Establishing Reference Intervals for $CD4^+$ and $CD8^+$ T Lymphocyte Subsets in HIV Negative Adults in Nairobi, Kenya 2008.

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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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DEDICATION

I dedicate this thesis to my dear wife Grace and our daughter Imani for their love, endless support and patience. May God bless the work of our hands.

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ABBREVIATIONS AND ACCRONYMS

AIDS	Acquired Immuno Deficiency Syndrome
ADE	AIDS Defining Events
AFMH	Armed Forces Memorial Hospital
ARV	Antiretroviral drugs
ART	Antiretroviral Therapy
CCR5	Cysteine-Cysteine linked Chemokine Receptor 5
CD	Cluster of Differentiation
CD4	Cluster of Differentiation antigen number 4
$CD4^+$	CD4 positive effector T lymphocytes
$CD8^+$	CD8 positive suppressor T lymphocytes
CXCR4	Cystine-Xylose linked Chemokine Receptor 4
DNA	Deoxyribonucleic Acid
FACS	Flourescent Assorted Cell Sorter
Gp120	Glycoprotein of 120 Daltons
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency Virus
KAIS	Kenya AIDS Indicator Survey
KDHS	Kenya Demographic Health Survey.
NBTS	National Blood Transfusion Services
NHRL	National HIV Reference Laboratory
Nef	Negative Factor gene
PCR	Polymerase Chain Reaction

RNA	Ribonucleic Acid
SPSS	Statistical Package for Social Sciences
TLC	Total Lymphocyte Count

ABSTRACT

The Human Immunodeficiency Virus infections have grown to pandemic proportions with morbidity of more than 65 million people and mortality of over 25 million people. The health of infected individuals deteriorates rapidly to the AIDS stage and consequent fast death when not treated. The advent of anti retroviral treatment has brought hope for living longer lives for those who are infected and living with the virus. In Kenya, the decision on when to start therapy is largely based on the baseline $CD4^+$ and $CD8^+$ T-cell count whose reference intervals are of a Caucasian population. Information on $CD4^+$ and $CD8^+$ T-cell count reference values for the Kenyan population is seldom available.

A cross-sectional study was carried out to establish reference intervals for $CD4^+$ and $CD8^+$ T -cell subsets for select reference blood donors as a local guideline to reference intervals. The study assumed there was no significant difference between the Kenyan population living in Nairobi and Western countries Caucasian $CD4^+$ and $CD8^+$ T-cell counts. These reference intervals could be used to influence change in policy on when and how to treat people infected with HIV among Nairobi residents. This can possibly be replicated to the whole country after further studies for different regions in order to have a nationwide reference interval guideline.

A total of 424 reference blood donors were recruited as study subjects after passing a donor recruiting interview. After preliminary testing 17 (4.5%) blood donor samples were excluded due to positive serological tests and a further 7(1.7%) were eliminated due to extreme values. Thus a total of 400 (215 (54%)

males, 185 (46%) females) were considered. The $CD4^+$ and $CD8^+$ T-cell count values obtained from the study were subjected to statistical treatment and reference intervals were determined using the mean±2SD based on a normal distribution. Reference intervals obtained included $CD4^+$:$CD8^+$ 1.8 (0.7-4), $CD4^+$ % 40 (27 – 53), $CD4^+$ absolute count 790 (392 – 1,405) cells/µL, and $CD8^+$ absolute count 500 (202 – 1,131) cells/µL. The $CD8^+$ % data did not attain a Gaussian distribution and the reference interval was determined as the median and the 2.5^{th} and 97.5^{th} interval at 95% CI i.e. 26% (15 – 50). Overall, Females had a higher mean ratio, $CD4^+$ % and absolute count than males and was statistically significant ($P<0.0001$) whereas males had a higher $CD8^+$ % than females that was statistically significant ($P<0.001$). In both sexes, the $CD4^+$ absolute mean counts were always higher than the $CD8^+$ absolute mean counts irrespective of age. There was no significant difference between the age groups for all the T-cell subsets. Reference mean $CD4^+$ values obtained in this study were much lower than those of Burkina Faso but compared well with intervals from Tanzania and current BD multiSET values from a Caucasian population used in the FACsCalibur machine in Kenya. The reference intervals obtained in this study though different, they are relatively comparable to the USA based reference intervals and can be used to compliment these values. Country wide reference intervals would add value to decision making about HIV/AIDS therapy especially where differences in geographical conditions exist.

CHAPTER 1

1.0 INTRODUCTION

The Human immunodeficiency virus (HIV) is transmitted primarily through unprotected sex and/or unsafe sexual practices, leading to a condition known as Acquired Immuno-Deficiency Syndrome (AIDS). The virus can also be transmitted through sharing of contaminated needles among drug users, and contact with infected blood, blood products, or other body fluids. Since 1981 when the first AIDS case was reported (Barré-Sinoussi *et al.*,1983**)**, HIV infections have increased to pandemic proportions with morbidity of more than 65 million people and mortality of over 25 million worldwide by the end of 2008 (WHO, 2007). Recently the annual number of AIDS deaths has however declined from 2.2 million in 2005 to 2.0 million in 2007, in part, due to increased access to anti-retroviral treatment (UNAIDS, 2008). In addition, there have been changes in sexual behavior that have contributed to declines in the number of new HIV infections. The number of annual new HIV infections declined from 3.0 million in 2001 to 2.7 million in 2007 (UNAIDS, 2008). An estimated 370,000 children younger than 15 years became infected with HIV in 2007 and those living with HIV increased from 1.6 million in 2001 to 2.0 million in 2007 in the same age group (UNAIDS 2008). The increasing number of deaths in children due to AIDS threatens to reverse many of the recent gains of child world health organization survival programmes (WHO, 2007).

Sub-Saharan Africa continues to be greatly affected by the HIV pandemic despite increased measures targeting prevention and control. By the end of 2007, an estimated 1.9 million people were newly infected with HIV, bringing to about 22 million the

number of people living with HIV. This represented two thirds of the global number of HIV-infected cases, which resulted in approximately 75% of all AIDS-associated deaths occurring in the region (UNAIDS, 2008). Women account for half of all people living with HIV worldwide, and nearly 60% of HIV infections in sub-Saharan Africa. An estimated 2.3 million children were living with HIV by 2006, and orphaned nearly 12 million children in sub-Saharan Africa (UNAIDS, 2008).

The Kenya AIDS Indicator Survey (KAIS) of 2007 reported an HIV prevalence of 7.4% (1.4 million cases) in the country, almost all among adults aged 15-64 years (KAIS, 2007). Whereas a study conducted in 2003 had reported a prevalence of 6.7% among 15-49 year olds (Kenya Demographic Health Survey, 2003), the KAIS (2007) study reported 7.8% in that age bracket. The increase in prevalence between 2003 and 2007 was attributed to several factors, including increased survival rate of AIDS patients due to accessibility of ARV drugs. The KAIS (2007) survey also included 50-64 years age group previously assumed as low risk whose prevalence was greater than among the youngest Kenyans. This however may have reflected a cumulative lifetime exposure to HIV (National AIDS and STI Control Programme, 2008). The survey also noted a significant increase in HIV infections among rural men (58%) from 2003-2007 and may contribute to the overall increase in prevalence. The KAIS prevalence rates were however much lower from previous rates of 10% nationally in the late 1990's (National AIDS and STI Control Programme**,** 2008). According to KAIS (2007), more women (8.7 %) aged 15-64 years than men (5.6%) were infected with HIV. Nyanza province exhibited the highest HIV prevalence of 15.3% among the eight provinces in Kenya and was twice the national level. Other provinces included Nairobi (9.0%), Coast (7.9%), and Rift Valley (7.0%). Prevalence in

Eastern was 4.7% and 3.8% in Central province of the adult population infected. North Eastern province had the lowest adult HIV prevalence at 1% (National AIDS and STI Control Programme, 2008). Approximately 55,000 new adult infections occurred with an estimated 85,000 deaths in Kenya by the end of 2006. The advent of antiretroviral (ARV) treatment brought with it new hope by allowing infected individuals to have longer, more productive and comparatively healthy lives therefore averting over 57,000 deaths since 2001. About 430,000 adults are in need of ARV drugs and 23,000 children born of HIV infected mothers while another 200,000 children need Cotrimoxazole prophylaxis (National AIDS and STI Control Programme, 2007).

Monitoring the $CD4^+$ and $CD8^+$ levels in HIV-infected individuals is important as an indicator for the initiation of ARV treatment, and for monitoring performance for AIDS cases on ARVs. Unfortunately, Kenya and most of Africa has never developed reference intervals of the $CD4^+$ and $CD8^+$ T-cells, instead using values developed in the Western nations. This lack of reference intervals for T-cell subsets has complicated interpretation of test results for HIV cases, making it hard to define normal individual variations known to be closely linked with genetic diversity, health and nutritional status of a population.

Current technology in Kenya for the enumeration of $CD4^+$ and $CD8^+$ T-cells is done by flow cytometry immunophenotyping that simultaneously measures and analyzes multiple physical and chemical characteristics of single cells or other biological particles, as they flow in a fluid stream past optical and electronic sensors (WHO, 2007). This technology has become a routine and indispensable procedure in the evaluation and prognosis of HIV infected individuals towards initiation and follow-

up on Anti-retroviral treatment. The FACSCount and FACsCalibur (Becton Dickinson Immunocytometry systems, San Jose, CA, USA) machines are among the available flow cytometry equipments currently used in government hospitals. The FACsCalibur is the more advanced of the two utilizing the single-platform technology and CD45 PanLeucogating technique as recommended by Centres for Disease Control and prevention guidelines for the determination of absolute $CD4^+$ T-cell counts thus providing both $CD4^+$ and $CD8^+$ T-cell absolute and percentage values(CDC, 2007). A combination of four monoclonal antibodies i.e. anti-CD3/CD8/CD45 and CD4 are used with different colors in TruCOUNT tubes to rapidly measure multiple lymphocyte subsets in a single tube (Riemann *et al.*, 2000). Comparative reference values for $CD4^+$ and $CD8^+$ T-cell absolute count, percentage and ratio are provided by Becton Dickinson Immunocytometry systems after conducting studies on reference Caucasian adults (BD San Jose, CA, USA, 1991). However, BD recommends that laboratories to establish their own reference values for the MultiTEST CD3/CD8/CD45/CD4 reagent parameters that can be affected by sex, age of patient and preparative technique (BD San Jose, CA, USA, 1991). Race of patient (Prince *et al.*, 1985) and individual variations of epitope expression (Angadi *et al.*, 1990) can also have an effect.

