THE EFFECTS OF NUTRIENT SUPPLEMENTATION ON CD4$^+$ T CELL SUBSETS IN ZAMBIAN ADULTS

By

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A thesis submitted to the University of Zambia in fulfillment of the requirements of the degree (Doctor of Philosophy–Ph.D.) in Immunology

Statement of Own Work

I, **Caroline Cleopatra Chisenga,** confirm that the work presented in this thesis is my own.

It has been done in accordance with the guidelines for the theses for the University of Zambia and it has not been submitted elsewhere for a degree at this or another University. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: ……

Date: …….

Dedication

It is with my deepest gratitude and warmest affection that I dedicate this thesis to my loving husband

Mr. Abraham Chansa Chisenga,

Who for all his sacrifices too numerous to point out has been a constant pillar for my encouragement and wisdom.

To you I say may God continue to prosper and richly bless you.

Quote

"Even though it is the case that poverty is linked to AIDS, in the sense that Africa is poor and they have a lot of AIDS, it's not necessarily the case that improving poverty - at least in the short run, that improving exports and improving development - it's not necessarily the case that that's going to lead to a decline in HIV prevalence."- **Emily Oster**

Certificate of Completion of Thesis

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Abstract

Background: Although human immunodeficiency virus (HIV) infection is characterized by the progressive depletion of $CD4^+$ T cells, information on the prognostic value of T cell subsets and their response to nutritional intervention is scarce.

Objective: To estimate the prognostic value of T-cell subsets in Zambian patients initiating antiretroviral therapy (ART), to assess the impact of nutritional interventions on T-cell subsets in *in vitro* and *in vivo* and to establish the pattern of T-cell distribution in a healthy population.

Methods: Five studies were conducted that is, cross-section (HIV-negative adults), retrospective (NUSTART HIV-infected adults), randomised double blind (NUSTART HIV-infected adults), case-control (HIV-infected and HIV-negative adults) and the *in vitro* selenium dose response study (HIV-negative adults) between April 2013 and May 2015. Immunophenotyping was undertaken to characterize T-cell subsets using the markers CD3, CD4, CD8, CD45RA, CCR7, CD28, CD57, CD31, $\alpha4\beta7$, Ki67, CD25 and HLA-DR.

Results: In the cross section study of 51 healthy adults, the majority of the $CD4^+$ T-cells were naïve and least being cells expressing proliferating marker (Ki67). Retrospective results for the 267 records reviewed showed that there was a significant increase in total CD4 count at $p<0.0001$ from baseline to twelve weeks of receiving ART plus nutritional support. For the randomised double blind study, among 181 adults enrolled, 36 (20%) died by 12 weeks after starting ART. In univariate analysis, patients who died had fewer proliferating, more naïve and fewer gut homing $CD4^+$ T-cells compared to survivors; and more senescent and fewer proliferating $CD8^+$ T-cells. In a

multivariate Cox regression model high naïve $CD4^+$, low proliferating $CD4^+$, high senescent $CD8^+$ and low proliferating $CD8^+$ subsets were independently associated with increased risk of death. Recent $CD4^+$ thymic emigrants increased less between recruitment and 12 weeks of ART in the intervention group compared to the control group.

Case-control results for 50 healthy adults and 50 HIV-infected adults receiving ART treatment plus nutritional intervention for three months showed that by three months T-cells were not comparable to the healthy population. And lastly, in the in vitro selenium dose response study, most of CD4 and CD8 T cell subsets showed significant response to selenium. These results mean that selenium might have transcriptional effects.

Conclusions: Although we found that high naïve $CD4^+$ and high senescent $CD8^+$ T cells were not protective against early mortality in HIV-infected adults, most of the T cells in the healthy population equally were naïve. Thus, measuring specific $CD4^+$ T-cell subsets should be considered for prognostic significance in patients initiating ART in Zambia. Furthermore, although there was some good response in the *in vitro* study, only thymic output responded to this nutritional intervention.

Acknowledgments

I would like to sincerely thank all those who made this work possible: my wise and encouraging supervisors **(Prof. Paul Kelly, Prof. Suzanne Filteau and Dr. Andrew J. Prendergast)**, the energetic and diligent NUSTART team, TROPGAN team, SACORE administrators, SACORE PhD scholars and my supportive TROPGAN colleagues and friends, **Patrick Kaonga** and **Mpala M. Lisulo** for the excellent technical assistance in this study.

List of Publications During the Course of Study

1. **Chisenga CC**, Filteau S, Siame J, Chisenga M, Prendergast AJ, Kelly P (2015) T-Cell Subsets Predict Mortality in Malnourished Zambian Adults Initiating Antiretroviral Therapy. PLoS ONE 10(6): e0129928. doi:10.1371/journal.pone.0129928.
2. **C. C. Chisenga** and P. Kelly (2014) The Role of Selenium in Human Immunity. Medical Journal of Zambia, Vol. 41, No. 4: 181-185.
3. Zulu JM, Lisulo MM, Besa E, Kaonga P, **Chisenga CC**, Mumba C, Simuyandi M, Banda R and Kelly P. (2014). Improving Validity of Informed Consent for Biomedical Research in Zambia Using a Laboratory Exposure Intervention. PLoS ONE 9(9): e108305. doi:10.1371/journal.pone.0108305.
4. **C. C. Chisenga**, Suzanne Filteau, Andrew J. Prendergast, Paul Kelly. Selenium responsive α4β7 integrin expression on T lymphocytes in Zambian adults with and without Se deficiency. Manuscript under review.

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List of Abbreviations

AICD	Activated Induced Cell Death
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral Drugs
BMI	Body Mass Index
CCR7	C-Chemokine Receptor 7
CD	Cluster of Differentiation
DAIDS	Division of AIDS
DNA	Deoxyribonucleic Acid
DSMB	Data and safety Monitoring Board
FACS	Fluorescence Activated Cell Sorter
FACS	Fluorescence-Activated Cell Sorter
Fe	Iron
GALT	Gut Associated Lymphoid Tissue
HAART	Highly Active Anti retroviral Therapy
HIV	Human Immunodeficiency Virus
IRIS	Immune Reconstitution Inflammatory Syndrome
LNS	Lipid-based nutrient supplement
LNS-VM	Lipid-based nutrient supplement with vitamins and minerals
NF-KB	Nuclear factor Kappa B
NGO	Non-governmental organisation
NUSTART	Nutritional Support for African Adults Starting Antiretroviral Therapy
OIs	Opportunistic Infections
SAEs	Serious adverse events
Se	Selenium
TB	Tuberculosis
TCR	T cell Receptor
UNAIDS	United Nations Acquired Immune Deficiency Syndrome
WHO	World Health Organization

Thesis outline

Chapter 1 of this thesis is a brief outline and scope of the work.

Chapter 2 of this thesis outlines the main Nutritional Support for Africans Starting Antiretroviral Therapy (NUSTART) trial and the concepts that gave birth to the sub study.

In **chapter 3**, a detailed review of the literature describing the global effects of the HIV is given. The focus in this chapter is on prevalence, pathogenesis, mortality, coverage of antiretroviral therapy (ART) and the dynamics of the $CD4^+$ and $CD8^+$ T cell following infection. Finally, what is known about the impact of nutritional interventions on immune reconstitution is described.

Chapter 4 set out the study justification, aims and objectives.

Chapter 5 describes the methods for analysing T cell subsets using flow cytometry as well as statistical analysis. Other details such as gating strategies, panel design and antibody titrations are provided in the appendices.

Chapter 6 is the results chapter. Firstly, the normal distribution of T cell subsets in healthy Zambian volunteers is described (Cross sectional study). Secondly, a sub-study of the NUSTART trial, including the relationship between T cell subsets and mortality, and the impact of the nutritional intervention on T cell subsets (RCT and retrospective studies) is described, thirdly, T cell subsets of the healthy Zambian volunteers are compared to the HIV-infected adults in order to understand the impact of ART plus nutritional supplementation after 3 months (Survey). Finally, the impact of selenium on T cell subset marker expression during *in vitro* incubation is described.

Chapter 7 is the discussion chapter. In this chapter, the relationship of all the studies is explained including the strengths and limitations of each study.

Chapter 8 provides the overall conclusions and future work from the studies conducted.

Chapter 1: Introduction

Although data from previous studies show that there is $CD4^+$ and $CD8^+$ T cell depletion in HIV-infected individuals, data on how the immune system reconstitutes is still unclear. Studies of micronutrient supplementation in HIV-infected patients starting ART have yielded conflicting results on its effects on morbidity and mortality. There has been little research showing the effects of micronutrients on immune reconstitution especially in malnourished HIV-infected adults.

Therefore, this thesis describes research designed to understand;

1. The pattern of distribution for T cell subsets in a healthy population.
2. The immunological changes which occur during initiation of antiretroviral therapy, and the impact of nutritional supplements on T cell subsets.
3. T cell recovery after ART treatment.
4. T cell responses to soluble selenium (Se) in *in vitro*.

Part of the work was nested in the NUSTART trial, described below, which was a phase III randomised controlled trial of a lipid-based nutrient supplement (LNS) with or without vitamins and minerals given during the first 12 weeks after referral for ART. The NUSTART trial was initiated in 2010 in Lusaka Zambia at the University Teaching Hospital and in Mwanza, Tanzania.

Chapter 2: Background

2.1. NUSTART main trial and sub-study overview

The work described in this thesis was a sub-study of a randomised controlled trial of a nutritional supplement for patients initiating ART, the NUSTART trial.

NUSTART was a randomised controlled trial of a vitamin and mineral intervention for HIV-infected adults initiating ART. Participants were recruited in Lusaka, Zambia and Mwanza, Tanzania from August 2011 to December 2013. NUSTART was a phase III individually randomised double blind controlled trial which compared, in a two-stage protocol, vitamins and minerals in a lipid-based nutritional supplement (LNS-VM) with unfortified LNS given from recruitment (referral for ART) until 6 weeks after starting ART. In the first stage, during the first two weeks after ART initiation, the active and control supplements were given with minimal calories i.e. 30 g/day, 100 kcal/day, then from 2-6 weeks after ART initiation, patients were given 250 g/day, in two 125 g sachets, comprising 1360 kcal/day in a calorie-rich supplement. Participants in this trial were allocated to receive nutritional supplements as shown in Table 2-1.

Table 2-1: Nutritional composition of trial supplements – amounts per day[1,2]

Nutrient	First phase supplement (from recruitment to 2 weeks of ART)		Second phase supplement (from 2 to 6 weeks of ART)	
	LNS-VM (30 g)	LNS (30 g)	LNS-VM (250 g)	LNS (250 g)
Calories (kcal)	139	168	1397	1416
Protein (g)	2.4	2.3	55	55
Fat (g)	11.0	10.9	97.5	97.5
Potassium (mmol)	30	0.9	32	15.8
Phosphorus (mmol)	47	0.4	38	9.3
Magnesium (mmol)	16	0.3	17	5.7
Calcium (mg)	29.8	5.0	140	115
Iron (mg)	0.4	0.4	14.7	8.4
Zinc (mg)	21	0.2	21	3.8
Copper (mg)	3.6	0.06	3.6	1.2
Manganese (mg)	4.2	-	4.2	-
Iodine (μg)	420	-	420	-
Selenium (μg)	180	-	180	-
Chromium (mg)	75	-	75	-
Retinol (as palmitate) (mg)	1800	-	1800	-
Vitamin D (μg)	10	-	10	-
Vitamin E (mg)	45	-	45	-
Vitamin K (mg)	95	-	95	-
Vitamin C (mg)	120	-	120	-
Thiamin (mg)	2.4	-	2.4	-
Riboflavin (mg)	3.3	-	3.3	-
Niacin (mg)	39	-	39	-

Pyridoxine (mg)	3.6	-	3.6	-
Folate (mg)	600	-	600	-
Vitamin B12 (mg)	4.5	-	4.5	-
Pantothenic acid (mg)	9	-	9	-

[1]Where nutrient contents are provided for both LNS and LNS-VM, these are values from analysis by the manufacturer, accounting for inter-batch variability; where values for only LNS-VM are given, these where not assessed in the prepared foods but refer to amounts added, that is, they do not include those innate to the LNS.

[2]ART=antiretroviral therapy; LNS=lipid-based nutritional supplement, LNS-VM=LNS with added vitamins and minerals.

2.1.1 NUSTART Trial Study Objectives

Primary:

- To assess the effect on mortality between referral for ART and 12 weeks after starting ART of a lipid-based nutritional calorie supplement (LNS) with additional vitamins and minerals (LNS-VM) compared to LNS alone. Both LNS and LNS-VM were given in two stages, with lower calories and no iron (Fe) initially, and a higher calorie intake later to permit weight gain.

Secondary:

- To determine if LNS-VM, compared to LNS alone, could:
  - Decrease admissions to hospital during the study period;
  - Increase body mass index (BMI) and lean body mass;
  - Increase serum phosphate;
  - Reduce total serious adverse events (SAE);
  - Increase grip strength, a measure of functional lean body mass;
  - Cause appetite to return more rapidly, enabling nutritional recovery;
  - Increase adherence to ART;
  - Improve immune function;
  - Improve functional status and/or quality of life;
  - Improve program retention;
- To assess the effect of the intervention on serum electrolyte shifts and iron status early in ART.

2.1.2 NUSTART Participant Eligibility, Recruitment and Location

Male and female patients were recruited from six different clinics (Chilenje, Kanyama, Mtendere, Kamwala, Chipata and Chawama) that manage ART initiation and support. The support staff (nurses) in various clinics confirmed eligibility using patient HIV, $CD4^+$ and BMI results. Eligible candidates were informed about the study and if willing transported to University Teaching Hospital (UTH). At UTH, participants were provided with detailed talks using the information sheet and consent was obtained if willing to participate and interested individuals were recorded (mobile number, house number and landmarks) and appointments for next visits set. If a participant declined to participate, recruitment form was completed and any reasons for not participating listed.

Inclusion criteria were:

- At least 18 years old
- ART-naive
- BMI < 18.5 kg/m^2. In the presence of clinical oedema and a BMI < 20 kg/m^2, BMI was re-measured after loss of oedema, and the patient considered eligible if BMI < 18.5 kg/m^2 and ART had not yet been initiated
- Requiring ART was determined by CD4 count < 350/μL or stage 3 or 4 disease
- Willing to undertake intensive ART follow-up in the study clinic
- Providing written, fully informed consent (thumbprint was accepted)

Exclusion criteria was the non-fulfilment of inclusion criteria above plus:

- Participation in a potentially conflicting research protocol
- Pregnancy (by self-report)

The interventions were not expected to have any adverse effects during pregnancy. However, the exclusion of women known to be pregnant was to avoid the changes in anthropometry and body composition found especially in late pregnancy. Women were also asked to exit from the trial and excluded them from body composition analyses if they were pregnant (either by self report or after examination).

In Zambia it is policy to give a small micronutrient supplement (240 µg vitamin A, 2.5 µg vitamin D and 0.5 mg each of thiamine, riboflavin, pyridoxine and pantothenic acid) to patients entering HIV care, whether they require ART or not. This supplement was withheld for the period (usually <16 weeks) of the NUSTART intervention, and re-introduced after exit from the trial. Open label pyridoxine was given to all patients also starting TB therapy in order to prevent drug-induced neuropathy (damage to peripheral nerves due to isoniazid). In addition, patients (estimated < 1%) with critically low serum electrolytes (phosphate < 0.65 mmol/l, potassium < 2.5 mmol/l, magnesium< 0.45 mmol/l) were admitted for close observation and were given oral doses of the minerals as required according to a standard algorithm.

2.1.3 Assessment for Adherence

Adherence to both LNS-VM and LNS was monitored by questionnaires at all visits and by asking participants to keep empty LNS packages and bring them to their next clinic visit. All participants were given dietary advice to increase their caloric intake and to eat a variety of foods to provide micronutrients. Those too poor to afford such foods were referred to the ART and food security program in Zambia and to local services such as non-governmental organisations (NGOs) and church groups that support HIV-affected families.

2.1.4 Randomization and Blinding

Randomization was in blocks of 16 and stratified by country with a separate computer-generated randomization list for each country. The statistician from the Data Safety and Monitoring Board generated and held the code. Two codes were prepared: (1) an **allocation code** (letters A to H) indicating the contents of the packets (intervention vs. control) known only to the producer and the DSMB statistician, and (2) a **randomization code** related to study ID numbers and held by the DSMB statistician and study pharmacists. The manufacturer (Nutriset SA, Malaunay, France) supplied nutriset satches labelled with the allocation code. The clinic pharmacists further labelled packets with study ID numbers when dispensing. Participants were given sequential IDs (within clinics) after having signed consent forms and completed baseline assessments.

2.1.5 Clinical Outcomes

Before initiating ART, as part of routine care, patients were screened, commenced on treatment for opportunistic infections, and counselled regarding lifelong treatment adherence. During this pre-ART period, the first stage study interventions were introduced. Medical care was provided primarily by local health services though study staff treated and referred as necessary during patients' study visits. Patients were seen weekly from referral for ART for 2 weeks, then optional weekly visits until the ART initiation visit, then at 2, 6, 8, and 12 weeks after starting ART. Additional optional visits were available at 1 and 4 weeks after ART initiation and patients who were ill came for unscheduled visits at any time. In addition to standard medical care, other tests done were as summarized in Table 2-2 below.

Table 2-2: Summary of study tests

	Consent	**Baseline**	**Pre-ART**				**ART**						
Time (weeks)	**N/A**	**0**	**1**	**2**	**(3)**	**...**	**0**	**(1)**	**2**	**(4)**	**6**	**8**	**12**
Nutritional intervention		x	x	x	x	x	x	x	x	x	x		
Weight		x	x	x	x	x	x	x	x	x	x	x	x
CD4 count		x											x
Haemoglobin		x											x

Some visits were optional, and patients decided to attend if they chose to; these are denoted in brackets.

Information sheet

On the information sheet, participants' characteristics were filled in as follows;

a) Sex

b) Age

c) Weight

d) Height

e) Education

f) Occupation

g) Marital status

Ascertainment of outcome and follow-up

The primary endpoint for the main NUSTART trial was mortality between the time of recruitment (referral for ART) and 12 weeks after starting ART. NUSTART study participants were followed up weekly before starting ART and at weeks 2, 4, 6, 8 and 12 after ART initiation. Whether the patient was still alive, and the dates of death if not, were ascertained by phone contact or home visit if the patient failed to return to the next scheduled visit.

2.1.6 Ethics

Ethical approval was obtained from the research ethics committee of the London School of Hygiene and Tropical Medicine, the University of Zambia Biomedical Research Ethics Committee (refer to appendix 6 and 7), and the Medical Research Coordinating

Committee of NIMR, Tanzania. The NUSTART trial was registered as PACTR201106000300631. Funding was provided for the NUSTART trial by the European and Developing Countries Clinical Trials Partnership grant # IP.2009.33011.004. All participants provided written or thumbprint informed consent. Medical care of patients was according to national guidelines and provided through the local health services. Patients with low levels of serum electrolytes according to **Division of AIDS (**DAIDS) criteria were provided appropriate electrolyte therapy.

2.2 Sub-study overview

The sub-study was designed to explore the effects of the nutritional supplement on absolute $CD4^+$ and $CD8^+$ T cells and their subsets, and also to determine whether an association exists between $CD4^+$ and $CD8^+$ T cell subsets and mortality.

2.3 Hypothesis tested in this sub-study

Combined lipid-based nutritional supplementation with added minerals and vitamins (LNS-VM) is associated with improved early (12 week) absolute $CD4^+$ and $CD8^+$ T cell reconstitution and their subsets among malnourished HIV-infected adults starting ART compared to a lipid-based supplement (LNS) alone.

Chapter 3: Literature Review

3.1 Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV) is a lentivirus that belongs to the family of retroviruses. About 75% of the HIV-infections worldwide are transmitted via sexual intercourse (Quinn, 1996). In humans, HIV infects immune cells like $CD4^+$ T cells, macrophages and dendritic cells. The first case of acquired immunodeficiency syndrome (AIDS) was reported in 1981 (Dehne et al., 1992). Two types of HIV (HIV-1 and HIV-2) exist and both are known to infect humans. Of the two, HIV-1 is widely distributed whereas; HIV-2 is almost entirely restricted to West Africa (Schim and Aaby, 1999).

The major contributor to increased susceptibility to infectious disease during HIV-infection is a lowered CD4 cell count due to destruction by the HIV (Dunham et al., 2008). Delayed treatment often result in depletion of these immune cells ($CD4^+$), and ultimately in AIDS, which is characterised by the development of opportunistic infections and tumours (Quagliarello, 1982).

About 0.8% of adults aged 15–49 years worldwide are infected with HIV, with Sub-Saharan Africa being most severely affected as shown in Figure 3-1 (WHO, 2013).

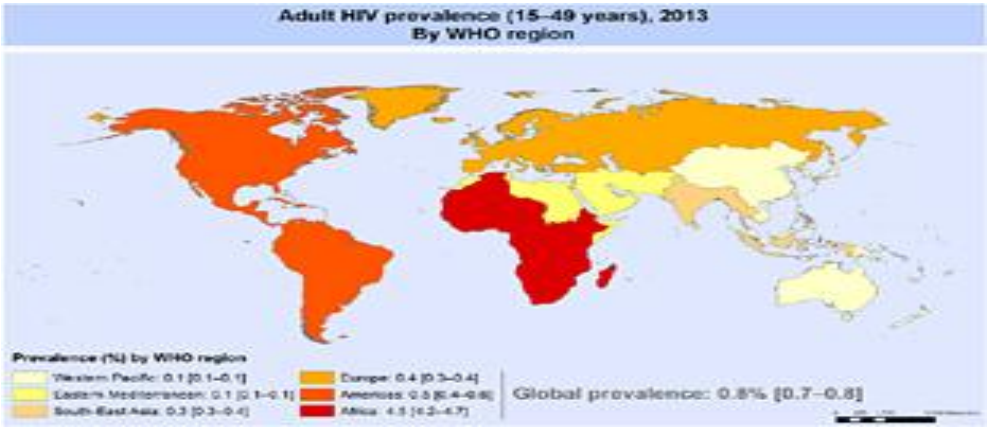


Figure 3-1: HIV prevalence (WHO, 2013)

About 35 million people are living with HIV globally (WHO, 2013).

According to the UNAIDS 2013 global report (Figure 3-2), the number of people on antiretroviral treatment has tripled in all regions of the world. However, in Eastern Europe, Central Asia, the Middle East and North Africa the rise is lower (UNAIDS, 2013).

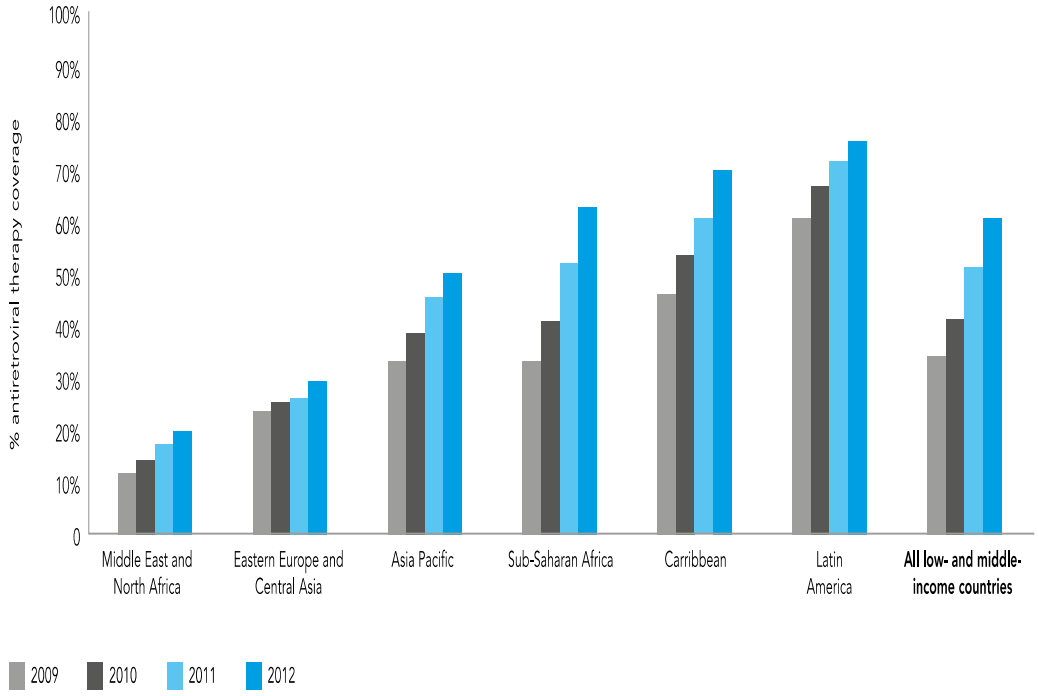


Figure 3-2: Global percentage of people receiving ART by region, 2009–2012 (UNAIDS, 2013)

In Sub-Saharan Africa, possibly because of increased access to antiretroviral therapy (ART), there has been a slow but steady decrease of new HIV-infections since 2001 (Figure 3-3) (Li et al., 2012; DHS, 2014).

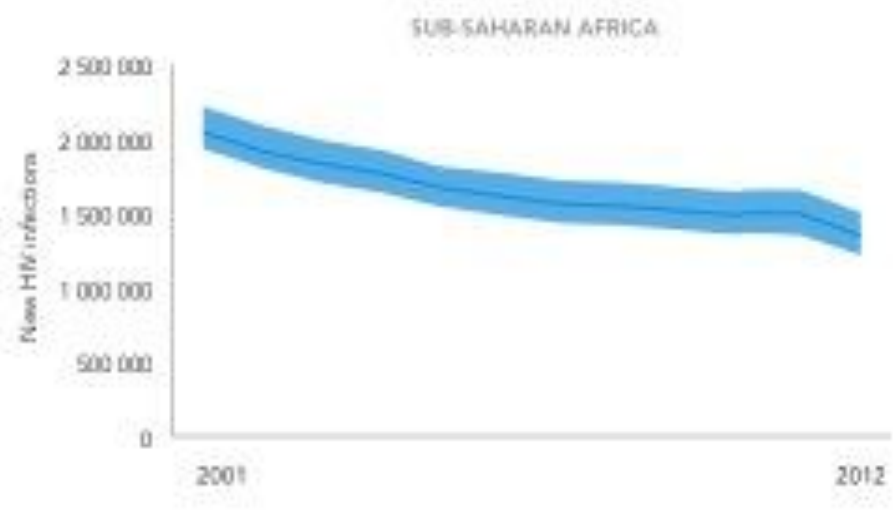


Figure 3-3: Global new HIV infections (UNAIDS, 2013).

