 5 years. Malnutrition can present as non-oedematous (marasmus) or oedematous disease (kwashiorkor or marasmic-kwashiorkor), with marasmus being more common in HIV-positive children (Zaidi *et al.,* 2013). In addition, HIV-positive children with malnutrition are at higher risk of infectious comorbidities (such as tuberculosis, respiratory tract infections, gastroenteritis and candidiasis) and other complications such as persistent diarrhoea and poor oral intake (Rose *et al.,* 2014).

When compared with HIV-negative counterparts, HIV-infected children (including asymptomatic children) have additional nutritional requirements to ensure normal

growth and development and require high-energy, high-protein, nutrient-dense diets. Calorie intake needs to be increased, with children requiring up to 150% of the recommended daily allowance of calories, and micronutrient requirement is up to five times that of an HIV-negative child (Zaidi *et al.,* 2013). It is recommended that— compared with HIV-negative children— asymptomatic HIV-positive children have a 10% increased calorie intake, which is best given through additional household foods, as part of a balanced, varied diet. HIV-positive children with chronic lung disease, chronic TB or chronic diar- rhoea require approximately 20–30% increased calorie intake, which might be provided through increased household foods or through nutritional supplementation. Children with severe complications of HIV infection, such as severe acute malnutrition or stunted growth, require 50–100% increased energy intake each day, until weight is recovered (Smith *et al.,* 1992). These increased nutritional demands reflect the increased nutrient cost of immune system support and prevention of muscle wasting. In resource-limited settings (RLS) where food availability is limited, it is clear that the increased nutritional requirements are unlikely to be met (Geissler *et al.,* 2006).

In recent years, there has been a dramatic increase in the proportion of HIV-positive individuals on treatment: for example, in one hyperendemic region in Southern Africa, since the ART programme ‘scale-up 2004 to 31% in 2011. Use of ART has led to a decrease in morbidity and mortality in HIV-positive children in RLS. Despite widespread advances in the availability of ART, there remains a disparity between the number of children who require ART and the number receiving treatment; furthermore, initiation of therapy occurs late in children with already advanced disease. Although

early initiation of antiretroviral treatment is advocated in these guidelines, this remains controversial, and delaying initiation of treatment until after the acute phase might be prudent (Prendergast *et al.*, 2011).

Initiation of ART is associated with a sustained improvement in growth responses, especially in younger children, even in the absence of complete viral suppression. The direct effect of ART on growth, as well as the decreased frequency of opportunistic infections in children receiving ART, means that absence (or late initiation) of ART treatment can be considered a risk factor for development of malnutrition in HIV-positive children. This is a controversial area as there have been several reports showing that severe malnutrition was precipitated in the months following ART initiation (Prendergast *et al.*, 2011). One study reported that approximately 10% of HIV-positive children were hospitalized for severe malnutrition within 12 weeks of starting ART. Furthermore, an unusually large proportion of these children developed oedematous malnutrition (kwashiorkor), and it has been argued that this is because a degree of immune competence is required to develop oedema (Zaidi *et al.*, 2013).

Hematological abnormalities and malnutrition are therefore present in HIV infected children and are associated with both HIV disease and ART treatment. The impact of long term ART on the hematological profiles and nutritional status of Kenyan HIV/AIDS infected children is not known. Hence, this study was conducted to determine the pattern and predictors of hematological abnormalities and changes in nutritional status in HIV-1 infected Kenyan children. To address this issue, this study;

which was nested within an existing prospective cohort study was conducted to investigate the changes in hematological parameters i.e., white blood cells, platelets, hemoglobin level and anthropometric measurements observed in HIV infected children who were on ART for three years.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was conducted at the Kenyatta National Hospital (KNH) comprehensive care center which offers outpatient services to all HIV infected patients including children and those requiring post-exposure prophylaxis. Clinical procedures were conducted at KNH comprehensive care center while laboratory investigations were done at the University of Nairobi pediatrics laboratory in the department of pediatrics and child health.

3.2 Study Design

This was a retrospective cohort study which was nested within the Pediatric Adherence (PAD) study. The pediatric adherence study was a prospective cohort study initiated in 2004 at Kenyatta National Hospital Comprehensive Care Clinic in Nairobi, Kenya. The aims of the pediatric adherence study were to define the pattern and predictors of virologic and clinical response to HAART in HIV-1 infected Kenyan children; to define the frequency of drug resistance mutations to the first-line NNRTI based triple therapy and the effect of such mutations on long-term virologic response in HIV-1 infected Kenyan children.

3.3 Study Subjects

Participants for this study were children enrolled in the PAD study and who had at least 3 years of follow up and for whom hematological profile was available for both baseline and 3 year follow up visits. Children enrolled into the PAD study were drawn from the KNH pediatric wards and clinics.

3.4 Inclusion and Exclusion Criteria

Children enrolled into the parent study (PAD) must have met the following eligibility criteria for inclusion into the study:

i. Confirmed HIV-1 infection and antiretroviral drug naive.
ii. Symptomatic with WHO clinical stage 3 or stage 2 with CD4 percentage < 15% of total lymphocytes.
iii. Age between 18 months and 12 years.
iv. Consent from a parent or legal guardian.
v. Willing to stay in Nairobi for at least one year after initiation of ART.

However, the current study was only interested in children who had attained three years of follow up into the main study. The total sample population for the pediatric adherence study was 170 children; however, only 86 were included in the current study. The illustration below shows how the inclusion/exclusion criterion for the current study was done.

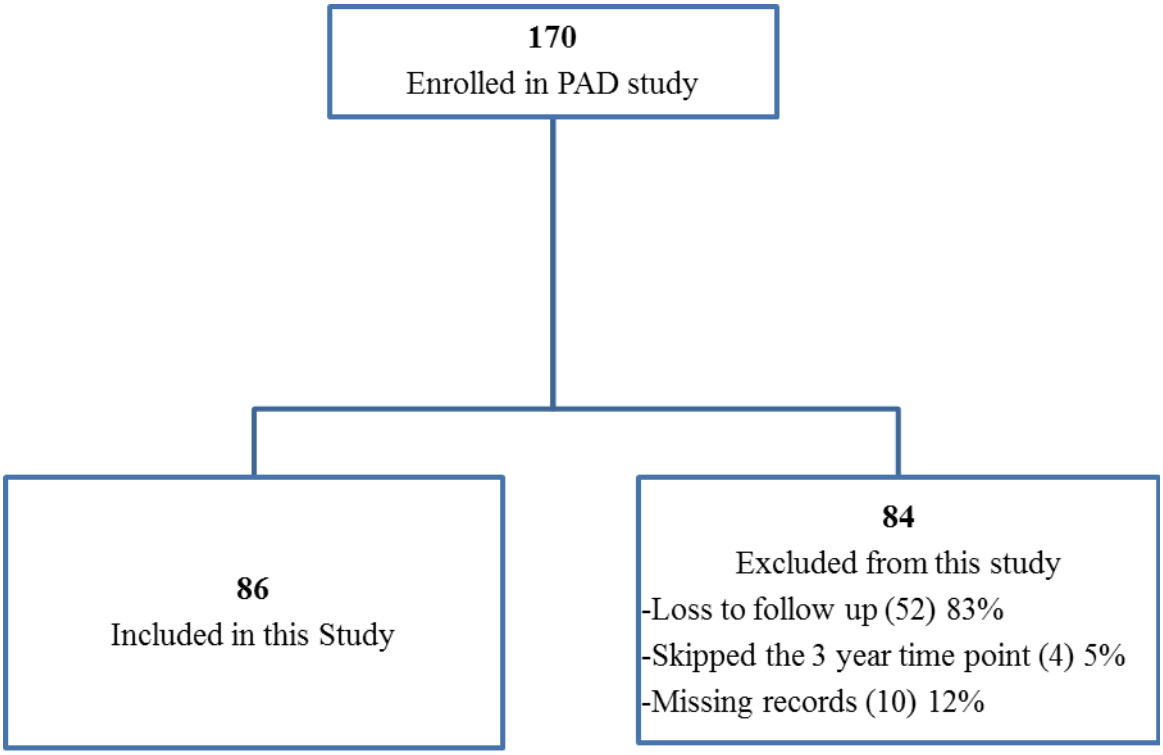

Figure 3. 1: The Inclusion and Exclusion Criteria used for the Study

Of the 170 children who were recruited into the pediatric adherence study, 84 were excluded from this study because of the following reasons: 70(83%) were lost to follow up, 4(5%) skipped the three year time point and 10(12%) had incomplete records. The remaining 86 children were included in this study and this comprised the sample size.

3.4.1 Sample size Calculation

The sample size was calculated using the following sample size calculation formulae (Moore & McCabe, 1989).

$$n = \frac{(Z_{\alpha/2} + Z_{\beta})^2 2\sigma^2}{(\mu_1 - \mu_2)^2}$$

Where:

n= Minimum sample size required

α=Type I error (0.05)

β=Type II error (0.10)

Zα= Standard normal deviate associated with type I error (1.96)

Zβ= Standard normal deviate associated with type II error (1.28)

μ1=Mean Hb estimate at baseline (11.9g/dL)

μ2=Mean Hb estimate at final (11.0g/dL)

μ1 - μ2=Effect size difference to be detected as statistically significant

Running the above figures on Stata ver 12.0 (SataCorp Inc.) gives a sample size of 79 subjects. Therefore, the minimum sample size required to detect 0.9g/dL Hb difference as significant was 79 samples. However, since the available data was more than required, all the 86 available samples were considered for analysis and were included in this study. The study adopted one sample comparison where the mean estimates were compared between baseline and final.

3.5 Data Collection

3.5.1 Laboratory Methods

HIV-1 antibody testing for the children was performed at the research clinic prior to enrollment using 2 rapid tests in parallel, Unigold (Trinity Biotech, Ireland) and Determine (Abbott, Japan) provided by the National AIDS and STI Control Program (NASCOP) of Kenya. Those who turned positive through the rapid antibody test were referred to the study for further screening according to the inclusion/exclusion criteria. Viral load testing, hematology assays and CD4 counts and serum albumin levels were conducted at baseline and then quarterly at each follow-up visit at the University of Nairobi pediatrics laboratory in the department of Pediatrics. The protocol of how CD4 testing was performed is attached on appendix vi. Specimen used for hematology profile assay was whole blood samples collected in the standard EDTA (purple top or lavender) vacutainer or microtainer using the standard vein puncture procedure for sample collection. Samples are transported from the research clinic in a cooler box are maintained at room temperature in the laboratory on a roller mixer until the time of analysis. CD4 counts were performed by flow cytometry on a BD FACSCount machine at the department of pediatrics laboratory, University of Nairobi. Plasma HIV-1 RNA was performed at Julie Overbaugh's laboratory at the Fred Hutchinson Cancer Research Center, Seattle Washington while the hematological profile assays were done using the MS4 Haematology analyzer at the department of pediatrics laboratory, University of Nairobi.

On receiving the EDTA sample in the lab, its appearance was checked for hemolysis, clots and whether the volume was enough for the run (in case of any of these irregularities, the samples are rejected). The sample was then placed on the roller mixer waiting for analysis. After setting up the MS4 hematology analyzer machine and selecting the right analysis mode and typing the correct patient identification number, the sample was thoroughly mixed and then placed on the machine for analysis. Results of the complete blood profile were automatically displayed once the counting was over they were then be printed. Among the hematological parameters generated and which were used in this study are the total white blood cells, neutrophils, platelets and hemoglobin. The generated results were then taken to the research clinic for the patient's clinical management and for entry into the patient results database. The protocol of how hematological parameters' testing was performed is attached on appendix v. For quality purposes, the University of Nairobi Pediatric Department laboratory performed both internal quality control and external quality assessment for hematology test and CD4 counts monitoring. A control sample was run every morning before running any patient samples as an internal quality control measure. The obtained control values were then plotted on Levy-Jennings charts which were evaluated on a monthly basis. The enrolled External Quality Assessment program for the MS4 hematology analyzer was Human Quality Assessment Scheme (HuQAS) which had three events in a calendar year. On each of the events, 5 samples were analyzed the same way as the patient samples. For this study, all the laboratory data was extracted from the medical records of the patients from the research clinic and used for analysis.