Kenya is among countries that have adopted the World Health Organization (2002) guidelines which recommend anti-retroviral treatment for patients with World Health Organization stage IV disease or a $CD4^+$ T-cell count of less than 200 cells/µl (WHO, 2002). Revised 2003 guidelines stated that in patients with World Health Organization stage III disease, treatment could be considered when the $CD4^+$ count is

below 350 cells/µl and initiated before the $CD4^+$count drops to below 200 cells/µl (WHO, 2004).

A possible difficulty to the application of the World Health Organization guidelines and usage of foreign reference values is the fact that certain ethnic groups, such as the Chinese (Kam *et al.*, 1996) and Ethiopians (Tsegaye *et al.*, 1999) have lower $CD4^+$ T-cell counts than Caucasians and may acquire opportunistic complications at correspondingly lower $CD4^+$ counts (Wong *et al.*, 1998). Information on $CD4^+$ T-cell counts and $CD4^+$ treatment threshold for the Kenyan population is hardly available from the local population and clinicians have to work with such recommended values. This however, has not stopped them from making substantial progress to providing anti-retroviral treatment to HIV patients especially since a $CD4^+$ count of $\leq$200 cells/µL still remains applicable. Establishing a minimum threshold level at which anti-retroviral treatment should be initiated urgently for defined patient populations would however still be useful. This minimum threshold value may vary from population to population and would need to be re-determined for each individual service or cohort (Ho, 2007).

1.1 Problem statement

There is lack of adequate information on reference values for $CD4^+$ and $CD8^+$ T–cell subset populations in Kenya. Reference intervals allow interpretation of laboratory results by comparing them to a set of reference data that enables the health practitioner to make a decision depending on the diagnosis. Therefore, local $CD4^+$ and $CD8^+$ T–cell reference intervals would serve as a point of comparison of local data of the Kenyan population. Such values would reflect the diversity of Kenyan

population in terms of geographical distribution and ethnic backgrounds especially in subsequent nationwide studies. The fact that there are differences for $CD4^+$ and $CD8^+$ T–cell counts between and among different racial groups around the world is well established. Based on these variations in reference intervals for lymphocytes in different populations, the real issue would be to find out whether local $CD4^+$ and $CD8^+$ T-cell reference intervals are significantly different or comparable to values from Europe and in North America that are used to guide therapy for the Kenyan population. The USA based company (BD San Jose, CA, USA, 1991) however acknowledges these variations and recommends that each laboratory or health institutions develop their own reference intervals. It is from the foregoing that no individual or collective effort has been made to establish and publish such local reference intervals. This study is intended to make this attempt and fill the existing knowledge gap.

1.2 Justification

Previous studies in some African and Asian countries have shown that significantly lower or higher $CD4^+$ and $CD8^+$ T-cell values exist from those of the Caucasian population. Adult Ethiopians had significantly lower $CD4^+$ and $CD8^+$ T-cell counts than Dutch Caucasians (Tsegaye *et al.*, 1999). Values from an Asian population showed significant interracial variability in which Indians had a higher $CD3^+$, $CD4^+$ and $CD19^+$ counts than the Chinese or Malays (Wee *et al.*, 2004). Due to the lack of such information from the Kenyan population it would be difficult to know whether their values are significantly variable or comparable to those used for comparison from foreign populations. Studies of absolute $CD4^+$ T-ell counts in French and West

African HIV-infected adults suggested that where thresholds of 200 and 500 cells/µl for $CD4^+$ are applied in Europe, thresholds of approximately 250 and 700 cells/µl $CD4^+$count may be more suitable in West Africa and would give a more precise guideline in administering anti-retroviral treatment (Tsegaye *et al.*, 1999). Lugada *et al* in Uganda concluded that higher T-cell counts existed from the western population based reference values (Lugada *et al.*, 2004). A mean $CD4^+$ absolute count of 1,256cells/µl from healthy Ugandans was obtained by Tugume *et al,* and was a much higher count than most Caucasian reference intervals (Tugume *et al.*, 1994). These values are the highest estimate in the East African region. As stated before, these variations could be attributed to several factors including ethnic or environmental etiology and /or differences in study designs and laboratory performance in the different studies.

Gitura *et al* in Kenya, showed a positive correlation of total lymphocyte count (TLC) cut-off value of 1,900cells/mm^3 to a $CD4^+$ T-cell count of <200 cells/µl as a guide to initiation of ARV treatment but was significantly higher than the World Health Organization cut-off value of total lymphocyte count of 1,200 cells/µl. (Gitura *et al.*, 2007). He concluded that such differences in total lymphocyte count results could lead in the underestimation of advanced stage of disease and to withholding anti-retroviral treatment to persons who need it. This study by Gitura *et al,* (2007), however deals with HIV positive individuals and a subsequent correlation of total lymphocyte count and $CD4^+$ cells cut off values rather than reference intervals. However, my study deals with HIV negative individuals and subsequent direct enumeration of $CD4^+$ and $CD8^+$ T-cells by flow cytometry technology to establish their reference intervals. Such enumeration of these $CD4^+$ and $CD8^+$ T-cells allows a

more accurate inference on patient results and especially when compared to locally established reference intervals.

From the fore going, it would then be essential to determine local reference intervals on the Kenyan population and in this case from blood donors within Nairobi. Establishing local reference intervals of $CD4^+$ and $CD8^+$ T-cells is also in line with international recommendations that suggest developing reference intervals from the local population.

1.2 Research questions

- What are the $CD4^+$ and $CD8^+$ sample estimates and reference intervals derived from blood donors in Nairobi?
- Would the local $CD4^+$ and $CD8^+$ reference intervals established in this study be different from the USA based reference intervals?

1.3 Hypothesis

This being a cross-sectional study, the hypothesis was not testable.

1.3.1 Null hypothesis

There is no significant difference between Kenyan and Western countries adult $CD4^+$ and $CD8^+$ T-cell reference intervals.

1.3.2 Alternative hypothesis

There is a significant difference between Kenyan and Western countries adult $CD4^+$ and $CD8^+$ T-cell reference intervals.

1.4 Objectives

1.4.1 General objective

To establish reference intervals for $CD4^+$ and $CD8^+$ T-cells among HIV-negative adults (16-64 yrs) in Nairobi, Kenya.

1.4.2 Specific objectives

- To determine reference intervals for $CD4^+$ and $CD8^+$ T-cells, from a select population of adult (16 – 64 yrs) HIV-negative blood donors in Nairobi region.
- To determine the effect of sex and age on $CD4^+$ and $CD8^+$ T-cells values among a select population of adult (16-64 yrs) HIV-negative blood donors in Nairobi, Kenya.
- To compare reference intervals for $CD4^+$ and $CD8^+$ T-cells intervals for Nairobi with those of other countries in Africa and the Western nations.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 The CD4 structure and function

The CD4 molecule is a 58 kilo Dalton monomeric glycoprotein that can be detected on the cell surface of about 60 % of circulating T-cells, on T-cell precursors within the bone marrow, thymus, and on monocytes, macrophages, eosinophils, dendritic cells and microglial cells of the central nervous system (Andrea *et al.,* 2007). The CD4 glycoprotein consists of 428 amino acids that are distributed into 3 major segments of 372 amino acids in the extracellular segment, 23 amino acids in the transmembrane segment, and 33 amino acids in the cytoplasmic segment (Wu *et al.*, 1996). Within the extracellular part of CD4, four regions have been characterized that represent immunoglobulin-like domains. These domains are categorized by their relation to the CD4 membrane in such a manner that there is a two domain segment called the membrane distal fragment which is domain 1, and domain 2, as well as two other domain segments referred to as the membrane proximal fragment (Brady *et al.,* 1993). Domain 1 is of significant interest since the binding site for HIV glycoprotein120 (gp120) resides within its amino terminal (Moebius *et al.,* 1992). This domain is homologous to the variable domain of antibodies that exhibit the structure of two Beta sheets four or five strands long (Wu *et al.*, 1996). Domains 1 and 3 structures are consistent with the variable domain of the antibodies, whereas the structures of domains 2 and 4 are consistent with the constant. The variable domain is often accompanied by a joining region and is classified as the V-J region

where CD4 binds to major histocompatibility complex (MHC) class II molecules (Wu *et al.*, 1996). Under a normal virus free environment these termini are used by CD4 for antigen recognition when associated with extracellular MHC class II. This occurs in antigen-presenting cells and its close association with the T-cell receptor and through intracellular signaling mediated by its association with the lymphocyte tyrosine kinase $p56^{lck}$ (Turner *et al.*, 1990). The structure of the antigens consists of a V-J domain region that is homologous to the MHC class II complex thus allowing for its recognition by CD4 (Wu *et al.*, 1996). The bonding between CD4 and gp120 occurs at the domain 1 terminus that has a V-J domain structure. Structurally, gp120 is variable but it is however recognized by the CD4 protein and an electrostatic bond occurs between the molecules. The most important aspect of this bond is the structure of the CD4 protein. Past studies showed that when the shape of the domain 1 was changed, the rate of the CD4 molecule binding to HIV-gp120 changed between 50 to 200 fold (Wu *et al.*, 1996). This indicates that the structure of the CD4 protein is an important characteristic in its ability to bind with gp120. Mutations in the structure of the CD4 protein have had a protective function to individuals against HIV infection, even after repeated exposure to HIV (Wu *et al.*, 1996).