HIV/AIDS related mortality remains a leading global cause of mortality (Ortblad et al., 2013) (Figure 3-4). However this picture is changing with the scale-up of ART.

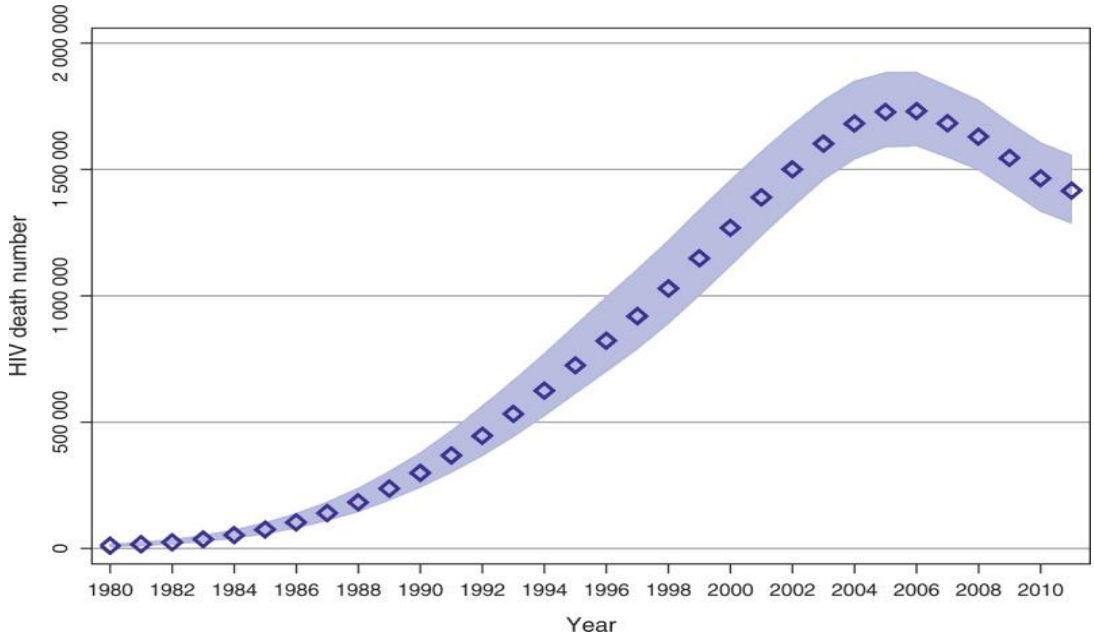


Figure 3-4: Global HIV/AIDS death from 1980-2010 (Ortblad et al., 2013)

There has been a decline in the annual number of AIDS-related deaths in people receiving ART, from a high of 2.3 (2.1–2.6) million in 2005, to 1.6 (1.4–1.9) million (Figure 3-5) in 2012 (UNAIDS, 2013).

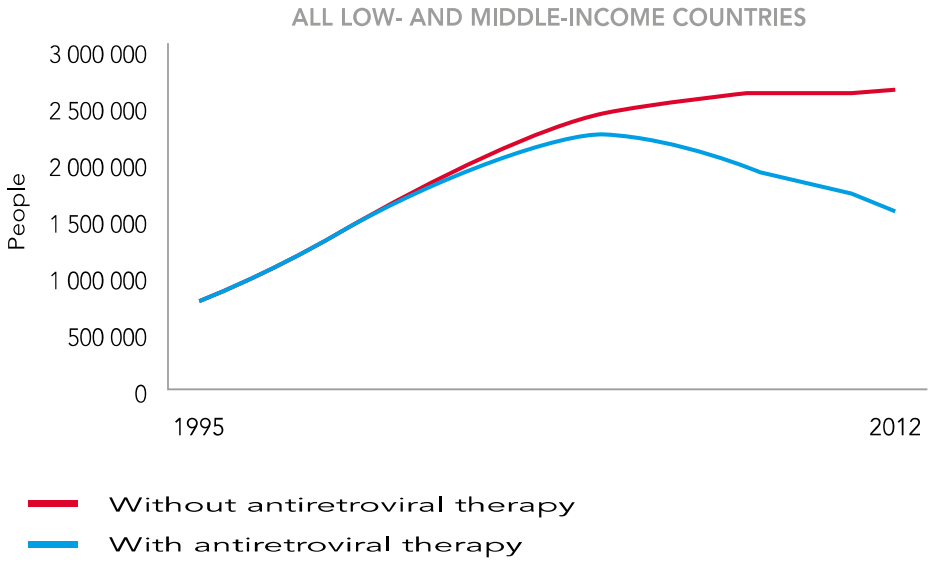


Figure 3-5: Estimated numbers of AIDS-related deaths, with and without antiretroviral therapy, in low and middle-income countries 1995–2012 (UNAIDS, 2013)

3.2 Lymphocyte Development

Lymphocytes differentiate from stem cells in the bone marrow (Figure 3-6). B cells develop entirely in the bone marrow. T cells mature in the thymus (located above the heart) (Janeway et al., 2001).

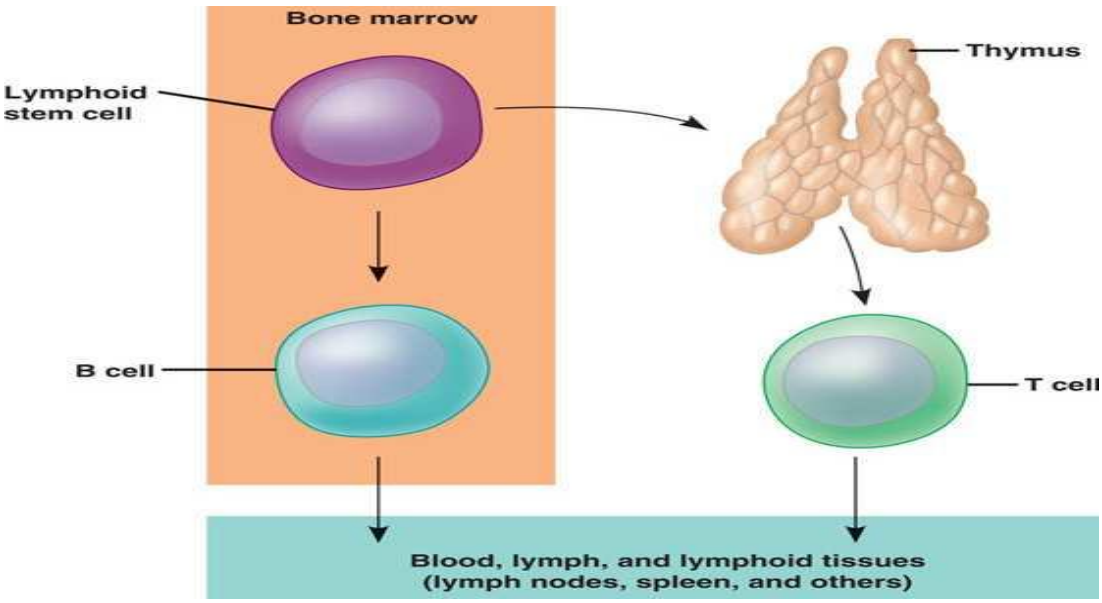


Figure 3-6: Lymphocyte development gotten from http://bio1152.nicerweb.com/Locked/media/ch43/43_10LymphocyteDevelop_L.jpg

3.3 $CD4^+$ T Lymphocyte Development

The thymus is a specialized organ for T cell development, in which T cell receptors are generated with a huge range of antigen specificity by the process of gene rearrangement (Petrie et al., 1992). Two lineages of T cells (αβ and γδ) are generated in the thymus through a process that is highly regulated and limits the production of cells bearing non-functional or auto reactive T cell receptors (TCRs). These two lineages are characterized by the expression of distinct αβ– or γδ–TCR complexes. Finally, key cell-surface molecules, namely the CD4 and CD8 co-receptors are expressed. Immature subsets of thymocyte precursors lack expression of both CD4 and CD8 and are known as double negatives (DN). Thus, at this critical stage progenitors become committed to the αβ or γδ T cell lineage (Petrie et al., 1992; Dudley et al., 1995; Ciofani et al., 2006). The αβ lineage thymocytes then progress to the CD4 CD8 double positive (DP) stage and undergo further positive and negative selection. This process produces major histocompatibility complex (MHC)-restriction and self-tolerance, in CD4 and CD8 single positive (SP) T cells (Starr et al., 2003). The resulting mature γδ T cells, and αβ T cells comprising the $CD8^+$ cytotoxic, $CD4^+$ helper, and regulatory lineages, form the foundation of cellular immunity.

Consequently, $CD4^+$ T cells mature into functionally heterogeneous subsets from a common lineage via a process of division, migration, selection, differentiation, and proliferation (Starr et al., 2003). $CD8^+$ cells, also known as cytotoxic T cells, kill infected cells (cancer cells or cells infected with the virus). During HIV-infection, activated $CD8^+$ cells kill $CD4^+$ cells infected with the HIV. Thus, as many cells are infected, many are destroyed resulting in depletion of $CD4^+$ cells beyond the minimum requirement (Lyles et al., 2000; Masel et al., 2000).

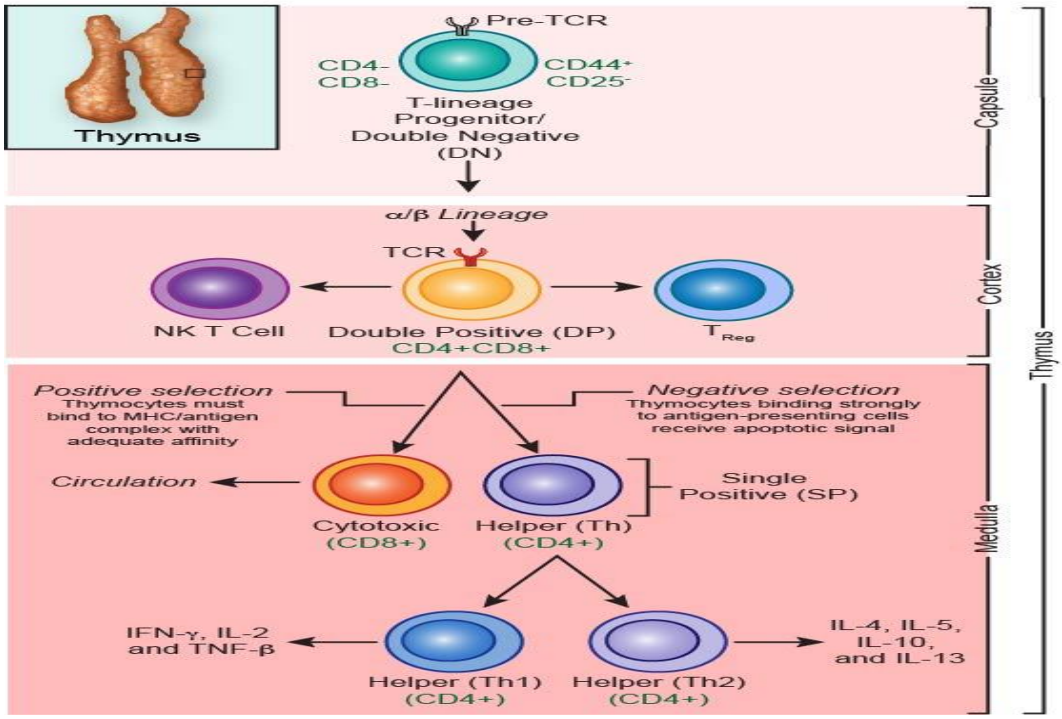


Figure 3-7: CD4$^+$ T lymphocyte development (source: https://mynotebook.labarchives.com)

3.4 HIV Immunopathogenesis

HIV-1 usually gains entry to its human host by crossing mucosal surfaces (Quinn, 1996) and subsequently disseminates throughout the lymphatic tissues (LTs). LTs are reservoirs where the virus is produced and stored throughout the long course of infection (Tenner-Racz et al., 1988; Parmentier, et al., 1990; Embretson, et al., 1993). Following infection, HIV infects and replicates in the vaginal or rectal memory T cells expressing CD4$^+$ and CCR5$^+$ markers (Mogensen et al., 2010). Highest depletion of CD4$^+$ and CCR5$^+$ cells occurs in the gut associated lymphoid tissue (GALT) where the majority of these cells reside. Throughout the course of infection, CD4$^+$ T cell count in blood slowly decreases, and at the AIDS-defining level of 200 cells/μL, opportunistic tumours and infections (OIs) appear that eventually claim the lives of most infected individuals (Pantaleo, et al., 1993).

Progression to AIDS and death is correlated with the amount of viral RNA in the blood

stream (Mellors, et al., 1996), which in turn reflects virus production in LT (Haase, et al., 1996). Increased HIV replication leads to immune cell activation and is directly proportional to the viral load. Hyper-activation is denoted by expression of activation markers (CD38 and HLA-DR) on T cells (Mogensen et al., 2010). Additionally, bacterial products (such as lipopolysaccharide) entering the blood system from a compromised GALT during HIV-infection also contribute to a continuous immune activation shown by the red line in Figure 3-8 (Mogensen et al., 2010). Highly active antiretroviral therapy (HAART) has also contributed to persistent immune activation due to virus latency, in that latently infected cells might lead to the regeneration and maintenance of the viral reservoir without cell death (Tyagi and Bukrinsky, 2012).

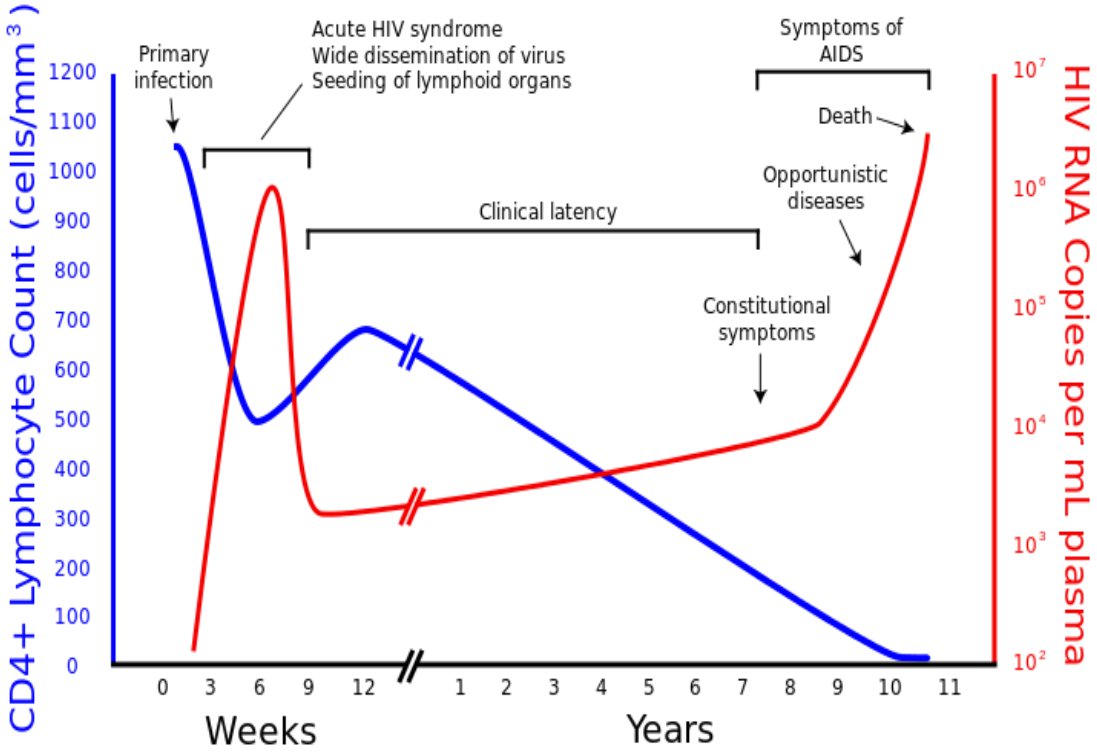


Figure 3-8: HIV pathogenesis (Mogensen et al., 2010)

The biggest current challenge is failure of ART to eradicate the virus. Even when the virus is suppressed to undetectable levels for many years, it quickly re-emerges if the treatment is stopped (Chomont et al. 2009).

The memory $CD4^+$ T lymphocytes are the main reservoirs of latent HIV. These cells harbour a set of integrated proviruses that are unable to complete their lifecycle because of the lack of suitable conditions and are called latent or "transiently silent" proviruses (Siliciano et al., 2003; Siliciano et al., 2011).

Latent proviruses are well protected from antiretroviral therapies that target actively replicating viruses, and from the host's immune system, which fails to differentiate infected cells from uninfected cells due to the lack of viral activity in latently infected cells (Pierson et al., 2000). These hibernating latent proviruses wait for favourable conditions and consequently are the source of replication-competent viruses (Tyagi and Bukrinsky, 2012).

Evidence from Brenchley and Douek confirm another potential mechanism that could be central in HIV pathogenesis by the activation of the innate immune system (Brenchley and Douek 2006). Massive depletion of $CD4^+$ T cells (and perhaps macrophages and dendritic cells) by HIV-1 in mucosal lymphoid tissues result in disruption of the different immune components constituting the mucosal barrier in the gut. This barrier prevents the translocation of intestinal flora and confines pathogens to the lamina propia and the mesenteric lymph nodes. Compromising the integrity of the gut often results in microbial translocation to the systemic immune system (Brenchley and Douek, 2006). Previous studies also report that HIV-1-infection is associated with a significant increase of plasma lipopolysaccharide (LPS) levels, an indicator of microbial translocation, which is directly correlated with immune activation (Brenchley and Douek, 2006). Thus, bacterial translocation may result in an overwhelming activation of the innate immune response.

Activation of T cells also increases intracellular nuclear factor kappa B (NF-κB) levels. NF-κB enhances the transcription of integrated virus and, therefore, the production of

new virions that infect new targets (Kawakami et al., 1988). It is therefore essential to understand that the consequence of immune activation in HIV-infection goes far beyond the simple loss of virus-specific $CD4^+$ T cells and extends to a global decline of the immune resources.

HIV-infection also results in a deregulation of haematopoiesis (lower numbers of progenitor cells and decline in their ability to generate new cells) (Marandin et al., 1996; Jenkins et al., 1998; Moses et al., 1998). The capability of the thymus to manufacture new fully differentiated ($CD27^+$, $CD28^+$, $CCR7^+$ and $CD45RA^+$) cells is reduced significantly in HIV-infected individuals (Douek et al., 1998). This could be as a result of several reasons such as the direct infection of the thymic stroma and thymocytes by HIV (Schnittman et al., 1990; Stanley et al., 1993) and the atrophy of the thymus in HIV-infected subjects, which is similar to age-related 'thymic involution' (Kalayjian et al., 2003) and is associated with thymosuppressive effects of proinflammatory cytokines such as IL-6 (Sempowski et al., 2000; Linton et al., 2004). Hence, it is clear that HIV research activities are progressively enabling researchers to understand the sophisticated mechanisms that link HIV-infection to the onset of immunodeficiency. The infection and depletion of $CD4^+$ and $CD8^+$ T cells represent the most fundamental events in HIV-infection. However, in recent years, the role-played by chronic immune activation and surface marker expression during HIV pathogenesis is becoming increasingly apparent. Paradoxically, immune activation markers are directly linked to HIV disease progression.

3.5 $CD4^+$ T cell Dynamics During HIV-Infection

Since $CD4^+$ cells and their subsets are the target cells of the HIV, their loss, exceeding replacement, leads to T cell deficiency (Ho et al., 1995; Wei et al., 1995; Dunham et al., 2008). The peripheral blood CD4 count is therefore, a very useful marker of the severity of HIV-related immune dysfunction, and there is extensive literature on the relationship between CD4 cell counts and morbidity and mortality in HIV-infection. However, the proportionate losses of T cell subsets, which bear specific cell surface markers, have not been thoroughly studied. The following section describes the cell surface markers that are relevant to the work in this thesis.

3.5.1 Ki67

Ki67 is a nuclear antigen that is expressed exclusively by cells that are in cell cycle and is frequently used as a surrogate marker for T cell proliferation. Ki67 expression increases in late G1 phase of the cell cycle and remains elevated throughout mitosis (Gerdes, et al., 1984). Although the function of Ki67 is not entirely defined, Ki67 molecules expression may be important for cellular proliferation and survival.

Ki67 is not expressed in resting cells and, therefore, makes this molecule a suitable marker of cell cycle progression. Ki67 expression among naïve $CD4^+$ T cells from HIV-infected individuals demonstrates that naïve $CD4^+$ T cells from HIV-infected patients have qualitative defects in cell cycle progression (Sieg, et al., 2003). Rapid turn over of the Ki67 marker on T cells indicates increased immune activation and if it is in HIV infected individuals, increased virus (Zack et al., 1990; Bukrinsky et al., 1991).

3.5.2 Cluster of Differentiation 31

The HIV is known to cause thymic T cell production impairment and a sustained immune activation (Mackall et al., 1995; Hellerstein et al., 2003; Hakim et al., 2005; Lee et al., 2006). The thymus is the site of T cell development, producing a variety of T cells including natural T-regulatory cells, and naïve T cells. Cluster of differentiation 31 (CD31), also known as platelet endothelial cell adhesion molecule-1 (PECAM-1) is expressed on T cells, platelets, monocytes, granulocytes and endothelial cells. It can mediate T cell adhesion and inhibit T cell activation (Tanaskovic et al., 2010). CD31 expression on $CD4^+$ T cells seems to indicate recent thymic emigrants (Nickel et al., 2005; Bofill et al., 2006). Naïve $CD45RA^+$ T cells co-expressing CD31 (a sure recent thymic emigrant definition) have a higher T cell receptor excision circles (TREC) content than $CD45RA^+$ cells lacking CD31 expression (Kimmig et al., 2002; Junge et al., 2007).

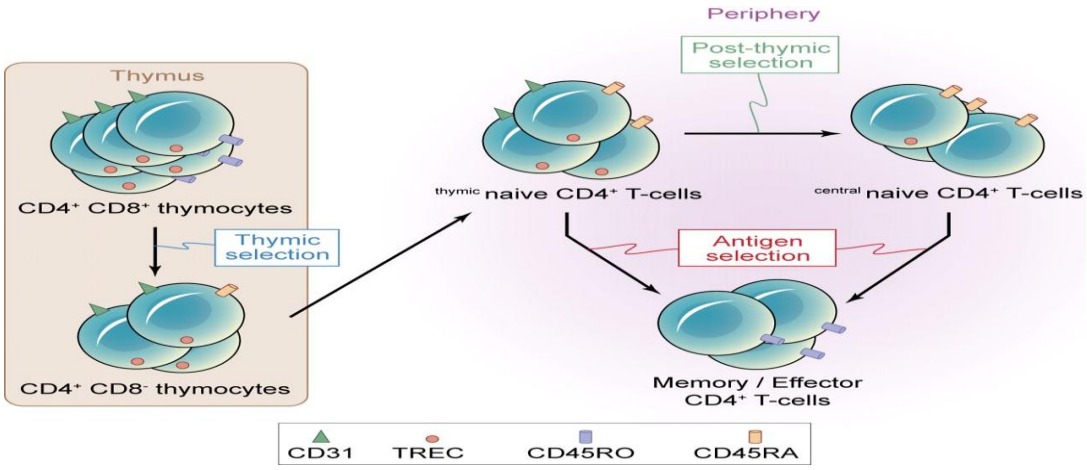


Figure 3-9: Post thymic development of $CD4^+$ T cells (Kohler and Thiel 2009)

CD31 expression on $CD4^+$ T cells is lost after repetitive TCR stimulation *in vitro* (Stockinger et al., 1992; Demeure et al., 1996) and so may also be lost after post-thymic peripheral expansion. Most CD8 cells express CD31 as such; expression of CD31 is relatively high on $CD8^+$ T cells compared to $CD4^+$ T cells (Stockinger, et al., 1992).

3.5.3 CD45RA

CD45 is a protein tyrosine phosphatase regulating src-family kinases, and is expressed on all hematopoietic cells. CD45 can be expressed as one of several isoforms by alternative splicing of exons that comprise the extracellular domain. CD45RA is expressed on naïve T cells, as well as the effector cells in both CD4 and CD8. After antigen experience, central and effector memory T cells gain expression of CD45RO and lose expression of CD45RA (Sanders et al., 1988). Thus, either CD45RA or CD45RO is used to generally differentiate the naive from memory populations (Sanders et al., 1988).

CD45 is also highly glycosylated and naïve T lymphocytes express large CD45 isoforms and are usually positive for CD45RA. CD45RA is found on naïve T cells and CD45RO is located on memory T cells (Murphy, 2012). During early HIV-infection, there is a reduction in naïve $CD4^+$ T cells ($CD45RA^+$), which are progressively depleted because of their frequent activation and differentiation into memory cells caused by chronic and high antigenic stimulation (Bandera et al., 2010). This increase in proliferating $CD4^+$ T cells correlates with high viral load (Koopman et al., 2009).

3.5.4 CC-Chemokine Receptor 7

Chemokines are key controllers of leukocyte trafficking (Murphy 2012). The expression of most chemokines is induced during infection and inflammation (Forster et al., 2008). Certain chemokines, including CC-chemokine ligand CCL19 and CCL21 are constitutively expressed and control cell movement during homeostasis (Rot et al., 2004). CCL19 and CCL21 are ligands for the CC-Chemokine Receptor 7 **(**CCR7) that are expressed by various subsets of immune cells (Rot et al., 2004). CCR7 and its ligands are involved in homing of various subpopulations of T cells and antigen-

presenting dendritic cells (DCs) to the lymph nodes. For example, CCL19 and CCL21 have been shown to increase the permissiveness of human resting memory $CD4^+$ T cells to HIV-1-infection (Rot et al., 2004). Although an interaction of different subsets of the immune and non-immune cells is required for efficient functioning (i.e. the ability of the cells to actively migrate to and within tissues) of the immune system.