3.5.2 Classification of Malnutrition, Hematological Abnormities, Serum Albumin, and Viral Suppression

The anthropometric measurement (Z-scores) for weight-for-age (WAZ), height-for-age (HAZ), and weight-for-height (WHZ) were calculated using World Health Organization (WHO) reference data as standard. The children were classified as underweight, stunted, and wasted if their WAZ, HAZ, and WHZ Z-score values were less than −2.0 standard deviation (WHO, 2009). Classification of hematological abnormalities and albumin levels was done using the Division of Aids (DAIDS) Table for Grading the Severity of Adult and Pediatric Adverse Events Version 1.0, December, 2004. Thrombocytosis was defined as platelets of 500-700 x 109/L while extreme thrombocytosis was defined as platelets >700 x109/L. Viral load of less than 100,000 copies/ML was considered as viral suppression according to the WHO consolidated HIV treatment guidelines of June 2006

Table 3. 1: Classification of Hematological Abnormities and Low Serum Albumin

		Grading			
Abnormality	**Parameter**	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Anemia	Hemoglobin (gm/dl)	8.5-10.0 gm/dl	7.5-8.4 gm/dl	6.5-7.4 gm/dl	<6.5 gm/dl
Neutropenia	Neutrophils (mm3)	1000-1300 mm3	750-1000 mm3	500-750 mm3	<500 mm3
Thrombocytopenia	Platelets $X10^9$/L	100-125 $X10^9$/L	50-100 $X10^9$/L	25-50 $X10^9$/L	<25 $X10^9$/L
Low serum albumin	Serum albumin g/L	30– < LLN g/L	20 – 29 g/L	< 20 g/L	N/A

3.5.3 Clinical Procedures and Follow-up

After initiation of treatment all children were reviewed in the research clinic within two weeks to assess the presence of adverse drug reactions. Thereafter they were seen monthly for a period of 9 months then followed up quarterly for 3 years. At each follow up visit, children would get a full physical examination and information from the legal guardian was obtained by interview with regard to adherence, adverse drug effects, and inter-current illnesses at the research clinic. Blood was collected at baseline and quarterly during each follow up for viral load testing, CD4 count testing and hematology assays. Antiretroviral drug prescriptions were re-filled for a period of three months except in cases of poor adherence when there was need to have shorter follow up periods until adherence improved. All children received daily cotrimoxazole prophylaxis as required according to the WHO recommendations for prophylaxis against Pneumocystis *jiroveci* pneumonia. Children were entitled to free outpatient treatment for minor illness in the research clinic, while those requiring inpatient management were admitted at

Kenyatta National Hospital and managed by hospital staff. For all patients admitted, hospital records were obtained to extract information regarding the treatment and outcome of hospitalization.

During enrollment, social demographic information about the child caregiver was captured through an interview with the study clinicians. Such information included, who is the child's primary care giver, the age, gender, marital status, education status, employment status, and HIV status of the care giver. During enrollment, information about the child was also captured through an interview with the study clinicians, this information includes the age of the child, the gender, weight, height, head circumference, presence of inter-current illnesses (like pneumonia, tuberculosis), vaccination status, WHO clinical stage at enrollment, baseline CD4 count and CD4 percentage, baseline viral loads, and baseline hematological parameters and the type of antiretroviral medication that the child was initiated on.

At each follow up visit, socio-demographic information and clinical and immunological information, about the child is captured through an interview with the study clinicians and through laboratory tests. The following information was captured at each follow up visit, weight, height, head circumference, age, antiretroviral medication and antiretroviral adherence issues, general clinical examination e.g. presence of opportunistic infections like pneumonia, tuberculosis, treatment with other medications apart from antiretroviral drugs, side effects of the medication, switching of antiretroviral therapy and reasons for switch of therapy. Blood was collected at baseline and quarterly

during each follow up visits and the following laboratory tests were done; viral load testing, CD4 count testing, and hematology assays. For this study, the clinical and demographics data was extracted from the medical records of these patients and used for analysis.

3.5.3 Data Management

All the data collected at all visits was stored in electronic databases in the pediatric adherence study research clinic. Baseline data and data after three years of ART treatment were extracted for use in this study. Data relating to socio-demographic characteristics, immuno-hematological tests, drug regimen and clinical conditions of the HIV infected children at baseline and at three years' time point were extracted for analysis. Exploratory data techniques were used at the initial stage as a way of data cleaning to uncover the structure of the data and identify outliers or unusual entered values. Any missing values were confirmed with the data clerks and from the original hardcopy results in the patient files. Data was exported into statistical software, Stata version 12.0 (Stata Corp, College Station Texas) for further cleaning and validation in order to achieve a clean dataset that was used for analysis. Back up files were stored in CDs and/or flask discs; this was done regularly to avoid any loss or tampering.

3.6 Statistical Analysis

Variables that were extracted for analysis in this study were; baseline socio-demographic characteristics (such as age, gender, child's primary care giver, the marital status,

education and employment status of the child's care giver) clinical and immunological variables (such as white blood cells, neutrophils, thrombocytes, hemoglobin, CD4 counts, anthropometric measurement, ART regimen, opportunistic infections, WHO clinical staging). Descriptive statistics were used to summarize categorical variables while measures of central tendency (mean, standard deviation) were used for continuous variables.

Paired t-test was used to test for the mean difference for haematological parameters between baseline and after three years. Linear regression was used to assess the relationship between the changes in the haematological parameters and the correlates identified as significant ($p<0.05$) at bivariate analysis. Potential correlates included age, gender, CD4 counts, viral load, serum albumin, anthropometric measurements, clinical disease severity, and choice of ART. Coefficients and odd ratios from logistic regression model predicting the probability of neutropenia in HIV Infected children on 3 years of ART were reported. The results were presented in summary tables.

3.7 Ethical Considerations

The study received ethical approval from KEMRI/National Ethical Review Committee. The study observed adequate protection of the study subjects' confidentiality. A study code/ patient identification code was used to link the child's name and the child's data. However, the study instruments including the databases only bear the study code/patient identification code and not the patient's name. All study records and computers were stored in a locked room with access restricted only to selected study personnel.

Computer databases containing subjects' information were password protected and accessible only to selected study personnel. Study personnel explained the study in the Swahili which was the most widely spoken language. They also obtained written informed consent approved by ethical committee. The study approvals are attached as appendices I and II. Informed consent was obtained from caregivers whose children agreed to participate. The consenting process was done in a confidential area. Potential risks associated with participation and the subjects' right to withdraw from the study at any time point was explained. Written assent was obtained from children between the ages of 7 years and 12 years who could read prior to enrolment. For children of these ages who could comprehend but were unable to read, the assent form was read aloud to them and assent obtained.

CHAPTER FOUR

RESULTS

4.1 Baseline social-demographic characteristics of the HIV Infected children before ART initiation

Of the 86 children included in this study; 50 (58%) were female and 36(42%) were male. The median age of the study subjects at enrollment was 4.4 years (IQR 2.7-6.2). The biological parent was the primary caregiver for the majority of children 70(81.4%) and approximately two-thirds of primary caregivers were married 56(65.2%). About three quarters of the caregivers were unemployed 66(76.7%), and only a few had college education 13(15.1%) (Table 4.1).

Table 4. 1: Baseline characteristics of the HIV infected children before ART initiation (N=86)

Demographic	N (%)
Sex	
Female (F)	50 (58)
Male (M)	36 (42)
Age in years	
1-6 years	63 (73.3)
6-12 years	23 (26.7)
Child's Primary Care Giver	
Biological Parent	70(81.4)
Other Guardian	16(18.6)
Care Giver Marital Status	
Married	56(65.2)
Not currently married	30(34.8)
Care giver Employment Status	
Employed	20(23.3)
Unemployed	66(76.7)
Care Giver Education Status	
Primary	36(41.9)
Secondary	35(40.7)
College	13(15.1)
No formal education	2(2.3)

4.2 Baseline Disease Status and ART Treatment Regimen

At enrollment, 31(36.1%) of the subjects had TB, 38 (44.2%) had pneumonia while 17(19.7%) did not have TB or pneumonia. Based on the WHO classification, majority of the children 63(73.3%) were at stage III since they were enrolled from the words which means there might have been severely immunocompromised. The majority of children, 68 (79%), were started on AZT-based ART since it was the standard first line therapy. Children were put on D4T depending on their hematological parameters at the time of initiation. (Table 4.2)

Table 4. 2: Baseline Disease Status and ART Treatment Regimen of the Children

Variable	N (%)
Opportunistic Infections	
TB	31(36.1)
Pneumonia	38(44.2)
No TB or Pneumonia	17 (19.7)
WHO HIV disease Stage	
Stage II	13(15.1)
Stage III	63(73.3)
Stage IV	10(11.6)
ART Regimen Started on	
AZT Based therapy	68(79.1)
d4T Based therapy	18(20.9)

4.3 Changes in the Mean Hematological Parameters before and after Three Years of ART

The hematologic values monitored for clinical improvement and drug toxicity in HIV-1 infected individuals on ARV were hemoglobin, as a measure of anemia, total white blood cell (WBC) count as marker of immune status, neutrophil count, as a measure of susceptibility to bacterial infections, and thrombocytes, as a measure of blood clotting time. The mean hemoglobin concentration increased significantly from 10.4 g/dl (±SD 2.1) at baseline to 12.5 (±SD 1.2) after 3 years of ART, (p=0.0001). Mean WBCs count ($x10^9$/L) decreased significantly from baseline 9.9 (±SD 5.2) to 6.4 (±SD 2.1) after 3 years of ART (p=0.0001). The mean absolute neutrophil counts (/mm^3) decreased significantly from baseline 4.2 (±SD 3.2) to 2.7 (±SD3.2) after 3 years of ART (p=0.0001). The mean thrombocyte counts ($x10^9$/L) was unchanged from baseline, 297 (±SD170) to 283 (±SD84) after 3 years of ART, (p=0.4391). The mean CD4 percentage

increased significantly from baseline 10.2 (±SD10.2) to 28.6 (±SD10.8) after 3 years of ART (p=0.0001). (Table 4.3)

Table 4. 3: Changes in the Mean Hematological Values of HIV-1 Infected Children, before and after 3 Years of ART at KNH

Variables	Before ART Mean (±SD)	After 3 years ART Mean (±SD)	P-value
Hemoglobin (Hb) (g/dl)	10.4 (2.1)	12.5 (1.2)	0.0001
White blood cells ($x10^9$/L)	9.9 (5.2)	6.4 (2.1)	0.0001
Neutrophils (mm^3)	4.2 (3.2)	2.7 (3.2)	0.0018
Thrombocytes ($x10^9$/L)	297 (170)	283 (84)	0.4391
CD4 percentage (%)	10.2 (10.2)	28.6 (10.8)	0.0001

4.4 Patterns and Severity of Hematological Abnormalities before and after 3 Years of ART

Hematological abnormalities were present both before and after treatment with ART. Anemia (Hb ≤10g/dl) was found in 33 (38.4%) of subjects before ART treatment and in 5 (5.8%) of the subjects after 3 years of ART. Among the anemic cases, 21 (24.4%) and 5 (5.8%) had Grade 1*(Hb 8.5-10.0 gm/dl)* anemia before and after 3 years of ART, respectively. Neutropenia was also found before and after initiation of ART, the proportion of those with neutropenia increased from 8(9.3%) to 19(22.1%) after 3 years of ART. Of those with neutropenia, the proportion with Grade 1 neutropenia *(ANC 1000-1300 mm^3)* increased from 5(5.9%) to 14(16.3%) before and after 3 years of ART,

respectively. The proportion of those with thrombocytopenia (*THR <125 x 10^9/L)* decreased from 9 (10.6%) to 1(1.2%) before and after 3 years of ART, respectively. Thrombocytosis *(THR 500-700 x 10^9/L)* was seen in 8(9.4%) and 1(1.2%) before and after 3 years of ART, respectively. (Table 4.4)

Table 4. 4: Patterns and Severity of Hematological Abnormalities in HIV Infected Children before and after 3 years of ART at KNH

Variable	**Before ART N (%)**	**3 Years ART N (%)**	**P value**
Hemoglobin			
Hb > 10gm/dl	53 (61.6)	81 (94.2)	0.0001
Hb ≤10gm/dl	33 (38.4)	5 (5.8)	0.0001
Anaemia Severity			
Grade 1(8.5-10.0 gm/dl)	21 (24.4)	5 (5.8)	0.0001
Grade 2 (7.5-8.4 gm/dl)	4 (4.7)	0 (0.0)	0.0434
Grade 3(6.5-7.4 gm/dl)	5 (5.8)	0 (0.0)	0.0232
Grade 4(<6.5 gm/dl)	3 (3.5)	0 (0.0)	0.0801
Absolute Neutrophil count (ANC)			
ANC >1300mm^3	77 (89.5)	67(77.9)	0.0385
ANC ≤1300 mm^3	8 (9.3)	19 (22.1)	0.0209
Neutropenia Severity			
Grade 1(1000-1300 mm^3)	5 (5.9)	14 (16.3)	0.0285
Grade 2(750-1000 mm^3)	1 (1.2)	5 (5.8)	0.0969
Grade 3(500-750 mm^3)	2 (2.4)	0 (0.0)	0.1556
Thrombocyte Counts			
THR > 125 x 10^9/L	76 (89.4)	84 (98.8)	0.0168
THR < 125 X 10^9/L	9 (10.6)	1 (1.2)	0.0091
Severity of Thrombocytopenia & Thrombocytosis			
Thrombocytopenia: (THR <125 x 10^9/L)	9 (10.6)	1 (1.2)	0.0091
Thrombocytosis: (THR 500-700 x 10^9/L)	8 (9.4)	1(1.2)	0.0164
Extreme Thrombocytosis: (THR >700 x10^9/L)	3 (3.5)	0 (0.0)	0.0801