2.2 $CD4^+$ T-cell count as a surrogate marker

The levels of $CD4^+$ T-cells in blood circulation serve as a prognostic indicator in HIV infection because they correlate with the occurrence of the spectrum of opportunistic complications (Hanson *et al.*, 1995). The levels are used to determine the degree of immune deficiency in HIV-infected persons, and to monitor progress including follow up of patients on ARV treatment. To correlate accurately $CD4^+$

values with the degree of immune deficiency in HIV-positive patients in a particular region of the world, knowledge of health-associated reference intervals of T-cell subset from healthy individuals in the same environment is essential. Developing such reference intervals requires sampling carefully selected individuals, putting into account demographic variables, documentation of general health status, and ensuring absence of disease of interest. (Solberg and Gräsbeck, 1989). Thus, these health-associated reference values are results obtained from a group of individuals corresponding to a stated description of health, (Solberg, 1987). Further staging of HIV disease, its progression (Stein *et al.*, 1992), response to anti-retroviral therapy (ART) (Goldman *et al.*, 1996) and making decisions about initiation of treatment for opportunistic infections (Aboulker *et al.*, 1992) are all based on $CD4^+$ T-cell counts. Over 80% of patients with AIDS-defining illness such as HIV encephalopathy and *Pneumocystis carinii* pneumonia occur at a $CD4^+$ T-cell count <200cells/µl while other conditions such as cerebral toxoplasmosis manifest at a $CD4^+$ T-cell count below 50-100cells/µl (Hanson *et al.*,1995).

2.3 The $CD4^+$ T-cell absolute count and percentage

The key characteristic of the immunodeficiency induced by HIV is a profound selective loss of $CD4^+$ T-cells (Fahey *et al.*, 1990). It is from this characteristic that the ability of the $CD4^+$ T-cell counts to predict the future risk of progression to AIDS and death was established (Phair *et al.*, 1990). Further work showed that the $CD4^+$ T-cell count was one of the strongest predictors of clinical disease in HIV infected individuals and led to its use for clinical monitoring of HIV infection (Phair *et al.*, 1990). The $CD4^+$ absolute count is further used in antiretroviral drug

trials eligibility and as an endpoint measure of drug efficacy (CDC, 1989). Treatment guidelines regarding initiation of ART have generally relied on absolute $CD4^+$ T-cell count. The absolute $CD4^+$ T-cell count is obtained from both the total and differential white blood cell count. However, laboratory test error and other factors may contribute to variability in $CD4^+$ measurement leading to adverse clinical consequences especially in prophylaxis decisions which rely on a single $CD4^+$ T-cell count (Mee *et al.*, 1997). Some studies have shown that patients with a true $CD4^+$ T-cell count far above a given $CD4^+$ threshold may test below it with a high probability as a result of $CD4^+$ measurement error and both tests are below the threshold limit (Hoover *et al.*, 1992). Absolute $CD4^+$ counts have been favored in many investigations as it also takes into account lymphopenia which is thought to be part of disease progression in HIV-I infections. On the other hand, the $CD4^+$ percentage (%) which is measured directly is considered to be less variable and stable by exhibiting a coefficient of variation of 6-24% compared with 19-40% for the absolute $CD4^+$ T-cell count (Giorgi *et al.*, 1990). Several studies have concluded that $CD4^+$ % is a better predictor of clinical progress based on its rate of change rather than the absolute $CD4^+$ T-cell count (Taylor *et al.*, 1989). The $CD4^+$ % is also considered less costly and thus affordable to patients (Mee *et al.*, 1997).

Each of the two measures has been justified in several studies as the ideal predictor for development of opportunistic illness and death due to AIDS. For short term prediction of development of opportunistic infections within 90 days Gebo *et al* (2004) found that with an absolute $CD4^+$ $\geq$350 cells/µl, $CD4^+$ % did not provide

more prognostic information than did the absolute count regardless of receipt of HAART.

Though different values occur for correspondence between the two measurements in different studies, $CD4^+$ absolute and $CD4^+$ % values should be considered when decisions are made regarding which patients may benefit most from earlier initiation of therapy. Studies by Masur *et al* (1989), reported that a $CD4^+$ % of 10%, 20%, and 30% corresponded broadly with a $CD4^+$ T-cell count of 100, 200, and 300 cells/µl respectively, for the purpose of clinical prediction. Moore *et al* (2006) showed that $CD4^+$ % was not a better predictor in patient mortality than absolute $CD4^+$ T-cell count in those who started HAART. However, a $CD4^+$ % <15 was predictive of death in patients who were AIDS free and had previously initiated HAART at a $CD4^+$ absolute count of 200-350 cells/µl of peripheral blood Todd *et al* (2006) predicted that an absolute $CD4^+$ T-cell count of 240 cells/µl and a $CD4^+$ % of 9, the probability of an AIDS defining event (ADE) or death was 65% greater than that for a person with the same absolute $CD4^+$ count and a $CD4^+$ % of 24 independent of prior HAART exposure**.** Such inferences from the foregoing are made in comparison with a baseline reference interval for $CD4^+$ absolute count and $CD4^+$ % from a presumed reference population.

The present study intends to establish reference intervals for $CD4^+$ % and $CD4^+$ absolute counts from a local reference population which can be used in other studies as a basis of comparison and allow inferences on the predictive value of $CD4^+$% and $CD4^+$ absolute counts on the Kenyan population.

2.4 $CD4^+$ T-cell count variations

The reference interval is described as the interval between, and including, two reference limits i.e. the lower and upper by the clinical laboratory standards institute (NCCLS, 2000). Reference intervals form an important basis of comparison for patient observed values to determine their health status. This is according to their values being within or out of range of the reference interval of a particular parameter of health and in this case $CD4^+$ T-cell values in relation to HIV/AIDS.

Recent studies have revealed important differences in T-cell reference intervals between different populations in Asia (Wee *et al.,* 2004), Europe (Jentsch-Ullrich *et al.,* 2005), and Africa (Kassu, 2001, Menard, 2003, Tsegaye, 1999 and Urassa, 2003). They have revealed significant $CD4^+$ T-cell and $CD8^+$ T-cell count variations with regard to race, geographical location, gender, circadian changes, and physical exercise (Uppal *et al.,* 2003). The clinical implication of wide variations in $CD4^+$ T-cell intervals is that the threshold for therapy may have to be established separately for different populations and with such would not undermine clinical decisions with regard to therapy to the local population (Wee *et al.,* 2004).

Based on the differences in reference intervals for the different populations, there are concerns whether $CD4^+$ T-cell count thresholds used to guide therapy in Europe and in North America are appropriate for the African and Kenyan population in particular. Studies conducted in West Africa showed that a given percentage of $CD4^+$ T-cell but not $CD8^+$ T-cells, tended to be about one-third higher than in French adults living in France. Approximate equivalencies of absolute $CD4^+$ T-cell counts in HIV-infected adults living in France and West African countries suggest that where thresholds of 200 and 500 $CD4^+$ cells/µl are applied in Europe, thresholds of approximately 250

and 700 $CD4^+$ cells/µl may be more suitable in West Africa and would give a more precise guideline in administering ARV treatment (Tsegaye *et al.*, 1999). In Burkina Faso, healthy residents living in Nouna had the highest absolute $CD4^+$ T-cell counts with an upper limit of 1,082 cells/µl that has ever been published in the West African and Asian populations (Klose *et al.*, 2007). This supports the hypothesis that specific genetic factors do influence $CD4^+$ T-cell counts (Ahmadi, 2001, Hall *et al.*, 2002). However, such results could have been influenced by differences in study designs and laboratory performance in the different studies. Further interracial studies suggest significant differences exist from the Caucasian reference values that have been used on most Asian and African countries. The Asian population showed significant interracial variability in which Indians had a higher $CD4^+$ counts than the Chinese or Malays (Wee *et al.*, 2004).

Studies in Uganda concluded that higher T-cell counts existed from western population based reference intervals (Lugada *et al.*, 2004). This further suggested an ethnic or environmental etiology. A study in Kenya, showed a positive correlation of total lymphocyte count (TLC) cut-off value of 1,900 cells/µl to a $CD4^+$ T-cell count of <200 cells/µl as a guide to initiation of ARV treatment. This contrasts significantly with WHO cut-off value of TLC of 1,200 cells/µl equivalent to a $CD4^+$ T-cell count of <200cells/µl (Gitura *et al.*, 2007). Such a difference in TLC values could lead in the underestimation of advanced stage of disease and to withholding ARV treatment to persons who need it and thus could be having a devastating effect on them. In this case, it strongly suggests that the Kenyan population could be having a different T-cell count reference interval than those currently used.

The impact of age on T-cell subsets count is not well established with different studies reporting varying results. The $CD4^+$ T-cell count was seen to increase with age while the $CD8^+$ T-cell count decreased (Denny, 1992, Tollerud, 1990) while another study concluded that both $CD4^+$ and $CD8^+$ T-cell counts increased with age (Wiener *et al.*, 1990). A study in Kenya in 2001 recorded a decline of $CD4^+$ and $CD8^+$ T-cell counts with age until the age of ten years, which was the oldest age, among the children who participated in the study. It was also observed that at this age children did not attain the cell count found in adults (Embree *et al.*, 2001). A comparable study in neighboring Uganda observed a decline in $CD4^+$ and $CD8^+$ T-ell count throughout childhood until the age of 18 years (Lugada *et al.*, 2004). Klose *et al.* (2007) found no statistical significant influence of age on the distribution of lymphocyte subsets in adults in a Burkina Faso. This inconsistency seems to arise from studying subjects of different age ranges among children and adults. One probable way of solving this would be to include subjects of a wide range of ages with each age well represented (Wee *et al.*, 2004).

Sex specific differences in healthy adults on T-cell subset counts have been widely consistent in many studies. Males had significantly lower $CD4^+$ T-cell counts than females with a mean difference of 140 cells/µl (Klose *et al.*, 2007). Similar significant differences between males and females were reported in Singapore and Tanzania (Bisset *et al.*, 2004 and Urassa *et al.*, 2003). However, Wee *et al.* (2004) did not find any significant differences between the sexes and differences between sexes may be secondary to the differential influences of sex hormones (McMurry *et al.*, 1997). A probable mechanism for this outcome could be the effect of binding to cell receptors for the sex steroid present on T-cells (Stimson *et al.*, 1998).