3.5.5 Human Lymphocyte Antigen Class II

Human Lymphocyte Antigen Class II (HLA-DR) is a glycosylated cell surface transmembrane protein expressed on antigen-presenting cells and constitutively expressed on monocytes. Expression of HLA-DR by monocytes is essential for the presentation of peptides to CD4 positive T cells for initiating a specific immune response (Perry et al., 2004). Expression of HLA-DR on T cells is a marker of activation. Expression of HLA-DR on $CD4^+$ T cells is increased in HIV-positive individuals and becomes relatively more important marker with progression from the asymptomatic to the symptomatic disease stage (Kestens et al., 1994).

3.5.6 Alpha4 and Beta7 Integrins

The HIV enters into latency (a state of reversibly nonproductive infection of individual cells) during ART (Siliciano and Greene, 2011). However, replication continues in the gastrointestinal tract (GIT) and CD4 cells are depleted continually in this site (Brenchley et al., 2008; Hed et al., 2010). The body compensates this with the help of gut homing receptors (α4 and β7). T cells express several integrin family members that are involved in activation, trafficking, and retention in tissue (DeNucci et al., 2010). The α4 integrin on T cells associates with either the β1 subunit, to form α4β1 integrin, or the β7 subunit, to form α4β7 integrin. Both α4β1 and α4β7 are expressed at low

levels on naïve T cells (DeNucci et al., 2010). Lymphocyte migration to the intestine is through interaction of α4β7 with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is predominantly expressed on venules in the GALT and intestinal lamina propria (Berlin et al., 1995). Recent evidence also shows that HIV-1 gp120 binds and signals through α4β7 (Wang et al., 2009).

3.5.7 Cluster of Differentiation 25 IL-2R (Activation)

CD25 is a type I transmembrane protein present on activated T cells. In this study, CD25 was used as a marker of activation when HLA-DR could not be used due to fluorochrome overlap.

3.5.8 Cluster of Differentiation 57

HIV-infection is characterized by a generalized state of immune activation that is believed to play the predominant role in immunopathogenesis, aside from the overall depletion of CD4 T cells and breakdown of T cell homeostasis (Paiardini et al., 2013). This uncontrolled state of immune activation perturbs the proliferative capacity of T cells, and leads to increased expression of the phenotypic marker of senescence CD57 (Mojumdar et al., 2011).

CD57 is a terminally sulphated glycan carbohydrate that is commonly expressed on T cells in persons with chronic immune activation. $CD4^+$ T cell lymphocytes that are $CD57^+$ have generally undergone more rounds of cell division (replicative senescence) compared with $CD57^-$ memory T cells. Expression of CD57 correlates with T cells susceptible to activation-induced cell death (AICD) (Tarazona et al., 2000). AICD is a homeostatic mechanism that eliminates hyper activated cells from the sites of an immune response thereby limiting immune pathology (Prado-Garcia, et al., 2012). It is

mediated by pro-apoptotic genes (FasL/Fas) interactions after persistent TCR-engagement, which provokes T cells to kill each other and themselves (Mak and Saunders, 2006). Through this mechanism, exhausted ($CD28^{-}CD57^{+}CD4^{+}$) T cells are eliminated (Prado-Garcia, et al., 2012) as shown in Figure 3-10.

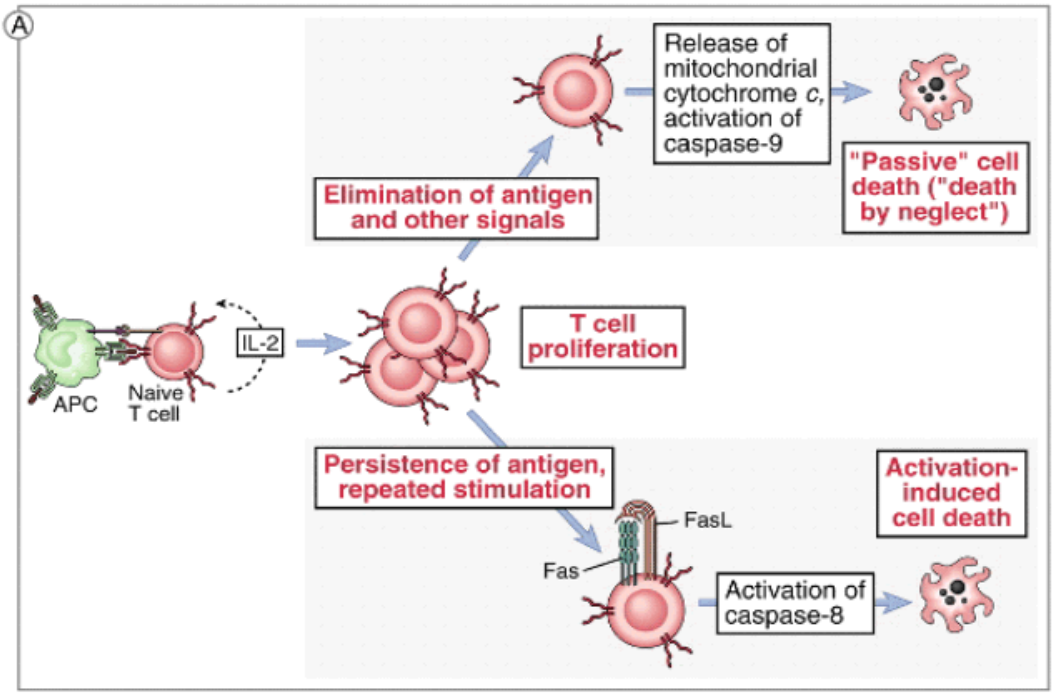


Figure 3-10: Summary of activation induced cell death (https://encrypted-tbn3.gstatic.com/images)

Furthermore, although $CD57^{+}CD4^{+}$ T cells are capable of producing IFN-γ, they are not able to proliferate in response to cognate peptide (Brenchley et al., 2003). Thus, $CD57^{+}$ T cells may not be "end-stage" effector T cells incapable of proliferation but may represent a highly differentiated subset of T cells capable of rapid ($CD8^{+}CD57^{+}$) cell division, cytotoxicity, and IFN-γ production, as well as secretion of IL-5 (Chong, et al., 2008). During infection, CD57 phenotypic marker identifies T cell subpopulations and proportions that are distorted or ageing (Mojumdar et al., 2011). Previously, it has been reported that activation and inflammation due to HIV-infection generate an accelerated replicative senescence of T cells (Appay et al., 2007; Appay and Sauce, 2008;

Mojumdar et al., 2011; Mendez-Lagares et al., 2013). Hence CD57 is used as an immunosenescence marker to identify late stage subsets lacking proliferative capabilities.

3.5.9 Cluster of differentiation 28

CD28 is a glycoprotein expressed on both resting and activated T cells, on almost all $CD4^+$ and on at least half of the $CD8^+$ T cells in humans (Mak and Saunders, 2006). HIV disease also leads to a profound depletion of $CD4^+$ cells co-expressing CD28 (Choremi-Papadopoulou et al., 2000). Absence of CD28 expression on T cells has functional consequences since CD28 is the primary co-stimulatory molecule for naïve T cells (Frauwirth and Thompson 2002; Ostrowski et al., 2003), which, upon engagement, enhances long-term T cell survival and prevents anergy induction (Chambers et al., 1999). Upon binding of the peptide major histocompatibility complex (pMHC) to the TCR, CD28 expression is up regulated on the T cell. The interaction of CD28 and its ligands is critical for antigen-induced T cell activation. Studies have demonstrated the existence of at least two members of the B7 receptor family that provide co-stimulatory signals: CD80 (B7-1) mice and CD86 (B7-2) mice. This interaction pushes cells from a resting state to enter the cell cycle. Studies have also shown that CD28 is essential for Treg development in the thymus and for Treg survival and homeostasis in the periphery (Tang et al., 2003; Tai et al., 2005).

Thus, lack of a potent co-stimulation (CD28) leads to lack of Tregs, which are the most effective mediators of self-tolerance (Guo et al., 2008).

3.5.10 Cluster of Differentiation 27

CD27 is expressed on the majority of resting $CD4^+$ and $CD8^+$ T cells in the peripheral blood and also appears to have a costimulatory role (Mak and Saunders, 2006). It is a member of the tumor necrosis factor receptor (TNF-R) superfamily and is expressed on T, B, and NK cells as a disulfide-linked homodimer. The CD27 ligand (CD70) belongs to the TNF superfamily and is expressed on the surface of activated T and B cells. Interaction of CD27 on a T cell and CD70 on a B cell enhances T cell activation and proliferation (Akiba et al., 1998). This in turn leads to the secretion of low amounts of IL-2.

3.5.11 7-Amino-Actinomycin D

Evaluation of cell viability is essential during HIV-infection. 7-Amino-Actinomycin D (7-AAD) is a fluorescent label for dead cells. It is excluded from live cells but penetrates dead or damaged cells to label DNA. Thus, using 7-AAD, the amount of dead (non viable) $CD4^+$ T cell populations in blood circulation can be identified. Accumulation of dead cells in blood circulation may be due to delayed elimination by phagocytic cells or the HIV increasing the level of cellular proteins, which prompt Fas-mediated apoptosis (Alimonti et al., 2003).

Finally, the summary of all monoclonal antibodies used and the populations of cells identifiable with these markers are as summarised in Table 3-1 below.

Table 3-1: Summary of antibodies and populations of cells

Antibody	Naïve	Cent. Memory	Effect. Memory	Effect	Senes	Gut homing	Activated homing	Recent thymic	Proliferation	Activation	Activated proliferating	Non Viable
CD3	+	+	+	+	+	+	+	+	+	+	+	+
CD4/CD8	+	+	+	+	+	+	+	+	+	+	+	+
CD25	-	-	-	-	-	-	+	-	-	-	-	-
CD27	-	-	-	-	-	-	-	-	-	-	-	-
CD28	-	-	-	-	-	-	-	-	-	-	-	-
CD57	-	-	-	-	+	-	-	-	-	-	-	-
7AAD	-	-	-	-	-	-	-	-	-	-	-	+
α4β7	-	-	-	-	-	+	+	-	-	-	-	-
CD45RA	+	-	-	+	-	-	-	-	-	-	-	-
CCR7	+	+	-	-	-	-	-	-	-	-	-	-
CD31	-	-	-	-	-	-	-	+	-	-	-	-
Ki67	-	-	-	-	-	-	-	-	+	-	+	-
HLA-DR	-	-	-	-	-	-	-	-	-	+	+	-

3.6 CD4 counts in healthy adults

Studies that have been conducted in Africa shows that CD4 counts vary in healthy individuals. This variation could be due to several predisposing factors, which are beyond the scope of this thesis to delve in. However, a few studies reviewed are summarized in Table 3-2 below.

Table 3-2: Mean CD4 counts in HIV-negative African population

First author/Country	Population sampled	Number	Mean CD4 cell count
Tsegaye 1999, Ethiopia	Healthy adults	142	775 +/- 225
Taylor 1991, Nigeria	Healthy adults	183	828 +/- 203
Crampin 2011, Malawi	Healthy adults	214	863 +/- 353
Ricard 1994, Guinea Bissau	Healthy adults	133	892 +/- 107
Menard 2003, Central African Republic	Healthy men	M 68 F 82	927 +/- 349 940 +/- 291
Levin 1996, Tanzania	Healthy adults	147	968 +/-
Tugume 1995, Uganda	Healthy adults	183	1256 +/-
Mair 2007, Senegal	Healthy adults	850	870+/-

3.7 ART and Immune Reconstitution Inflammatory Syndrome

Many people run a serious risk of suffering from Immune Reconstitution Inflammatory Syndrome (SE) if not properly managed after ART initiation (French and Colebunders, 2008). ART initiation in HIV-infected patients leads to recovery of $CD4^+$ T cell numbers and restoration of protective immune responses against a wide variety of pathogens, resulting in reduction in the frequency of opportunistic infections and prolonged survival (Murdoch et al., 2008). However, in a subset of patients, dysregulated immune response after initiation of ART leads to the phenomenon of IRIS (Antoneli et al., 2010). The paradoxical hallmark of the syndrome is worsening of an existing infection or disease process or appearance of a new infection/disease process soon after initiation of therapy. Reported risk factors for developing IRIS include the

rapid decline in viral load especially during the first three months of ART, low baseline CD4 count, rapid increase after ART initiation and initiation of ART soon after start of treatment for opportunistic infection (OI) (Shelburne et al., 2005; Antoneli et al., 2010).

3.8 Malnutrition in HIV

One of the common symptoms responsible for malnutrition in an HIV-infected person is reduced appetite, which could be due to difficulty with ingesting food as a result of infections like oral thrush or oesophagitis caused by *Candida,* a common opportunistic infection in HIV-infected people and fever, side effects of medicines, or depression (Kelly et al., 2004). Poor absorption of nutrients may be due to accompanying diarrhoea caused by bacterial infections like *Salmonella* or *Mycobacterium avium intracellulare;* viral like cytomegalovirus or parasitic infections like *Giardia, Cryptosporidium parvum,* and *Enterocytozoon bieneusi;* due to nausea/vomiting as a side effect of medications used to treat HIV or opportunistic infections. About 30–50% of HIV-infected patients in developed and nearly 90% in developing countries complain of diarrhoea and malabsorption (Smith et al., 1992). The GIT is the largest lymphoid organ in the body and is directly affected by HIV-infection. HIV causes damage to the intestinal cells through activation of T cells, which lead to villus flattening, reduced surface area for absorption, and enterocyte damage (Kelly et al., 2004). This can be measured as decreased D-xylose absorption (Kelly et al, 2004), and absorption of micronutrients is likely to be reduced, though this has only occasionally been measured. Fat malabsorption reduces availability of fat-soluble vitamins like vitamins A and E to immune cells, and these are important for proper functioning of the immune system. Nutrient requirements are increased during fever and infections that accompany HIV-infection, but the body utilizes them poorly and excretion is increased during

inflammation (particularly vitamin A and zinc). The combination of reduced intake due to anorexia, malabsorption, and increased resting energy expenditure leads to loss of weight and lean muscle tissue (Macallan et al, 1995). In their study, Kelly and colleagues comment that 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 (Kelly et al, 1997). 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 (Bijlsma 2000; Piwoz and preble 2000). Dietary intake also varies inversely with intensity of the virus, suggesting that viral replication directly or indirectly suppresses appetite (Arpadi, 2005). Malnutrition is frequent and is considered a marker for poor prognosis among HIV-infected subjects (Suttmann, 1995). Malnutrition and HIV-related immunosuppression form a vicious cycle and ultimately reduces the immunity of the patient.

There is abundant evidence that HIV causes reduced numbers of circulating CD4 cells, but the evidence that malnutrition causes reduced CD4 cell counts is much less convincing. Clear evidence that malnutrition causes reduced T-lymphocyte numbers (Chandra 1999) has been discredited as fraudulent (Stephens and Avenell, 2006). However there is other evidence of delayed cutaneous sensitivity and reduced bactericidal properties (Beisel, 1996), and impaired serological response after immunizations. Guarino and colleagues reported that approximately 30 to 60% of asymptomatic children infected with HIV malabsorb carbohydrates, 30% malabsorb fat, and 32% malabsorb proteins (Guarino et al., 1993).

Whereas micronutrient deficiencies may affect replication of the invading virus, they

also induce several metabolic alterations in the body, i.e. changes in whole-body protein turnover, increased urinary nitrogen loss, elevated hepatic protein synthesis, 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 (Duggal et a., 2012). It also includes hypertriglyceridaemia, elevated hepatic de novo fatty acid synthesis, decreased peripheral lipoprotein lipase activity, hyperglycaemia, insulin resistance, and increased gluconeogenesis. Serum concentrations of iron and zinc fall dramatically due to redistribution within the body, with accumulation in the liver (Friis and Michaelsen, 1998). Glutathione, which is the principle intra-cellular antioxidant, was reported to have been reduced in children with HIV-infection, especially those showing growth failure (Rodriguez et al., 1998).

During infection, reactive oxygen molecules and prooxidant cytokines are released from activated phagocytes (Schwarz, 1996) leading to increased consumption of vitamins like E, C, and β-carotene which serve as antioxidants and of minerals like zinc, copper, manganese, and selenium, which serve as components of antioxidant enzymes (Bendich, 1990). Deficiencies of antioxidants cause increased oxidative stress which leads to apoptosis of T cells and indirectly compromise cell-mediated immunity and may stimulate HIV replication (Schwarz, 1996). In cell cultures, HIV replication was shown to be inhibited by various antioxidants but stimulated by reactive oxygen radicals via activation of nuclear transcription factor cell gene (Kalebic et al., 1991).

HIV-infection in nutritionally deprived individuals intensifies the nutritional deficits and further enhances cellular oxidative stress. This affects the functions of transcription factors such as NF-kB and contributes to HIV replication and progression. Although

HIV attacks only a limited variety of T-lymphocyte subspecies, AIDS-induced malnutrition can lead to the secondary development of nutritional-acquired immune deficiency syndrome (NAIDS) through the action of pro-inflammatory cytokines. Also, malnutrition could hasten the development of AIDS in an HIV-infected person (Beisel, 1996).

Though antioxidants inhibit HIV replication, they may actually promote opportunistic infections by preventing the oxidative burst which is considered important for the bactericidal properties of phagocytes (Schwarz, 1996). Therefore, balanced nutrition and dietary consultation with experts helps in balancing immune effects, malnutrition, and HIV-infection (Olsen et al., 2014). FAO (2003) stated that "Food is not a magic bullet. It won't stop people from dying of AIDS but it can help them live longer, with more comfortable and productive lives." Evidence-based nutrition interventions should be part of all national HIV care and treatment programs. Routine assessment should be made of diet and nutritional status (weight and weight change, height, body mass index or mid upper arm circumference, symptoms and diet) for people living with HIV (WHO, 2010).

Finally, Baum and colleagues concluded that "intake of nutrients at levels recommended for the general population does not appear to be adequate for HIV-1-infected patients" (Baum et al., 1997)." An active non-HIV-infected adult requires approximately 2070 kcal/day including about 57 grams/day of protein. An HIV-infected adult requires 10 to 15 percent more energy per day and approximately 50 to 100 percent more protein (Woods, 1999; WHO, 1985).

3.9 Nutritional Deficiencies, BMI, ART and Mortality

The World Health Organization defines nutritional status as follows: Mild malnutrition (Body mass index (BMI), 17.00–18.49 kg/m^2), moderate malnutrition (BMI, 16.00–16.99 kg/m^2), and severe malnutrition (BMI, <16.00 kg/ m^2) (United Nations ACC/SCN, 2000). Food insufficiency, anorexia, increased basal metabolic rate; malabsorption, opportunistic infections and reduced intake may all contribute to malnutrition and weight loss during HIV-infection (Koethe et al., 2009). Anorexia is one of the symptoms suffered by HIV-infected patients and is the major contributor to lessened food intake (Koethe et al., 2009) and weight loss (Macallan et al, 1995).

Studies show that low BMI at ART initiation is an independent predictor of mortality (Moh, et al., 2007; Sieleunou, et al., 2009; Hanarahan, et al., 2010). A study conducted by Verguet and colleagues in the U.S, though not powered to comment on the effect of BMI on mortality, showed that there was an increase in mortality during the first 6 months of ART for individuals starting ART, especially those with low CD4 count (<100 cells/μL) compared to those above 100 cells/μL (Verguet et al., 2013). A study by Liu and colleagues concluded that poor nutritional status at ART initiation is independently correlated with a high risk of mortality within the first 3 months. Equally weight loss decreases in the first 3 months of ART were associated with a further increased risk of subsequent mortality (Liu et al., 2011). This is shown in Figure 3-11 below.

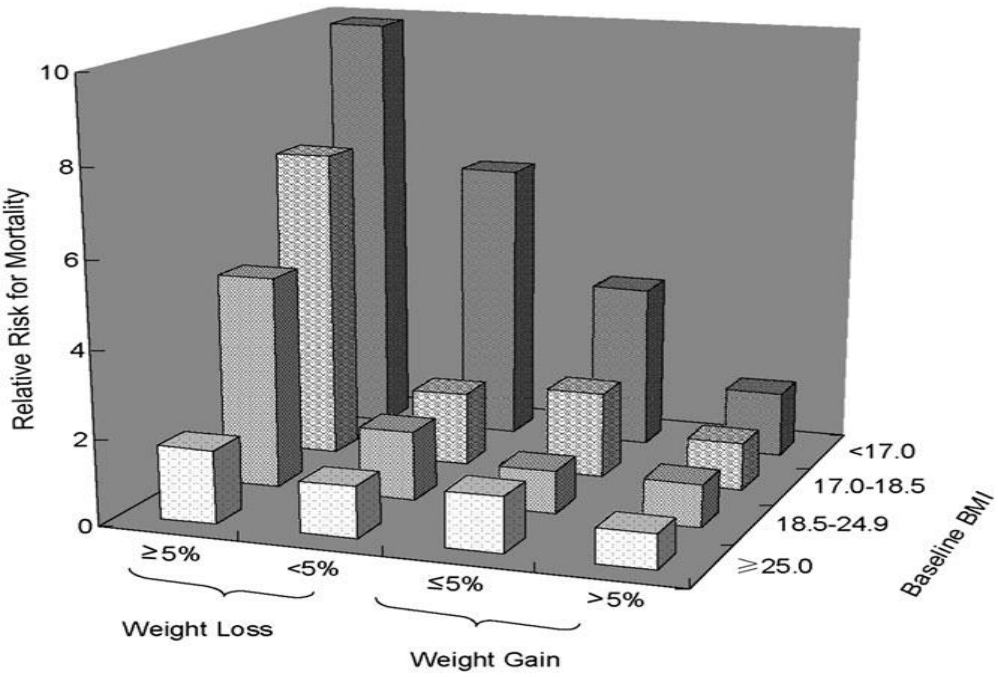


Figure 3-11: The combined effect of BMI and weight change after 3 months of ART on the risk of mortality (Liu et al., 2011)

Such results suggest that provision of nutritional interventions may be helpful before, and in the early weeks after ART initiation. Other researchers have also reported that HIV-infected patients starting ART with low BMI risk dying during the first few weeks (Stringer et al., 2006; Zachariah et al., 2006; Johannssen et al., 2009). Stringer and colleagues reported that in Zambia, patients commencing ART with a BMI of <16.0 kg/m^2 had a higher mortality rate in the first 90 days of therapy (adjusted hazard ratio (HR), 2.4; 95% confidence interval (CI), 1.8–3.2), compared to those initiating ART with BMI >16.0 kg/m^2 (Stringer et al., 2006). A study conducted in more than 1500 Malawians starting ART reported that participants with BMI <15.9 kg/ m^2 had a 6-fold increased risk of death at 3 months, in comparison with those with a BMI of >18.5 kg/m^2 (adjusted HR, 6.0; 95% CI, 4.6–12.7). For individuals with a BMI of 16.0–16.9 kg/m^2, the risk was 2-fold (adjusted HR, 2.4; 95% CI, 1.7–6.3) (Zachariah et al., 2006). Data from Tanzania showed that patients with BMI <16.0 kg/m^2 at the start of ART had

a double mortality rate compared to that of patients with BMI >18.5 kg/m^2 (adjusted HR, 2.1; 95% CI, 1.1–4.2) (Johannssen et al., 2009). This evidence suggests that a correlation exists amongst ART, mortality and BMI during HIV-infection.

Other authors report an increased risk of early mortality among HIV-positive patients starting ART in resource-constrained countries compared to high-income countries (Kelly et al., 1999; Zulu et al., 2008; Koethe et al., 2009). Increased early mortality rate is associated with the loss of immunocompetence and the evolving opportunistic infections common amongst HIV-positive individuals (Zulu et al., 2008). Koethe and colleagues reported that mortality in HIV-positive persons initiating ART were dependent on serum biomarker levels i.e. albumin, phosphates and ferritin (Koethe et al., 2011). Low levels of albumin and a body mass index of 16 prior to initiation of ART especially in HIV-infection, increases susceptibility to a number of infections as well as the mortality rates (Koethe et al., 2010).

3.10 The Impact of Nutrient Interventions During HIV-Infection

During HIV-infection a number of nutrient deficiencies occur, resulting from malabsorption, reduced dietary intake or compromised stores as demonstrated in Figure 3-12. Malabsorption of trace elements and macronutrients may result from GIT infections, disturbances in the gut barrier and an altered metabolism (Semba and Tang, 1999).

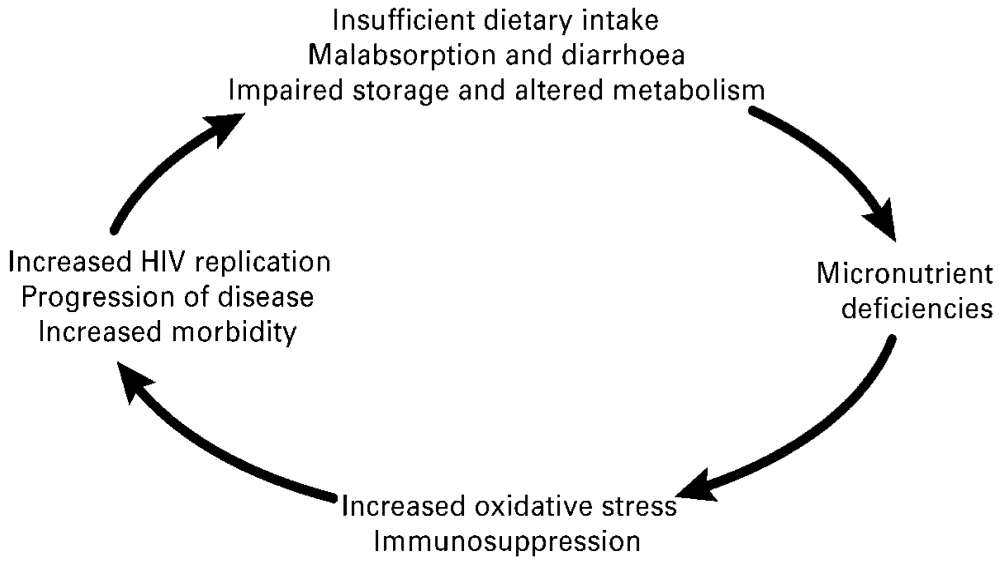


Figure 3-12: Impact of poor nutrition and HIV (Semba and Tang, 1999)

Individual micronutrient deficiencies may impact on the immune system response to infection. Humans use many metabolic control mechanisms to prevent oxidative damage from free iron (Puntarulo, 2005; Arosio et al., 2009). These controls involve both protein synthesis and adequate nutritional status in relation to key antioxidant micronutrients. Both protein synthesis and antioxidant micronutrient status are impaired in severe malnutrition (Ashworth et al., 2003), hence re-feeding malnourished people or animals without caution is associated with infection and death. Several randomized controlled trials have been undertaken to explore the potential for micronutrient treatment, both to restore micronutrient status and to improve immune function. These have recently been summarized in a Cochrane review as exemplified with forest plots below (Figures 3-13-3-21) (Irlam et al, 2012).