4.5 Changes in Nutritional Status before and after Three Years of ART

The mean anthropometric weight for age (WAZ), weight for height (WHZ), and height for age (HAZ) measurements (z-scores) improved significantly after 3 years of ART. WAZ increased from -2.3 (± SD 1.8) to -1.0 (±SD 1.0) (p =0.0001), WHZ improved from -1.2 (±SD 1.5) to -0.2 (±SD 1.0) (p=0.0001), and HAZ improved from -2.2(±SD 1.9) to -1.2 (±SD 1.5) (p=0.0001). (Table 4.5)

Table 4. 5: Changes in Nutritional Status before and after Three Years of ART

Nutritional Status	At Baseline	After 3 Years ART	P-Value
	Mean (±SD)	**Mean (±SD)**	
Weight for Age *(WAZ)*	-2.31 (1.8)	-1.00 (1.0)	0.0001
Weight for Height *(WHZ)*	-1.20 (1.5)	-0.16 (1.0)	0.0001
Height for Age *(HAZ)*	-2.2(1.9)	-1.18 (1.5)	0.0001

4.6 Level of Malnutrition in the HIV Infected Children before and after Three Years of ART

The proportion of those children who were underweight, as defined by WHO as WAZ ≤ -2SD, dropped significantly from 50 (58.8%) to 12(14.1%) (p=0.0001), after 3 years of ART, the proportion of those children who were stunted, (HAZ ≤ -2SD), dropped significantly from 42(51.9%) to 23(27.1%) (p=0.0028) and the proportion of those children who had wasting (WHZ≤-2 SD) dropped significantly from 15(20.0%) to 2(2.7%) (p=0.0009) after 3 years of ART. (Table 4.6)

Table 4. 6: Level of malnutrition of HIV Infected Children before and after 3 years of ART at KNH

Severity of Malnutrition	At Baseline	After 3 Years ART	P-Value
	N (%)	**N (%)**	
Underweight (*WAZ ≤-2 SD*)	50 (58.8)	12 (14.1)	0.0001
Stunted (*HAZ≤-2 SD*)	42 (51.9)	23 (27.1)	0.0028
Wasting (*WHZ≤-2 SD*)	15 (20.0)	2 (2.7)	0.0009

4.7 Changes in the Clinical and Immunologic characteristics after Three Years of ART

The mean HIV plasma viral load (log_{10} HIV-1 RNA copies/ml) dropped significantly (p=0.0001) from 5.77 (±SD 1.2) to 1.83(±SD 1.9) after 3 years of ART, with the proportion of those children who had high viral load (≥100,000 copies/ml) decreasing from 68(85%) to 8(11.1%) (p=0.0001). The levels of serum albumin, (which has been shown to predict disease progression and death, independently of traditional markers of HIV disease progression such as CD4 count and viral load) normalized in all children with the use of ART from a mean of 35.2 (±SD 6.7 mg/L) to 49.9 (±SD 6.5 mg/L) (p=0.0001). The proportion of children with severe immunosuppression as defined by CD4 percentage below 15% of total lymphocyte count dropped significantly from 66 (77.6%) to 6(7%) after 3 years of ART (p=0.0001). (Table 4.7)

Table 4. 7: Clinical and Immunologic characteristics of HIV Infected Children before and after 3 years of ART at KNH

Variable	At Baseline	After 3 Years ART	P-Value
Virology (Viral Load)	**Mean (±SD)**	**Mean (±SD)**	
Log10 HIV-1 RNA copies/ml	5.77 (1.2)	1.83(1.9)	0.0001
Monitoring Viral Load	**N (%)**	**N (%)**	
Viral Load (≥*100,000 copies/ML*)	68(85)	8(11.1)	0.0001
Viral Load (< *100, 000 copies/ML*)	12(15)	64(88.9)	0.0001
Serum Albumin	**Mean (±SD)**	**Mean (±SD)**	
Serum Albumin in *mg/L*	35.2 (6.7)	49.9 (6.5)	0.0001
Grading of Serum Albumin	**N (%)**	**N (%)**	
Serum Albumin (≥*34mg/L*)	40 (50%)	85 (100%)	0.0001
Serum Albumin (<*34mg/L*)	40 (50%)	0 (0.00%)	0.0001
CD4 lymphocyte %	**N (%)**	**N (%)**	
< 15%	66 (77.6)	6 (7)	0.0001
15-24%	13 (15.3)	22 (25.6)	0.0891
> 25%	6 (7.1)	58 (67.4)	0.0001

4.8 Comparison of d4T and AZT based ART Regimen Groups

The increase in the mean Hb concentration was higher in the group of patients who had been taking d4T than those who had been taking AZT based ART (2.9 g/dl Vs 1.8 g/dl) (p=0.0013). Neutropenia was observed more frequently in children who were started on AZT based therapy than in children who were started on d4T based therapy. Of the 19 children who were neutropenic after 3 years of ART, 18(94.7%) were on AZT based therapy and the other were on d4T based therapy (Table 4.8)

Table 4. 8: Comparing AZT and d4T based ART Regimen Groups

Variables	ART Regimen		p value
	d4T based therapy	AZT based therapy	
Increase in Hb	2.9g/dl	1.8g/dl	0.0013
Percent with Neutropenia n (%)	1(5.3%)	18(94.7%)	0.0001

4.9 Correlation of serum albumin with CD4 count and CD4 percent

Pre-treatment serum albumin levels correlated positively though weakly with pre-treatment CD4 cell count (correlation coefficient =0.23, p= 0.04) and CD4 percentage (correlation coefficient =0.29, p=0.008). (Table 4.9)

Table 4. 9: Correlation of serum albumin with CD4 count and CD4 percent

	CD4 count (cell/ul)		CD4% (total lymph)	
Serum Albumin (g/L)	Correlation coeff	p value	Correlation coeff	p value
	0.23	0.04	0.29	0.008

4.10 Predictors of Neutropenia in HIV Infected Children on ART

Predictors of development of neutropenia after 3 years of ART in children who were on AZT containing therapy were evaluated. These included baseline viral load, WAZ, HAZ, and WHZ z-scores, and CD4%. Children with CD4 percentage ≤ 15% were slightly more likely to develop neutropenia as compared to their counter parts (p= 0.015). (Table 4.10)

Table 4. 10: Predictors of Neutropenia in HIV Infected Children on ART at KNH

Variables	Statistics			
	Coefficient	**Odds Ratio**	**95% CI**	**p value**
AZT based therapy	1.8	6.12	0.75-49	0.089
Viral Load ≥200 copies	1.13	3.12	0.79-12	0.103
Low CD4 Percentage (≤ 15%)	-0.06	0.94	0.89-0.98	0.015
WAZ ≤-2SD	-1.2	0.28	0.03-2.3	0.235
HAZ ≤-2SD	-0.84	0.43	0.11-1.6	0.219
WHZ≤-2SD	-0.98	0.34	0.12-1.7	0.201

CHAPTER FIVE

DISCUSSION

5.1 Introduction

This study reports changes in most hematological and nutritional indices following three years of ART in HIV-1 infected children. Changes in hematological parameters are common in HIV infected children and can be the result of HIV disease progression or the adverse effects of ART. There is limited evidence of adverse effects of ARV prophylaxis on the hematological system in children, although until recently these effects were presumed to be short term, with levels of hemoglobin and neutrophils returning to normal after cessation of exposure (European Collaborative Study., 2004). One researcher, Le Chenadec *et al.,* (2003) showed that exposure to ARV in utero and postnatally was associated with lower levels of hematological parameters, in particular neutrophil counts, until eighteen months of age, but no longer-term data were available.

In Kenya and sub-Saharan Africa in general, there is also lack of data on HIV treatment related hematological abnormalities and malnutrition especially on children who have been on ART for a long period of time; hence the findings of this study partially fill this gap. In addition to the hematological abnormalities and malnutrition, this study has also given some insight about the efficacy of ART which was demonstrated by the significantly improved zscores (WAZ, HAZ and WHZ) from the baseline, increased mean serum albumin levels, increased mean CD4 value and CD4 lymphocyte percentage

and significantly reduced HIV-1 RNA copies. Significant changes in hematological parameters and associated hematological abnormalities and nutritional status were observed in this study.

5.2 Changes in Hemoglobin levels in HIV-1 Infected Children

In this study, there was a significant increase in mean Hb concentration after the initiation of ART from 10.4g/dl to 12.5 g/dl (p=0.0001) between baseline and after three years of ART. This difference could be attributed to the fact that the study population had a longer follow up period. After three years of ART therapy the overall mean Hb level increased by 2.1mg/dl from baseline. On further analysis, the increase in the mean Hb concentration was higher in those patients who had been taking d4T than AZT based ART (2.9 g/dl Vs 1.8 g/dl). These findings were similar to those found by a study in Ethiopia conducted to investigate the hematological abnormalities in children on ART for a period of 6 months. This study showed a higher increase in mean Hb concentration in those patients who had been taking d4T than AZT based ART (3.5 g/dl Vs 0.6 g/dl) (Muluneh *et al.,* 2007). When compared to the mean change of Hb with the meta-analysis of the six prospective, randomized controlled trials (Sullivan *et al.,* 2007) which showed a decrease by 0.4g/dl at 6 months and 0.2g/dl at 12 months in AZT group but an increase by 0.45g/dl at 6 months and 0.58g/dl at 12 months in d4T group, the finding of this study showed higher increase in both regimens. This difference could be due to relatively low baseline prevalence of anemia in the latter study which was conducted in developed country and partly due to the difference in study design and size of study

populations. Also, the improvement in mean Hb concentration in this study subjects could be attributed to the fact that some of the study subjects received hematinics and the fact that being on treatment reduced the severity of opportunistic infections and improved the nutritional status of the children which could have caused the Hb to increase.

5.3 Prevalence of Anemia in HIV-1 Infected Children

The prevalence of anemia before initiation of treatment in this study (38.4%) was consistent with reports of other studies that ranged from 36.9% to 95% depending on the stage of the disease (Moyle *et al.,* 2002). However, the findings of this study were on the lower side when compared to the following studies which found the prevalence of anemia to be 73% to 94% (Ellaurie, *et al.,* 1994; Eley *et al.,* 2002; Erhabor, *et al.,* 2005; Adetifa, *et al.,* 2006). Compared to the findings of a review of 32,867 HIV patient medical records that showed a prevalence rate of 36.9% (Sullivan *et al.,* 1998), the findings of this study are slightly higher (38.4%). This difference could be attributed to the higher prevalence of anemia in the local context and possibly due to the difference in age and size of the study population.

The prevalence of anemia in children who were on ART has been reported to be between 18-36% (Ellaurie *et al.,* 1990; Mathews *et al.,* 1991), however, this study showed a lower prevalence of 5.8% for the children on ART. These finding are similar

to those reported by Ezeonwu *et al.,* (2014) in Enugu, Nigeria and Bachou *et al.,* (2006) (8.0%) in Kampala, Uganda who reported a prevalence of 3.0% and 8.0% respectively.

Although ART treatment improves anemia in most HIV positive children, AZT based ART has been shown to be a risk factor for the development of anemia in HIV positive children as it causes myelosuppression (Ezeonwu *et al.,* 2014). Okechukwu *et al.,* 2010 found that children on AZT based ART were at higher risk for anemia than those on d4T based ART. Zidovudine intake is also an independent predictor of persistent anemia in those who had anemia prior to commencement of zidovudine based ART (Ezeonwu *et al.,* 2014). The low prevalence of Anemia could be attributed to the fact that this study population was being followed up closely with improved care and ability to access quality health care. This could also be attributed to the fact that some of the study subjects received iron supplements and the fact that being on treatment reduced the severity of opportunistic infections and improved the nutritional status of the children. The hemoglobin increments and subsequent reduction in the prevalence of anemia in this is similar to the findings in Malawi where iron supplementation in HIV infected children increased hemoglobin levels and reduced the prevalence of anemia by 40 % but iron was noted to increase the risk of malaria (Esan *et al.,* 2013).

Neutrophils are the most numerous and important cellular component of the innate immune response and deficiencies in neutrophil function can lead to overwhelming

bacterial infections. Their vital role in a competent immune system adds to the concern of possible long-term adverse effects of ARV exposure (Le Chenadec *et al.,* 2003).

Previous results from the European Collaborative Study (2004) showed gender and race differences in CD4 and CD8 T cell and absolute lymphocyte counts in uninfected and HIV-infected children, and gender and race may also be associated with neutrophil patterns and levels. In this study, the mean absolute neutrophil counts decreased significantly from baseline 4.2 cells/mm^3 to 2.7 cells/mm^3 after three years of ART ($p=0.0001$) resulting in a significant increase in the proportion of the children with Neutropenia.