Anti-retroviral (ARV) treatment has been shown to suppress circulating levels of HIV ribonucleic acid (RNA) leading to a $CD4^+$ T-cell rebound (Gross *et al.*,2001) and in turn dramatic reductions in HIV-related morbidity and mortality (Garcia *et al.*, 2004; Phillips *et al.*, 2001). The impressive effects of antiretroviral treatment led to a hit early/hit hard approach with guidelines stating an initiation of treatment when the $CD4^+$ T-cell count decreased to less than 500 cells/µl (Hammer *et al.*, 1995, Ho *et al.*, 1995). However, the long term side effects of these drugs are substantial and have led to a paradigm shift away from the hit early/hit hard approach. Currently, it is recommended to delay the initiation until the $CD4^+$ T-cell count falls to or less than 350 cells/µl but before the count reaches 200 cells/µl (d'Arminio *et al.*, 2000).

2.5 $CD8^+$ T-cell structure, activation and function

Most T-cell progenitors migrate to the thymus during embryonic and early postnatal life where the thymus micro-environment is necessary for early differentiation where they become functionally competent, and are then exported into the peripheral lymphoid tissues and circulation (Owen *et al.*, 1969). Once outside the thymus, the $CD4^+$ and $CD8^+$ T-cells represent the circulating T-helper and T-suppressor populations respectively (Reinherz *et al*., 1980). The $CD4^+$ antigen is predominantly expressed on 55-70% of peripheral T-cells, while the $CD8^+$ antigen is expressed on 25-40% of circulating T-cells.

The $CD8^+$ molecule is a transmembrane glycoprotein that serves as a co-receptor for the T-cell receptor on cytotoxic T lymphocytes (CTL) and is about 13,463.2 Da. It is composed of CD8-α and CD8-β chains belonging to the immunoglobulin family with a variable (IgV) like extracellular domain connected to the membrane by a thin stalk,

and an intracellular tail (Leahy *et al.*, 1992). The extracellular IgV-like domain of CD8-α interacts with the α_3 portion of the Class I MHC molecule allowing the T-cell receptor of the cytotoxic T-cell and the target cell bind closely together during antigen-specific activation ((Leahy *et al.*, 1992).

The use of tetrameric peptide-bound class I MHC molecule complexes to identify antigen specific $CD8^+$ T-cells has enabled new insights into the interaction between viruses and cytotoxic T lymphocytes (Kelleher *et al.*, 2000). These enables a better understanding of HIV-specific $CD8^+$ T-cell populations through the different stages of HIV-1 infection by examination of their activation status represented by CD38 and Ki67 expression. The level of susceptibility to apoptosis is indicated by Bcl-2 and CD95 expression and differentiation state using CD28, CD27, CD45RA, and perforin expression (Appay *et al.*, 2002a). Two pathways exist for the natural death of $CD8^+$ T-cells where the extrinsic pathway or "death by design" is represented by CD95 and the intrinsic pathway or "death by neglect," governed by Bcl-2 family which includes both proapoptotic i.e. Bax, Bik and antiapoptotic i.e. Bcl-2, Bcl-xL members (Strasser *et al.*, 2000).

During primary HIV-1 infection, $CD4^+$T-cells are progressively depleted and the immune system responds appropriately by activating HIV-specific $CD8^+$ T-cells expressed by high levels of CD38 activation marker (Appay *et al*, 2002b). This is in order to prevent viral spread by mounting a strong HIV-specific $CD8^+$ T-cell response and a corresponding reduction in viraemia (Koup *et al.*, 1994, Panteleo *et al.*, 1994). The magnitude of each $CD8^+$ T cell expansion may vary according to the antigen, with strong responses often seen toward *nef* epitopes (Appay *et al*, 2002a).

HIV-specific $CD8^+$ T cells express a high level of CD95 during acute infection, while this is much lower in resting cells in the chronic phase. This is explainable in that their susceptibility to apoptosis is reduced due to up-regulation of Bcl-2 and down-regulation of CD95. HIV-specific $CD8^+$ T cells may thus be highly prone to apoptosis during primary infection, presumably through either of the main apoptotic pathways, and may be more protected from cell death and hence more stable during chronic infection. This susceptibility to apoptosis is closely related to the activation status of the cells, and therefore also to the antigen load present in the patient (Kuroda *et al.*, 1999).

The $CD8^+$ reference intervals obtained in this study would be used in relation to acute or chronic stage of HIV infection interpretation and their influence on the $CD4^+$:$CD8^+$ ratio.

2.6 HIV interaction and entry into host cells

Through the typical transmission routes from one infected partner to another HIV gains entry into the body and comes into contact with a number of cells. The virus's envelope protein complex controls the key process of viral tropism and facilitates the membrane fusion process that allows entry into the target host cells (Chan and Kim *et al.*, 1998). Viral tropism determines which cell types HIV infects and includes a variety of immune cells such as $CD4^+$ T-cells, macrophages and microglial cells. HIV entry to macrophages and $CD4^+$ T-cells is mediated through interaction of the virion envelope glycoprotein 120 (gp120) with the CD4 molecule on the target cells and also with chemokine coreceptors. HIV enters macrophages and $CD4^+$ T-cells by the adsorption of glycoproteins on its surface to receptors on the target cell followed

by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell (Wyatt and Sodroski *et al.*, 1998). Earlier studies identified the CD4 molecule as a primary and necessary receptor for HIV-1, HIV-2 and SIV (Dalgleish and Klatzmann *et al.*, 1984). This binding however induces conformational changes in the gp120 that contribute to the formation or exposure of the binding site for the chemokine receptors (Dragic *et al.*, 1996). Several studies indicated the chemokine receptor CCR5 as a necessary coreceptor for monocytotropic (M-tropic) HIV-1 isolates (Deng 1996, Doranz 1996, Dragic *et al.*, 1998). These isolates that are transmitted and that predominate during the early years of infection interact with CCR5 as an obligate coreceptor, and rare individuals that are genetically deficient in CCR5 expression are relatively resistant to HIV-1 infection (Connor *et al.*, 1997). The chemokine receptor CXCR4 (Fusin) was described as being the coreceptor used by T-cell-tropic (T-tropic) HIV isolates (Feng *et al.*, 1996). These isolates were mainly those arising later in the course of infection and often used CXCR4, in addition to CCR5 (Feng *et al.*, 1996, Connor *et al.*, 1997). This raised the possibility that the chemokine receptors represented the primordial, obligate receptors for this retroviral lineage (Connor *et al.*, 1997). Thus the use of CD4 as a receptor seemed to have evolved subsequently, thus allowing the high affinity chemokine receptor–binding site of primate immunodeficiency viruses to be sequestered from host immune surveillance (Wyatt *et al.*, 1998*)*. Once HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease and protease are injected into the host cell (Chan and Kim *et al.*, 1998).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study area

The study was conducted in Nairobi province which lies at the southern end of Kenya's agricultural heartland, 1.19^0 S of the Equator and 36.59^0E at an altitude between 1,600 and 1,850 metres above sea level. The average daily temperature ranges from 29º C in the dry season to 24º C during the rest of the year. The population estimate by 2007 was 3.0 million people in the urban part and approximately 4.0 million people in the metropolis. The National Blood Transfusion Services (NBTS) and the National HIV Reference Laboratory (NHRL) are located on Kenyatta National Hospital (KNH) grounds in Nairobi. The study subjects were selected from different donor centres within Nairobi.

3.2 Study population

This study focused on the adult population of Nairobi who qualified as reference blood donors of ages 16-65 years old. A set of selection criteria specifying the desired state of health was applied to the intended reference blood donors. In particular according to recommendations from CLSI the disease and/or conditions of interest for which the reference intervals are to be determined must be absent and at a minimum a questionnaire should be used to evaluate the health status of each reference subject (NCCLS, 2000). Those who met the criteria were recruited into the

study. The reference blood donors included in the study sample were among an estimated population of 1.5 million adults in Nairobi.

3.3 Study design

This was a cross-sectional study and the target blood donors were selected by non-randomized sampling technique. Subjects who attended the blood donation sites from 22ndSeptember to 23rd October 2008 were included in the study. For any of these subjects that met the inclusion criteria, a standard questionnaire (Appendix A) was administered to the subjects to capture socio-demographic characteristics and health status. Following interviews the cases were assigned a code number and data questionnaires safely stored away by either the researcher or a trained laboratory technologist.

3.4 Inclusion criteria

Any male or female adults of ages 16-65 years and residents of Nairobi were included. Consenting subjects were classified as “presumed healthy blood donors” if they did not show or report any signs and symptoms of disease. These symptoms included anaemia, diabetes, epilepsy, heart disease, jaundice and high blood pressure. Haematological results were satisfactory if found to be within reference intervals including haemoglobin levels, absolute and differential leucocytes count. All subjects were tested and found negative for HIV, Hepatitis B Virus (HBV), Hepatitis C Virus (HCV) and Syphilis.

3.5 Exclusion criteria

Any individual outside the blood donor age bracket and/or was a non resident of Nairobi was excluded. Subjects who did not consent to the study or those who showed and/or reported signs and symptoms of disease were also excluded. Subjects were further excluded if they had a blood transfusion in the last one year, major operation in the last two years, any body piecing in the last one year, or on medication or had been on medication in the last one month. Female subjects were excluded if they were pregnant or had delivered in the past six months. Any subject whose hematological values were outside of the normal ranges was also excluded.

3.6 Sample size

The study by Reed *et al*,(1971), has been quoted by the clinical laboratory standards institute which recommends a minimum sample size of 120 for randomly selected subjects in determining reference intervals accurately (NCCLS, 2000). Therefore from predetermined eleven (11) blood donor sites within Nairobi from which about 74% of the donors were to be obtained from institutions was considered. To adjust for the clustering effect on non-randomized sampling a study design effect of three (3) was included and raised the minimum number of subjects to 360. To cushion against sampling error I projected to have more study subjects to at least 420.

3.7 Sampling procedure

Selection of subjects was done upon arrival at the blood donor site and the potential subject was consented before the questionnaire was administered. Personal

identifiers were removed and a unique identification number assigned to each specimen.

Any potential subjects with indications of disease were referred to a government hospital for treatment, follow-up evaluation and care, by counselors based at NBTS.