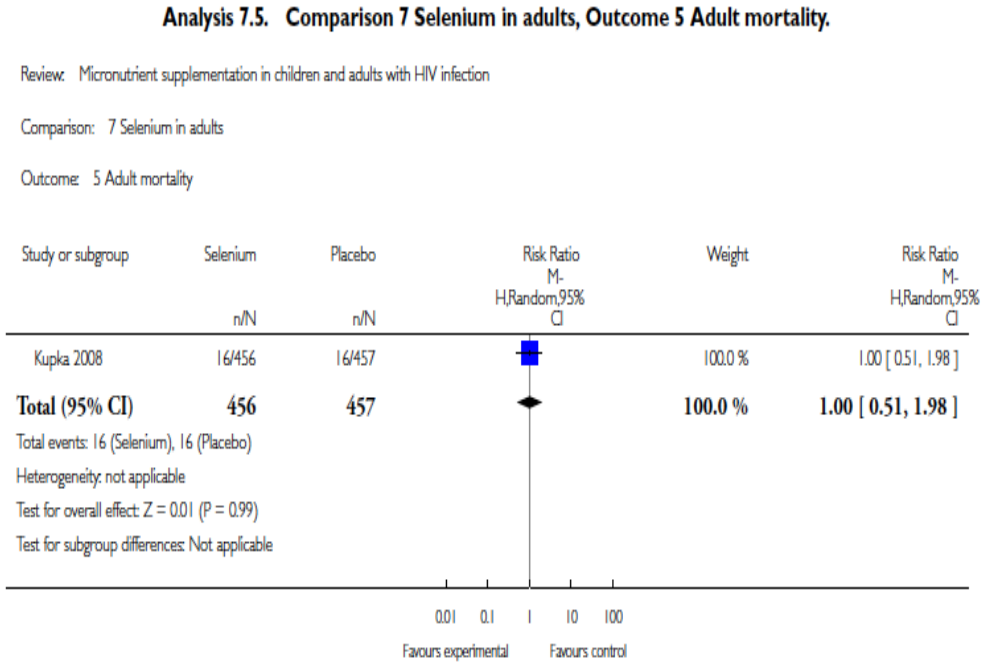


Figure 3-13: Selenium supplementation and mortality in adults with HIV-infection

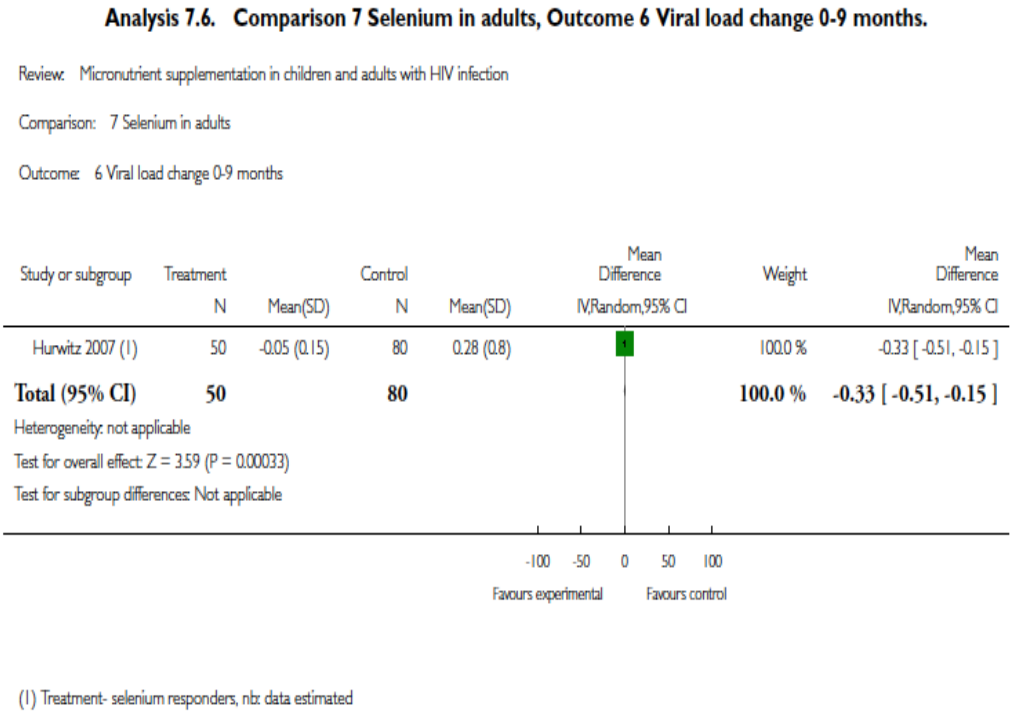


Figure 3-14 Selenium supplementation and viral load in adults with HIV-infection

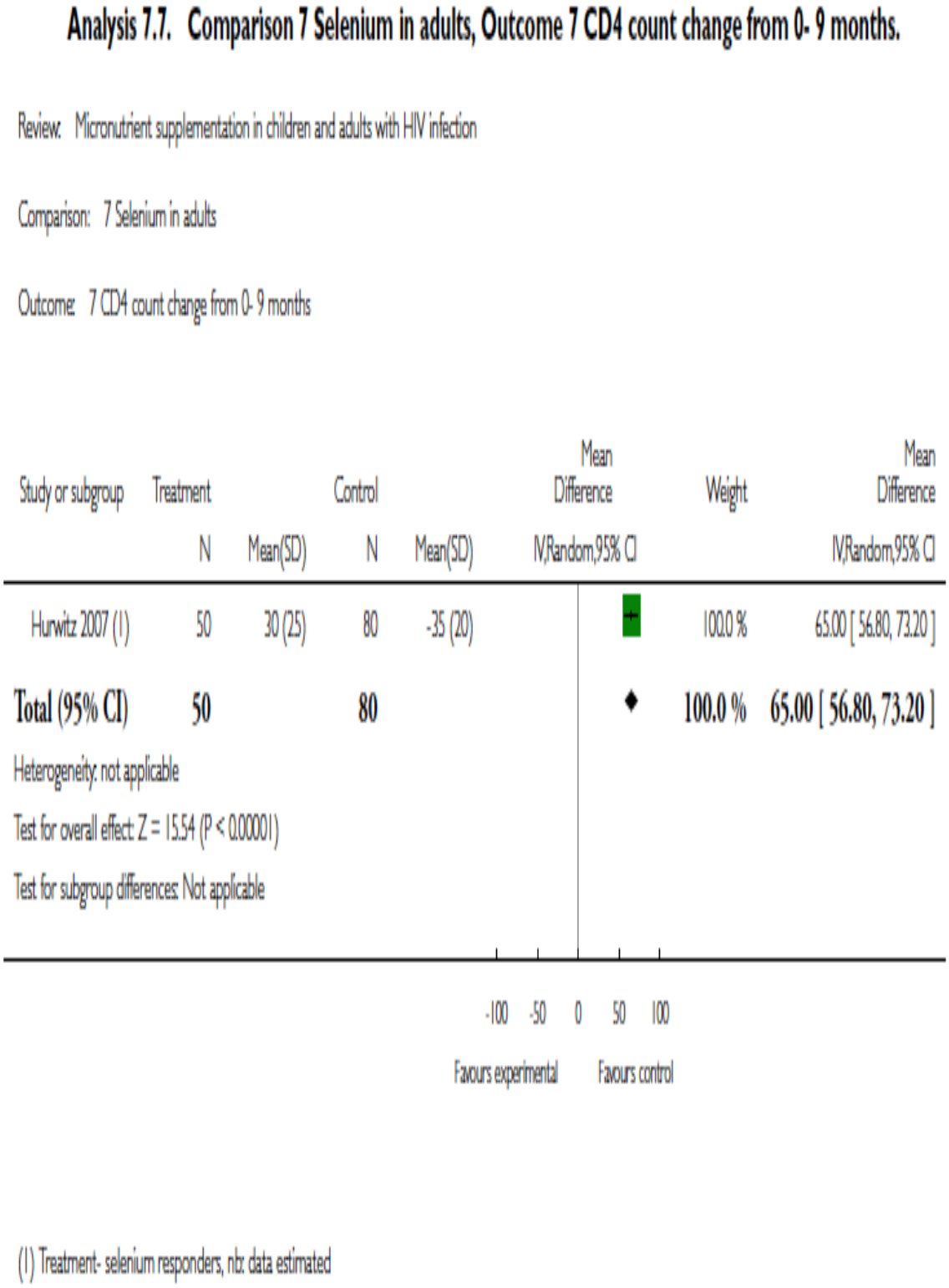


Figure 3-15: Selenium supplementation and CD4 count change in adults with HIV-infection

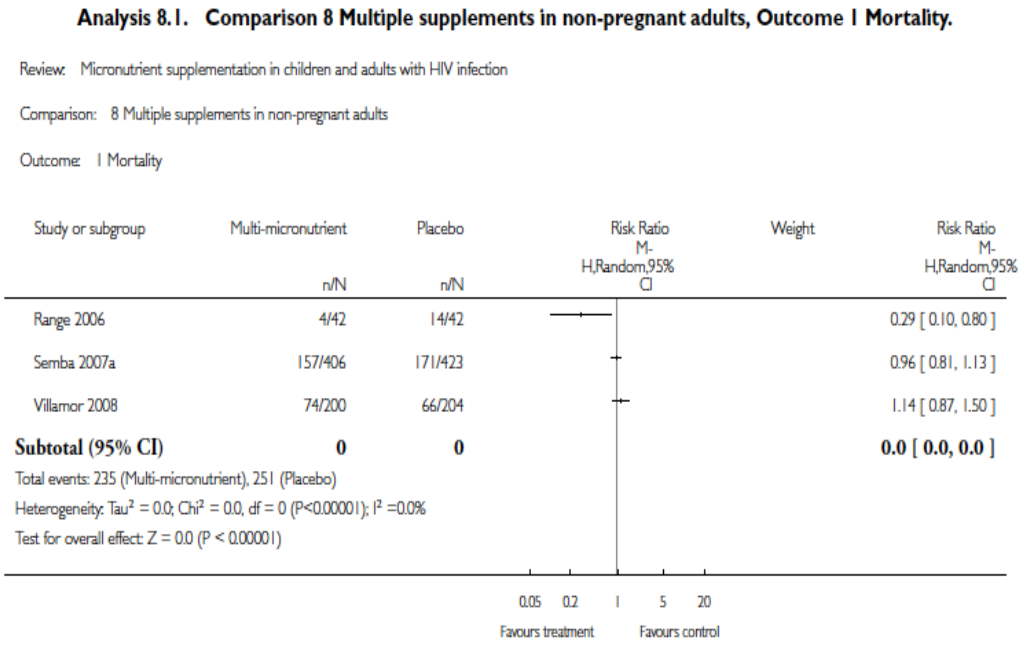


Figure 3-16: Micronutrient supplementation and mortality in adults with HIV-infection

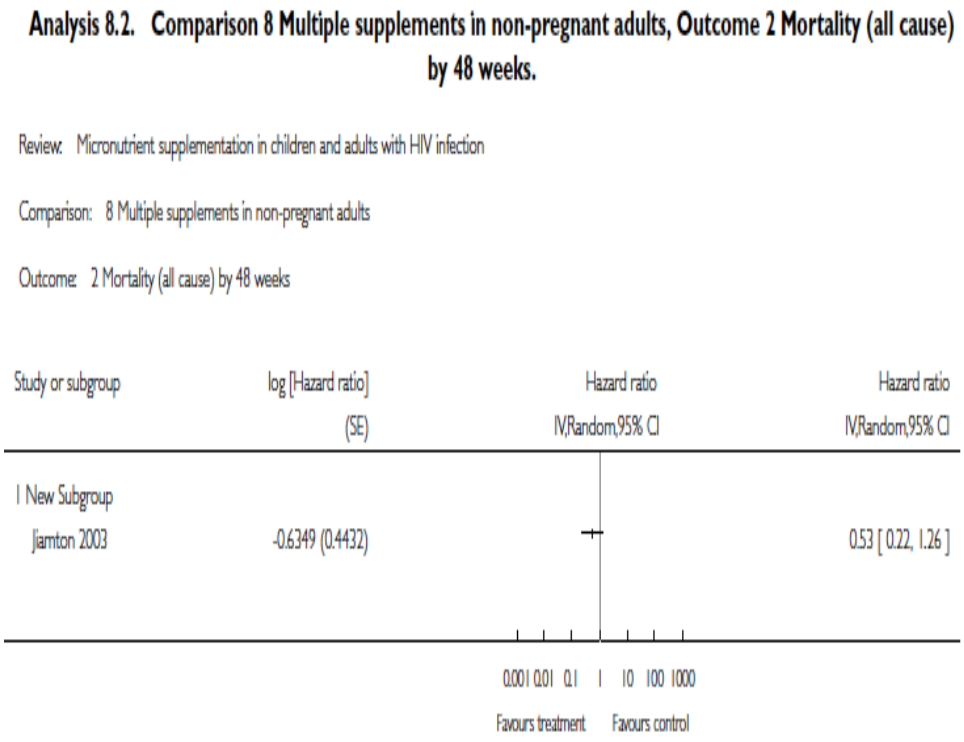


Figure 3-17: Micronutrient supplementation and mortality in adults with HIV-infection

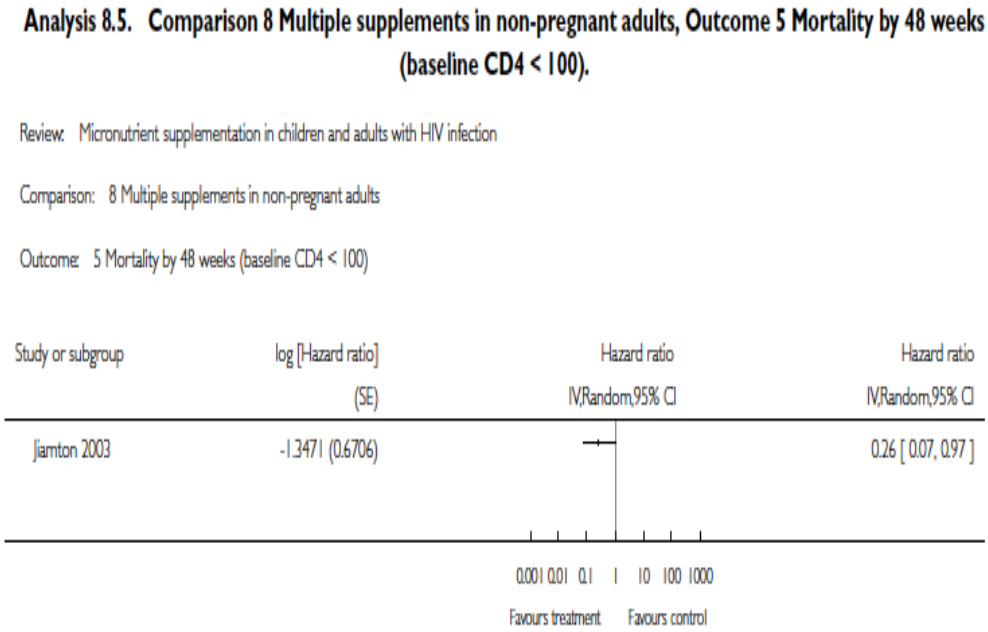


Figure 3-18: Micronutrient supplementation and mortality in adults with HIV-infection (severe immunodeficiency)

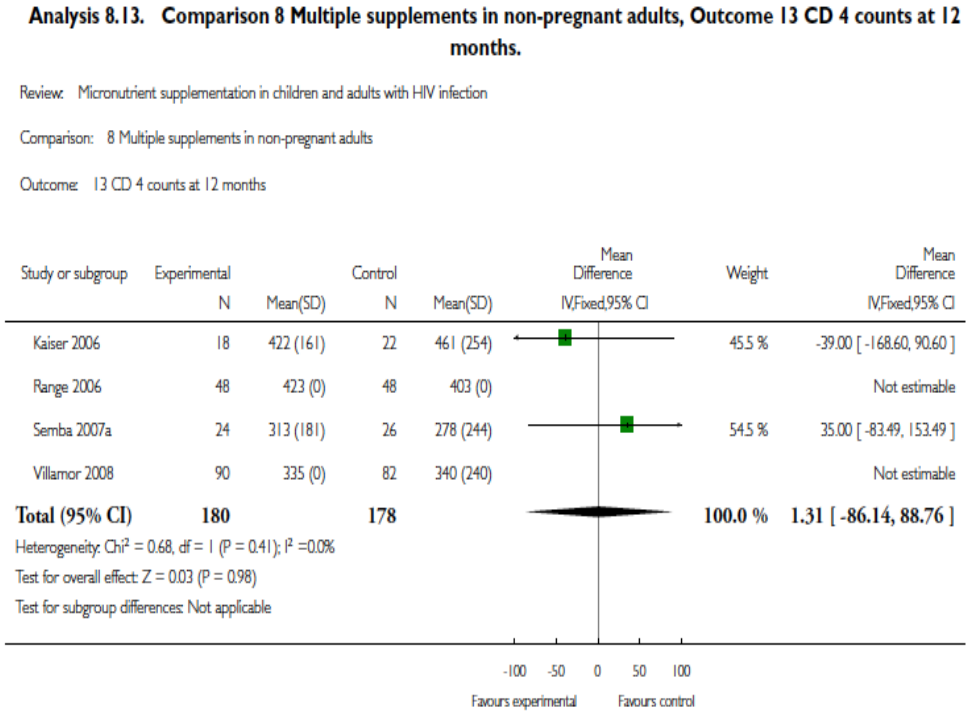


Figure 3-19: Micronutrient supplementation and CD4 counts in adults with HIV-infection

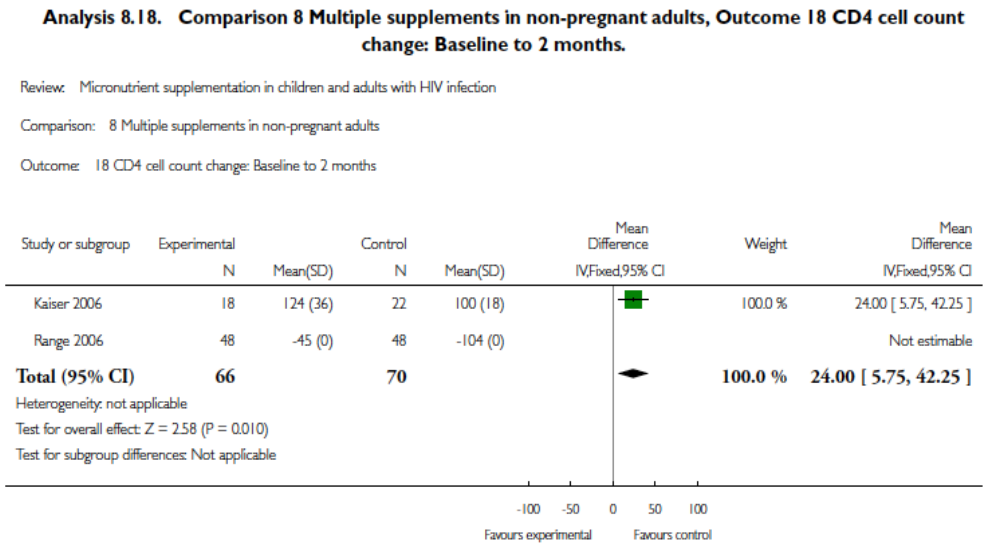


Figure 3-20: Micronutrient supplementation and CD4 change at 12 months in adults with HIV-infection

A study conducted in Mwanza Tanzania reported a reduction in mortality (RR 0·29; 95 % CI 0·10, 0·80; interaction ratio 0·52) in HIV-positive TB patients allocated to receive a combination of multivitamins, minerals and zinc (Range et al., 2006). A similar study conducted in South African on TB patients showed no significant effects following supplementation with vitamin A and zinc (Visser et al., 2011). In another study, micronutrient supplementation increased HIV in the vagina of HIV-positive women (McClelland et al., 2004). A question that remains unanswered is whether supplementation during HIV-infection should be encouraged seeing there is increased risk of HIV transmission during supplementation.

However, vitamin supplementation in HIV infected patients under oxidative stress has been found to reduce lymphocyte DNA damage (Jaruga et al., 2002). Kelly and colleagues also reported reduced mortality (p=0.029) in HIV-infected individuals following micronutrient supplementation compared to participants that received

placebos (Kelly et al., 1999). Results from the main NUSTART trial also showed that the bulk of mineral given at the start of ART did not reduce mortality or any serious adverse events but showed an increased CD4 count as a secondary outcome (Filteau et al., 2015). These and other studies demonstrate that there is urgent need for further investigation into the effect of micronutrient supplementation as regards HIV-infection. It is also clear that micronutrient supplements have a potential benefit for people living with HIV-infection. However, in order to understand the magnitude of this benefit and how supplements should be positioned alongside the proven advantages of antiretroviral drugs, robust evidence base to guide policy and practice is required.

3.11 CD4 Reconstitution During ART

In a Zambian study, Chi and colleagues reported that good adherence to ART results in an increased CD4 count (Chi et al., 2009). Results from this study (Chi and colleagues) demonstrated that at less than 80% ART adherence for 18 months, there was an increase of 185 cells/μL vs. 217 cells/μL; $P<0.001$. At 24 months (213 cells/μL vs. 246 cells/μL; $P<0.001$), 30 months (226 cells/μL vs. 261 cells/μL; $P<0.001$) and 36 months (245 cells/μL vs. 275 cells/μL; $P<0.01$) (Chi et al., 2009). Another study conducted in Europe reported a significant increase in CD4 count, which was also dependent on the duration of ART and the median CD4 count at starting ART was 204 cells/μL (Mocroft et al., 2007). A yearly mean increase in CD4 count of 100 cells/μL was observed after starting ART. For patients with initial CD4 count less than 500 cells/μL, lower yearly increases in CD4 count of about 50 cells/μL, were seen at 5 years after starting ART (Mocroft et al., 2007).

A study in US, recorded an average improvement of 138 cells/μL (SD = 125) CD4 cells during ART treatment (Ross-Degnan et al., 2010). Smith and colleagues in a study

conducted in U.K reported that the median increase in CD4 cell counts after 6, 12, and 24 months of HAART, as 114, 181, and 248 cells/µL, respectively (Smith et al., 2004). All studies reviewed are summarised in Table 3-3 as shown below.

Table 3-3: Summarizes $CD4^+$ T cell Changes During ART

First author/ Country	Mean increase	Duration
Ross-Degnan, 2010, USA	138 cells/µL	**6 months**
Chi, 2009, Zambia	217 cells/µL	**18 months**
Mocroft, 2007,UK	100 cells/µL	**12 months**
Smith, 2004, UK	248 cells/µL	**24 months**
Stringer, 2006, Zambia	175 cells/µL	**12 months**

3.12 CD4 T cell Reconstitution and Nutrition in HIV

A considerable amount of literature addresses the relationship between CD4 T cell reconstitution and nutrition during HIV-infection. A study in Rwanda hypothesized that poor nutritional status was associated with smaller gains in CD4 count in Rwandan women initiating ART (Kiefer et al., 2011). Seven hundred and ten ART-naive HIV-positive and 226 HIV-negative women were enrolled in 2005 with follow-up every 6 months. The aim was to assess changes in CD4 count at 6, 12, and 24 months after ART initiation. Nutritional status measures taken prior to ART initiation were: BMI, height adjusted fat free mass (FFMI), height adjusted fat mass (FMI), and sum of skin fold measurements. Findings from this study showed mean changes in CD4 count for HIV-positive women from pre ART initiation at 6, 12, and 24 months post initiation as 71,

89 and 153 cells/µL, respectively (Kiefer et al., 2011). It was concluded that no nutritional variable was found to be associated with change in CD4 count.

Forty HIV-infected patients in the United States of America taking HAART regimen were prospectively randomized to receive micronutrient supplements or a placebo twice daily for 12 weeks (Kaiser et al., 2006). The mean absolute CD4 count increased by an average of 65 cells in the supplemented group versus a 6-cell decline in the placebo group at 12 weeks (p=0.029). The absolute CD4 count increased by an average of 24% in the micronutrient group compared to a 0% change in the placebo group (p=0.01). It was concluded that micronutrient supplementation significantly improves CD4 cell count reconstitution in HIV-infected patients taking HAART (Kaiser et al., 2006).

Evidence from a study on Kenyan women showed that micronutrient supplementation when starting ART resulted in higher $CD4^+$ (+23 cells/µL, p=0.03) and $CD8^+$ (+74 cells/µL, p=0.005) counts compared with a placebo group (McClelland et al., 2004). And finally, Hurwitz et al., reported an increase in CD4 count of 27.9 ± 150.2 cells/µL following micronutrient supplementation with selenium (Hurwitz et al., 2007).

In Switzerland macro and micro nutritional intervention on HIV-infected individuals with a body mass index <21 kg/m^2 or $CD4^+$ T cells <500 µL had no significant effect on lymphocyte CD4 counts/L (Berneis et al., 2000). Another study in South India evaluated the effects of the high-calorie, high-protein macronutrient supplement, Indian mix, among HIV-infected adults. From the 361 participants who completed 6 months of follow-up, a slower decrease in $CD4^+$ cell counts among patients who were provided supplements was observed (Swaminathan et al., 2010). This study concludes that supplementation did not result in significantly increased weight gain compared with standard care among patients with HIV disease (Swaminathan et al., 2010).

The effect of nutritional deficiencies on mortality has also been widely investigated in HIV-infected individuals. A study by Kelly and colleagues revealed that supplementation with vitamin A, C and E plus Se and zinc, showed no changes in $CD4^+$ cell count (Kelly et al., 1999). However, there is a paucity of studies addressing the association between micronutrients with vitamins and minerals and $CD4^+$ T cell reconstitution.