5.5 Prevalence of Neutropenia in HIV Infected Children

Neutropenia is well described in children infected with HIV and occurs in approximately 10% to 50% of cases (Miguez-Burbano *et al.,* 2005). It is generally caused by inadequate blood cell production because of bone marrow suppression by HIV infection mediated by abnormal cytokine expression and alteration of the bone marrow microenvironment. Neutropenia is frequently observed in advanced stages of HIV infection after development of AIDS, and has been associated with certain types of myelosuppressive antiretroviral medications used to treat HIV infection (Consolini *et al.,* 2007).

In this study, neutropenia was seen both before and after initiation of ART treatment in this study. The prevalence of neutropenia before ART (9.3%) was lower than after ART

treatment (22.1%). In this study, the prevalence of pre-treatment neutropenia (9.3%) was found to be half the prevalence of neutropenia in a study by (Adetifa *et al.,* 2006) who reported a prevalence of 17.5%. In a study by (Erhabor *et al.,* 2005) the prevalence of neutropenia was 24%. A higher prevalence in this study could be attributed to the fact that 80% of children in this study had advanced and severe immune suppression compared to 77 % in this study. Notably, this study has also shown that neutropenia is strongly associated with AZT based ART treatment regimen, where 94.7% of all the cases of neutropenia were patients who were taking AZT based ART. However, the mean difference in the change of neutrophil counts between the two groups was not statistically significant (p=0.5712). The prevalence of neutropenia after ART treatment in this study was higher than that found by the Ethiopian based study (Muluneh *et al.,* 2007) which found a prevalence of neutropenia of 4.7% after 6 months of ART. However, the short duration of follow up (6 months) and the small size of the study population (64 children) in this study could limit making a valid comparison between the findings. Findings of this study are similar to those of a meta-analysis which reported 26-46% prevalence of neutropenia in AZT recipients and the prospective randomized comparative trial of d4T and AZT in children which found neutropenia of 20% over one year period among AZT recipients (Moyle *et al.,* 2004, Mark *et al.,* 1998).

5.6 Changes in Thrombocyte Counts in HIV Infected Children

Thrombocytopenia occurs often in patients with HIV infection and is one of the most common hematological abnormalities found in children with HIV infection (Labrune *et*

al., 1989). Thrombocytopenia is associated with rapid progression of disease in patients with HIV infection. There is also an association between thrombocytopenia and mortality; one study showed a mortality rate of nearly 40% in children with HIV infection and thrombocytopenia (Miguez-Burbano *et al.,* 2005). Furthermore, thrombocytopenia complicates treatment of HIV infection and associated malignancies because the medications used often affect the platelet level (Consolini *et al.,* 2007). In this study, there was no significant change in the mean thrombocyte counts between baseline, $297x10^9$/L and after three years of ART $283x10^9$/L (p=0.4391). These findings are almost similar to those of Kibaru *et al.,* 2015, who found the mean platelet count to be $255x10^9$/L before ART and 279 $x10^9$/L after 6 months of ART.

5.7 Prevalence of Thrombocytopenia in HIV-1 Infected Children

Thrombocytopenia was found before and after 3 years of ART. The prevalence of pretreatment thrombocytopenia was found to be 10.6% which was similar to the prevalence of thrombocytopenia found by (Erhabor *et al.,* 2005) who reported 10% prevalence. In their study, (Adetifa *et al.,* 2006) found the prevalence of thrombocytopenia to be 12.5% while Suarez *et al.,* 1994 reported a higher prevalence of 27%. The prevalence of thrombocytopenia after three years of ART treatment (1.2%) was lower as compared with the findings of the randomized comparative trial of AZT and d4T in children (Sullivan *et al.,* 1998) which showed a 7% prevalence and an Ethiopian based study (Muluneh *et al.,* 2007) on hematological abnormalities in children on ART which showed a prevalence of 7.8%.

5.8 Changes in Nutritional Status and Prevalence of Malnutrition

This study demonstrated that malnutrition occurs in HIV positive children as did a study by Bachou *et al.,* 2006 who documented 43.0% prevalence of malnutrition in HIV-1 infected ART naïve children. The use of ART has been shown to improve the nutritional indices of HIV positive children (Ezeonwu *et al.,* 2014). In this study, the proportion of those children who were underweight dropped from 58.8% to 14.1% after 3 years of ART, the proportion of those children who were stunted dropped from 51.9% to 27.1% and the proportion of those children who had wasting dropped from 20.0% to 2.7% before and after 3 years of ART. The prevalence of malnutrition before ART initiation in this study was closer to that observed by a study conducted in Ethiopian on HIV positive ART naïve children which found the prevalence of those underweight to be 41.7%, stunting 65% and those with wasting 5% (Berihun *et al.,* 2013). The findings are however higher than those documented by a community based study on HIV positive orphans in Kenya which reported 29.3% stunted, 13.2% underweight and 3.4% wasting (Semba *et al.,* 1992). These differences could be attributed to the different geographical locations and possibly due to the differences in age and size of the study population. Equally, majority of the caregivers in this study were unemployed and of low economic status which could have contributed to high levels of malnutrition. The effect of ART on nutritional status could be attributed to a reduction in viral load and subsequent decrease in body metabolic rate that usually accompanies infectious processes. The pre-treatment prevalence of malnutrition in this study is comparable to a study done in Uganda which reported a 47% prevalence of underweight in HIV-positive children (Beach *et al.,* 1992)

and that done as a baseline evaluation of the first 145 HIV-infected children to receive ART in Botswana which reported 59% underweight and 75% stunted (Anabwani *et al.,* 2005).

5.9 Serum Albumin as a predictor of HIV disease progression

Based on the World Health Organization classifications, HIV infection and AIDS may be staged using clinical criteria and CD4 counts. Equally, CD4 counts and CD4 percentage have been used as the traditional markers of HIV disease progression (Olawumi *et al.,* 2006). These have been indispensable in the management of both newly diagnosed patients and monitoring disease progression. The need for alternate methods of staging and monitoring the disease are necessary due to the cost and inaccessibility of these tests. Since, changes may occur in the concentrations of some biochemical markers which serve as indicators of the evolution of the disease and its complications, serum albumin levels were measured in HIV-1 infected children and evaluated as a possible predictor of CD4 count in staging HIV disease and monitoring disease progression in HIV-1 infected children. Results from this study showed that serum albumin can be a maker of severity of disease, because pre-treatment albumin level correlated positively though weakly with pre-treatment CD4 cell count (correlation coefficient =0.23, p= 0.04) and CD4 percentage (correlation coefficient =0.29, p=0.008). This is consistent with some previous studies in industrialized countries (Shafer & Vuitton 1999, Sabin *et al.,* 2002) and one African study (Olawumi *et al.,* 2006). Therefore, serum albumin, which is a cheaper test in terms of cost, would be a very

useful test for predicting the severity of HIV infection in cases where CD4 counts and CD4 percentage are not available. Also, serum albumin can be useful for the clinical monitoring of response to antiretroviral therapy, and more so because it can also reflect the level of nutrition as well as improvements in metabolism and liver function.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The findings of this study have demonstrated that the prevalence of hematological abnormalities and malnutrition remains high in HIV-infected children in sub-Saharan Africa, even in an HIV care programme which is supposed to have a high standard of care. A better acknowledgement of this problem is needed, that should lead to a better healthcare management of HIV-infected children, with active routine anthropometric measurements easy to perform to allow an earlier detection of hematological abnormalities and malnutrition leading to an appropriate nutritional package. This study strengthens the World Health Organization recommendation on the need for a nutritional assessment and support that should be an integral part of the care plan of HIV-infected children (WHO 2009). Indeed, an early detection of growth impairment could detect, for example, poor treatment response, poor adherence to treatment, and could prevent morbidity and mortality risks. Further studies about associated factors with malnutrition, such as differences in sex need to be examined more closely in prospective designs. Moreover, food supplementation and multivitamin use may improve the health status of the children and these needs to be examined properly. Equally, nutritional interventions should be tailored and assessed to improve growth, especially at time of ART initiation

that could lead to an optimization of their clinical response and survival of ART-treated children.

Notable in this study, anemia, neutropenia and thrombocytopenia were seen both before and after ART treatment among the study subjects; with neutropenia being the most prevalent. However, in the absence of local reference ranges, it is possible that the abnormalities were overestimated or underestimated in this study. Majority of those who developed neutropenia in our study were on AZT based ART. Nonetheless, ART resulted in increase of the mean Hb concentration irrespective of the regimen used.

This study confirms that in HIV-infected children, ARV therapy and disease progression are associated with changes in hematological and nutritional parameters and that hematological abnormalities and malnutrition are common manifestations in children with HIV/AIDS. These changes have a significant impact on clinical outcomes and quality of life of the HIV infected children.

6.2 Conclusions

The main objective of this study was to determine the changes in hematological profiles and nutritional status and the related hematological and nutritional abnormalities in HIV-1 infected children who were on prolonged ART at the Kenyatta National Hospital.

The following conclusions can be made from the study:

1. The mean hemoglobin concentration increased significantly between baseline and after 3 years of ART administration, with the proportion of children with anemia decreasing significantly.
2. The mean absolute neutrophil count decreased significantly between baseline and after 3 years ART administration, with proportion of those who had neutropenia increasing significantly.
3. There was no significant increase in platelet counts between baseline and after 3 years ART administration, with the proportion of those who had thrombocytopenia decreasing significantly.
4. The mean anthropometric measurements, weight for age (WAZ), weight for height (WHZ), and height for age (HAZ) (z-scores) improved significantly after 3 years of ART administration.
5. The proportions of those who were underweight, stunted, and had wasting dropped significantly after 3 years of ART administration.

The null hypothesis was therefore not accepted.

6.3 Limitations of the study

This study, like other hematological abnormalities studies in the sub-Saharan African had one main limitation. There were no locally established, age and HIV specific reference ranges for grading of hematological abnormalities in the HIV-1 infected

children. DAIDS toxicity tables derived from the western populations were used which may over or under estimate hematological abnormalities in this cohort of African children. This problem has been reported by other African based studies (Zeh *et al.,* 2011). There is need for locally-derived age-specific clinical laboratory reference ranges of healthy and HIV-infected Africans in sub-Saharan Africa. Reference values from North American and European populations are being used for African subjects despite previous studies showing significant differences (Zeh *et al.,* 2011). Use of normal hematologic ranges and grading systems derived from African infants may be useful to avoid overestimation of toxicities. Another limitation in this study was that children included in this study had access to pediatric HIV healthcare at Kenyatta National Hospital which is an urban area where the standard of care may be higher than that offered in rural areas. This makes the results difficult to extrapolate to rural areas. Furthermore, since 50 % of HIV-infected children not initiated on ART die before their second birthday (Newell *et al.,* 2004), the sickest children could not have survived until the study period, leading to a survivor bias. So, the selection of the study population may not have been a representative of a birth cohort of HIV-infected children in sub-Saharan Africa, leading to an underestimation of the prevalence of malnutrition and hematological abnormalities associated with HIV before ART initiation. Another limitation is that there were possible measurement errors in weight and height; the study limited this by using a standard measurement protocol following the WHO recommendations (WHO, 2006). However, peripheral oedema, sign of severe malnutrition, was not collected, despite their effect on increasing artificially weight.

6.4 Recommendations

The following recommendations emanating from the study can be made:

1. Physicians giving care to HIV infected children should routinely investigate and treat hematological abnormalities before and after initiating ART treatment. Routine monitoring of hematological parameters in children with HIV/AIDS will be important to detect the abnormalities at the earliest, find the aetiology and treat appropriately. These measures will reduce the morbidity and mortality among children infected with HIV/AIDS.
2. Malnutrition in HIV-infected children has been associated with metabolic complications including electrolyte disorders, micronutrient deficiencies, and severe infections, which contribute to the high mortality rates among HIV-1 infected children in resource limited settings in sub-Saharan Africa (Musoke & Fergusson 2011). Clinicians already face the challenges of identification and treatment of metabolic complications in children on ART in resource limited settings and these challenges are intensified by malnutrition. Studies are therefore needed to determine the incidence, risk factors, and effect of the metabolic complications of ART in HIV-infected children in resource limited settings.
3. Large scale and longitudinal studies are recommended in order to strengthen and explore in depth the problem of hematological abnormalities and malnutrition associated with HIV disease progression and ART treatment.

4. Integration of HIV/malnutrition services and further research to determine optimal ART timing, role of supplementary feeding and antimicrobial prophylaxis are urgently required.
5. Further studies on a larger population of children, to ascertain the role of other factors, such as malaria and micronutrient deficiency, which may contribute to anemia, neutropenia, thrombocytopenia and malnutrition in HIV-1 infected children are recommended.