3.8 Blood Specimen collection.

The specimens were collected from 0900Hrs to 1400Hrs in order to ensure they were transported and processed on the same day. Experienced laboratory technologists drew blood from an arm by venipuncture. At the completion of the blood collection a total of 8Mls of blood was collected as follows, 2ml of blood was drawn for $CD4^+$ and $CD8^+$ T-cell count an additional 3ml of blood sample was collected for HIV, syphilis, and hepatitis B and C testing and a final 3ml blood sample was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) for haematological testing. The CD4+ collection tubes are designed to stabilize CD4+T-cells for up to 7 days.

3.9 Labeling and transport of blood specimens

A unique random identification number (ID No) was assigned to the consenting subjects. The unique number was affixed to the $CD4^+$ tubes, the red top tubes, and on the inclusion questionnaire script. The unique identification number was assigned as for instance, **014644508,** where: 014 was the team number, 6445 the donor number and 08 the year. All samples were transported at room temperature of 18^0C-22^0C and packed safely to avoid leakages and contamination. To further ensure their integrity

and timely arrival for testing and analysis handling personnel were trained on these requirements.

3.10 Blood Specimen testing and analysis

All hematological testing was carried out at the hematology department at the Armed forces memorial hospital laboratory (AFMH). This was performed to determine whether individual samples were within satisfactory reference values and samples not conforming were excluded from the study. Serological testing for HIV 1/2, Syphilis, Hepatitis B and C was done at the National Blood Transfusion Services centre (NBTS). Any specimen that tested positive was excluded from the study. The $CD4^+$ and $CD8^+$ T-cell counts for all the samples collected was done at the National HIV Reference Laboratory (NHRL) irrespective of haematological or serology results. All results whose samples did not pass the hematology and serology screening tests were eliminated from further analysis.

3.10.1 Hematological testing

This was done using a hematology analyzer that provided haematological parameters. Briefly, the hematology analyzer was standardized against a blood control where the machine automatically dilutes a whole-blood sample, lyses, counts and gives a printout result of absolute numbers of leukocytes (expressed as number of cells x $[10^{9}]$ per liter), erythrocytes (number of cells x $[10^{12}]$ per liter), platelets (number of cells x $[10^{9}]$ per liter), lymphocytes (number of cells x $[10^{9}]$ per liter), mononuclear cells (number of cells x $[10^{9}]$ per liter), and granulocytes (number of cells x $[10^{9}]$ per

liter). Heamoglobin (in grams per deciliter) and heamatocrit (in percentage) values were also included.

3.10.2 HIV testing

This was done at NBTS using a combination of 4th generation (Vironostika® HIV 1 / 2 antigen / antibody) and 3rd generation (Murex HIV.1.2.O) HIV Enzyme Immuno-Assay (EIA) in a parallel testing algorithm. The microelisa plates are coated with HIV-1gp 160, HIV-1 ANT70 peptide, HIV-2 envelope peptide and anti-HIV-1 p24 each well contains a horse radish peroxidase (HRP) - labeled conjugate of the same HIV antibody/antigen mixture. The specimen diluent added to the wells dissolves the conjugate sphere. The test sample or control containing the HIV antibody or antigen is incubated into the microelisa wells. If HIV-1 and/or HIV-2 antibody is present in the sample a solid phase antigen/anti-HIV/enzyme labeled antigen complex is formed. If HIV-1 antigen is present in the sample a solid phase antibody/HIV-antigen/enzyme labeled antibody complex is formed. Following a wash procedure and incubation with tetramethylbenzidine (TMB) substrate, colour develops which turns yellow when the reaction is stopped with sulfuric acid. If anti HIV-1, anti HIV-2, anti HIV-1 group O and/or HIV antigen is present in the sample, an intense colour develops. However, when the sample is free of anti-HIV and HIV antigen, no or low colour forms with the addition of the substrate. Samples showing discordant results were to be repeated and ribonuclease polymerase chain reaction (RNA-PCR) was to be carried out on samples showing discordance twice.

3.10.3 Syphilis testing:

Serum samples were screened at NBTS using Rapid plasma reagin (RPR). The RPR test is a nontreponemal testing procedure for the serologic detection of syphilis (Portnoy *et al*. 1972). The RPR card antigen suspension is a carbon particle cardiolipin antigen that detects reagin, an antibody-like substance present in the sera from syphilitic persons and occasionally in sera of persons with other acute or chronic conditions. When a specimen contains an antibody, flocculation occurs with a co-agglutination of the carbon particles of the RPR card antigen, which appear as black clumps against the white background of the plastic-coated card. Nonreactive specimens appear to have an even light-gray color.

In addition, all positive samples were retested using *Treponema pallidum* Hemaglutination Assay (TPHA) which was to identify false positive RPR samples. The Syphilis TPHA test is an indirect hemaglutination test for the detection and titration of specific antibodies against *Treponema pallidum*. Avian erythrocytes are sensitized with antigens of the Nichol's strain of Treponema pallidum. In the presence of positive syphilitic serum, the red cells aggregate to form characteristic patterns on the surface of the micro-plate wells. Antibodies directed against other non-pathogenic Treponema are absorbed by an extract of Reiter's Treponema present in the cell suspension, thus greatly reducing false positives. Other non-specific reactions can be detected and eliminated with non-sensitized control cells.

3.10.4 Hepatitis B and C virus (HBV and HCV) testing

This was done using anti-HBV and anti-HCV Enzyme Immuno-Assay (EIA).The detection of anti-HBV and anti-HCV antibodies in plasma or serum is based on the

use of third-generation EIAs, that detect mixtures of antibodies directed against various HBV and HCV epitopes. Recombinant antigens are used to capture circulating antibodies onto the wells of microtiter plates, micro-beads, or specific holders adapted to closed automated devices. The presence of anti-HBV and anti-HCV antibodies is revealed by anti-antibodies labeled with an enzyme that catalyzes the transformation of a substrate into a colored compound. The optical density (OD) ratio of the reaction (sample OD/internal control OD) is proportional to the amount of antibodies in the serum or plasma sample.

3.10.5 $CD4^+$ and $CD8^+$ T-cell count enumeration

This was done using the flow cytometry technology utilizing the single-platform technique which is considered a reliable and robust method and has less interlaboratory variability (Wee *et al.*, 2004). Blood specimens remained and transported at room temperature (18-25^{0}C) to the NHRL. Samples were analyzed within a time period of 24 hours after collection.

Enumeration of absolute, percentage and CD4:CD8 ratio values of T lymphocyte subsets from each CD4+ tube of whole blood was done using four colour MultiTEST CD3 FITC/CD8 PE/CD45 PerCP/CD4 APC reagents and BD FACsCalibur™. When whole blood was added to the reagent, the fluorochrome-labeled antibodies in the reagent bind specifically to leukocyte surface antigens. During acquisition, the cells travel past the laser beam and scatter the laser light. The stained cells fluoresce. These scatter and fluorescence signals, detected by the instrument, provide information about the cell's size, internal complexity, and relative fluorescence intensity. MultiTEST reagents employ fluorescence triggering, allowing direct

fluorescence gating of the lymphocyte population to reduce contamination of unlysed or nucleated red blood cells in the gate.

When TruCOUNT Tubes were used, a known volume of the sample is stained directly in a TruCOUNT tube. The lyophilized pellet in the tube dissolves, releasing a known number of fluorescent beads. During analysis, the absolute number (cells/µl) of positive cells in the sample can be determined by comparing cellular events to bead events.

3.11 Data analysis

Values obtained were subjected to statistical treatment using the SPSS version 12.0 by calculating the mean, median standard deviation (SD) and standard error of the mean (SE) and determination of normality of the data by the Kolmogorov-Smirnov test. Stratification of the values into male and female subgroups allowed further analysis and comparison. Reference intervals were determined using the mean±2SD depending on the distribution of the values obtained.

To determine the effect of sex and age on $CD4^+$ and $CD8^+$ T-cell count among the reference blood donors, the values obtained were subgrouped into males and females and into different age groups. The student t-test was used to compare the two means obtained from data of both sexes to draw inferences on the outcome. Analysis of variance (ANOVA) was used to compare the differences among the age groups. Statistical significance based on P-value of < 0.05 was used to determine the variations between the sexes and among the age groups.

Finally, values obtained were compared with the current reference values being used as guidelines for anti-retroviral treatment. The $CD4^+$ and $CD8^+$ absolute and

percentage values were compared against other published reference values to make inferences on different population values.

CHAPTER 4

4.0 RESULTS

A total of 424 reference blood donors were sampled from different sites within Nairobi between 23rd September 2008 and 23rd October 2008. All recruited donors fulfilled the inclusion criteria, and gave both oral and written consent. The donors included 232 (55 %) males and 192 (45%) females out of whom 17 (4%) donors were excluded from the study due to positive results for HIV, syphilis, Hepatitis B, or Hepatitis C (Appendix C). The remaining 407 donor results consisting of 222 (55%) males and 185 (45%) females were tested for outliers and extreme values using SPSS Ver. 12.0 statistical software and 7(1.7%) had extreme values and were excluded from further analysis. The immediate cause of these extreme values was not possible to elucidate but could have been attributed to either a clinical or biological phenomenon in the patient. However chance could have had a role as possibly due to equipment error. Probable outlier values were retained if they were within the mean ± 3SD or by use of the normal Q-Q plots thus none were excluded.

4.1 Determination of $CD4^+$ and $CD8^+$ reference intervals

A total sample size of 400 donors consisting of 54% (215) males and 46% (185) females had an age range from 18 – 60 years, with a mean age of 26.3 ± 7.6SD years (Table 1). The mean age for males was 27.9 ± 8.2SD years whereas that for females was 24.3 ± 6.3SD years and whose difference was not statistically significant (t=4.58, df=398, P=0.100). The 400 reference donors were used to determine the reference intervals whereby the data was first tested for normal distribution using the goodness

of fit test of Kolmogorov-Smirnov. Where $P<0.05$ the values were concluded to have a poor fit to the normal distribution and conversely $P>0.05$ the values were concluded to have a good fit to the normal distribution. Among the T-cell subsets, only $CD4^+$ % values exhibited a normal distribution whereas the other T-cell subset variables showed non-normal type of distribution.

Table 1: Age and sex distribution of reference blood donors by site in Nairobi.