3.13 Selenium and HIV

Jons Jacob Berzelius, a Swedish national, discovered selenium (Se) in 1817 (Bowrey et al., 1999; Stone et al., 2010). Amounts of Se present in the soil fluctuate and it is incorporated into the food chain via uptake into plant crops (Peters and Takata, 2008). Eggs, meat and fish contain the highest (87.6-737 μg/kg) Se levels and fruits and vegetables the lowest being at 1.7-24.9 μg/kg (Ventura et al., 2006). Brazil nuts contain up to 254 μg/100g while crabmeat has about 84 μg/100g (Bowrey et al., 1998).

Se in the form of selenite, selenate, or selenocysteine is absorbed in the duodenum (Bowrey et al., 1998). Red blood cells rapidly absorb Se. In the presence of thiols, Se is reduced to hydrogen selenide and it is transported bound to alpha and beta globulins (Bowrey et al., 1998). Se is also found in small amounts in liver (0.63 μg/g) and kidney 0.39 μg/g tissues (Bowrey et al., 1998).

3.13.1 Selenium and Human Requirements

Although the essential role of Se in human health is established, the actual human requirement is still uncertain. Brown and Arthur reported that a human body weight of about 60 kg needs 41 μg of Se per day (Brown and Arthur, 2001). A study by Baum and

co-workers defined Se deficiency as a serum concentration level of 85 μg/L or less (Baum et al., 1997). Fairweather-Tait and colleagues reported that dietary Se intake varies from 7 μg/day to about 4990 μg/day (Fairweather-Tait et al., 2011). In the USA, dietary Se intake stands at 70 μg/day for men and 55 μg/day in women (Khan et al., 2012). In Europe, a reduction over several years of average serum Se concentrations to 0.63-1.69 μmol/L has been interpreted as a reflection of the reduction in imported wheat with high Se content from North America (Brown and Arthur, 2001). The observed uncertainty in Se requirements is a consequence of differences in the amounts present in food chains and soils (Johnson et al., 2010). Rayman suggests that on average, women need about 53 μg/day while men about 60 μg/day (Rayman, 2004). Data from Finland indicate that 70 μg/day Se supplementation increase serum Se concentrations (Brown and Arthur, 2001).

3.14 The Role of Selenium in Human Physiology

3.14.1 Selenium and Oxidative Stress

Se can be incorporated in selenoproteins in form of a modified amino acid, selenocysteine. Selenocysteine is incorporated into proteins in the liver, from which it is bound and transported by albumin (Brown and Arthur, 2001). About 100 selenoproteins have been assumed to exist in mammalian tissues, of which about 30 have been characterized in full. Se-dependent enzymes include classical glutathione peroxidase, (GPx1), gastrointestinal glutathione peroxidase (GPx2), plasma glutathione peroxidase (GPx3) and phospholipid hydrogen peroxide glutathione peroxidase (GPx4) (Brown and Arthur, 2001; Reeves and Hoffmann, 2010). The marked preponderance of antioxidant enzymes among Se dependent enzymes strongly suggests that Se has important antioxidant effects. Hydrogen peroxide (H_2O_2) is toxic to all cells, especially

when Se is deficient (Brown and Arthur, 2001). Adequate detoxification of free radicals results in a decrease in oxidative stress due to reactive oxygen species (ROS) with a consequent decrease in cellular pathology (Brown and Arthur, 2001).

3.15 Transcriptional Effects of Selenium

Nuclear factor Kappa B (NF-$_K$B) controls the transcription of pro-inflammatory cytokine genes (Tak and Firestein, 2001; Youn et al, 2008). Increased replication of several viruses is seen when NF-$_K$B is activated (Schreck et al. 1991). In a study conducted in Japan by Maehira and colleagues on 141 healthy individuals, human hepatoma cell lines were grown for 3 days in medium containing 2% foetal calf serum with the aim of investigating the regulatory effect of Se on NF-$_K$B (Maehira et al., 2003). Following this, cells were cultured in the same type of medium containing sodium selenite for another 3 days (Maehira et al., 2003). Se, at a concentration of 0.5~1 μmol/l which is about half the serum concentration for healthy individuals, was sufficient for maximum inhibition of activated NF-$_K$B. As Se concentrations in this range are common in the serum of subjects with elevated C-reactive proteins (CRP) levels (Maehira et al., 2003), they postulated that low Se might contribute to NF-$_K$B-mediated inflammation.

3.16 Impact of Selenium on Immunity

3.16.1 Innate Immunity

Se deficiency has been shown to impair the phagocytic function of neutrophils and macrophages (Arthur et al., 2003), Although the number of circulating neutrophils was normal in Se deficient experimental animals, the ability to kill an ingested organism was compromised (Arthur et al., 2003), and this defect was attributed to a drop in the

cytosolic GPx1 activity in the neutrophils. Thus, instead of the organism being killed, neutrophils were killed.

In another experimental system, *Listeria monocytogenes* was administered orally to mice to assess the effect of Se on innate immunity (Wang et al., 2009). Mice were infected with *L. monocytogenes* and categorised into four classes as follows: Group I, non-infected Se-adequate; Group II, non-infected Se-deficient; Group III, infected Se-adequate; and Group IV, infected Se-deficient. Glutathione activity was lowered in Se deficient mice as compared to replete mice, and *L. monocytogenes* persisted in-group IV compared to group III. These data suggest that the innate immune response in Se deficient mice is defective in *L. monocytogenes* infection (Wang et al., 2009). Another interesting outcome of this experiment was the discovery of increased numbers of natural killer cells and production in the Se supplemented mice.

3.16.2 Humoral Immunity

A study conducted in rats infected with *Trypanosoma brucei brucei* suggested that Se supplementation could play a role in the humoral immune response. Antibody titers were increased in supplemented rats compared to the control group ($p<0.05$). Mortality was 100% in the infected non-supplemented group but was reduced to 40% in rats that received 4 and 8 parts per million (ppm) Se in feeds (Eze et al., 2011).

In a double-blinded study conducted by Broome et al., 22 individuals with a low plasma Se measure were chosen as study participants. These participants were allocated to receive either a 50 μg, or 100 μg or placebo every day for a period of 15 weeks (Broome et al., 2004). An oral live attenuated polio vaccine was administered at week 6

to all twenty-two subjects. Se supplementation increased poliovirus antibody titers in adults challenged with the poliovirus.

3.16.3 T Cell Immunity

Several studies highlight the importance of Se to proper functioning of the immune system, particularly in $CD4^+$ T cell functioning and mounting responses against infection. In the aforementioned study by Broome and colleagues a T lymphocyte ($CD3^+$) immune response was enhanced in persons that received the sodium selenite by day 7 giving rise to an effective cellular elimination of the poliovirus. An increase in the clearance of the poliovirus in subjects that received the sodium selenite was made possible by an increased proliferation and differentiation of the T cells and $CD4^+$ cells (Broome et al., 2004). However, in participants receiving placebos there was no detectable increase in Se plasma level or change in T cells. A mutation also occurred in the polio viral genome of the placebo group, giving rise to a more virulent form of a virus, which did not occur in the supplemented group. Interferon gamma (IFN-γ) increased in both groups (placebo and supplemented), but the increase was much higher in the group that received 100 μg Se/day. Still in the supplemented group, peak levels for IFN-γ production was at day 7 while in the placebo group the reported peak was at day 14 (Broome et al., 2004).

In another study, mice were randomised to receive low (0.08 mg/kg), medium (0.25 mg/kg), or high (1.0 mg/kg) Se diets for 8 weeks (Hoffmann et al., 2010). These mice were challenged with Lymphocytic choriomeningitis (LCMV) gp66–77 with the aim of assessing the role of Se on CD4 T cell proliferation and maturation. There was a skewing towards a Th1 response with an increase in IFN-γ production in mice on a high Se diet as opposed to the other two groups. IFN-γ was also found to play a role in macrophage activation and increased inhibition of viral replication

3.17 Selenium, Cardiomyopathy and Coxsackie Virus

In China, Keshan and Kashin-Beck diseases are human conditions that are associated with Se deficiency (Su et al., 1979; Rayman, 2000; Rayman, 2012). This may be as a result of a direct effect of Se deficiency on cardiac muscle (Broome et al., 2004), but Keshan and Kashin-Beck diseases may also result from defective surveillance allowing the pathogenic coxsackie virus to develop enhanced virulence (Broome et al., 2004).

Beck and colleagues carried out an experiment in Se deprived mice. Se deficient mice were inoculated with a benign strain of coxsackie type of the virus. A virulent type of a virus evolved following mutations in its genome. The resulting virus was reported to cause a type of myocarditis in mice similar to that of humans. When isolated, the mutated virulent virus was inoculated in mice that had sufficient Se. Heart damage was still induced, which demonstrated that Se deficiency causes permanent damage or mutation to the virus (Beck et al., 1998).

3.18 HIV

During HIV-infection a number of micronutrient deficiencies occur, perhaps resulting from malabsorption. Malabsorption of trace elements and micronutrient is attributed to gastrointestinal tract infections, mucosal damage, and altered metabolism (Semba and Tang, 1999).

3.18.1 Observational Data

Several studies report observational data. Rayman (2004) has reported a correlation between low Se and reduced CD4 T cells of HIV infected individuals.

Death rates in HIV-infected persons with lower levels of Se are reported to be 20 times higher ($p<0.0001$) than in HIV-infected individuals with sufficient Se levels (Baum et

al., 1997). It seems plausible that this mortality effect might be mediated through oxidative stress (Baum et al., 1997), although it is possible that both are merely markers of some other pathophysiological process. Glutathione activity increases helper T cell count, and cytotoxic cell activities that act against the HIV replication rate and lower HIV viral load (Stone et al., 2010). However, direct evidence for this is conflicting.

A cross-sectional study was conducted in 400 HIV-1-seropositive women to assess, among other things, the relationship between plasma Se level and CD4 count. In univariate analysis, an association existed between low Se and low CD4 count (Drain et al., 2005). No association was found using multivariate analysis

During HIV-infection interleukin-2 and IFN-γ levels are reduced. IL-2 is required in differentiation and activation of effector cells. Activation and proliferation of natural killer cells is equally dependent on IL-2 production. A compromise in IL-2 cytokine level leads to rapid progression of HIV disease and a decrease in T lymphocytes (Baum et al., 2000). Therefore, in HIV-infected individuals Se supplementation is a plausible adjunct for therapy and combating free radicals constantly produced following chronic immune activation (Baum et al., 2000; Drain et al., 2005).

3.18.2 Clinical Trial Data

In a double-blinded randomized controlled trial conducted by Hurwitz and colleagues in Miami USA, 200 μg of Se was given to 262 participants for 9 months. One hundred and twenty one received supplementation (with 200 μg Se) over a 9-month follow-up assessment while 141 received a placebo. An increase of 32.2 +/- 24.5 μg/L in serum Se concentration was recorded in the treatment group as opposed to 0.5 +/- 8.8 μg/L in placebo. HIV viral load was reduced ($p<0.02$) and CD4 counts increased ($p<0.04$)

compared to placebo (Hurwitz et al., 2007). Furthermore, 200 μg supplementation every day resulted in an increased CD4 cell count and a reduction in the viral load (Hurwitz et al., 2007). There were no side effects attributed to the supplementation. In the same study, the number of hospitalisations reduced (RR = 0.38; p=0.002) (Hurwitz et al., 2007).

In contrast, a study that was carried out in Tanzania on HIV-positive pregnant women, using the same amount of Se supplementation (200 μg) did not show any reduction in the viral load nor an increase in CD4 cell counts but reduced the chances of CD4 decline to >50 cells/mm3 (Kupka et al., 2008). A decrease in the mortality rates and a slight increase in the birth weight of the infants born from HIV-positive women were reported (Kupka et al., 2008). This reduction was attributed to the increased supply of Se via the umbilical cord, placenta and breast milk to the infants.

In another double-blinded, randomized trial conducted among 400 HIV-1 women in Mombasa Kenya, McClelland tested whether multivitamin plus Se supplementation decreases genital HIV-1 shedding. The odds of detection of vaginal HIV-1-infected cells were higher (2.5-fold; p=0.001) among women who received micronutrients in comparison to placebo. Supplementation also resulted in higher CD4 (+23 cells/mL, p=0.03) and CD8 (+74 cells/mL, p=0.005) counts compared with placebo but did not alter the plasma viral load (McClelland et al., 2004).

During HIV-infection, Se deficiency increases susceptibility and accelerates progression of the disease. To confirm that Se supplementation can be used to increase body Se, two small studies were conducted in France and U.S on people living with HIV. In a study conducted in France, reduced serum Se levels increased from an average of 0.75 +/−

0.27 μmol/L to 1.63 +/− 0.27 μmol/L following 21days of Se supplementation (Stone et al., 2010). Whilst in the U.S, serum Se in people living with HIV increased from a mean of 1.55 +/− 0.38 μmol/L as compared to 2.47 +/− 0.25 μmol/L in the control group (Olmstead et al., 1989). Based on these and other related trials, it is probable that supplementation could be useful in boosting the serum Se levels (Broome et al., 2004; Stone et al., 2010).

Brief overview of the effects of Se on CD4 count is as summarized in Table 3-4 below.

Table 3-4: Effects of Selenium on Absolute CD4 count

First author & year	Study design	Cohort & amount given	Findings
Kamwesiga, 2015	A multicenter, double-blinded, placebo-controlled, randomized clinical trial	Of the 300 participants, 149, HIV-infected adults (≥21 years) received 200µg Se once-daily or identical placebo.	CD4 depletion was reduced by 43.8% [95% Confidence interval (CI) 7.8–79.8% decrease] in the treatment arm – from mean 3.97 cells/µL per month to mean 2.23 cells/µL per month.
Kupka, 2008	Randomized, double-blind, placebo-controlled trial	Women between 12 and 27 wk. of gestation received 200 µg Se or placebo as supplements from recruitment until 6 months after delivery.	No significant effect on maternal CD4 cell counts or viral load. Selenium was marginally associated with a reduced risk of low birth weight [relative risk (RR) 0.71; 95% CI: 0.49, 1.05; *P*=0.09]
Hurwitz, 2007	Double-blinded randomised controlled trial	121 received 200 µg Se over 9-months while 141 received a placebo.	HIV viral load was reduced (*P*<0.02) and CD4 counts increased (*P*<0.04) compared to placebo
Drain, 2006	Cross-sectional	400 HIV-1 seropositive women	Association between low Se and low CD4 count
Broome, 2004	Double-blind study	22 healthy individuals with low Se received either a 50 µg or 100 µg or placebo every day for a period of 15 weeks	Effective cellular elimination of the poliovirus in persons that received the sodium selenite

Chapter 4. Statement of the Problem

4.1 Statement of the Problem

A good number of studies have explored the prognostic value of T cell subsets in HIV-treated individuals and immune reconstitution following nutritional interventions (Table 4-1). However, data on the prognostic value of T cell subsets in malnourished untreated HIV-infected adults and the effects of minerals (Table 4-2) on $CD4^+$ and $CD8^+$ T cell subsets is inadequate. From the literature reviewed, the following knowledge gaps are apparent:

a. The prognostic value of T cell subsets in untreated malnourished HIV-infected adults initiating ART

b. The effects of micronutrient interventions on $CD4^+$ and $CD8^+$ T cell subsets in African HIV-infected adults on ART.

c. There are no data on whether T cell subsets after immune reconstitution approach normal levels.

d. *In vitro* response of T cell subsets to individual micronutrients.

e. No studies have separately analysed the effects of minerals (potassium, phosphorous and magnesium), which are important for rebuilding lean tissue mass on T cells.

4.1.1 Study Justification 1

Although the prognostic value of T cell subsets have been investigated, only a few markers (at most six) have been used (Table 4-1). It is likely that the prognostic value of certain markers might have been overlooked. In addition, while immune reconstitution as regards nutritional supplementation has been investigated in HIV-infected individuals, none has been conducted in HIV-infected individuals receiving bulky minerals (Table 4-2). Therefore this study set out to investigate two fundamental issues;

1. The prognostic value of T cell subsets using 18 different monoclonal antibodies to categorise the $CD4^+$ and $CD8^+$ T cells by stage of life cycle (Proliferation, senescence, naïve, central memory, effector memory and effector), by gut homing potential and thymic production.
2. $CD4^+$ and $CD8^+$ T cell subset responses to a cocktail of micronutrients with or without vitamins and minerals (*in vivo)* and selenium *in vitro.*

It is envisaged that the cellular subsets associated with early mortality after ART initiation may provide insight into the mechanisms contributing to immunological dysfunction and impaired immune reconstitution.

Eventually, the information derived from this study would provide insight into the optional composition of any nutritional rehabilitation trends for HIV-infected patients.

Table 4-1: T cell subsets and Mortality

First Author & Year	Study Design & Population	Antibodies Used	Findings	Mortality
Hunt, 2014	64 case patients who died within 12 months of treatment-mediated viral suppression were each matched to 2 control individuals total number of controls, 128	CD3, CD4, CD8, CD28, CD45RA, CD31, CCR7, CD57, CD27, HLA-DR, CD38, CCR5,	A higher percentage of CD38+HLA-DR+ cells in the CD8+ T-cell population was a predictor of mortality before but not after adjustment for proximal CD4+ T-cell count. Frequencies of senescent, naive, and CMV-specific T cells did not predict mortality.	Gut epithelial barrier integrity markers predict mortality in patients with ART-suppressed HIV infection and a history of AIDS
Lee, 2014	141 ART-suppressed participants	CD4, CD8, CD57, CD28	Abnormally low proportions of $CD28^{-}CD8^{+}$ T cells expressing CD57	Low proportions of $CD28^{-}CD8^{+}$ T cells expressing CD57 predict increased mortality during treated HIV infection
Rainwater-Lovett, 2012	Prospective, observational study, 149 HIV-infected children	CD3, CD4/CD8, HLA-DR, CD38, CCR7, CD45RA	Increase in CD8+ effector T cells increased the odds of overall mortality	Effector T cell subsets may be more accurate predictors of early mortality
Ronsholt, 2012	101 HIV-infected patients	CD4/8, CD45RA, CD62, CD45RO, HLA-DR, CD38	High level of memory CD8+ T cells, was an independent predictor of increased overall survival and immune activation showed no prognostic value	T cell activation was not associated with increased risk of death.
van Dijk, 2011	A prospective cohort study, 267 HIV-infected children	CD3, CD4	Younger age, higher viral load, lower CD4+ T-cell percentage and lower weight-for-age z-scores at ART initiation was associated with higher risk of mortality	Higher HIV viral load was associated with an increased risk of mortality
Hunt, 2011	Prospective cohort study, 451 HIV-infected adults	CD4, HLA-DR, CD38	Higher CD8+ T cell activation levels during early ART	T cell activation independently predicted mortality
Srinivasula,	41 HIV-infected patients	CD4/8, CD38,	CD4/CD8 Activation & proliferation was	Not reported

2011		HLA-DR, Ki67, CD45RA, CD27	associated with viral load	
Lederman , 2011	60 immune failure, 188 immune, success & 21 healthy subjects	CD4/8, CD45RA, CCR7, CD38, Ki67	CD4/8 activation was used to described immune failures & success	Not reported
Rickabaugh, 2011	Cross- section 47 HIV seronegative & 19 HIV seropositive	CD4, CD45RA, CD31, CD28, CD27, 7AAD	Partial explanation both for the faster disease progression of older adults and the observation that viral responders to ART present with clinical diseases associated with older adults	Not reported
Ganesan, 2010	466 HIV-infected subjects	CD4/8, CD45RO, CCR5, CCR7, CD27, CD28, Ki67	Expression of Ki67 on $CD4^+$& $CD8^+$ determined fast progression to AIDS	Not reported
Murray, 2010	13 HIV-infected Double-blind, randomized placebo-controlled trial	CD4/8, Ki67, HLA-DR, CD27, CD38, CD45RO	Reduction in immune activation during HIV-infection	Not reported
Schacker, 2010	348 patients from multiple AIDS Clinical Trials Group studies	CD4, CD45RA, CD62L	Lower baseline naive CD4 percent was associated with greater time spent at lower CD4 T-cell counts after initiating ARV	Naïve cells identifies individuals at lower risk of morbidity and mortality
Cicala, 2009	Not mentioned	CD4, CD45RO, CCR5, Ki67, α4, β7	α4β7 T cell subsets are prone to highly productive infection	Not reported
Huang, 2008	Retrospective, 13 HIV-infected patients	CD4/8CD57, CD28	CD4+CD57+ T-cells population were more susceptible to cell death	Not reported
Elrefaei, 2004	31 HIV-infected & 10 HIV negative	CD4, CD28, CCR7, CD45RA	During HIV-infection, there is an impaired maintenance of central memory $CD4^+$ T cells	Not reported
Ostrowski, 2003	107 HIV-1–infected patients and 65 control subjects	CD4, CD8, CD28, CD45RA, CD45RO	Concentration of CD4+ cells markedly reduced	50% reduction in CD4+CD28+ cells predicted increased mortality

Hazenberg, 2003	Prospective cohort study of 102 HIV-infected participants	CD4, CD8, CD70, CD27, CD38, CD45RO, HLA-DR, Ki67	Increased T cell activation has predictive value for HIV-1 disease progression	Not reported
Choi, 2002	130 HIV-infected people	CD4, CD8, CD28, HLA-DR	CD28/HLA-DR expressions on CD4+ T cells were more predictable in the evaluation of the disease progression	Not reported
McCloskey, 2001	Cohort of 112 HIV-infected children	CD3, CD4, CD8, CD14, CD45, CD38, CD95, CD45RA, CD45RO, HLA-DR	Perturbations in the lymphocyte pool determined HIV disease progression	Not reported
Giorgi, 1999	37 HIV-1–infected men	CD3, CD4, CD8, CD45RA, CD45RO, CD38, HLA-DR, CD62	Expression of CD38 antigen on CD4+ and CD8+ T cells, was strongly associated with shorter subsequent survival	Not reported
Giorgi, 1993	98 HIV-infected men	CD4, CD8, CD38, HLA-DR, CD57	CD38+ CD8+ levels had prognostic value for progression to AIDS	Not reported

Table 4-2: Summary of Interventional Studies and Effects on Total CD4/CD8 Count

First Author	Study Design/Population	Nutritional Intervention	CD4 count	Mortality
Low income				
Swaminathan, India (2010)	636 HIV+ patients; prospective interventional	100 mg/d of whole wheat & soybean 6months	No significant effect	Not reported
Ndekha, Malawi (2009)	491 HIV+ patients; RCT	245 g peanut-based 3.5 months	No significant effect	No significant effect
Cantrell, Zambia (2008)	636 HIV+patients; A pilot study	Home-based adherence	No significant effect	Not reported
Kelly, Zambia (2008)	276 HIV+ and 224 HIV-; A cluster RT	Micronutrients 1.9 y	No significant effect	Decreased
Kupka, Tanzania (2008)	913 HIV+ pregnant women; RCT	Selenium + prenatal iron, folic acid and vitamins 6 months	No significant effect	No significant effect
Villamor, Tanzania (2008)	471 HIV+ and 416 HIV- patients with TB RCT	Micronutrients 30 months	No significant effect	No significant effect
McClellard, Kenya (2004)	400 HIV+ women RT	Micronutrients 6 wks.	No significant effect	Not reported
Jiamton, Thailand (2003)	481 HIV+patients RCT	Micronutrients 48 wks.	No significant effect	Improved survival
High income				
Sattler, United States (2008)	59 HIV+ patients	High-calorie protein 12 wks.	Significantly increased	Not reported
Hurwitz, United States (2007)	310 HIV+ patients	High selenium yeast 9 months	Increased	Not reported
Kaiser, United States (2006)	40 HIV+ patients	Micronutrients 12 wks.	Increased	Not reported
Burbano, United States (2002)	186 HIV+ patients	Selenium 2 years	Increased	Not reported

4.1.2 Study Justification 2 (Selenium)

Studies reviewed above (Table 3-4) also show a positive correlation between serum Se levels and CD4 counts overall. Thus, since Se is one of the micronutrients for which there is best direct evidence of a benefit on immunity, it is rational to explore the direct effects of Se on T cell phenotypes.

4.2 Conceptual Framework

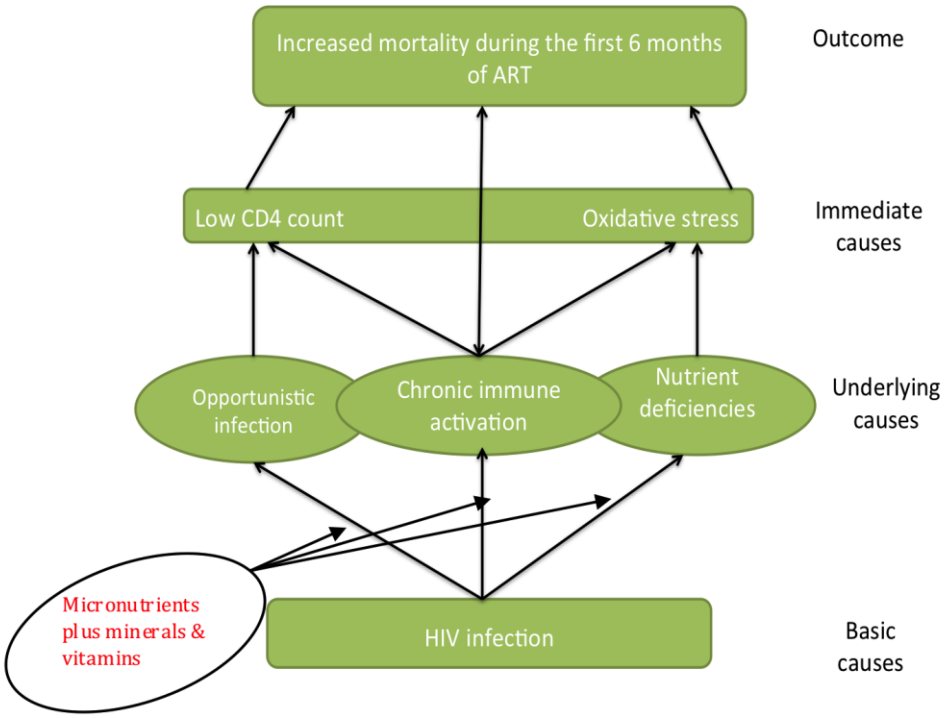


Figure 4-1: Study Conceptual Frame Work

4.3 General Objective

To determine the prognostic value of, and the effects of nutritional supplements on, $CD4^+$ and $CD8^+$ T cell subsets in malnourished HIV-infected Zambian adults.