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APPENDICES

Appendix i: KEMRI SSC Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400009, Fax: (254) (020) 2720530
E-mail: director@kemri.org, info@kemri.org, Website: www.kemri.org

ESACIPAC/SSC/100850 10th September, 2012

John Gatimu

Thro'

Director, CPHR
NAIROBI

Forwarded 11/9/12

REF: SSC No. 2328 (Revised) – The hematological profile of HIV infected children receiving antiretroviral therapy over a three year period in Kenyatta National Hospital, Nairobi, Kenya

I am pleased to inform you that the above mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **194th** meeting held on **4th September, 2012** and has since been approved for implementation by the SSC.

Kindly submit 4 copies of the revised protocol within 2 weeks from the date of this letter i.e, 24th September, 2012 to SSC for onward transmission to ERC office.

We advise that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix ii: Ethical approval by KEMRI/National Ethical Review Committee.

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-203691, 0733-403003, Fax (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1

October 31, 2012

TO: MR. JOHN GATIMU (PRINCIPAL INVESTIGATOR)

THROUGH: DR. YERI KOMBE,
THE DIRECTOR, CPHR
NAIROBI

Dear Sir,

RE: SSC PROTOCOL No. 2328 – 2ND REVISION: HEMATOLOGICAL PROFILE OF HIV INFECTED CHILDREN RECEIVING ANTIRETROVIRAL THERAPY OVER A 3 YEAR PERIOD IN KENYATTA NATIONAL HOSPITAL, NAIROBI, KENYA
(*VERSION DATED OCTOBER 2012*)

Reference is made to your letter dated 11th October 2012. The ERC Secretariat acknowledges receipt of the revised proposal on October 17, 2012.

This is to inform you that at the 209th meeting of KEMRI Ethics Review Committee held on 30th October 2012, the Committee made note of the revisions and determined that the issues raised at the initial review are adequately addressed. Consequently, the study was granted approval for implementation effective the **30th day of October 2012** for a period of one year.
Please note that authorization to conduct this study will automatically expire on **October 29, 2013**.

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **September 17, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix iii: Manuscript publication approval by AJHS at KEMRI.

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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KEMRI/AJHS/371/2014 21st March, 2014

John Gatimu
Institute of Tropical Medicine and Infectious Diseases,
Jomo Kenyatta University of Agriculture and Technology,
Box 62000-00200
NAIROBI

Dear Sir,

AJHS/2013/371 – Hematological profile of HIV infected children receiving Anti-retroviral therapy over 3 years period in Kenyatta National Hospital, Nairobi Kenya by John Gatimu et al

This is to inform you that the above manuscript has been approved for publication in the African Journal of Health Sciences.

It was noted that your study was on Hematological profile of HIV infected children receiving Anti-retroviral therapy over 3 year period in Kenyatta National Hospital, Nairobi Kenya

Thank you for taking interest in the journal.

Yours faithfully,

DR. HUDSON LODENYO
EDITOR-IN-CHIEF
AFRICAN JOURNAL OF HEALTH SCIENCES

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In Search of Better Health

Appendix iv: The Aims, Research design and Methods for the parent study

AIMS, RESEARCH DESIGN AND METHODS FOR THE PARENT STUDY

A. SPECIFIC AIMS

The burden of pediatric HIV-1 infection globally is highest in sub-Saharan Africa with over two million children infected currently (1). Following increased access to highly active antiretroviral therapy (HAART), survival of HIV-1 infected African children is expected to improve. However, most such children initiate treatment at more advanced disease stage and greater level of immunosuppression than their counterparts in Europe and the U.S. (2). In addition, initial (first-line) HAART regimens in Africa typically have a non-nucleoside reverse transcriptase inhibitor (NNRTI) backbone and do not include ritonavir-boosted protease inhibitors (PIs), which are considered the most potent antiretrovirals available(3). Failure to adequately suppress HIV-1 can rapidly lead to emergence of drug-resistance and negatively impact long-term response to HAART (4).

The overall hypothesis of this proposal is that clinical disease stage; level of immunosuppression at presentation, and choice of initial HAART regimen will impact long-term response and selection of drug resistant virus. For this GRIP application we propose to utilize and extend a recently accrued pediatric HIV-1 treatment cohort in Nairobi to determine the pattern and correlates of long-term response to HAART and to determine levels of resistance to first line regimens in HIV-1 infected children failing therapy. Our specific aims are:

Aim 1

Define the pattern and predictors of virologic and clinical response to HAART during 5 years of follow-up in HIV-1 infected Kenyan children. Potential predictors include baseline CD4% and viral load, clinical disease severity, adherence, and choice of non-nucleoside reverse transcriptase inhibitor (NNRTI).

Hypotheses: This aim will test the following hypotheses:

H1: HIV-1 infected children with baseline CD4% < 5% and or/advanced clinical disease stage, and suboptimal adherence will have lower rates of virologic success, poorer clinical outcomes and higher mortality at 5 years post-HAART initiation compared to those without these characteristics.

H2: HIV-1 infected children initiated on efavirenz will have higher rates of virologic success and better clinical outcomes at 5 years post-HAART initiation than those on nevirapine.

Aim 2

Define the frequency of mutations associated with resistance to the first-line NNRTI based triple therapy being introduced in Kenya and the effect of such mutations on virologic success in HIV-1 infected children. To this end, all children who experience virologic failure will have genotyping done at the point of treatment failure. A subset of children who achieve sustained virologic suppression will also have genotyping performed on the last plasma sample available. The frequency of mutations to first-line

drugs will be compared between children failing therapy and those achieving virologic response.

B. BACKGROUND

Efficacy of HAART in children

Level of immunosuppression at HAART initiation

As HIV treatment programs in Africa scale, up the immediate focus is to rapidly increase access to therapy. In Kenya, less than 20% of the ~ 20000 HIV-1 infected children that require HAART are currently accessing treatment (1). One key consequence of the delayed/limited access to HAART is that most HIV-1 infected children in Kenya will be started on treatment at very advanced disease stage and severe immunosuppression.

The level of immunosuppression at HAART initiation has recently been shown to affect survival in children from an HIV-1 treatment program in Cote'dIvoire (5). In that program, survival in the children with baseline CD4 percentage $\geq$ 5% was 98% at 24 months, significantly higher than the 73% survival observed in those with CD4 percentage <5% at baseline ($p<0.01$). Furthermore, 8 out of the 9 children (89%) who died while receiving HAART had CD4 percentage <5% at baseline and remained low despite good virologic response. Similar findings have been reported from Thailand where 3 of the 4 (75%) children who died in a cohort with 107 children had CD4 percentage < 4% at baseline and which persistently remained low despite good virologic response (6).

The scenario of children initiating HAART with very severe immunosuppression is likely to be common in other African settings and warrants further investigation to define its long-term implications. A currently accruing cohort of 100 HIV-1 infected children in Nairobi, (the Pediatric HAART Adherence Study) provides an excellent opportunity to study long term effects given that, at baseline, nearly 40% of the children have a CD4 percentage below 5% (Appendix 1). If children with such profound immunosuppression are shown to respond poorly to the recommended initial (first-line) HAART regimens currently being scaled up, it may be important to revise treatment guidelines to use more potent initial antiretroviral drug regimens for this subgroup in an effort to improve survival. It will also be important to identify HIV-1 infected children early and initiate therapy before they reach such advanced levels of immunosuppresion, but such efforts will take time and may not directly benefit the already existing pool of children with CD4 percentage below 5%.

Choice of initial antiretroviral drug regimen

The second factor that is likely impact the efficacy of HAART in Kenyan children is the choice of initial antiretroviral therapy. Unlike Western settings where HAART is individualized, all HIV-1 Kenyan infected children are initiated on a standard first-line regimen (4, 7). Currently this consists of two NRTIs (zidovudine and lamivudine) and one NNRTI (nevirapine or efavirenz). Nevirapine is recommended as first-line for children both below and above 3 years while efavirenz can only be used for children

above 3 years. The first-line regimen specifically excludes protease inhibitors due to difficulties in storage (liquid formulations are heat labile and require refrigeration) and cost-implications. In contrast, under U.S. guidelines ritonavir-boosted protease inhibitors are strongly recommended as initial therapy based on data suggesting their superior efficacy in children (8). In addition, nevirapine is considered an alternative NNRTI choice to efavirenz for children above the age of 3 years.

There is increasing evidence to suggest that protease inhibitor (PI) based regimens and especially lopinavir/ritonavir result in better virologic response compared to other combinations (9-10). In a recent study with lopinavir/ritonavir in combination with two nucleoside reverse transcriptase inhibitors NRTIs), 88% of antiretroviral naïve children achieved plasma HIV-1 RNA levels <400 copies/ml at 48 weeks(9). Several studies show that addition of a protease inhibitor to NNRTI based regimens (quadruple therapy), results in 78%-87% virologic success levels in children (11, 12). The major setback of protease inhibitor therapy is potential poor adherence associated with poor palatability of liquid formulations (13).

Among the NNRTIs, efavirenz has shown superior virologic success rates compared to nevirapine in a number of studies making it the strongly recommend NNRTI for children above 3 years (14, 15). The highest rate of virologic success with efavirenz and 2 NRTIs was observed recently in the Thai National Access to Antiretroviral Program in which 91% children achieved plasma HIV-1 RNA levels <50 copies /ml at 48 weeks (6). In the same cohort, the corresponding success rate for nevirapine was 64%, significantly lower than that from efavirenz ($p = 0.001$).

A different study in adults, the 2NN study, compared nevirapine once or twice daily to the use of efavirenz (16). The virological success rates were equivalent, and this has lead support to the notion that in adults the two NNRTI differ in toxicity but not antiviral activity. However, the half-life of nevirapine in children is shorter than in adults, and for this reason alone, it may prove less effective. Other studies with nevirapine in children have yielded much lower success rates ranging from 25% to 50% (17-19). In one of these studies nevirapine resistance was documented in all children failing therapy (18). This finding is consistent with the fact that a single point mutation in the reverse transcriptase gene is sufficient to confer class resistance for all NNRTIs while multiple mutations are required for resistance to develop against protease inhibitors (20).

Thus, although the Kenyan guidelines may potentially result in higher levels of adherence by using less complex NNRTI regimens and sparing PIs, it remains important to evaluate the long-term efficacy of NNRTI-based and in particular nevirapine-containing regimens in HIV-1 infected children.

Adherence to therapy

Adherence to therapy is the third key factor that may impact efficacy of pediatric HAART in sub-Saharan Africa. The importance of adherence to therapy in influencing virologic outcome has been well documented in children (21, 22). Watson et al found that 52% of children with good adherence achieved virologic response compared to only 10% of children with poor adherence (23).

Overall, adherence to HAART in Western studies is variable but two prospective studies, the Paediatric European Network for treatment of HIV/AIDS (PENTA)-5 trial, and the Paediatric AIDS Clinical trial Group (PACTG)-377, have recently demonstrated ~70% full adherence overall (24, 25). Although there was optimism about high levels of adherence in Africa, research in adults indicates that adherence in the long term may still pose a significant challenge in this context as it did in the Western Europe and the U.S (26). While a few studies have reported high levels of adherence in the short-term, there is currently inadequate African data on long-tem adherence to HAART particularly in children and this remains an important research priority.
In a study from adults in Senegal, the proportion of patients reporting above 80% adherence declined from 95% at 1 month to 80% at 18 months. In the same study, the proportion achieving undetectable viral load similarly fell from 79% at 1 month to 60% at 18 months (27). A similar trend where adherence fell markedly over time has been described in Cameroon (28). This limited evidence calls into question how long the high levels of adherence being reported will be sustained and highlight the need for long-term studies to address this question in HIV-1 infected children.

The schematic diagram below summarizes the factors that will be investigated in the proposed study and their relation to pediatric HAART efficacy:

Figure 1: Factors likely to impact response to HAART

Antiretroviral Drug resistance
Currently most children enrolled into treatment programs in Kenya are on the first-line antiretroviral drug regimens consisting of two NRTIs and one NNRTI (7, 29). However within the next few months to years an increasing number of children may experience treatment failure on first-line regimens necessitating the use of second-line regimens. The change from the first- to second-line antiretroviral regimens takes into account drug history and availability of alternatives as stipulated in the Kenyan National guidelines. It is recommended at least two of the drugs preferably from different classes be replaced

(7). But data on drug resistance in the setting of pediatric treatment in Africa are rare and it is therefore unclear how frequently such a shift in drug regimens will be needed.