Site	Included n (M/F)	Mean age ±SD yrs	Age (yrs)	
			Lower	Upper
Kencom [a]	74 (51/23)	30 ± 8.7	19	60
Catholic university	135(52/83)	21 ±2.7	18	38
Huruma centre	7(4/3)	25 ±5.4	19	34
Stanbic Bank	40 (25/15)	31 ±6.9	23	53
Marist Int. College	25 (13/12)	23 ±7.7	18	56
CFC Life Ins. Company	18 (12/6)	28.8 ±6.4	21	43
Bomb blast Memorial [b]	29 (16/13)	28.8 ±6.2	20	50
Nazarene University	21(10/11)	24 ±6	18	51
KUSCO	17(13/4)	30.9 ±8.8	23	53
University of Nairobi	18(7/11)	26 ±7.5	19	42
Fidelity Ins. company	16(12/4)	34.5 ±8.4	24	47
TOTAL	**400 (215/185)**	**26.3 ±7.6**	**18**	**60**

[a] Kenya Commercial Bank area, Moi Avenue.
[b] Former USA embassy grounds, off Haille Selasie Avenue.
n– Total donors
M/F – Males/Females

To attain normality the other T-cell subset variables were power transformed using the square root transformation determined by the SPSS Ver. 12.0 statistical software and attained normality (Table 2). Unlike other T-cell subset variables, $CD8^+$ % values failed to attain normality and thus its determination of reference interval was done using the median (range 2.5^{th} to 97.5^{th} percentile).

Table 2: Test of normality of $CD4^+$:$CD8^+$ Ratio, $CD4^+$, $CD8^+$ % and absolute counts using Kolmogorov-Smirnov test before and after power transformation of the data.

T-cell subset	Kolmogorov-Smirnov [a] before power transformation			Kolmogorov-Smirnov [a] after power transformation		
	Statistic	Obs.	P-value	Statistic	Obs	P-value
$CD4^+$:$CD8^+$ Ratio	0.075	400	<0.0001	0.036	400	0.200*
CD4+ % [b]	0.024	400	0.200*			
CD4+ Abs Cnt	0.077	400	<0.0001	0.036	400	0.200*
CD8+ % [c]	0.097	400	<0.0001			
CD8+ Abs Cnt	0.097	400	<0.0001	0.039	400	0.148

* This is a lower bound of the true significance.
[a] Lilliefors Significance Correction
[b] Original data had normal distribution thus not transformed
[c] Original data failed transformation thus non-normal distribution
Abs Cnt- Absolute count
Obs - Observations

Reference values for $CD4^+$ and $CD8^+$ T-cell subsets were determined and expressed as ratios, relative percentage and mean absolute counts of cells per microlitre of blood (cells/µl). Reference intervals were determined as mean ± 2SD and back transformed to the original values as shown in Table 3.

Table 3: Reference intervals for T-cell subsets after power transformation to original values for combined and separate sexes

		$CD4^+$ Abs Cnt Cells/µl	$CD8^+$ Abs Cnt Cells/µl	$CD4^+$ %[a]	$CD4^+$:$CD8^+$
Combined sexes (n=400)	Mean ± SD	7.4 ± 0.7	1.86 ± 0.08	40 ± 6.7	1.2 ± 0.13
	Ref. intervals (Mean ± 2SD)	7.4 (6– 8.8)	1.86 (1.7–2.02)	40 (27–53)	1.2 (0.9 -1.5)
	Ref. intervals *	790 (392-1,405)	500 (202–1,131)	40 (27–53)	1.8 (0.7 -4)
	95%CI of mean	754 - 790	470 - 523	3 -40	1.5 - 1.6
Males (n=215)	Mean ± SD	7.2 ± 0.7	1.86 ± 0.08	38 ± 6.4	1.1 ± 0.13
	Ref. intervals (Mean ± 2SD)	7.2 (5.8–8.6)	1.86 (1.7–2.02)	38 (25 – 51)	1.1(0.8–1.4)
	Ref. intervals *	720 (371–1,348)	501 (202–1,131)	38 (25 – 51)	1.4(0.5–3.1)
	95% CI of mean	681- 742	470 - 523	37 - 39	1.4 – 1.5
Females (n=185)	Mean± SD	7.6 ± 0.7	1.85 ± 0.08	42 ± 6.2	1.2 ± 0.12
	Ref. interval (Mean ± 2SD)	7.6 (6.2 – 9)	1.85 (1.7–2.0)	42 (30 –54)	1.2 (1 –1.4)
	Ref. intervals *	863 (438–1,516)	493 (202-1,024)	42 (30 –54)	1.8 (1 – 3)
	95% CI of mean	817-895	470 - 523	41 - 43	1.7 – 1.8

[a] Data not power transformed, * Back transformed data to original values
Abs Cnt- Absolute count

The overall absolute $CD4^+$ T-cell mean value and reference interval obtained from the study was 790 cells/µl (Range 392 – 1,405). A total of 3% (n=12) of the group were below the lower reference limit of 392 cells/µl of which 4.2% (n=9) males and 1.6% (n=3) females were below this limit. On the other hand a total of 2.8% (n=11)

of the subjects were above the upper reference limit of 1,405 cells/µl of which 0.5% (n=1) males and 5.4% (n=10) females were above this limit.

The $CD8^+$ absolute T-cell reference interval for the entire group was obtained as 500 cells/µl (Range 202 – 1,131). The entire group reference mean and interval were comparably similar to males who had 501cells/µl (range 202 – 1,131) whereas females had a slightly lower mean of 493cells/µl (Range 202 – 1,024).

The overall $CD4^+$ % obtained was 40% (Range 27 – 53) for the entire group with females having a comparably higher reference interval of 42% (Range 30 – 54) than the entire group. Conversely males had lower values overall for $CD4^+$ %. In comparison with the established reference interval 4% (n=9) and 1% (n=2) of males and females respectively were below the lower reference limit of 27 %. Three percent (n=5) of females had reference values above the upper reference limit of 53%.

The results obtained established a mean value and reference interval for $CD4^+$:$CD8^+$ratio as 1.8 (Range = 0.7 – 4) where 67% (n=268) of the donors had a $CD4^+$:$CD8^+$ratio of between 1 and 2, including 68% (n = 147) males and 65% (n = 121) females. For 15% (n=32) of males they had a $CD4^+$:$CD8^+$ ratio <1 while 29% (n= 58) of females had a ratio >2.

As observed from the results the mean absolute counts for $CD4^+$ T -cells were higher than the $CD8^+$ T-cell mean absolute counts irrespective of sex in over 70% of the donors, resulting in a $CD4^+$: $CD8^+$ ratio of between 1.0 and 2.0.

The $CD8^+$ % reference value and interval were determined from the median and the 2.5^{th} and 97.5^{th} percentile at 95% confidence interval as shown in Table 4.0. The attained reference values for the total group were 25% (Range15– 44) at 95%CI

where males had the overall higher reference values of 26% (Range 15 – 50) than the entire group.

Table 4: $CD8^+$ % reference intervals as percentiles to the median at 95% CI.

$CD8^+$ %			Ref interval in percentile	
Sex	**n**	**Median**	**2.5^{th}**	**97.5^{th}**
Males	215	26	15	50
Females	185	24	13	37
Total	400	25	15	44

4.2 Effect of sex and age on T-cell reference values.

There was a statistically significant difference between the sexes for the T-cell subsets except for $CD8^+$ mean absolute count when the means were compared for the two sexes using the student t-test (Table 5). Both assumptions of equal and unequal variances for the means were used and did not exhibit any variation in the P-value. The mean difference for $CD4^+$ mean value for males against females was found to be statistically significant (t=-5.88, df 398, P<0.0001). Differences in the proportions of males and females below the lower limits and above the upper limit had a direct effect on the respective means of the two sexes.

The results for $CD8^+$ absolute counts did not show any significant difference (t=0.39, df 398, P=0.700) between males and females despite males having a slightly higher reference mean and 4% (n=8) of these being above the upper reference limit of 1,131 cells/µl. Males had a lower $CD4^+$ % count than females whose mean difference of -4.47 was statistically significant (t=-7.02, df 398, P<0.0001). Females showed a

higher mean $CD4^+$:$CD8^+$ ratio that was also statistically significant (t=-5.49, df 398, P, 0.0001).

Table 5: Student t-test for comparison of means with equal variances assumed between males and females for the different T-cell subset variables.

Variable*	t-test for equality of Means [a]						
	t	df	P-value	Mean Diff.	SE Diff.	95% CI of the mean Difference	
						Lower	Upper
$CD4^+$ Abs Cnt	-5.88	398	0.0001	-0.41	0.07	-0.54	-0.27
$CD8^+$ Abs Cnt	0.39	398	0.700	0.003	0.01	-0.01	0.02
$CD4^+$ %	-7.02	398	0.0001	-4.47	0.64	-5.72	-3.22
$CD4^+$:$CD8^+$	-5.49	398	0.0001	-0.07	0.01	-0.09	-0.04

* Variable values in power transformed state.
[a] Equal and unequal variances assumed

The median difference of reference values obtained for $CD8^+$ % between males and females were subjected to the Mann-Whitney U test whereby the difference between the two sexes was statistically significant (Mann-U test 16158, Z=-3.235, P=0.001). To determine the effect of age, the data was classified into three different age groups; 18 – 27, 28 – 37, and above 37 years. Progressive age increase in the combined group did not have a significant influence on the $CD4^+$:$CD8^+$ ratio, $CD4^+$, and $CD8^+$ percentage and mean absolute T-cell subset values (P>0.05) as shown in Table 6.

Table 6: Test of statistical significance by ANOVA for T-cell variables between the age groups.