4.3.1 Specific Objectives

1. To describe $CD4^+$ and $CD8^+$ T cell subpopulations (differentiation, senescence, thymic and homing) and surface markers in healthy Zambian adults.
2. To determine whether baseline markers profile predict mortality.
3. To define the immunological restitution marked by T cell surface marker expression in adults receiving vitamins and mineral supplementation on ART.
4. To determine if surface expression changes differ in trial participants receiving micronutrients plus minerals and vitamins and those receiving micronutrients only.
5. To determine the effects of Se *in vitro* on expression of T cell gut homing, senescent, differentiation and thymic output markers in adult HIV-negative volunteers.
6. To determine whether T cell subsets after immune reconstitution approach normal levels.

4.4 Research Question

What is the prognostic value of, and the effects of nutritional supplements on, $CD4^+$ and $CD8^+$ T cell subsets in malnourished HIV-infected Zambian adults?

4.5 Study Design

In order to address the research question, this research included five distinct designs: a cross-sectional study, retrospective study, survey, *in vitro* dose response study, and a randomized controlled phase III trial.

Chapter 5: Research Methods

5.1 T Cell Subset Profile in Healthy Zambian Adults (Cross Sectional Study)

In order to understand and describe the $CD4^+$ and $CD8^+$ T cell subsets distribution in a healthy population, a cross sectional study was undertaken and sample size calculated based on a study by Tsegaye et al.,1999 Ethiopia.

5.1.1 Sample Size Calculation-Cross Section Study

$$\mathbf{n = (Z\delta/\Delta)^2}$$

n=Minimum sample size

δ=Standard deviation

Δ=Effect size

Z=1.96

Table 5-1: Sample Size Calculations

CD4 Std dev.	Effect size	Power	Sig.	Number	Loss to follow up	Total
225	65 cells/μL	90%	5%	46	10%	51

5.1.2 Study Participants and Area

Participants were identified amongst staff of the University Teaching Hospital (UTH) between February and March 2015. Participants were taken to the recruitment center at the UTH-TROPGAN lab where further details were provided through the information sheet and the consent form filled in if participants were willing. A total of 52 healthy volunteers consented and were recruited. Of the 52 participants; 20 were lab scientists, 22 medical students from Ridgeway Campus, 4 UTH-OPD data clerks, 1 nurse and the rest in administration (accountants).

Inclusion criteria

- 18 to 65 yrs old (either male or female)
- HIV- negative
- HbsAg negative
- HCV negative
- Syphilis-non reactive
- Normal full blood count results
- Providing written, fully informed consent (thumbprint was accepted)

Exclusion criteria were; not providing consent, pregnancy and having any chronic disease.

5.1.3 Sample Collection and Transportation

Following consent, 10 mls blood were collected into three EDTA tubes for haematology testing at the UTH haematology Laboratory, CD4 and CD8 testing at UTH-Virology Laboratory and for HIV, syphilis, HbsAg and anti-HCV testing at Blood Bank. All the blood samples were delivered to the Laboratory within 1 hour of collection.

5.1.4 Haematological Analysis

Haemoglobin measurements were undertaken immediately on whole blood on a Sysmex xt 4000i automated haematology analyser (Sysmex Corporation, Kobe, Japan).

5.1.5 Total CD4 and CD8 Estimation

Briefly, CD4 and CD8 testing was done on the FACSCaliber flow cytometer (Becton Dickinson, San Jose, U.S.A) with MultiSET V2.2 software. CD3/CD4/CD45 TruC v2.0 (BD Biosciences) were used, and 15000 events were acquired. Antibodies used were

conjugated to the following flourochromes as follows; CD45-PerCP CD4-PE CD3-FITC.

5.1.6 $CD4^+$ and $CD8^+$ T Cell Subset Testing (Immunophenotyping)

Briefly, 100 μL of whole blood was stained for each panel of markers; cells were fixed, washed, and resuspended in 250 μL paraformaldehyde or 250 μL BD Cell Fix diluted according the manufacturer's (BD Biosciences) instructions and 50-100,000 lymphocytes acquired on a FACSverse flow cytometer (Becton Dickinson, San Jose, U.S.A.). Intracellular Ki-67 staining was carried out following nuclear membrane permeabilisation (BD Perm/Wash™ buffer from Becton Dickinson, San Jose, U.S.A). Unstained cells and fluorescence minus one (FMO) controls were used to set all gates. Analysis of markers was carried out using BD FACSuite™ software v1.0.2. Sample processing steps are summarised in the appendices also other details on panel design, principle and setup related issues are provided in appendix 10.

5.1.7 Qualitative Testing HIV-1/HIV-2, HCV and HBsAg

The ARCHITECT System Abbott i2000 (Abbott, Germany) was used to qualitatively detect HIV P24 antigen and antibodies to HIV-1/HIV-2, antibodies to anti-HCV, HBsAg and syphilis in plasma. The ARCHITECT qualitative assay is a chemiluminescent microparticle immunoassay (CMIA) for the qualitative detection of antibodies/antigens in serum or plasma. The presence or absence of antibodies or antigens in the sample was determined by comparing the chemiluminescent signal in the reaction to the cutoff signal, which was determined from an active calibration. Samples with a chemiluminescent signal greater than or equal to the cutoff signal were considered as reactive.

5.2 Overview of the NUSTART trial and the sub-study reported here.

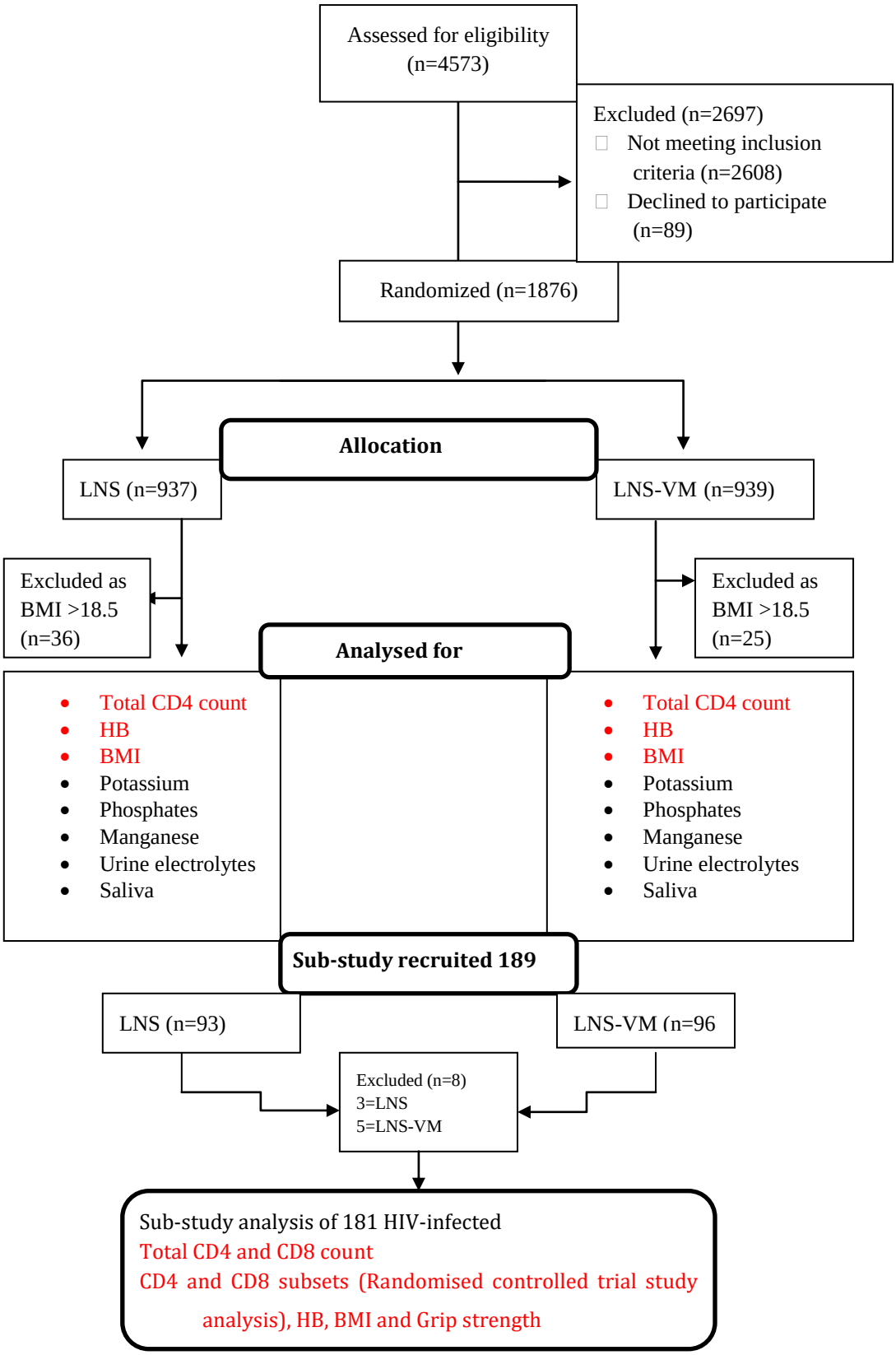


Figure 5-1:Relationship Between NUSTART Main Trial and the Sub-Study

5.2.1 An Overview of the Study (sub-study) Design and Sample Size

Embedded in the sub study was a Retrospective study. This study aimed at understanding the level of increase in absolute CD4 count following 12 weeks of receiving nutritional supplements and also to establish the need to explore the T cell subsets. All adults recruited into the NUSTART trial in Lusaka between March 2011 and April 2012 who had completed their twelve weeks of receiving ART plus nutritional support were eligible for this study. The inclusion and exclusion criterion were the same as those of NUSTART. CD4 results for all the 267 NUSTART study participants who had completed their twelve weeks were collected from their respective files entered and analyzed using STATA version 12.

Secondly, in order determine whether baseline $CD4^+$ and $CD8^+$ T cell subpopulations predict mortality and also to understand the effects of the nutrients on the $CD4^+$ and $CD8^+$ subsets, a sub-study of the RCT was conducted. Patients were followed up and evaluated at 12 weeks following ART initiation and immunophenotyping was carried out on baseline and 12 week blood samples. CD4 results for 267 NUSTART study participants who had completed their twelve weeks were used and sample size calculated. Briefly, a mean CD4 of 147 at 12 weeks and standard deviation (SD) of 144 was obtained. Assuming an increase of about 100 CD4 cells/μL at the end of twelve weeks in the study, the sample size calculation was done.

$$\mathbf{n=(2\delta^2/\Delta^2)(Z\alpha+Z\beta)^2}$$ **(Kadam and Bhalerao, 2010).**

n=Minimum sample size; δ=Standard deviation; Z1-β=Power of study; Zα=Accepted error; Δ=Effect size; Z=1.96

Table 5-2: Sample Size Calculations

Std dev. 1	Std dev. 2	Effect size	Power	Sig.	Number per arm	Loss to follow up	Number per arm	Total
144	102	100 cells/μL	80%	5%	25	50%	38	76
144	102	100 cells/μL	90%	5%	33	50%	50	100
144	**144**	**100 cells/μL**	**90%**	**5%**	**44**	**50%**	**66**	**132**

Based on this calculation, the sample size was 132.

5.2.2 Study Subjects and Area

Patient selection was the same as for the parent study. All the participants who consented to be part of the NUSTART between April 2013 and December 2013 were recruited into this study. Their inclusion and exclusion criteria followed that of NUSTART as described in section **2.1.2**.

5.2.3 Sample Collection and Transportation

At baseline and at week 12 following ART initiation, 3-5 ml of peripheral blood was collected. Blood samples were collected for haematology plus CD4 and CD8 count with surface maker testing using EDTA containers. Blood samples reached the laboratory within 1h of collection and CD4 and CD8 immunophenotyping and full blood count measurement were undertaken on whole blood. Full blood count was measured on a Sysmex xt 4000i automated haematology analyser (Sysmex Corporation, Kobe, Japan) and Immunophenotyping as described for Phase I. However, CD4 and CD8 absolute counts were done using the lyse no wash protocol on the FACSVerse. Details are provided in the appendices.

5.3 Experimental *In Vitro* Sample Prosessing (Exploratory)

About 10 ml of blood was collected in EDTA and lithium heparin tubes tubes from HIV negative volunteers. Part of the blood was processed immediately for $CD4^+$ and $CD8^+$ T cell subset analysis as shown in the cross sectional study. Another part of the same sample was incubated for 18-24 hrs. in the incubator with different Se concetrations as follows; 180 μmol/L, 200 μmol/L and 220 μmol/L.

Figure 5-2: Selenium Measuring and Sample Incubation

Incubated samples were stained with monoclonal antibodies as described in phase I above and analyzed using the FACSVerse as described in Chapter 5. To establish the baseline Se level in healthy volunteers before incubating samples with Se, blood samples in lithium heparin tubes were spun down and plasma samples collected. Plasma samples were measured on the Optimal Emission Spectrometer 7000 DV (Optima) serial number 080C1020202 PerkinElmer United States for the determination of Se levels.

5.3.1 Method for Selenium Measurement in Plasma

Five working standard solutions were prepared using PerkinElmer Pure Instrument Calibration Standard 4 (N930021). Plasma samples were diluted (1:9 v/v) with an aqueous solution containing 2% Nitric acid, 3M HCl and 1% Triton. The instrument was started, brought to operational conditions and stabilized. The sample introduction system was checked and the wavelengths were tuned to 196.026nm, which is the standard for Se. The instrument was standardized with the five working standard solutions (multi-point linear fitting). Samples were measured with standardization blanks, control samples, and quality control samples (Seronorm L-1). The detection limit ranges for Se using ICP-OES are 1-10ppb.

5.3.2 Calculation

The data were exported to an Excel spreadsheet. Correction for standardization blanks, drift correction, and dilution factor application were preset on the instrument software (WinLab32). Concentrations were reported in mg/L and converted to µmol/L.

5.4 Survey

The overall aim of this study was to determine whether ART plus nutritional supplementation was able to restore CD4 and CD8 T cell proportions as obtaining in the healthy volunteers. Fifty-one individuals were randomly selected from the 88 participants that had completed the 3 months of receiving ART plus nutritional support (at 80% power). These individuals were matched 1:1 with the healthy adult volunteers for cross section study. Ultimately, there were 51 HIV+ and 51 HIV- calculated as shown in Table 5-3 below.

$$n = \frac{2\sigma^2}{(\mu_2-\mu_1)^2} f(\alpha,\beta)$$

Table 5-3: Survey Sample Size Calculation

M1	M2	CD4 Std. dev.	Effect size	Power	Sig.	Number per arm	Loss to follow up	Total	
800	670	227	130 cells/μL	80%	5%	49	10%	98	

All participants enrolled in the cross section study as described in **5.1.3** were included in this study.

5.5 Data Analysis

Data were entered and analysed using STATA software version 13 (StataCorp LP, College Station, Texas, USA). T cell markers were determined to be non-parametrically distributed using Shapiro-Wilk W test for normality, so with medians and interquartile ranges presented and variables compared using the Mann-Witney U test, or the Wilcoxon rank sum test for paired variables and Friedman or Kruskal-Wallis test with Dunn's correction for multiple comparison to test three or more matched and unmatched groups respectively. For parametric analyses, paired or unpaired *t*-test was performed comparing two groups. *P* values less than 0.05 were considered statistically significant. As follow up was short and uniform, model of death was constructed using unconditional logistic regression. For multivariate analysis, T cell markers were dichotomised around the median.

5.6 Ethical Considerations

5.6.1 Study Approval

The University of Zambia Biomedical Research Ethics Committee (UNZAREC) approved the sub-study (Ref. No. 009-01-11-for the whole PhD work).

5.6.2 Validation of Experiment (Quality Assurance and Procedures)

Validation of laboratory procedures was done before running experiments on the FACSVerse. At the start, three different blood samples were sent to different labs within UTH and one lab outside UTH i.e. (virology, adult center of excellence, paediatric center of excellence and CIDRZ) to run CD4s and CD8 testing. Other samples were also run on the FACSVerse and all the results compared for consistency. However, samples were run parrarel with CIDRZ lab and occasionally with UTH virology throughout the experiment.

5.6.3 Benefits

There may be no direct benefit to you from participating in the study. However, you may find out that you have the co-infection of the HIV and HSV-2 or either of the two and be referred for management, which is free. Counselling will be done before and after VCT.

5.6.4 Risks

There are no risks except some tolerable pain when drawing venous blood. Steps have been taken to reduce the risk. You may however, feel some discomfort with some questions that are unusual.

5.6.5 Reimbursement

You will be paid transport money to and from home (bus fare). If you are injured as a result of being in this study, treatment will be available at UTH. The researcher will pay the costs of such treatment. Additionally, lunch will be provided in an event that the procedure takes long that it covers the lunch hours.

5.6.6 Confidentiality

The results of all the study will be discussed with you, and kept confidential unless you wish otherwise. Except for this disclosure, all information obtained in this study will be considered confidential and used only for research purposes. Your identity will be kept confidential as far as the law allows.

5.6.7 Injury Clause

In the event that you become injured during the course of the research study, immediately notify the principal investigator or the chairperson of the Research Biomedical Ethical committee of the University of Zambia, School of Medicine on telephone number 256067 or P.O BOX 50110, Ridgeway campus, Lusaka. If you believe that your injury directly resulted from the search procedures of this study, you can file a complaint with the principal investigator.

5.6.8 Right to Refuse or Withdraw

Your participation in the study is entirely voluntary, and you are free to refuse to take part or withdraw at anytime without affecting or jeopardizing your future medical care.

Other ethical considerations have been provided in the appendices.

Chapter 6: Study Results

6.1 Normal distribution of T cell subsets in HIV Negative Adults

Figure 6-1 describes the flow of participants enrolled in the cross sectional study and the experiments done.

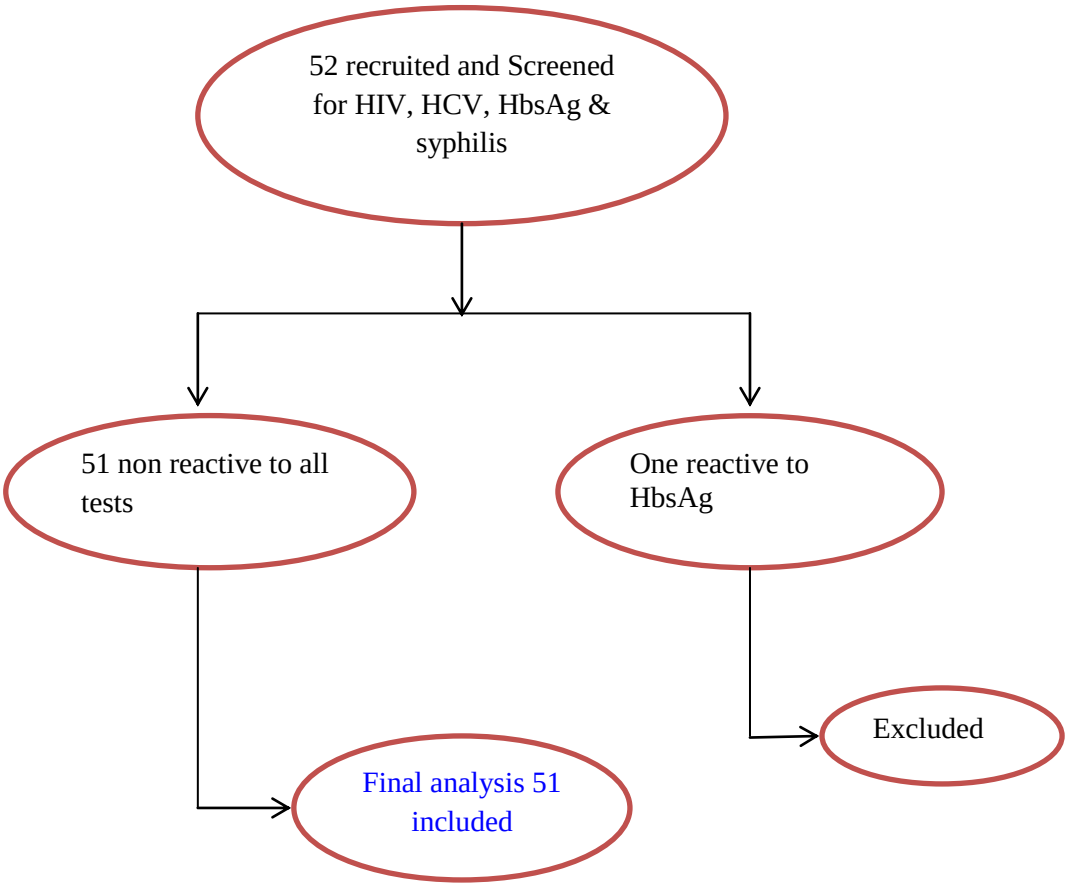


Figure 6-1: Flow diagram for the participants enrolled in the study

Table 6-1: Baseline Characteristics for the Participants

Variable	Females N=17	Males N=34	*p* value
Sex	17 (33%)	34 (67%)	<0.001
Age (Years)	34 (27, 45)	23 (28, 34)	0.01
Absolute CD4 count (Cells/µL)	1042 (864, 1270)	671 (545, 899)	0.003
Absolute CD8 count (Cells/µL)	1531 (1248, 1740)	1346 (1101, 1801)	0.42
Hb (g/dl)	13.3 (11.3, 14)	15.6 (14.8, 16.6)	0.0001
BMI (Kg/m^2)	25.03 (23.3, 28.88)	23.18 (21.18, 25.5)	0.07
Grip strength (Kg)	25.9 (23.8, 27.5)	44.7 (39.9, 47.7)	0.0001
Education University College Secondary Primary	 8 (47%) 6 (35) 2 (12%) 1 (6%)	 22 (64%) 6 (18%) 6 (18%) 0	0.21

Occupation			0.22
Employed	11 (64%)	23 (68%)	
Students	4 (24%)	11 (32%)	
Business	1 (6%)	0	
Unemployed	1 (6%)	0	
Marital status			0.23
Married	10 (59%)	17 (50%)	
Single	6 (35%)	17 (50%)	
Divorce	1 (6%)	0	

1 Mann–Whitney U-tests were used for non-parametric variables and Pearson chi square for categorical variables. Continuous characteristics are expressed as median (interquartile range) and dichotomous characteristics are expressed as n (%). g/dl=grams per decilitre; Hb= haemoglobin; BMI= body mass index

Table 6-2: CD4 and CD8 Subset Reference Intervals in Healthy Population Stratified by Sex

Variable	**Female N=17**	**Male N=35**
CD4	**Median (95% RI)**	**Median (95% RI)**
Absolute count	1042 (514-1799)	671 (68-1333)
Homing	413 (168-1134)	239 (17-673)
Homing activated	247 (89-628)	161 (21-413)
Thymic	31 (19-45)	28 (9-55)
Senescent 1	30 (1-191)	18 (5-278)
Senescent 2	10 (0-156)	6 (0-133)
Naïve	904 (184-1435)	573 (64-1283)
Central memory	51 (4-309)	43 (4-478)
Effector memory	1 (0-48)	0 (0-56)
Effector	55 (0-497)	9 (0-270)
Proliferating	22 (3-106)	12 (0-59)
Activated	4 (0-29)	7 (0-49)
Proliferating activated	3 (1-15)	4 (0-36)
CD8		
Absolute count	1531 (21-2264)	1346 (323-2603)
Homing	537 (11-1488)	627 (140-1436)
Homing activated	0 (0-0)	0 (0-4)
Thymic	146 (1-724)	38 (4-254)
Senescent 1	426 (3-700)	283 (69-1136)
Senescent 2	193 (0-691)	163 (0-1125)
Naïve	227 (3-754)	256 (60-1579)
Central memory	154 (2-494)	204 (37-1145)
Effector memory	435 (0-981)	316 (0-890)
Effector	695 (0-934)	472 (0-999)
Proliferating	28 (1-73)	29 (0-150)
Activated	4 (0-21)	14 (0-41)
Proliferating activated	1 (0-12)	2 (0-1281)

Values are medians, 95 percentiles are in parentheses. RI=Reference interval

In this study, there were more males than females, and the majority of these were in formal employment with a median age of 29 (IQR, 25-37) Table 6-1. Hb, BMI and grip strength results for both male and female participants were within the normal ranges, which are Hb range for females (12 - 15 g/dl) and 13.5 – 18 g/dl in males, BMI (18.5 – 25 kg/m^2). As expected, grip strength differed substantially by sex. Absolute CD4 was 824 (IQR, 558-1044) and CD8 1371 (IQR, 1136-771) were also

within normal ranges (CD4 400 – 1200; CD8 150 – 1000), most of the individuals were married and had attained university education.

Generally, in both females and males, CD4 and CD8 absolute values were within the reference intervals (Table 6-2). T cell subset ranges are also shown in Table 6-2.