Studies documenting HIV drug resistance in children

Several pediatric studies have shown that resistance to HAART is associated with virologic failure(30-32). In these studies, which mainly involve HIV-1 subtype B, the prevalence of resistance mutations has been shown to increase from none to few at baseline to high levels at treatment failure. Children failing treatment have often demonstrated higher frequency of mutations than controls with no evidence of virologc failure. In most studies the reverse transcriptase gene is more frequently involved than the protease gene. In general the most common mutations identified include M184V mutation associated with lamivudine resistance and K103N and Y181C mutations associated with nevirapine resistance. In a study of 84 children where majority of the children were black with non-B HIV-1 subtype, 90% of those failing therapy had mutations involving the reverse transcriptase gene while 33% had resistance involving the protease inhibitor gene(33). Consistent with other studies, more than 90% of the children exposed to lamivudine had M184V mutation. Resistance to zidovudine and nevirapine in this cohort was 64% and 75% respectively. Previous dual NRTI therapy has been associated with higher level of resistance than triple therapy (HAART) in studies from children in Brazil and adults in Uganda (34,35). Table 1 summarizes selected studies on HAART resistance in children.Table 1. A summary of selected studies on resistance to pediatric HAART

Study	Sample size, design	Failing therapy n (%)	Time to failure	Frequency of key mutations in RT In children failing therapy
Eshleman, PACTG 377 (31)	181	70 (38)	12 weeks	Baseline – 16 (25%)$_a$ At failure –47 (81%)$_a$
Aboulker, PENTA- 7 (32)	21	14 (70)	12 weeks (5) 72 weeks (9)	Baseline - 2 (10%) 72 weeks - 5 (25%)
Pillay, PENTA-5 (30)	113	55(49)	-	Baseline - 0 28 weeks - M184V (65%)
Mullen (33)	26	All	Retrospective	19 (90%)
Brindeiro (36)	52	All	Retrospective	M184V - 85% M41L - 39% K103N - 15%

a Refers to resistance to study drugs only. RT –Reverse transcriptase.

HIV-1 Subtypes and resistance mutations:

There are conflicting data regarding the effect of subtype on resistance mutations with most studies showing no difference between subtype B and non-B subtype and others reporting significant differences; however the data are still quite limited. In the PENTA 5 study, there was no significant difference in virologic response and primary resistance

mutations between subtypes although there were significant differences in secondary PI mutations (30). In a Brazilian study, mutations associated with resistance to zidovudine (K70R, D67N and K21Q) were significantly higher in subtype B relative to subtype non-subtype-B, but there was no difference between subtypes in resistance to lamivudine (M184V)(36). A recent study on adults from Uganda reported a higher prevalence of resistance mutations in subtype D (21 of 33) than subtype A (7 of 25)(35). The frequency of resistance mutations following single dose nevirapine has been found to be higher for subtype C than subtype A or D (37).
Thus, due to limited studies involving small numbers of children and with inconsistent findings, the effect of HIV-1 subtype on pattern of antiretroviral drug resistance in children is still unclear. This holds relevance to HIV-1 infected children in Africa where there is predominance of nontype-B and NNRTIs form the backbone of initial HAART regimens.

Rationale for resistance testing in current cohort of children
There is randomized control trial evidence in HIV-1 infected adults showing that salvage regimens chosen on basis of genotypic testing result in superior virologic response compared to decisions based on guidelines and expert advice alone (38-41). Following this finding, resistance testing has been recommended as standard of care in HIV-infected adults when changing from one regimen to another. While resistance testing for HIV-1 in Africa may not be implemented widely for some time, its is critical that when such technology becomes more readily available, we have data on resistance patterns to the treatments in common use so that we are poised to help guide its implementation and maximize its utility. Findings from our study will describe the pattern and nature of resistance mutations in HIV-1 infected children failing therapy in Kenya, where HIV-1 epidemic is driven by non-subtype B HIV-1.

D. RESEARCH DESIGN AND METHODS

This study will leverage an existing cohort that we established in Nairobi (the Paediatric Adherence Study cohort) in order to determine long-term response to HAART, patterns of viral response and resistance during a further 4-year follow-up period. This will enable comprehensive long-term assessment of pediatric compliance over a total 5-year follow-up period. The ongoing Pediatric adherence Study has enrolled 80 HIV-1 infected children with an anticipated total enrollment of 150 children as part of the proposed GRIP cohort.

Aim 1

To define pattern and predictors of long-term response to HAART in HIV-1 infected children in a resource-poor setting. Potential predictors include baseline viral load, CD4 percent, drug adherence, choice of NNRTI, and clinical disease severity at presentation.

Overview of Study Design
We propose to follow up the entire cohort of 100 HIV infected children on HAART. Children will be seen once quarterly for an additional period of 4 years to make a total

observation time of approximately 5 years. In addition, as part of the GRIP pediatric efficacy study 50 additional children will be recruited for 4-year follow-up.

Study subjects

Children enrolled into the Pediatric Adherence Study are drawn from the Kenyatta National Hospital (KNH) pediatric wards and clinics and must have confirmed HIV-1 infection and meet following eligibility criteria:

Antiretroviral drug naive.

Symptomatic with WHO clinical stage 3 or stage 2 with CD4 percentage < 15%.

Age between 18 month and 12 years.

Consent from a parent or legal guardian.

Willing to stay in Nairobi for at least one year after initiation of HAART.

Children severe neurological impairment due to HIV-1 associated encephalopathy are excluded from the study.

Procedures and Follow-up

The ongoing Pediatric Adherence Study has a randomized design and includes 9 months of follow-up. Children were initiated on NNRTI based HAART according to the Kenyan national guidelines, and randomized to either medication diary or standardized counseling (see appendix 1). After initiation of treatment all children were reviewed in the research clinic within two weeks and at monthly intervals for a period of 9 months. At each follow up visit, children had a full physical examination and information regarding adherence, adverse drug effects, andintercurrent illness obtained by interview. Plasma HIV-1 RNA full blood count and liver function tests were measured at baseline and quarterly. T cell lymphocyte subsets were measured at baseline and after 6 months.

In the proposed study these infants will be followed quarterly for an additional 4 years. At each follow up visit, children will have a full physical examination and questionnaire administered to gather information on adherence, adverse drug effects, andintercurrent illness. Antiretroviral drug prescriptions will be refilled for a period of three months at every visit unless there is need to have shorter periods in cases of poor adherence. Additional plasma HIV-1 RNA will be performed once every 6 months. Lymphocyte subset determinations, complete blood counts, liver function tests and urea/creatinine will be determined quarterly. All children will receive daily cotrimoxazole prophylaxis as required according to the WHO recommendations for prophylaxis against *Pneumocystis* pneumonia (29). Children will be entitled to free outpatient treatment for minor illness in the research clinic, while those requiring inpatient management will be admitted to Kenyatta National Hospital (KNH) and managed by hospital staff. For all patients admitted, hospital records will be obtained to abstract information regarding the treatment and outcome of hospitalization.

Response to therapy

Monitoring for response during treatment will be done using clinical, immunologic (CD4 count and percent) and virologic (plasma HIV-1 RNA) criteria. Clinical parameters used will include change in weight for age z-scores, weight for height z-scores and body mass index z-scores from baseline (pre-HAART) to the last follow-up visit (post-HAART). In addition, frequency of infections (bacterial infections, oral

thrush, pneumonia and other opportunistic infections) both treated as outpatients and those requiring hospitalization will be documented.

Definition of good response

For the proposed study long-term response will be measured at 5 years post-HAART initiation or latest available after 2 years post-HAART initiation using the following definitions:

a. Clinical response: Sustained improvement of anthropometric parameters, reduction of opportunistic infection and progressive neurodevelopment measured by achievement and retention of developmental milestones.

b. Immunological response: sustained increase in CD4+ T-cell count and percentage.

c. Virologic response: Achievement of undetectable plasma HIV-1 RNA levels (less than 400 copies/ml), or sustained decrease of at least 1.0 log plasma HIV- RNA from baseline within 6 months after initiation of therapy.

Monitoring for Drug Toxicity

Before initiating HAART, parents/caregivers are taught how to identify side effects and encouraged to bring the child to the study clinic as soon as they suspect development of any such outcomes. In addition, liver function tests and complete blood counts are performed at 3-month intervals to detect hepatotoxicity that may arise following nevirapine use or anemia from zidovudine. Serious adverse drug effects necessitate withdrawal of drugs suspected to be responsible for toxicity. Serious adverse drug reactions that necessitate report to the Ethics and Research committee at the KNH and the Human Subjects Division at the University of Washington include grade 3-4 nevirapine associated rash, 3-fold or greater elevation of liver transaminases (either AST or ALT), anemia less than 7g/dl and neutropenia less than 1500/µl.

Aim 2

To define the frequency of drug resistance mutations to the first-line NNRTI based triple therapy being introduced in Kenya and the effect of such mutations on long-term virologic response in HIV-1 infected Kenyan children.

Overview of Study Design

For this aim, archived plasma samples collected from the cohort of children in the Paediatric Adherence Study at baseline, and quarterly thereafter will be analyzed retrospectively. Genotypic resistance testing to detect mutations in the reverse transcriptase will be performed. The frequency of mutations to study drugs at baseline and at treatment failure will be compared between children failing therapy and those achieving virologic response in a cohort of HIV-1 infected Kenyan children.

Timing of genotypic testing

a. Treatment failure samples

The following are examples of situations we anticipate in this study based on previous US/European studies.

i. Children with initial rapid viral suppression by 3 months followed by rebound by month 6: genotyping will be done on the month- 6 sample.
ii. Children with slower decline to suppressed by month 6 followed by rebound by month 9: genotyping will be done on the month 9 sample.
iii. Children with no evidence of viral suppression at any time point (primary resistance): genotyping will be done at baseline and month 3.
iv. Children with viral suppression by either month 3 or 6 remaining suppressed on subsequent samples: genotyping will only be done on a subset of these children. If viral rebound occurs then genotyping will be done at the point of rebound.

b. Baseline samples

For children with resistance mutations found at treatment failure, we shall go back and test archived samples collected at baseline to determine whether the mutations were present before initiating HAART. A subset of children with virologic success will also undergo genotypic resistance at a time-point matched with those failing treatment. Similarly baseline samples for this subset shall only be genotyped if resistance mutations are demonstrated.

Resistance testing and antiretroviral drug regimen.

This will be done while the child is still on the antiretroviral drug regimen thought to be failing (i.e. before any changes are made). Previous studies have shown that HIV-1 reverts to the wild type which is more efficient in replication within a matter of weeks when drug pressure is removed (41).

Change to Second line Regimen

Change to second antiretroviral therapy in this cohort will be based on clinical and immunologic criteria as follows:
a. Clinical deterioration evidenced by:
i. Lack of weight gain or loss of weight.
ii. New or continuing serious opportunistic infections after 6 months of treatment.
iii. Neurological deterioration evidenced by stagnation or regression of developmental milestones.
b. Immunologic failure: Persistent decline in CD4 percent reported from 2 consecutive samples taken 3 months apart. For children without clinical deterioration, a drop in CD4% of $\geq$ 5%, confirmed by a repeat test within 3 months will be considered evidence of immunological failure.
Any child failing to respond as expected will first be re-evaluated to determine the cause of poor response. If poor adherence is suspected, the caregiver will be referred to trained adherence counselors. Children with true treatment failure will be changed to second line antiretroviral drug regimens.
The choice of second-line antiretroviral therapy following treatment failure in the proposed study will primarily be based on the Kenyan national guidelines (7). These guidelines recommend as second-line the use of two nucleoside reverse transcriptase

inhibitors not included in the first-line regimen and a protease inhibitor. The choice of drugs will therefore include lopinavir/ritonavir (Kaletra) as the protease inhibitor and d4T or ddI and abacavir. Since the proposed study includes resistance testing in cases of virologic failure, the results of such testing will be made available to the clinical team making decisions on treatment changes. In cases where the antiretroviral drug regimen is changed due to toxicity only the offending drug will be substituted.

Laboratory Methods

HIV diagnosis, HIV-1 viral load, and laboratory monitoring for toxicity:

HIV-1 antibody testing for children in KNH is performed using 2 rapid tests in parallel, Unigold and Determine provided by the National AIDS Control Program of Kenya. CD4 counts will be performed by flow cytometry or FACSCAN (Becton Dickson) at the department of pediatrics laboratory, University of Nairobi. Plasma HIV-1 RNA is performed at Dr. Julie Overbaugh's laboratory at the Fred Hutchinson Cancer Research Center, Seattle Washington using a transcription-mediated amplification (TMA) method developed by Gen-Probe(42).

b) HIV-1 genotyping: (Drug resistance testing)

Genotypic resistance testing will be performed onsite using methods similar to those routinely used in clinical laboratories to detect resistance mutations. Our in-house method has been developed by Julie Overbaugh's laboratory and relies on cDNA synthesis and nested PCR followed by direct sequencing. The mutations to the first line antiretroviral medications used in this study cluster within a ~700 base region of the reverse transcriptase (RT) gene. Thus, sequencing using two primers in each direction yields overlapping sequence for the region of interest. Because there are at least three different HIV-1 subtypes in our cohort, we have designed primers for both amplification and sequencing that bind to conserved regions of subtypes A, D and C (43, 44). To accommodate for sequence variation, a nested PCR that amplifies a ~900 base pair fragment spanning the region of interest was developed. The first round primers are degenerate (corresponding to positions 2075-2094 and 4332-4351 in HXB2) and never differ from any of the known HIV-1 sequences in the Los Alamos database by more than 2 bases. The second round primers also typically do not differ from known subtype A, D, and C sequences by more than 2 bases and correspond to positions 2610-2634 and 3507-3529 in HXB2. We have shown that we can detect a mutant genome present at 20% of the population as has been shown by other groups for subtype B (45, 46). For sequence studies, we will perform cDNA synthesis and nested PCR to amplify ~900bp fragment of RT. We will use our viral load data to ensure that we sample ~100 proviral genomes, which will allow us to readily detect resistance mutations present at >20%. The Stanford and International AIDS Society-USA web sites will be used to aid analyses and detection of drug resistance mutations.