Dependent variable	Age group in years (n)	Dependent variable mean	df	F	P-value
$CD4^+$:$CD8^+$	18-27 (269)	1.6	2	0.716	0.489
	28-37(96)	1.54			
	>37(35)	1.54			
	Total (400)	1.6			
$CD4^+$ %	18-27 (269)	39.51	2	0.239	0.788
	28-37(96)	40.05			
	>37(35)	39.84			
	Total (400)	39.67			
$CD4^+$ Abs Cnt (cells/µl)	18-27 (269)	772	2	0.086	0.917
	28-37(96)	775			
	>37(35)	793			
	Total (400)	790			
$CD8^+$ Abs Cnt (cells/µl)	18-27 (269)	501	2	0.979	0.377
	28-37(96)	522			
	>37(35)	522			
	Total (400)	501			

Abs Cnt- Absolute count

4.3 Comparison of Kenya data with reference values from other regions.

The reference values from this study were compared with USA derived reference intervals that are currently used for decision making as to whether to prescribe antiretroviral treatment or not. These were derived from the FACsCalibur System using the Becton, Dickson (BD) MultiSET software. Table 7 shows comparison of

BD MultiSET values with those obtained in our study and reference individuals within and outside this range.

Table 7: Present Kenyan study versus BD MultiSET, USA derived reference intervals and reference individuals within and out of range of the USA reference intervals.

T-cell subset	Present Kenyan study	BD MultiSET (USA)	Ref. subjects within BD Ref. Interval	OOR* Ref. subjects	
				Lower (%)	Upper (%)
$CD4^+$ %	27 – 53	31 - 60	359 (90%)	41 (10%)	-
$CD4^+$ Abs. Cnt (cells/µl)	392 –1,405	410 –1,590	380 (95%)	16 (4%)	4 (1%)
$CD8^+$ %	15 - 50	13 - 41	381(95%)	6 (2%)	13 (3%)
$CD8^+$ Abs. Cnt (cells/µl)	202 –1,131	190 –1,140	390 (97.5%)	6 (1.5%)	4 (1%)

*OOR: Out Of Range of the USA reference intervals

Majority of the study's reference individuals i.e. 95% and 97.5% were within the USA derived reference intervals for $CD8^+$ percent and $CD8^+$ absolute count respectively. Despite the Present study having a higher lower reference limit for that $CD8^+$ absolute count the results showed that 1.5% (n=6) and (n=4) 1% of the reference individuals were below and above the lower and upper limits respectively when compared against the BD MultiSET (USA) values of. 190 cells/µl. Ten percent (10%) of the $CD4^+$ percent reference individuals in the present study were below the reference limit of 31% from the BD MultiSET values and none of them was above the 60% upper limit. Over 95% of the reference individuals in this study had absolute $CD4^+$ counts within the USA derived values and only 16 (4%) and 4 (1%) of these individuals were below and above the lower and upper reference limits

respectively. From this study 2% (n=6) of reference individuals were below the lower reference limit and 3% (n=13) were above the upper reference limit for $CD8^+$ %. Overall, approximately 95% of the present Kenyan study reference individuals were within the reference intervals provided by the USA based BD MultiSET.

These values were also compared with other studies in different regions of the world. Table 8 shows mean reference values for different countries from African, Asian and American regions. Uganda had the highest mean absolute count for $CD4^+$ (1, 256cells/µl) and also mean absolute $CD8^+$ count and the $CD4^+$:$CD8^+$ ratio. Kenyan values were also compared against Becton, Dickson (BD) values from the USA provided in the reagent insert that were relatively higher in both mean percentage and mean absolute counts. However, despite differences in means the reference intervals between the two were comparable especially that 95% of the present study values were within the USA derived reference intervals.

Table 8: Reference mean values of present study compared with other published reference T-cell subsets values from other regions.

Country	Total No. (M /F)	$CD4^+$T-cells		$CD8^+$ T-cells		$CD4^+$:$CD8^+$ ratio
		Percent (%)	Abs cnt cells/µl	Percent (%)	Abs cnt cells/µl	
Kenya	400 (215/185)	40 (27 – 53)	790 (392 – 1,405)	26* (15 - 50)	500 (202 – 1,131)	1.6 (0.6 - 4)

Uganda	183 (114/69)	NA	1,256 (559 – 2,333)	NA	668 (253 – 1,396)	2.16 (0.68 – 4.4)
Ethiopian	142 (92/50)	NA	761 (366 – 1,235)	NA	637 (311 – 1,618)	1.2 (0.4 – 2.4)
India	94 (55/39)	40 (31- 50)	865 (430 – 1,740)	31 (20 – 43)	552 (218 – 1,396)	1.7 (0.39 – 3.0)
BD MultiTEST (USA) [a]	164	45 (33 – 58)	941 (404 – 1,612)	24 (13 – 39)	511 (220 -1,129)	NA

[a] Values provided from BD MultiTEST reagent product insert San Jose, California, 1991,

* Median value and 2.5^{th} and 97.5^{th} interval

M/F: Males/Females

Abs cnt- Absolute count
NA: Not available

CHAPTER 5

5.0 DISCUSSION

Africans in general are considered to have lower $CD4^+$ T-cell counts than Caucasian populations as expressed by Tsegaye *et al.,* (1999) and Tugume *et al.,* (1995). The main purpose of this study was to find out whether any differences existed for $CD4^+$ and $CD8^+$ reference intervals between Kenyan African population and those

currently used as references obtained from a Caucasian population (BD systems, 1991). This was done by establishing local Kenyan African $CD4^+$ and $CD8^+$ reference intervals and the results of which can possibly be used to guide anti-retroviral therapy.

The reference intervals obtained in this study were from 400 HIV negative reference individuals where males were slightly more than females by 7% where the lower proportion of females was likely due to less frequent participation in blood drives either due to cultural and other reasons. The low prevalence of transfusion transmittable infections of 4% in this group demonstrated that the National blood transfusion services screening questionnaire as a method for eliminating donors with pathological conditions or acute illnesses was positively accepted and applied in this study. The rigorous screening process employed at the National blood transfusion services blood bank resulted in collection of blood specimens from "presumed healthy adults"

The study adopted the square root transformation of the data as proposed in the Clinical Laboratory Standards Institute guidelines (NCCLS, 2000) to allow proper comparison of the mean values with the current reference values provided by BD MultiSET, USA (BD systems, 1991) that are used for daily interpretation of reference intervals for local individuals.

Results from this study found that despite a 5% difference in the distribution of absolute $CD4^+$ values within 500 - 1000 cells/µl between males and females the presence of more women values (30%) above 1000 cells/µl than males (13%) may have directly caused a significant difference ($P<0.0001$) in their means. These findings agree with those from a study in Tanzania to determine gender differences

in $CD4^+$ T-cells, which showed that males were more likely to have $CD4^+$ T-cells <500cells/µl (Urassa *et al.,* 2003). Such sex variation in absolute $CD4^+$ count could complicate prophylaxis and initiation of antiretroviral treatment decisions (Urassa *et al.,* 2003). Thus a pragmatic approach to decision making on this basis could be used in the management of HIV infected individuals especially if local reference values have clearly established sex differences. A significant difference in $CD4^+$ % between males and females was also found in this study ($P<0.0001$). These findings agree with recent findings from a study on adult HIV vaccine trials volunteers in Kericho (Kenya) which found a significant difference ($P<0.05$) (Rukia *et al.*, 2008). Males had significantly higher ($P=0.001$) CD8% count than females which was also in agreement with findings on Indian adults (Uppal *et al.,* 2003). However this was not the same for adults in Kericho (Kenya) who did not present any significant difference (Rukia *et al.*, 2008). Females had a higher $CD4^+$:$CD8^+$ ratio than males ($P<0.0001$) and these results agreed with work done in Ethiopia (Kassu *et al.,* 2001, Tsegaye *et al.,,* 1999) and India (Saxena *et al.,* 2004, Ray *et al.,* 2006). There were significant differences ($P<0.05$) between the sexes for all the T-cell subsets except for $CD8^+$ absolute count which was also similar to study reports of Indian men showing no difference from the women (Uppal *et al.,,* 2003). Saxena *et al.,* (2004) reported the presence of high absolute $CD4^+$ counts influenced levels for $CD4^+$:$CD8^+$ ratio and $CD4^+$ % counts. It is from this basis that the significant differences seen between males and females in this study are as a result of observed significantly high mean $CD4^+$ absolute counts in females.

However, studies conducted on adults in Nigeria and neighboring Uganda did not find any significant difference between the sexes for any of the T-cell subsets (Olumuyiwa *et al.,* 2005, Tugume *et al.,* 1995).

Attempts to explain variations in $CD4^+$ T-cell counts between males and females found that HIV seronegative women had 28% higher $CD4^+$ T-cell counts than that of men and suggested that the sex difference could be due to diurnal variation (Bofill *et al.,* 1992). This was explained by the different times of sample collection where male samples were obtained in the morning hours while female samples were collected during the afternoon and significantly higher counts were established. Significant sex differences in the $CD4^+$ absolute counts where women had a 3.5% higher count than males as observed in this study could not probably be explained by diurnal variation as all the samples were collected between 0900hrs – 1400hrs. A possible explanation for the observed gender difference in $CD4^+$ counts could be caused by other factors such as sex hormones effect since the circulating lymphocytes have receptors for androgens and estrogens (Grossman *et al.,* 1985).

The study on Kenyan reference individuals did not establish any age related significant changes in the whole group ($P>0.05$) for any of the T-cell subsets. There was no age related difference in similar studies conducted previously in India (Uppal *et al.,*2003), Kuwait (Kaaba *et al.,* 2004) and in the Central African Republic (Menard *et al.,*2003) where T-cell values did not show significant changes with progressive age. Similar observations were made among Nouna residents in Burkina Faso (Klose *et al.,* 2007) where no significant difference was seen in any of the T-cell subsets either in terms of absolute or relative cell numbers.

However, healthy adult donors from Switzerland, indicated an age-related differences where a significant decrease in the absolute levels of peripheral blood $CD4^+$ and $CD8^+$ T-cells was observed with age (Bisset *et al.,* 2004). This confirmed earlier observations that a generalized drop in T-cell numbers is a hallmark of the aging immune system (Sansoni *et al.,* 1993, Utsuyama *et al.,,* 1992). Other investigators also reported decreases in both absolute and proportional levels of $CD4^+$ and $CD8^+$ T-cells (Xu *et al.,* 1993). However, such discrepancy among different studies could most likely be reflecting differences in donor recruitment strategy or other causes for drops in these values. (Stulnig *et al.,* 1995).