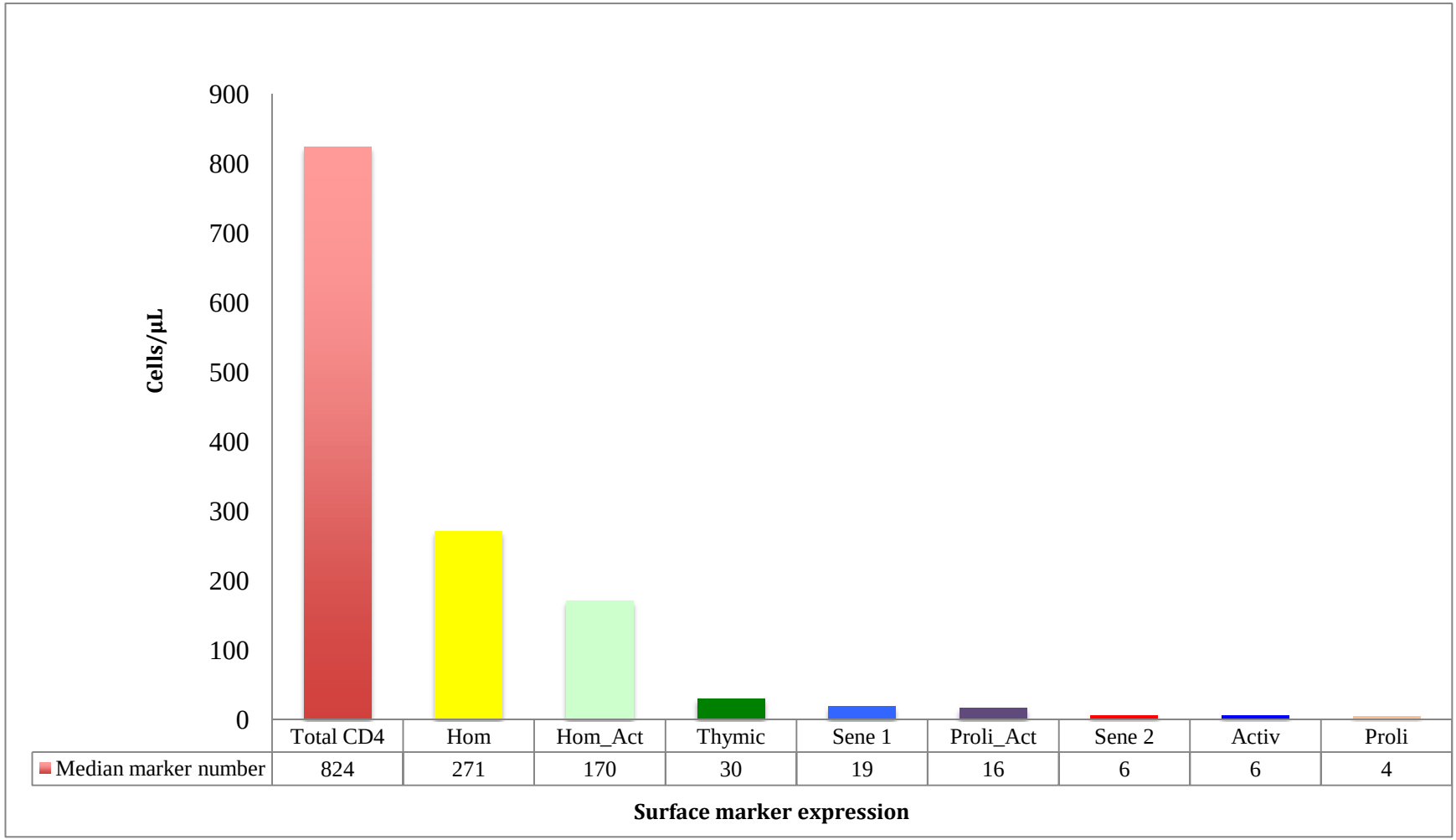


Figure 6-2 Total CD4 and subset distribution in healthy Zambian adults using surface marker expression

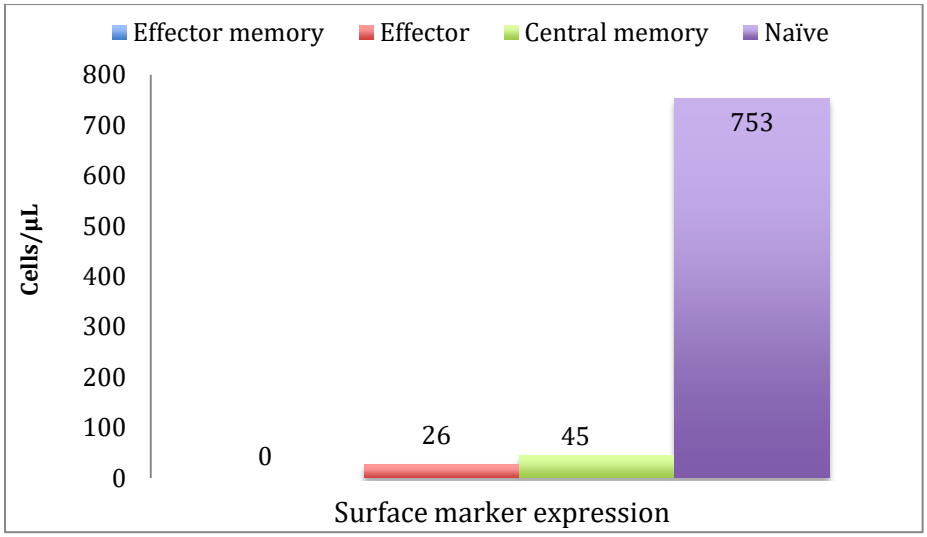
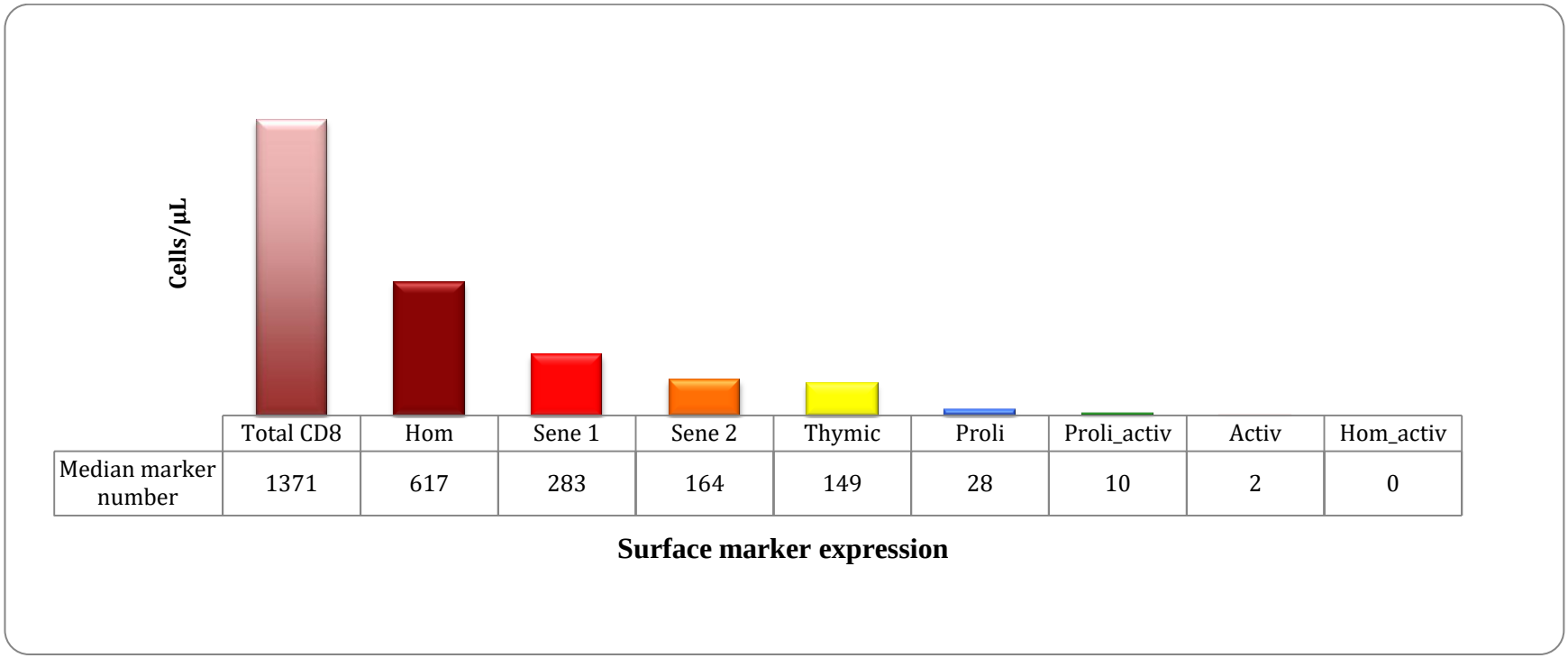


Figure 6-3: Distribution of functional status of CD4 cells in healthy Zambian adults.

Figures 6-2 and 6-3 show $CD4^+$ T cell subset distribution in healthy adult volunteers. Non-viable ($CD4^+7AAD^+$) $CD4^+$ T cells were completely absent in the healthy participants i.e. 0 (IQR, 0-0) (data not shown). These results show that majority of the $CD4^+$ T cells in healthy adults were naïve| cells ($CCR7^+CD45RA^+$) 753 (IQR, 418-885), followed by the recent thymic emigrants ($CD31^+CD45RA^+$) at median of 30 (IQR, 22-35), gut homing cells ($\alpha4^+\beta7^+$) 271 (IQR, 198-408) and gut homing and activated ($\alpha4^+\beta7^+CD25^+$) $CD4^+$ T cells 170 (IQR, 129-269).



	Total CD8	Hom	Sene 1	Sene 2	Thymic	Proli	Proli_activ	Activ	Hom_activ
Median marker number	1371	617	283	164	149	28	10	2	0

Figure 6-4: Shows total CD8 and subset distribution in healthy adults using surface marker expression

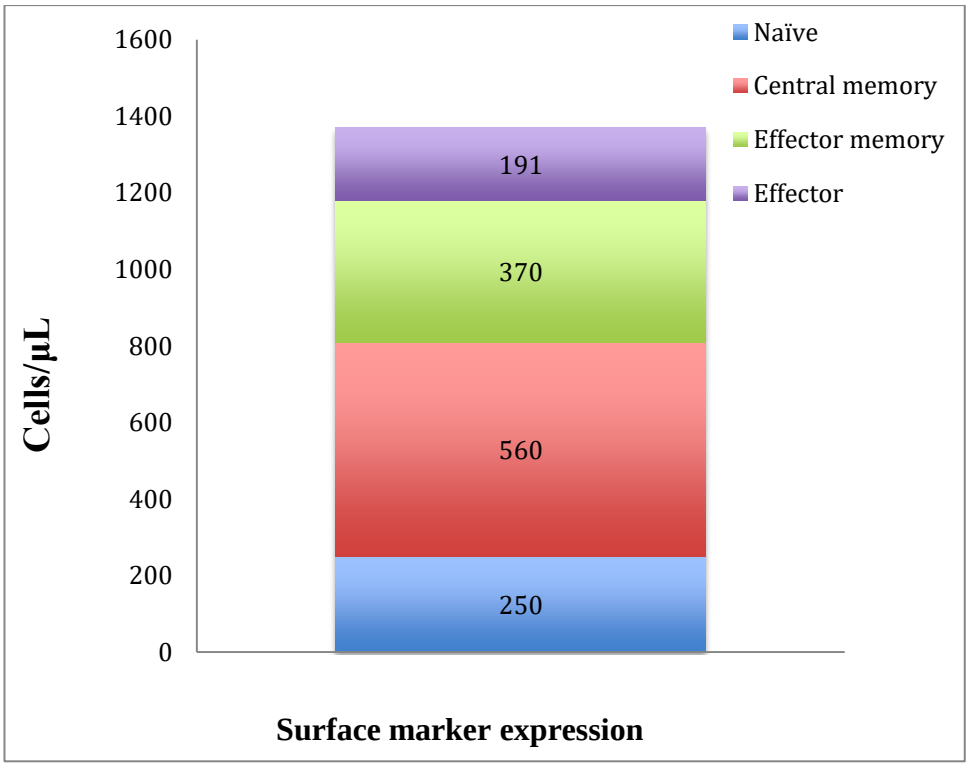


Figure 6-5: Distribution of functional status of CD8 cells in healthy Zambian adults.

In the $CD8^+$ T cell subsets (Figure 6-4), there were no homing activated cells i.e. 0 (IQR, 0-0). Similarly, expression of activation markers was minimal 2 (0, 6), as were proliferating markers 28 (IQR, 17-38), non-viable cells 0 (0, 0) and cells expressing both proliferation and activation markers 10 (IQR, 4-19).

$CD8^+$ recent thymic emigrants T cells were moderately expressed i.e. 149 (105, 381), central memory 191 (IQR, 109-282), senescent 2 ($CD57^+CD28^-$) 164 (IQR, 36-332) and moderate expression of naïve $CD8^+$ T cells 255 (IQR, 127-401).

Senescent 1 ($CD57^+$ only) $CD8^+$ T cells were 283 (IQR, 182-452) and effector memory cells at 374 (IQR, 185-481). Thus, in the $CD8^+$ T cells population, majority of the subset cells are gut homing cells 617 (IQR, 429-985) followed by effector cells 564 (IQR, 305-732).

Table 6-3: CD4 and CD8 Subset in Healthy Population Stratified by Sex

	CD4 Males (N=34)	CD4 Females (N=17)		CD8 Males (N=34)	CD8 Females (N=17)	
Variable	**Median (IQR)**	**Median (IQR)**	***P* value**	**Median (IQR)**	**Median (IQR)**	***P* value**
Absolute number	671 (545, 899)	1042 (864, 1270)	0.003	1346 (1101, 1801)	1531 (1248, 1740)	0.42
Homing	239 (183, 354)	413 (324, 471)	0.01	627 (475, 1004)	536 (393, 758)	0.33
Homing and activated	160 (122, 257)	247 (139, 385)	0.07	0 (0, 0)	0 (0,0)	0.22
Thymic	28 (20, 34)	31 (28, 36)	0.21	254 (106, 396)	146 (84, 366)	0.48
Senescent 1	18 (12, 38)	31 (13, 55)	0.63	282 (231, 394)	426 (75, 497)	0.99
Senescent 2	6 (1, 16)	10 (4, 17)	0.46	163 (46, 297)	193 (24, 439)	0.48
Naive	573 (411, 806)	904 (657, 1006)	0.02	256 (134, 587)	227 (83, 380)	0.30
Central memory	43 (14, 90)	51 (14, 99)	0.72	204 (112, 388)	154 (84, 228)	0.23
Effector memory	0 (0, 1)	1 (0, 9)	0.01	316 (73, 455)	435 (348, 514)	0.08
Effector	9 (0, 93)	55 (15, 261)	0.01	472 (106, 628)	695 (533, 776)	0.04
Proliferating	4 (1, 6)	3 (3, 7)	0.30	29 (18, 38)	28 (17, 43)	0.91
Activation	7 (2, 12)	4 (2, 14)	0.85	2 (0, 6)	1 (0, 5)	0.47
Proliferating and activated	12 (8, 21)	22 (14, 28)	0.10	14 (5, 21)	4 (2, 17)	0.08

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

Stratification by sex showed very interesting results for the same population. In the $CD4^+$ population, females had higher absolute $CD4^+$ count, gut homing, naïve, effector memory and effector $CD4^+$ T cells than males (Table 6-3). However in the $CD8^+$ population, only effector cells were significantly different between the females and males (Table 6-3). However, although females had higher CD4 and CD8 cells than the males, the overall distribution of both CD4 and CD8 was similar.

6.2 Retrospective analysis of overall changes in total CD4 count in NUSTART study

In this study, 267 files of NUSTART study participants that had completed their 12 weeks were reviewed. Baseline absolute CD4 and week 12 counts were recorded.

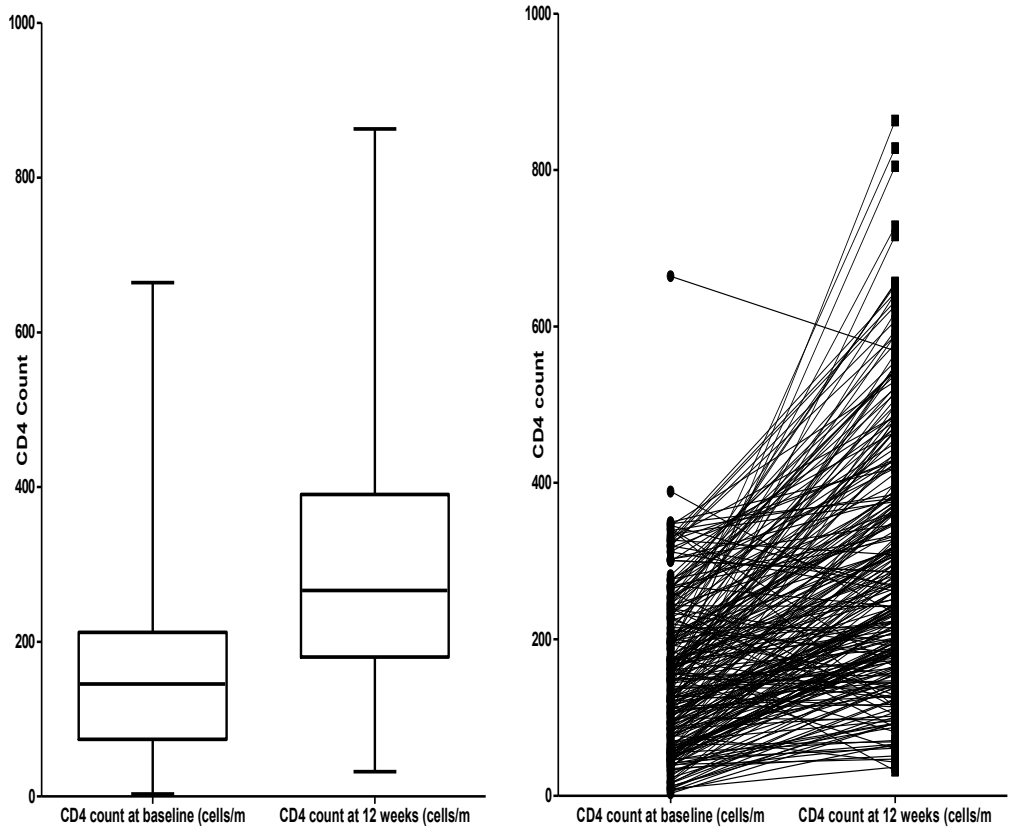


Figure 6-6: Shows baseline CD4 count and 12 weeks after ART and nutritional

supplementation at p<0.0001. There was a significant increase in median CD4 count after 12 weeks**.**

In this analysis, although the majority of individuals had an increase in absolute CD4 count, there were a few individuals whose CD4 count actually dropped after 12 weeks of ART and nutritional support. For others, the increase was almost three times the initial count. The median CD4 count at enrolment was 145 cells/μL interquartile range of 74-212. After 12 weeks, the median increased to 266 (IQR, 180-390) with the calculated delta change being 140 (IQR, 53-216).

6.2.1 Randomised Controlled Trial-NUSTART

The first aim of this study was to determine whether specific T cell subsets predict mortality in a cohort of HIV-infected Zambian adults initiating ART. The second aim was to assess whether T cell subsets change over time in patients randomised to LNS-VM compared to LNS alone.

A total of 189 HIV-infected adult men and women were enrolled in this sub-study and randomised to either LNS or LNS-VM. Participants were excluded from analysis if some baseline $CD4^+$ and $CD8^+$ T cell panel data were missing (n=3 LNS and n=5 LNS-VM). Of the remaining 181 participants, 90 were in the LNS and 91 in the LNS-VM group (Figure 8-1). Of 181 participants, 36 (20%) died at median 5.7 (IQR, 3.5-10.1) weeks after ART initiation; 145 (80%) survived through 12 weeks after initiating ART.

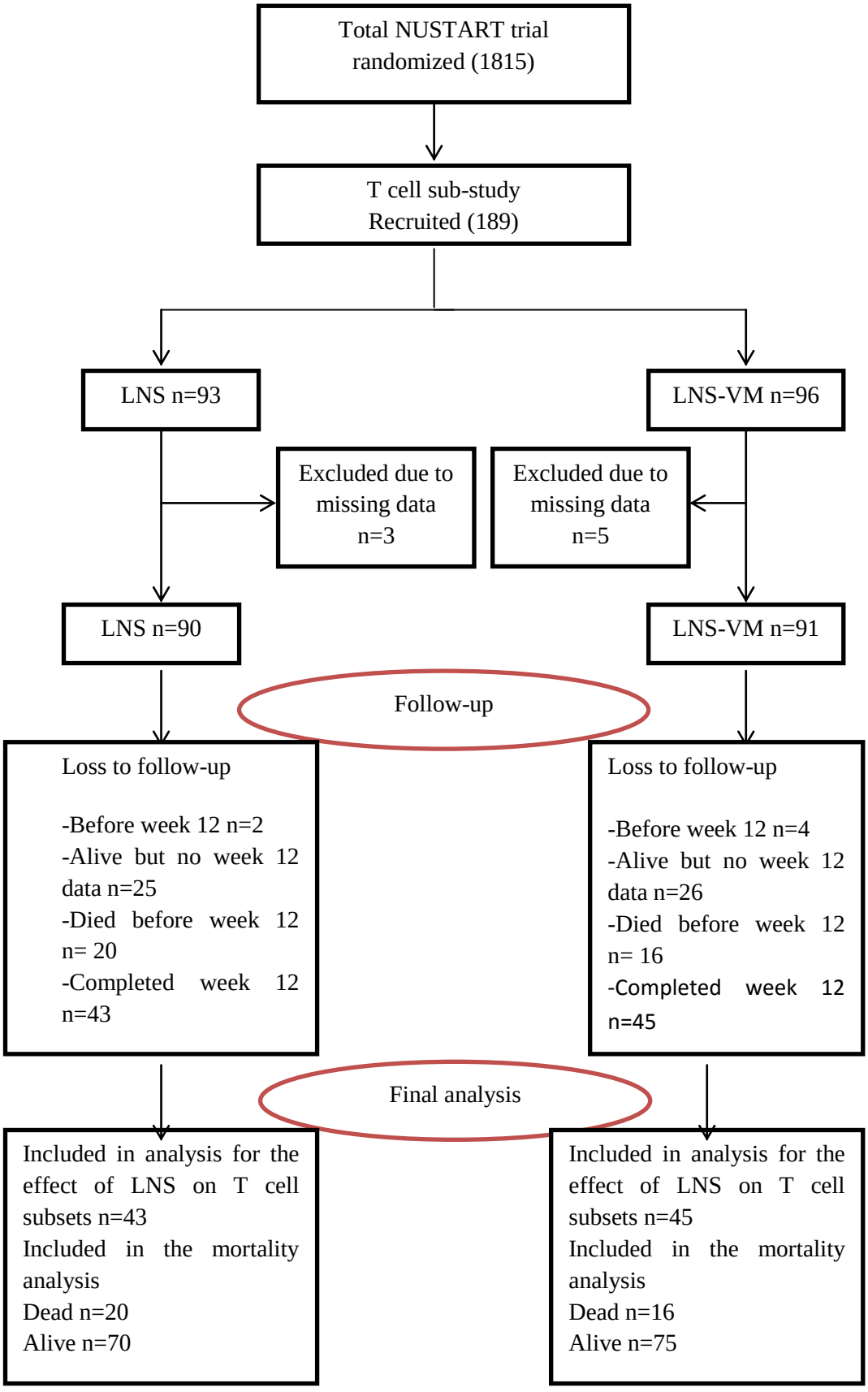


Figure 6-7: Flow of participants through the study

Table 6-4: Baseline Clinical and Nutritional Characteristics of HIV-infected Adult Zambian Men and Women

	Survivor Status			Clinical Trial	
Variable	**Died (n=36)**	**Survived (n=145)**	***P* value**	**LNS (n=90)**	**LNS-VM (n=91)**
Age (years)	36 (34, 42)	35 (30, 41)	0.10	36 (33, 42)	36 (31, 42)
Male	20 (56%)	82 (57%)	0.53	47 (51%)	60 (63%)
Haemoglobin (g/dl)	10.6 (9.2, 12.3)	10.3 (8.7, 11.6)	0.45	10.0 (8.5, 12.0)	10.7 (9.5, 11.4)
Total CD4 (cells/μl)	73 (39, 145)	125 (58, 234)	0.01	119 (66, 264)	87 (55, 205)
Total CD8 (cells/μl)	893 (516, 1270)	856 (583, 1370)	0.51	962 (596, 1304)	974 (674, 1542)
BMI (kg/m^2)	16.4 (15.6, 17.2)	16.7 (15.8, 17.8)	0.07	17.0 (16.0, 18.1)	16.6 (15.8, 17.6)
Grip strength (kg)	14.5 (8.7, 16.8)	20.2 (15.4, 24.9)	<0.0001	20.2 (14.9, 26.6)	20.0 (15.5, 24.1)
On TB treatment	6 (17%)	49 (34%)	<0.05	19 (21%)	36 (39%)
Education					
- No education	5 (14%)	19 (13%)	0.85	14 (15%)	10 (13%)
- Primary	18 (50%)	62 (43%)		37 (42%)	42 (44%)
- Secondary	12 (33%)	59 (41%)		36 (40%)	37 (38%)
- Tertiary	1 (3%)	4 (3%)		3 (3%)	2 (5%)
Occupation					
- Employed	14 (39%)	82 (57%)	0.17	47 (51%)	49 (52%)
Marital status					
- Single	18 (50%)	57 (39%)	0.45	32 (36%)	35 (38%)
- Married	16 (44%)	74 (51%)		52 (55%)	47 (50%)
- Widowed	2 (6%)	14 (10%)		6 (9%)	9 (12%)

Mann–Whitney U-tests were used for non-parametric variables and t tests for parametric variables. Continuous characteristics are expressed as median (interquartile range) and dichotomous characteristics are expressed as n (%). g/dl=grams per decilitre; BMI= body mass index; LNS=lipid-based nutritional supplement; LNS-VM=LNS with added vitamins and minerals

Groups randomised to LNS and LNS-VM was similar (Table 6-4). Those who died had lower baseline total $CD4^+$ cell count, lower grip strength and a small difference in BMI than survivors (Table 6-4).