Statistical methods

Sample size calculations

Aim 1

Sample size calculations are based on the first aim comparing survival in children initiating HAART with CD4 percentage < 5% to those with percentage ≥ 5% at baseline.

From Fassinou's study in Cote d'Ivoire, the survival at ~ 2 years in children with CD4 percentage below and equal to or above 5% at baseline was 73% and 98%, respectively(22).
We propose to recruit an additional 50 children into the current cohort in to make a total of 150 children.
The table below shows the power for hypothesis testing assuming 50 children with CD4% < 5% and 100 children with CD4 ≥ 5% at baseline, a 2-sided chi-square test, and $\alpha = 0.05$. This ratio of children with CD4% < 5% to children with CD4% ≥ 5% (1:2) reflects the distribution in the current pediatric adherence cohort (appendix 1). Given these assumptions, with 150 children there is high power to demonstrate a difference of 20% in mortality.

Table 4. Power determination

p_1 survival at 60 months CD4 < 5%	0.73	0.75	0.75	0.70
p_2 survival at 60 months CD4 ≥ 5%	0.98	0.95	0.90	0.90
Power (1 - β)	0.98	0.92	0.66	0.85

Aim 2
Based on previous studies, a virologic failure rate of ~ 40% is expected (5,31-32). The frequency of common resistance mutations in the failure group is expected to range from 60%-80%, while in the virologic success group between 10%-20% will develop resistance mutations (32-33). The scenario requiring highest power would be a frequency of 60% in children failing therapy and 20% in those achieving virlologic success. With these assumptions and 150 children there is high power (> 99%) to show difference in frequency of mutations between children in the two groups using a two-sided Chi-square test with $\alpha = 0.05$.

Statistical methods.
Aim 1.
Kaplan Meier survival analysis will be used to estimate and compare survival at 60 months between children with CD4 percentage ≥ 5% at baseline to that in children with CD4 < 5% at baseline. In addition Cox proportional hazards models will be used to define predictors of survival in this cohort. Multivariate logistic regression will be used to define predictors of virologic response.

Effect of age and the H2 Hypothesis
Our second hypothesis states that HIV-1 infected children initiated on efavirenz will have higher rates of virologic success and better clinical outcomes at 5 years post-HAART initiation than those on nevirapine. It is expected that Nevirapine will be used differentially in younger children because efavirenz is not licensed for use in children below the age of 3 years. Therefore, to compare these rates in children started on the

two regimens (nevirapine vs. efavirenz) we will use logistic regression adjusted for the age of the child.

Aim 2

The frequency of different resistance mutations in reverse transcriptase gene will be expressed in proportions with confidence limits. Chi-square tests will be used to compare the prevalence of resistance mutations in children with and without virologic response.

Timeline:

The pediatric adherence study cohort is currently accruing children. It is therefore anticipated that by December 2006, all 150 children will have completed at least 9 months of follow-up. The extension phase of the study will start as soon as children complete 9 months follow-up. This ranges from October 2005 through December 2006. We anticipate that the additional 4 years follow-up period will thus be completed by December 2010. There fore the proposed study will span 5 years from mid-2006 to mid 2011 with the last 6 months being used for final data compilation and analysis.

E. HUMAN SUBJECTS ISSUES

Human subjects' involvement and characteristics

One hundred HIV-1 infected children and caregivers who are already participating in a pediatric adherence cohort study will be enrolled for an additional follow-up duration of 4 years. An additional 50 HIV-1 infected children will be enrolled into the main study. Informed consent will be sought from parents or legal guardians above the age of 18yrs before children before children enroll for this additional follow-up.

Potential risks to subjects

a. Confidentiality.

This is a potential risk since personal information regarding the child and family including HIV-1 status will be obtained

b. Provision of antiretroviral drugs after study completion.

Children will require lifelong treatment with antiretroviral drugs. In the proposed study children will receive antiretroviral therapy from the President's Emergency Program for AIDS Relief (PEPFAR), which is funded for 5 years starting from 2004. There is a subsidized fee for receiving the medication that will be paid by the project while children are in follow-up. Efforts are underway to provide antiretroviral treatment at no cost to children and this is expected to be in place the completion of the proposed study. However this is still likely to cause distress to caregivers who are unable to pay for the subsidized fee.

c. Adverse effects from antiretroviral drugs.

A few children may develop side effects from use of antiretroviral drugs. Caregivers have been taught how to recognize common side effects and advised to bring children to the research clinic or other heath facility as soon as they observe such signs. To date the level of serious side effects has been low (about 12%) and in all cases children have improved following substitution of the offending drug.

Adequacy of protection against risks

Recruitment and Informed consent and assent

Study personnel will explain the study in the Swahili which is the most widely spoken language in Kenya. They will also obtain written informed consent approved by the local IRB (Kenyatta National Hospital) and the University of Washington Human Subjects Division, prior enrolment for extended follow-up. Informed consent will be obtained from caregivers whose children agree to participate. The consenting process will be done in a confidential area. Potential risks associated with participation and the subjects' right to withdraw from the study at any time point will be explained. Written assent will be obtained from children between the ages of 7 years and 12 years who can read prior to enrolments. For children of these ages who can comprehend but are unable to read, the assent form will be read aloud to them and assent obtained.

Preservation of confidentiality:
Subjects' confidentiality will be protected at all levels. All interviews will be conducted in a confidential setting. The study instruments including questionnaires will bear a study code and not the patient's name. Since information obtained form the study will be useful for future clinical management of the children, the study code will be linked to the child's name and this information kept safely under lock and key. At the end of the study only findings relevant to the child's clinical management (e.g. lab results, clinical progress) will be summarized for inclusion in the child's medical records. All study records and computers will be stored in a locked room with access restricted to study personnel. Computer databases containing subjects' information will be password protected and accessible only to study personnel.

Potential Benefits of the Proposed Research to the Subjects and Others
HIV-1 infected children participating in the research will receive antiretroviral therapy and laboratory monitoring at no cost to them for an additional period of 4 years. The parents and caregivers will of participating children will receive ongoing adherence counseling and support during the period of study. Transport costs to and from the clinic will also be reimbursed during the period of study.

Importance of the Knowledge to be Gained
This study will provide critical information on long-term response and adherence to highly active antiretroviral therapy in HIV-1 infected children in Kenya and sub-Saharan Africa in general. It will also provide information regarding the magnitude of antiretroviral drug resistance to the triple drug combination being introduced in Kenya and the effect of such resistance on effectiveness of therapy. Such information will be useful to clinical teams and treatment programs in helping to devise strategies to maintain high levels of adherence and also make decisions regarding choice of antiretroviral drugs in case of children failing first line drug regimens.

Inclusion of Women and Minorities
Since this is a study of HIV-1 infected children and their caregivers based in an international site, approximately 120 out of the 150 caregivers will be Kenyan women.

Inclusion of Children

This being a study on HIV-1 infected children; the primary study subject will be Kenyan children between the ages of 18 months and 13 years. The age upper age limits is adjusted upwards to accommodate children who have been followed up in the ongoing pediatric adherence study for approximately one year.

Appendix v: Clinical and Socio-demographics Data Extraction Form

Clinical and socio-demographic data collection from medical records of HIV-1 infected children in KNH

Key:

Variable	Variable Description
IDNum	Child Identification Number
AgeM0	Age of the Child
Sex	Gender of the child
WAZM0	Weight for Age Zscores at Baseline (Month 0)
WHZM0	Weight for Height Zscores at Baseline (Month 0)
HAZM0	Height for Age Zscores at Baseline (Month 0)
HAZM39	Height for Age Zscores at 3 years (Month 39)
WAZM39	Weight for Age Zscores at 3 years (Month 39)
WHZM39	Weight for Height Zscores at 3 years (Month 39)
Child Caregiver	The primary child caregiver/gurdian
Caregive Marital	The marital status of the child's care giver
Caregiveedeuc	The Education status of the child's care giver
TB	If the child had Tuberculosis (TB)
Pneu	If the child has Pneumonia (Pneu)
WHO Stage	WHO HIV disease classification stage
ART Given	HAART the child has been put on

Clinical and socio-demographic Data Collection Template

IDNum	Age	Sex	WAZ m0	WHZ m0	HAZ m0	HAZ M39	WAZ M39	WHZ M39	Child caregiver	Caregiv Marital	Caregive educ	TB	Pneu	WHO stage	ART Given
1															
12															
33															
65															
86															

Appendix vi: Laboratory Data Extraction Form

Laboratory data collection from medical records of HIV-1 infected children in KNH

Key:

Variable Code	Variable Definition
ID Number	Participant Identification Number
HBm0	Hemoglobin at Baseline (Month 0)
HBm39	Hemoglobin after 3 years (Month 39)
WBCm0	White Blood Cell Count at Baseline (Month 0)
WBCm39	White Blood Cell Count after 3 years (Month 39)
NEUTm0	Absolute Neutrophil Count at Baseline (Month 0)
NEUTm39	Absolute Neutrophil Count after 3 years (Month 39)
CD4m0	CD4 T Cell Count at Baseline (Month 0)
CD4m39	CD4 T Cell Count after 3 years (Month 39)
CD4Pm0	CD4 T Cell Percentage at Baseline (Month 0)
CD4Pm39	CD4 T Cell Percentage after 3 years (Month 39)
THRM0	Thrombocytes at Baseline (Month 0)
THRM39	Thrombocytes after 3 years (Month 39)
vloadm0	HIV-1 RNA Copies (Viral Load) at Baseline (Month 0)
vloadm39	HIV-1 RNA Copies (Viral Load) after 3 years (Month 39)
ALBM0	Serum Albumin at Baseline (Month 0)
ALBM39	Serum Albumin after 3 years (Month 39)

Laboratory Data Collection Template

IDNum	HB M0	HB M39	WBC M0	WBC M39	NEUT M0	NEUT M39	CD4 M0	CD4 M39	CD4P M0	CD4P M39	THR M0	THR M39	VLOAD M0	VLOAD M39	ALB M0	ALB M39
1																
27																
62																
86																

Appendix vii: Laboratory Protocol for Measurement of Hematological Profiles.

UNIVERSITY OF NAIROBI

PAEDIATRICS AND CHILD HEALTH DEPARTMENT LABORATORY

PROTOCOLS

HAEMATOLOGY **SOP Number 6.0, Version #2**

		Date Prepared
	Prepared by : Mercy Ngure **Signature :**	14.01.2010

		Date Approved
	Approved : **Head of Department**	24.01.2010
	Effective Date: 01.02.2010	

Background and Introduction

MS4 is a 3-part Haematology analyzer

Objective

To provide step-by-step instruction on how to run whole blood samples for the determination of Total blood count values on the MS4 3-part differential Haematology analyzer in a manner that ensures the obtained results are quality, accurate and reproducible

Responsibility

Staff Technologist assigned to run and operate the MS4 Haematology analyzer

Frequency of Testing

Whenever there are samples requiring the determination of the Total blood count values

Specimen Collection and Handling

Specimen should be whole blood samples collected in the standard K_3EDTA vacutainer or microtainer using the standard vein puncture procedure as described in the SOP # 9.0 for sample collection

Samples should be maintained at room temperature in the Lab preferably on a roller mixer until the time of analysis.

Sample Type

Whole blood (anticoagulated) collected in K_3EDTA (purple top or lavender) vacutainer or microtainer.

Equipment and Materials

- MS4 analyzer
- MS4 card
- Roller mixer
- MS4 diluent solution
- MS4 rinse solution
- MS4 lyse solution
- MS4 Detergent solution
- 10% bleach solution
- Latex gloves

Calibration

Calibration of the machine is done twice a year every time the machine is serviced by the service engineers.

Quality Control

A control samples MUST be run every morning before running any patient samples as an Internal Quality control measure. The obtained control values are then plotted using a spread sheet program to generate a Levy-Jennings curve which is evaluated on a monthly basis. Refer to the SOP # 2.0 for running Controls.