Reference intervals obtained from Kenyan reference donors in Nairobi were not different from those provided by BD Company from Caucasian adults in California using the FACsCalibur though statistical significance was not done. Approximately 95% of all the reference values established in this study were within the USA derived reference intervals. Continued use of the USA derived reference intervals on the Kenyan African population seemed appropriate enough.

On the other hand reference intervals for $CD4^+$:$CD8^+$ ratio, $CD4^+$ percent and mean absolute counts obtained from adults in Kericho (Kenya), were all higher than those obtained in this study though statistical difference was not established. The exception was for $CD8^+$ mean absolute count where Nairobi reference subjects had a higher mean count of 501cells/µl than the Kericho reference subjects. A possible explanation to these T-cell count variations could be attributed to differences in altitude since Kericho is situated at approximately 2,042 metres above sea level making it a high altitude environment while Nairobi is approximately 1,680 metres above sea level. Similar T-cell variations due to altitude differences were noted on

values obtained from Dar es Salaam, Tanzania, which were lower than those from rural Northern Tanzania (Urassa *et al.,* 1996). Such variations in these populations due to differences in altitude and other factors could be valid causes of the observed differences and would be better understood in subsequent studies.

Like other studies done in Africa and Asia that report different values for $CD4^+$ T-cell levels compared to standard values for Western countries (Kaaba *et al.,* 2004). This study's reference individuals established values that were lower to those generated by the FACsCalibur using MultiSET software and TruCOUNT tubes for a study done on Caucasian donors in San Jose, California USA (BD systems, 1991). However this study's reference intervals were however higher than Chinese and Switzerland subjects but much lower than Dutch (Tsegaye *et al*., 1999) and Kuwait (Kaaba *et al.,* 2004) subjects. The $CD4^+$ mean absolute counts from this study were comparatively higher i.e. 790cells/µl than Ethiopian subjects mean absolute counts of 761cells/µl (Tsegaye *et al.,* 1999) but much lower than Indian absolute counts of 865cells/µl (Ray *et al.,* 2006). A study on an Asian population using a similar FACsCalibur system had higher percentage and mean absolute counts for $CD4^+$ and $CD8^+$ with values of 838cells/µl and 642 cells/µl respectively than reference values obtained in this study (Wee *et al*., 2004). These variations in $CD4^+$ and $CD8^+$ T-cells have been understandably associated with ethnicity, gender, diet, geographical area, as well as being dependent of genetic and environmental factors including differences in enumeration methods (Wee *et al.,* 2004).

The enumeration of peripheral blood $CD4^+$T-cell absolute count, percentage, and $CD4^+$:$CD8^+$ ratio values are among the best surrogate markers for the assessment of the risk for progression to AIDS in HIV-infected individuals. They are clinically

useful in assessing the risk of developing certain AIDS-related opportunistic infections and for timing the initiation of antiretroviral and prophylactic antimicrobial therapies (Coetzee *et al.*, 1994).

Assumptions to this study included that the NHRL participation in the South African external quality control program was appropriate for the calibration and control of the FACsCalibur machine. This study also assumed there were insignificant changes in the blood specimens during transportation.

The study was strengthened partly by enumeration of $CD4^+$ and $CD8^+$ T-cell counts using the FACsCalibur system under the single platform technology which is considered a reliable and robust method and has less interlaboratory variability (Wee *et al.*, 2004).

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CHAPTER 6

6.0 CONCLUSION

This study established reference values and intervals for $CD4^+$ and $CD8^+$ T-cells from the local blood donor community within Nairobi.

The study also determined the effect of sex on $CD4^+$ and $CD8^+$ references values and intervals as indicated from previous studies in different populations however no age related differences were established in this study.

This present Kenyan study also concludes that the reference intervals obtained are comparable to the U.S.A based comparison intervals.

Local reference intervals are also necessary for successful evaluation of vaccines and drugs internationally and also for advancing basic health care services in the developing nations.

6.1 RECOMMENDATIONS

The following are some of the recommendations:

1. The reference intervals obtained in this study though different, they are relatively comparable to the USA based reference intervals and can be used to compliment these intervals.
2. Adoption of such local reference intervals for patient management is preferable since they are established from the local population.
3. Further exhaustive studies with regard to $CD4^+$ and $CD8^+$ reference intervals and HIV therapy and other factors affecting the same. This would allow an insight to adjusting HIV therapy and a change in treatment policy for the same.
4. Allocate resources and develop national reference intervals for not only Immunohematological parameters but also such parameters in biochemistry and hematology.

6.2 LIMITATIONS

The results of this study need to be interpreted with caution as this study is neither exhaustive nor conclusive in relation to $CD4^+$ and $CD8^+$ reference intervals. This study was limited in that all the information on the donor recruitment form (Appendix A) was filled out by the donors and their health status was not independently verified through a clinical checkup. It is therefore possible that some donors did not remember recent infections or the use of medications. Recent seroconversion would not have been detected by the ELISA methods used and thus could have been included in the analysis of samples from HIV negative individuals. The study subjects were blood donors within Nairobi region whose distribution is not entirely representative of the larger Nairobi population. Only sex and age as factors affecting $CD4^+$ and $CD8^+$ reference values were analyzed in this study.

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APPENDICES

Appendix A : Inclusion questionnaire script

Donation number:……. ABO GROUP…….

Rhesus……

Date of birth…….. Sex…… Marital status……

Contact No…… Email…… Postal address…….

Weight (Kg)…….. HB (g/dl)……. BP (mmhg)……

Signature …….. Date…….

Indicate your state for the following (yes/no)

- Transfusion in the last one month……
- Donated in the last four months……..
- Major operation in the last 2 years…….
- Dental work in the last 1 week……
- Body piecing in the last one year……..
- Currently under medication of unwell…….
- Multiple sexual partners (self)……
- Not sure about my sexual partner……..
- Sexual contact with suspicious partner……..
- STD infection in the last 1 year……..
- Pregnant or breast feeding……

Do you have any of the following conditions (yes/no).

- Anaemia or bleeding disorder……..
- Diabetes…….
- Epilepsy……..
- Heart disease……..
- Stomach ulcers……..
- High blood pressure……..
- Jaundice ……..

Signature…….. Date……

Appendix B: Informed consent script

Hello, my name is…… and I Am working with the National Blood Transfusion Services in the Ministry of Health. We are conducting a study on healthy blood donors resident in Nairobi to determine their CD4+ and CD8+ T-cell counts. This study will aid the Ministry of Health formulate guidelines on when to start Anti Retroviral Therapy (ART) on patients infected with HIV.

A CD4+ T-cell count

During infection with HIV the CD4+ T-cells are the main target into which the virus will develop. These cells play an important role in defending the body from disease causing organisms. Therefore, when these cells are continuously destroyed by the virus our body's immune system becomes weak and infected people finally develop AIDS.

It is possible to count these CD4+ T-cells in order to determine how many are left before one develops AIDS. By knowing the number, doctors are able to determine when one should start their ART in order to fight the virus. However, reference values of these CD4+ T-cells have to be determined from the normal local population so as to know when the count is normal or low. The current reference values used are from a different population in Western countries and we need to have our own values from the Kenyan population and in this case from Nairobi residents.

Procedure of participation

Participation in this study is completely voluntary. If you agree to take part, I will ask you to fill out a blood donor recruitment card. Feel free to ask any question or clarification on anything you do not understand. I will take your blood pressure

and haemoglobin level to make sure you are further fit to donate blood. I shall then ask you to go ahead and donate blood from a vein in your arm equivalent to half a litre or 500Mls. All the materials that will be used are clean and completely safe. They have never been used before and will be thrown away after use.

After donation, small volumes of blood will be collected into three tubes from the blood bag. I will put a study number, but not your name, on the tubes with your blood so that nobody else will be able to know your results. The blood will be sent to the National Blood Transfusion Services to be tested for HIV, Syphilis and Hepatitis B and C. Once the results are satisfactory the blood will also be taken to the National Reference laboratory for CD4+ and CD8+ cell counts. Here is some information on these infections and an appointment card with your study number, the date when the results will be ready and place to collect them.

Benefit to the participant

Your participation in this study will be highly appreciated. Your participation will be part of a contribution in helping to provide quality health care to those infected with HIV. You will further benefit fro free counseling, knowing your health status through a series of free specialized tests. You also gain the opportunity to learn and ask questions concerning the stated diseases and their prevention.

Incase of any further information you might need you can contact me or any of the study in charge.

1. Lead study investigator: Robert M. Gatata

P.O BOX 1509-01000

Thika.

Tel. 0722397812

2. National Reference Laboratory

The laboratory manager,

P.O BOX 19361-00200

Nairobi.

Tel.2729549

Please sign the consent script to confirm your consent to voluntarily participate in the study and have understood the terms and conditions.

Signature…….. **Date……**

Appendix C: Serology results of excluded blood samples

	SEROLOGY TEST			
SEX	**HIV1& 2**	**VDRL**	**HEP B**	**HEP C**
FEMALE	NEG	NEG	**POS**	NEG
FEMALE	**POS**	NEG	NEG	NEG
FEMALE	NEG	NEG	**POS**	NEG
FEMALE	NEG	NEG	NEG	**POS**
FEMALE	NEG	NEG	NEG	**POS**
FEMALE	NEG	NEG	**POS**	NEG
MALE	NEG	NEG	**POS**	NEG
MALE	**POS**	NEG	NEG	NEG
MALE	**POS**	NEG	NEG	NEG
MALE	**POS**	NEG	NEG	NEG
MALE	**POS**	NEG	NEG	NEG
MALE	**POS**	NEG	NEG	NEG
MALE	NEG	NEG	**POS**	NEG
MALE	NEG	**POS**	NEG	NEG
MALE	NEG	NEG	**POS**	NEG
MALE	NEG	**POS**	NEG	NEG
MALE	NEG	NEG	**POS**	NEG
FEMALES =6	**F = 1**	**F=0**	**F=3**	**F=2**
MALES =11	**M = 5**	**M=2**	**M=4**	**M=0**
TOTAL = 17	**T = 6**	**T=2**	**T=7**	**T=2**
4% (17/400)	**1.4%**	**0.50%**	**1.70%**	**0.50%**

NEG- NEGATIVE
POS- POSITIVE
F-FEMALES
M-MALES
T-TOTAL