The control sample may also be re-run in the course of the day if a trend is noted on a parameter either repeatedly reading high/low values for a number of consecutive runs on different samples as a trouble shooting measure

The enrolled External Quality Control program for the MS4 analyzer is Human Quality Assessment Scheme (HuQAS) which has three events in a calendar year. On each of the events, 5 samples are analyzed same way as the patient samples. Both the obtained results and performance evaluation are exchanged on-line using the DigitalPT computer program. Refer to the SOP # 2.4 for running EQA samples.

Safety Precautions

- Safety precaution for universal blood precaution should be used
- Refer to MSDS on each particular reagent in the MSDS appendix
- Appropriate safety precaution should be utilized for the biohazard materials used here
- Personal protection equipment (PPE) including proper usage of gowns and gloves

Preparation of Reagents, Controls and Samples

- The MS4 reagent pack solutions to be used on the instrument are ready for use. Check the level of the reagents in the packs to ensure there is sufficient solution for the day's work.
- Each reagent pack has a marked level below which the reagent must be replaced with a new pack
- For the preparation of controls, refer to SOP # 2.0 for running controls
- Check patient samples to ensure that they are fresh (collection date and time), in the correct specimen bottle (K_3EDTA) and that they are free from clots. Samples failing to meet these criteria MUST be rejected

Procedure / Method

1. On receiving the K3 EDTA sample, check for its appearance to check for hemolysis, clots and whether the volume is enough for the run. (in case of any of this irregularity, indicate on the requisition form)
2. Place the sample on the roller mixer NB: The MS4 will be in its standby "READY" screen
3. Select the analysis mode on the MS4 by pressing the analysis icon on the MS4 screen (the "analysis process" screen with the word "ANALYSIS" highlighted comes on)
4. To select the appropriate bank for running the sample, press the arrow key **(↓)** (BANK is highlighted), followed by the arrow key **(→)** (the BANK selection is

highlighted) then use the (↑) or (↓) arrow keys to select the appropriate BANK (e.g. Male, Female, New born)

5. Press the Enter key on the keyboard or the Enter Icon on the MS4 screen three times, the "FILE SCREEN" comes on
6. Press the Enter key on the keyboard or the Enter Icon on the MS4 screen once to Type in the Patient Name under the "PATIENT" typing space on the screen
7. Press the Enter key on the keyboard or the Enter Icon on the MS4 screen once to type in project name and/or ID number as applicable under the "COMMENT" typing space
8. Press the Enter key on the keyboard or the Enter Icon on the MS4 screen once and the following message is displayed…."***Place whole blood sampling tube then validate***"….
9. Thoroughly mix the blood by gently inverting the tube 8-10 times
10. Remove the cap and place tube into the whole blood sampling tube holder then validate by pressing the Enter key on the key board or the Enter Icon on the MS4 screen (NB: analysis of sample initializes)
11. Wait until the end of the analysis before removing the tube from the holder
12. Results are automatically displayed once the counting is over. To visualize all the parameters, change screen using the Left (←)of Right (→) arrows
13. To print generated sample report, press the printing command Icon on the MS4 screen

Reference Ranges and Expected Results

Parameter	Sex	Range	Units
WBC	Newborn	3.5-11.0	m/mm^3
	Male	4-10	m/mm^3
	Female	4-10	m/mm^3
Lymphocytes count	Newborn		m/mm^3
	Male	1.5-4.0	m/mm^3
	Female	1.5-4.0	m/mm^3
Lymphocytes percent	Newborn	20-40	%
	Male	15-40	%
	Female	20-40	%
Monocytes count	Newborn		m/mm^3
	Male	0.2-0.8	m/mm^3
	Female	0.2-0.8	m/mm^3
Monocytes percentage	Newborn	3-10	%
	Male	3-10	%
	Female	3-10	%
Granulocytes count	Newborn		m/mm^3
	Male	2.0-7.5	m/mm^3
	Female	2.0-7.5	m/mm^3
Granulocytes percentage	Newborn	30-70	%
	Male	30-70	%
	Female	30-70	%
Red Blood Cells (RBC)	Newborn	4.0-5.5	m/mm^3
	Male	4.0-5.9	m/mm^3
	Female	3.8-6.0	m/mm^3
Mean Cell Volume (MCV)	Newborn	73-106	fL
	Male	83-98	fL
	Female	80-100	fL
Hematocrit/Packed Cell Volume (HCT/PCV)	Newborn	35-45	%
	Male	38-54	%
	Female	33-54	%
Mean Cell Haemogrobin (MCH)	Newborn	35.6-45.4	pg
	Male	25-33	pg
	Female	25-32	pg
Mean Cell Haemogrobin Concentration (MCHC)	Newborn	28-36	g/dL
	Male	28-40	g/dL
	Female	28-36	g/dL
Red cell Diameter Width (RDW)	Newborn	8-12	%
	Male	8-12	%
	Female	8-12	%
Haemogrobin (HB)	Newborn	11-18.5	g/dL
	Male	12-18.0	g/dL
	Female	10-16.5	g/dL
Thrombocytes/Platelets (THR/PLT)	Newborn	150-450	m/mm^3
	Male	150-450	m/mm^3
	Female	100-450	m/mm^3
Mean Platelet Volume (MPV)	Newborn	6-13	fL
	Male	6-13	fL
	Female	6-13	fL

Appendix viii: Protocol for Measurement of CD4 counts by flow cytometry

UNIVERSITY OF NAIROBI
PAEDIATRICS AND CHILD HEALTH DEPARTMENT LABORATORY PROTOCOLS

Flow Cytometry **SOP Number1.1, Version #2**

		Date Approved
	Approved: **Head of Department**	
	Effective Date: 01.02.2010	

Background and Introduction

Absolute CD4 and CD3 T-lymphocyte counts are used to evaluate the immune status of patients with or suspected of developing immune deficiencies such as acquired immunodeficiency syndrome. The CD4 T-lymphocyte is the cellular parameter most closely associated with HIV disease progression and patient prognosis.

Objective

To provide step-by-step instruction on how to run samples for the determination of lymphocytes subset values on the BD FACScount cytometer in a manner that ensures the obtained results are quality, accurate and reproducible

Principle

A single test requires one convenient ready to use reagent tube which determines the absolute numbers of helper/inducer T lymphocytes (CD4/CD3). When whole blood is added to the reagent, fluorochrome-labeled antibodies in the reagent bind specifically to lymphocyte surface antigens. After a fixative solution is added to the reagent tube, the sample is run on the instrument. Here, the cells come in contact with laser light, which causes the fluorochrome-labeled cells to fluoresce. The fluorescent light provides the information necessary for the instrument to count the cells. The reagent tubes also contain a known number of fluorochrome-integrated reference beads which function as a

fluorescence standard for locating the lymphocytes and also as a quantitation standard for enumerating the cells.

Responsibility

Staff Technologist assigned to run and operate the FACScount system

Frequency of Testing

Whenever there are samples requiring the determination of the Lymphocytes subset values

Specimen Collection and Handling

- Specimen should be whole blood samples collected in the standard K_3EDTA vacutainer or microtainer using the standard vein puncture procedure as described in the SOP # 6.0 for sample collection
- Samples should be maintained at room temperature until the time of analysis.
- Samples MUST be run on the same day of collection

Sample Type

Whole blood (anticoagulated) collected in K_3EDTA (purple top or lavender) vacutainer or microtainer.

Equipment and Materials

- BD FACScount system
- FACScount software protocol diskette
- Voltex Mixer
- BD FACSflow fluid
- Distilled water
- 10% bleach solution
- BD FACScount reagent kits. Keep at 2-8^{o}C in the dark until use; do not freeze.
- 50uL micropipettor and corresponding sterile disposable tips
- Latex Gloves

Quality Control

Before running any patient samples, the BD FACScount control beads MUST be run on the instrument as an Internal Quality control measure. The obtained control values are then plotted using a spread sheet program to generate a Levy-Jennings curve which is evaluated on a monthly basis. Refer to the SOP # 1.0 for running Controls

The enrolled External Quality Control program for the BD FACScount cytometer is Human Quality Assessment Scheme (HuQAS) which has three events in a calendar year. On each of the events, 2 samples are analyzed same way as the patient samples. Both the obtained results and performance evaluation are exchanged on-line using the DigitalPT computer program. Refer to the SOP # 1.4 for running EQA samples.

Safety Precautions

- Safety precaution for universal blood precaution should be used. Dispose waste reservoir content, used reagent tubes, pipette tips, gloves and other biohazardous materials accordingly
- Refer to MSDS on each particular reagent in the MSDS appendix
- Appropriate safety precaution should be utilized for the biohazard materials and the formaldehyde solution used here
- Personal protection equipment (PPE) including proper usage of seamless gowns and gloves
- For protection against electrical shock, equipment should be connected to an approved power source
- Because the FACScount laser is fully contained within the instrument structure, there are no special work area safety requirements
- Keep hands clear of the sample holder during operation, except when a screen message prompts you to change tubes

Waste Generation, Handling and Disposal

- Follow all relevant Laboratory procedures in disposing waste generated by this method
- All liquid waste from assay is collected in a labeled waste container, which is mixed with 10% bleach. Waste bottles are then emptied out in a designated Lab "dirty" sink
- All used test tubes are discarded in designated yellow lined biohazard waste container for disposal
- All used pipettor tips are pre-soaked in a designated10% bleach container and then discarded in designated yellow lined biohazard waste container for disposal

Preparation of Reagents, Controls and Samples

- The BD FACScount reagent tubes to be used on the instrument are ready for use
- For the preparation of controls, refer to SOP # 1.0 for running controls
- Check patient samples to ensure that they are fresh (collection date and time), in the correct specimen bottle (K_3EDTA) and that they are free from clots. Samples failing to meet these criteria MUST be rejected
- Always run control beads before running any patient sample. Once the controls have passed, proceed with patient samples as follows;

1. Label the tab of the reagent tube with the patient accession number or number that identifies the tube of blood. You will require a reagent tube for each sample to be tested
2. Vortex the tube upside down for 5 seconds,
3. Vortex the tube Upright for 5 seconds,
4. Open the reagent tube with the coring station.
5. Mix the patient whole blood by inverting the tube five times.

6. Pipette 50 microlitre of the whole blood into the reagent tube. Change tip between tubes.
7. Cap the tubes and vortex upright for 5 seconds.
8. Incubate the tubes for 60 to 120 minutes at room temperature, place the reagent tubes in the workstation and close the cover to protect the reagents from light.
9. After the incubation time, uncap the tubes and pipette 50 microlitre of fixative solution into each reagent tube. Discard the caps and tips to soak in the appropriate 10% bleach container.
10. Recap the reagent tubes with new caps and vortex upright for 5 seconds
11. Run the tubes on the BD FACScount instrument within 48 hours of preparation. Store samples at room temperature in the workstation until they are run on the instrument. Vortex upright for 5 seconds immediately before running.

Procedure / Method

- You must enter an accession number for each sample before running the sample on the FACScount instrument

1. From the FACScount screen or CONTROL result screen, press [SAMPLE]. This displays the REAGENTS screen
2. Verify reagent lot code and reference bead counts and press [Confirm]. The SAMPLE screen is displayed
3. Type in the patient accession number (lab number identifying the blood sample) in the appropriate field (up to 15 characters). If the accession number from the previous run exists, press [Clr Acc#] to remove it. The instrument prompts you to move the CD4 tube in position
4. Vortex the reagent tube upright for 5 seconds
5. Uncap the CD4 tube and place in the sample holder then press [RUN]. The sample holder raises and analysis begins. When analysis is complete, the sample holder lowers.
6. Remove the reagent tube and recap. The patient results are displayed and printed automatically
7. To run the next sample, press [SAMPLE], enter patient accession number and follow step 3 through 6 as above.
8. To return to the FACScount screen, press [MAIN]

Analysis / Interpretation of Results

Reference Ranges and Expected Results

Parameter	Sex	Age	Range cells/uL
CD4+ Lymphocytes	Male	18 – 65	355 – 1213
	Female		470 – 1298
CD3+ T Lymphocytes	Both	18 – 65	688 – 1955

Limitations of Method

- The pipette used in the sample preparation procedure must be properly calibrated to ensure it is dispensing exactly 50 uL of blood
- Do not store blood longer than 48 hours before preparing
- Do not store whole blood on a blood rocker or other mixing device
- Do not refrigerate whole blood before preparing
- Store prepared samples at room temperature in the dark and run within 48 hours of preparation
- The reagents used in this test system are light sensitive. Minimize exposing the reagent tubes to light

Storage and Retrieval

- Two copies of the patient result are printed. One copy goes to the original patient's requisition form while the second copy goes to a duplicate patient requisition form for filing
- Filing is done in order of date and there is a separate file for each month

References

1. BD Biosciences Instrument User's guide
2. Schmidt RE. Monocolonal antibodies for diagnosis of immunodeficiencies. Blut. 1989;59:200-206
3. Giorgi JV, Hultin LE. Lymphocytes subset alterations and immunophenotyping by flow cytometry in HIV disease. Clin immunol newslett. 1990;10:55-61