Table 6-5: Immunological Risk Factors for Death within 12 weeks of Initiating Antiretroviral Therapy

	Univariate	**Multivariate**
Variable	**Unadjusted HR (95% CI)**	**Adjusted HR (95% CI)**
CD4 Total	0.51 (0.27 – 0.95)*	
CD4 Thymic emigrants	0.89 (0.49 - 1.59)	
CD4 Senescent	1.97 (1.03 - 3.76)*	
CD4 Naive	2.25 (1.78 - 4.29)*	2.88 (1.39 – 5.97)*
CD4 Central memory	1.46 (0.81 - 2.63)	
CD4 Effector memory	0.49 (0.27 - 0.94)	
CD4 Effector	0.70 (0.38 - 1.27)	
CD4 Activated	0.68 (0.36 – 1.30)	
CD4 proliferation	0.29 (0.14 – 0.60)*	0.43 (0.19 – 0.98)*
CD4 Proliferating & activated	0.68 (0.36 – 1.30)	
CD4 Gut homing	0.39 (0.20 - 0.76)*	
CD4 Gut homing activated	0.39 (0.20 - 0.76)*	
CD8 Total	1.13 (0.63 – 2.03)	
CD8 Thymic emigrants	1.42 (0.78 - 2.57)	
CD8 Senescent	3.03 (1.51 - 6.08)*	6.62 (2.94 – 14.88)*
CD8 Naive	0.64 (0.35 - 1.18)	
CD8 Central memory	1.16 (0.64 - 2.08)	
CD8 Effector memory	0.51 (0.27 - 0.95)*	
CD8 Effector	0.52 (0.28 - 0.98)*	
CD8 Activated	1.83 (0.99 - 3.38)*	
CD8 Proliferation	0.25 (0.12 - 0.54)*	0.12 (0.05 – 0.28)*
CD8 Proliferating & activated	1.79 (0.97 - 3.31)*	
CD8 Gut homing	0.72 (0.40 - 1.31)	
CD8 Gut homing and activated	2.07 (1.10 - 3.88)*	
BMI	0.66 (0.37 – 1.19)	
Hb	0.38 (0.14 – 1.07)	

Sex	0.97 (0.54 – 1.74)	
Grip strength	0.23 (0.11 – 0.50)*	0.23 (0.10 – 0.53)*

All variables were calculated using absolute numbers. Thirty-six participants died within 12 weeks of follow up. All variables were dichotomized; univariate logistic regression models were constructed comparing the upper half of the distribution with the lower half. Multivariate analysis was done using a Cox regression model, and was adjusted for, total CD4 and CD8 counts plus BMI, haemoglobin, sex and grip strength. HR=hazard ratio *=significant CI (confidence interval).

In univariate analysis of dichotomised variables, fewer total $CD4^+$, more senescent or naive, fewer proliferating, fewer gut homing, and gut homing activated $CD4^+$ T cells were all predictors of death (Table 6-5). More senescent, activated, activated proliferating, or activated gut homing, and fewer effector, effector memory or proliferating, $CD8^+$ T cells also predicted death (Table 6-5).

Using a Cox regression model that included all baseline cellular $CD4^+$ and $CD8^+$ subsets plus BMI, haemoglobin, grip strength and sex, higher numbers of circulating naïve $CD4^+$ and senescent $CD8^+$ cells were associated with increased mortality, whilst higher numbers of proliferating $CD4^+$ and $CD8^+$ cells were associated with reduced risk of mortality by 12 weeks (Table 6-5).

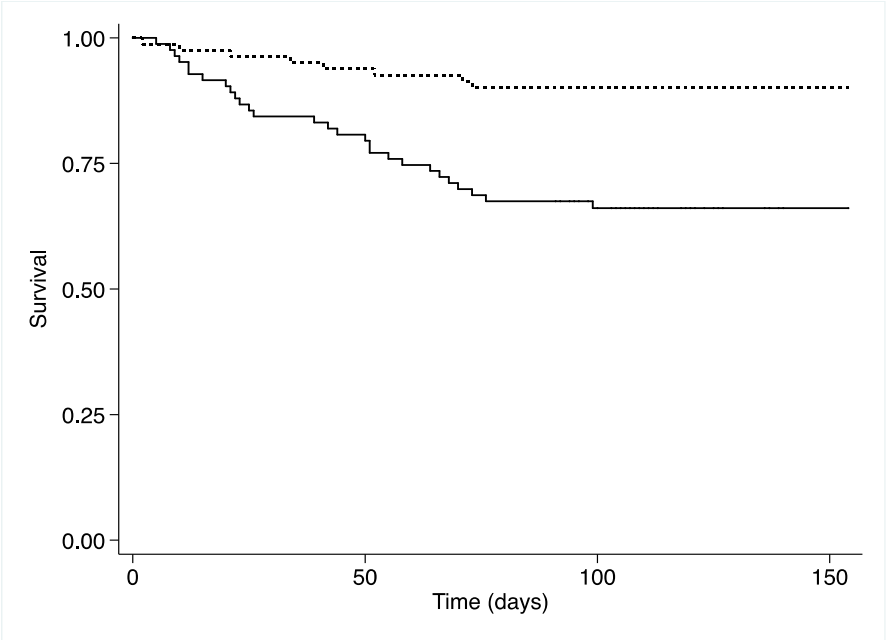


Figure 6-8: Kaplan-Meier plot of survival over time of follow up in patients with high (dashed line) or low (solid line) absolute number of $CD4^+$ cells expressing the proliferation marker Ki67 (p=0.0003 using log rank test).

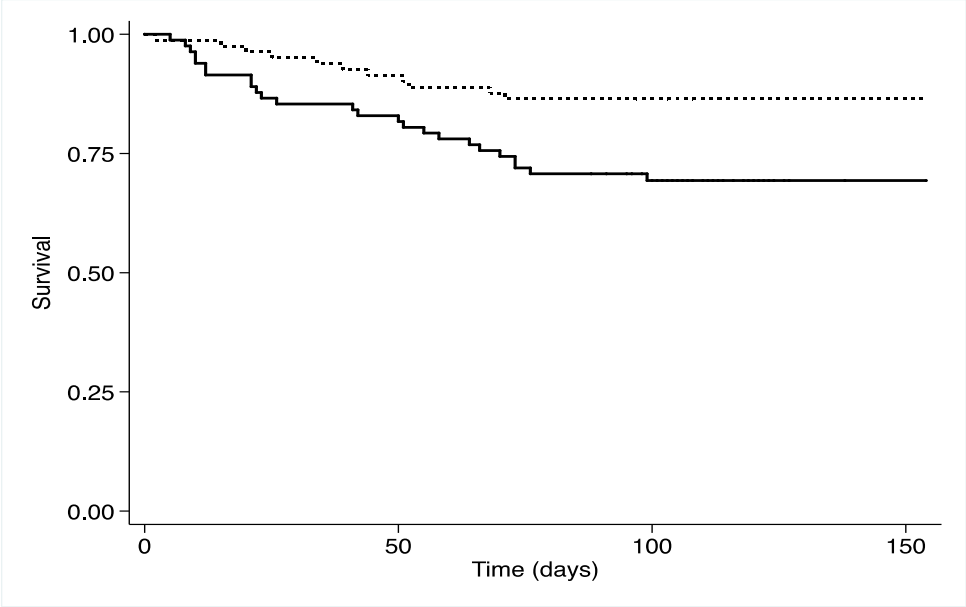


Figure 6-9: Kaplan-Meier plot of survival over time of follow up in patients with low (dashed line) or high (solid line) absolute number of $CD4^+$ cells expressing the naive markers CD45RA and CCR7 ($p<0.001$ using log rank test).

6.3 Analysis of CD4 and CD8 T Cell Subsets at Baseline and Week 12

Table 6-6: T Cell Subset Markers at Baseline and After ART and Nutritional Support

	CD4			**CD8**		
	Baseline	**Week 12**		**Baseline**	**Week 12**	
Variable	**Median (IQR)**	**Median (IQR)**	***P* value**	**Median (IQR)**	**Median (IQR)**	***P* value**
Total count	98 (64, 209)	259 (166, 389)	<0.0001	968 (630, 1529)	971 (656, 1322)	0.71
Thymic emigrants	6 (2, 32)	36 (10, 88)	<0.0001	279 (150, 497)	421 (267, 641)	0.001
Senescent	16 (6, 33)	1 (0, 6)	<0.0001	149 (0, 459)	323 (219, 523)	<0.0001
Naive	2 (0, 9)	9 (2, 51)	<0.0001	38 (17, 94)	105 (45, 324)	<0.0001
Central memory	1 (0, 5)	8 (2, 30)	<0.0001	0 (0, 13)	22 (2, 87)	<0.0001
Effector memory	61 (22, 136)	156 (87, 249)	<0.0001	229 (99, 393)	119 (56, 258)	0.01
Effector	14 (3, 39)	19 (7, 57)	0.03	592 (301, 1021)	498 (195, 784)	0.03
Activated	2 (0, 6)	8 (3, 21)	<0.0001	83 (7, 373)	372 (212, 571)	<0.0001
Proliferation	53 (17, 154)	86 (27, 236)	0.01	285 (0, 665)	745 (497, 1101)	<0.0001
Proliferating & activated	1 (0, 4)	4 (0, 14)	0.002	33 (10, 211)	83 (40, 187)	<0.004
Gut homing	44 (25, 95)	0 (0, 1)	<0.0001	509 (314, 814)	452 (287, 725)	0.20
Gut homing & activated	13 (6, 26)	4 (2, 8)	<0.0001	5 (0, 13)	4 (0, 9)	0.42

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

Together with an overall CD4 and CD8 T cell increase after 12 weeks of ART and nutritional supplementation, hemoglobin, BMI and grip strength also increased in HIV-infected adults. The baseline median Hb was 10.35 (IQR, 8.85-11.6), and increased to 12.4 (IQR, 11.05-13.6) after 12 weeks. These results suggest that ART with supplementation improves Hb. The baseline BMI was at 16.7 (IQR, 15.9-17.8) whereas at 12 weeks BMI was at 18.45 (IQR, 17.25-19.3). Similarly, ART with nutritional supplementation seemed to have improved the BMI. Participants' grip strength at enrolment was 20.2 with a median IQR of 15.4-25.1. Following 12 weeks of ART and nutritional supplementation, the grip strength increased to a median of 21.8 and IQR of 18.2-27.9. These results also indicate that there was an improvement in grip strength at 3 months of ART and nutritional support.

Among all participants in this sub study, the median CD4 count at week 0 (enrolment) was 98 (IQR, 64-209) and at week 12 (primary endpoint) was 259 (IQR, 166-389) (P<0.0001) Table 6-5. However, although there was an increase in absolute CD4 count in the majority of participants after 12 weeks, these results also show that a majority of people seeks medical attention late. The median numbers of homing cells at baseline were 44 (IQR, 25-95) and decreased to 0 (IQR, 0-1) after 12 weeks of receiving ART and nutritional supplementation. These results indicate that during HIV infection and before ART was commenced, there was a significant number of $CD4^+$ T cells homing to the gut and then reduce drastically after treatment. Similar to the above, there were more homing and activated $CD4^+$ T cells at baseline compared to 12 weeks after treatment and the intervention (Table 6-5). Baseline numbers of activated homing $CD4^+$ T cells were at 13 (IQR, 6-26) and after 12 weeks reduced to 4 (IQR, 2-8).

There were also few recent thymic emigrants at baseline median 6 (IQR, 2-32) compared to 12 weeks after the intervention i.e. 36 (IQR, 10-88). These results could indicate a strain in the thymus that probably is improved after ART and nutritional support. There was also a general increase in $CD4^+$ T cells expressing the senescent marker CD57 after the intervention 16 (IQR, 6-33) compared to baseline numbers 1 (IQR, 0-6). These results could indicate an improved response to the virus after 3 months of ART and nutritional support. Contrary to the findings for senescent 1, there was no significant difference in cells expressing the marker for senescent 2 ($CD4^+CD57^+CD28^-$) at baseline 8 (IQR, 2-23) and 12 weeks 5 (IQR, 1-19). These results suggest that there is as much activity at 3 months as there is at baseline in HIV-infected individuals. Naive $CD4^+$ T cells at baseline were few 2 (IQR, 0-9) compared to 12 weeks after the intervention 9 (IQR, 2-51). This could mean an improvement in the production and a reduction in the numbers of differentiating cells or the antigen.

There was an increase in the number of central memory cells from 1 (IQR, 0-5) at baseline to 8 (IQR, 2-30) after 12 weeks of receiving ART plus nutritional support. These results indicate that there is an improvement in the cells that mount a quick response to infection. Baseline effector memory cells were few 61 (IQR, 22-136) and doubled 12 weeks after intervention 156 (IQR, 87-249). These results show that there is good immune response to infection after 12 weeks of ART and nutritional support. Similar to effector memory $CD4^+$ T cells, effector $CD4^+$ T cells also increased after the intervention although the increase was not as much as in the effector memory cell population. This steady increase show a slow but sure improvement in the immune response to the infection. Baseline effector cells were at 14 (IQR, 3-39) and increased to 19 (IQR, 7-57) after 12 weeks. Proliferating and activated $CD4^+$ T cells

at enrolment were few 1 (IQR, 0-4) compared to 12 weeks after the intervention 4 (IQR, 0-14). These results show that there is an improved response to antigens after 12 weeks of receiving ART plus nutritional support. There was also an increase in the number of activated cells from 2 (IQR, 0-6) to 8 (IQR, 3-21) after the intervention. These results could mean that the viral load does not drop immediately after initiating ART with nutritional support assuming that activation is being triggered by HIV. The number of proliferating $CD4^+$ T cells also increased from 53 (IQR, 17-154) at baseline to 86 (IQR, 27-236) after the intervention. These results suggest that there is good clonal expansion following treatment.

In table 6-6, absolute $CD8^+$ T cells were comparable both at baseline 968 (IQR, 630-1529) and at 12 weeks 971 (IQR, 656-1322). Since these are cytotoxic cells that fight the virus, these results could mean that the amount of HIV after 3 months of ART plus nutritional support does not immediately go down. There was an insignificant change in the number of homing $CD8^+$ T cells after the intervention, also suggesting that there is still high activity in the gut after 12 week of ART and nutritional support. Gut homing $CD8^+$ T cells at baseline were 509 (IQR, 314-814) and after 3 months CD8+ T cells were at 452 (IQR, 287-725). Activated gut homing and $CD8^+$ T cells did not significantly decrease by 12 weeks of ART plus nutritional support i.e. from 5 (IQR, 0-13) at baseline and 4 (IQR, 0-9) at 12 weeks. This could mean that the high number of $CD8^+$ T cells still homing to the gut after the intervention could be channeled there for repopulation and not necessarily because of infection. There were few recent thymic emigrant $CD8^+$ T cells at baseline 279 (IQR, 150-497) compared to 12 weeks after intervention 421 (IQR, 267-641) in the participants. This may be an indicator of cell repopulation following destruction by the HIV. The

number of senescent 1 $CD4^+$ T cells doubled from median of 149 (IQR, 0-459) at baseline to 323 (IQR, 219-523) at week 12. These results show that probably programed cell death is slower than can be corrected within 3 months of treatment. The numbers of senescent 2 ($CD8^+CD57^+CD28^-$) cells both at baseline and week 12 were comparable after the intervention. There were few naïve cells at baseline 38 (IQR, 17-94) and this increased 105 (IQR, 45-324) by 12 weeks post ART and supplementation. This could mean that there was a reduction in the rate at which cells differentiate and also in their antigen encounter. Similarly, central memory cells were almost completely depleted at baseline 0 (IQR, 0-13) and increased to 22 (IQR, 2-87) by 12 weeks. These results could indicate a good immune response of the body to the antigens. Effector memory cells were 229 (IQR, 99-393) at baseline and reduced by 12 weeks to 119 (IQR, 56-258). This could mean that there is still more interaction between the effector cells and the antigen. The number of effector $CD8^+$ T cells is more at baseline 592 (IQR, 301-1021) than at 12 weeks 498 (IQR, 195-784). Since effector cells interact with their cognate antigen in the lymphoid tissues, the reduction could be an indication of reduced antigen in the lymphoid tissues after the intervention. There were few proliferating and activated $CD8^+$ T cells at baseline 33 (IQR, 10-211) as opposed to 12 weeks after ART and supplementation 83 (IQR, 40-187). A similar trend as above was observed for activated $CD8^+$ T cells, in that there were few activated $CD8^+$ T cells at baseline 83 (IQR, 7-373) compared to the number of cells at 12 weeks i.e. 372 (IQR, 212-571). This could mean that more cells are primed for antigen encounter after ART and nutritional support than before initiating ART with nutritional supplementation. There were also fewer proliferating $CD8^+$ T cells at baseline i.e. 285 (IQR, 0-665) as compared to 12 weeks 745 (IQR,

497-1101) after ART and supplementation. This could also be an indication of a good immune response to antigen.

In Summary,

There was a significant change in either the $CD4^+$ or $CD8^+$ T cell subsets from enrolment to 12 weeks after ART and supplementation. Cells either significantly increased or significantly decreased. In both the CD8 and CD4 senescent 2 subsets, there were no significant differences between baseline and after 12 weeks. There were no significant differences in absolute $CD8^+$ T cells, homing $CD8^+$ T cells and homing and activated $CD8^+$ T cells in the HIV sero positive individuals.

Table 6-7: Baseline T Cell Subset Markers in Patients Allocated to Receive Supplements with (LNS-VM) or without (LNS) Additional Vitamins and Minerals

	CD4			CD8		
	LNS-VM group (N=45)	**LNS group (N=43)**		**LNS-VM group (N=45)**	**LNS group (N=43)**	
Variable	**Median (IQR)**	**Median (IQR)**	***P* value**	**Median (IQR)**	**Median (IQR)**	***P* value**
Total count	87 (55, 205)	119 (66, 264)	0.26	974 (674, 1542)	962 (596, 1304)	0.46
Thymic emigrants	6 (3, 33)	7 (2, 32)	0.59	277 (155, 444)	279 (139, 542)	0.83
Senescent	2 (0, 6)	1 (0, 9)	0.59	157 (0, 491)	148 (0, 444)	0.66
Naive	2 (1, 15)	2 (0, 5)	0.20	36 (8, 55)	44 (22, 125)	0.12
Central memory	0 (0, 8)	1 (0, 4)	0.55	0 (0, 14)	0 (0, 10)	0.25
Effector memory	62 (18, 127)	61 (32, 172)	0.31	231 (141, 411)	187 (51, 381)	0.09
Effector	9 (3, 32)	22 (6, 43)	0.11	589 (394, 1060)	598 (252, 978)	0.59
Activated	2 (1, 9)	2 (0, 5)	0.48	88 (61, 464)	78 (10, 312)	0.76
Proliferation	39 (18, 137)	65 (13, 183)	0.62	324 (0, 704)	216 (0, 626)	0.49
Proliferating & activated	1 (0, 8)	0 (0, 3)	0.30	63 (13, 249)	24 (4, 128)	0.06
Gut homing	40 (23, 78)	65 (28, 102)	0.33	513 (330, 914)	505 (292,804)	0.89
Gut homing & activated	11 (4, 25)	15 (7, 27)	0.34	4 (0, 12)	6 (0, 14)	0.93

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

In the placebo arm the median baseline Hb was at 10 with (IQR, 8.5-12) while in the active arm Hb was at median 10.7 (IQR, 9.5-11.4). Thus there were no significant differences at baseline in the participants enrolled to the sub-study. Participants receiving placebos increased their Hb 12.5 (IQR, 11.2-13.6) as much as those in the active group 12.2 (IQR, 10.9-13.3). BMI baseline results were also similar. The median BMI for participants in the placebo group was 17 (IQR, 16-18.1) and 16.6 (IQR, 15.8-17.6) in the active group. Similarly, there were no significant differences in BMI after 12 weeks of receiving ART and supplementation in both groups. Median BMI at 12 weeks for the placebo group was 18.6 (IQR, 17.1-19.2) and 18.3 (IQR, 17.3-19.3) in the active group. Grip strength for both groups was comparable at baseline. Participants in the placebo arm had median grip strength of 20.2 (IQR, 14.9-26.6) and 20 (IQR, 15.5-24.1) in the active arm. After 12 weeks of ART and nutritional supplementation, the grip strength in both arms was the same. In the placebo group, the median grip strength was 20.7 (IQR, 17.8-26.4) and 21.8 (IQR, 18.5-28.1) in the active arm. Analysis of the $CD4^+$ and $CD8^+$ T cell subsets showed that all subsets were similar at enrolment in the participants (Table 6-5).

In Summary,

Before the start of ART, there were more absolute CD8 cells compared to the number of CD4. At enrolment, most of the CD8 were effector cells whereas in the CD4 population, majority of the cells were effector memory. In both the CD8 and CD4 population, central memory cells were almost completely absent at enrolment. And there was a considerable number of gut homing cells in both CD8 and CD4 populations at enrolment compared to 12 weeks after.

6.4 Sub Study Analysis of CD4 and CD8 Change by Arm

Table 6-8: Increase in T Cell Subset Markers in Patients Receiving Supplements with (LNS-VM) or without (LNS) Additional Vitamins and Minerals after 12 weeks.

	CD4			CD8		
	LNS-VM group (N=45)	**LNS group (N=43)**		**LNS-VM group (N=45)**	**LNS group (N=43)**	
Variable	**Median (IQR)**	**Median (IQR)**	***P* value**	**Median (IQR)**	**Median (IQR)**	***P* value**
Total count	257 (150, 389)	274 (186, 374)	0.61	1096 (720, 1424)	932 (590, 1214)	0.23
Thymic emigrants	25 (8, 83)	47 (18, 95)	0.045	375 (269, 521)	458 (242, 668)	0.40
Senescent	17 (6, 29)	11 (6, 43)	0.85	370 (266, 549)	287 (193, 408)	0.08
Naive	7 (1, 62)	13 (3, 35)	0.77	90 (40, 305)	111 (48, 455)	0.29
Central memory	7 (2, 27)	11 (3,30)	0.56	19 (0, 84)	22 (4, 90)	0.43
Effector memory	160 (96, 219)	155 (86, 274)	0.82	526 (205, 968)	104 (62, 223)	0.16
Effector	20 (6, 54)	19 (9, 69)	0.72	526 (205, 968)	412 (185, 765)	0.26
Activated	8 (3, 22)	8 (3, 20)	0.88	384 (240, 663)	354 (189, 532)	0.26
Proliferation	85 (23, 189)	86 (27, 300)	0.70	736 (445, 1037)	853 (555, 1173)	0.48
Proliferating & activated	4 (0, 10)	3 (0, 15)	0.98	104 (44, 224)	82 (39, 158)	0.41
Gut homing	0 (0,1)	0 (0, 1)	0.18	458 (314, 747)	427 (275, 662)	0.70
Gut homing & activated	4 (2, 7)	4 (2, 9)	0.34	4 (1, 8)	4 (0, 12)	0.95

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

There were no significant differences in absolute CD4 counts for the participants after 12 weeks of ART and nutritional support. The median absolute CD4 count in the placebo group at 12 weeks was 274 (IQR, 186-374) and 257 (IQR, 150-389) in the active group (Table 6-8). Homing $CD4^+$ T cells after 12 weeks of receiving ART plus nutritional supplementation was the same in both arms; 0 (IQR, 0-1) in the placebo group and 0 (IQR, 0-1) in the active arm. There was a significant difference between the placebo and the active groups in the $CD4^+$ T cell recent thymic emigrants after 12 weeks of receiving ART and supplementation. The median number of $CD4^+$ T cell recent thymic emigrants for the placebo group was 47 IQR 18-95 and 25 IQR 8-83 for the active group. The median CD4 count in senescent 1 in the placebo group was 11 IQR 6-43 and 17 IQR of 6-29) in the active group (p=0.85).

The numbers of central memory $CD4^+$ T cells after 12 weeks were comparable; 11 (IQR, 3-30) in the placebo group 7 (IQR, 2-27) in the active group after 12 weeks of ART and supplementation. After 12 weeks of receiving the intervention, the number of effector memory $CD4^+$ T cells increased proportionately and were comparable; 155 (IQR, 86-274) in the placebo group and 160 (IQR, 96-219) in the active group. After 12 weeks of receiving the intervention, although there was a slight decrease in the placebo group and a slight increase in the active group, the overall figure were comparable; 19 (IQR, 9-69) in the placebo and 20 (IQR, 6-54) in the active group after 12 weeks of ART and nutritional support. After 12 weeks of ART plus nutritional supplementation, proliferating and activated $CD4^+$ T cells similar in both the placebo and active groups: 3 (IQR, 0-15) and 4 (IQR, 0-10) respectively. Activated $CD4^+$ T cells were similar in both arms and increased proportionately at 12

weeks to 8 (IQR, 3-20) and 8 (IQR, 3-22) respectively. Similarly, there were no significant differences in the proliferating $CD4^+$ T cells after treatment between the two groups, although the numbers in both groups increased proportionately. The median number for proliferating $CD4^+$ T cells at 12 weeks was 86 (IQR, 27-300) in the active group and 85 (IQR, 23-189) in the active group.

The absolute number of $CD8^+$ T cells did not differ between the two groups; 932 (IQR, 590-1214) in the placebo group and 1096 (IQR, 720-1424) in the active group after 12 weeks of ART and nutritional supplementation. The number of homing $CD8^+$ T cells were also similar; 427 (IQR, 275-662) in the placebo group and 458 (IQR, 314-747) in the active group respectively. The median homing and activated $CD8^+$ T cell number was 6 (IQR, 0-14) in the placebo group and 4 (IQR, 0-12) in the active group. The number of homing and activated $CD8^+$ T cells was also similar: 4 (IQR, 0-12) in the placebo and 4 (IQR, 1-8) in the active group after 12 weeks of receiving ART plus nutritional support. The median number in the placebo group was 458 (IQR, 242-668) and 375 (IQR, 269-521) in the active group. After 12 weeks of taking ART plus nutritional support, there were no significant differences in the two arms. However, there was a proportionate increase in the number of senescent 1 $CD8^+$ T cells after 12 weeks compared to the baseline number. The median senescent 1 in the placebo arm after 12 weeks was 287 (IQR, 193-408) and 370 (IQR, 266-549) in the active group. After 12 weeks of ART and supplementation, senescent 2 $CD8^+$ T cells were similar. In the placebo group, the median number was 245 (IQR, 131-397) and 324 (IQR, 229-512) in the active group. Similarly, there were no significant differences in the naive $CD8^+$ T cells after 12 weeks of ART plus nutritional support in both the placebo group; 111 (IQR, 48-455) and 90 (IQR, 40-305) in the active

group. In the placebo group, the median number was 0 (IQR, 0-10) and 0 (IQR, 0-14) in the active group. At 12 weeks after receiving the intervention, the number of central memory $CD8^+$ T cells in the placebo arm was 22 (IQR, 4-90) and 19 (IQR, 0-84). Similarly, there were no significant differences between the two groups in the central memory cells. There were no significant differences in both groups after 12 weeks of receiving ART with nutritional supplementation. In the placebo arm, the median was 104 (IQR, 62-223) and 526 (IQR, 205-968) in the active arm. Effector $CD8^+$ T cells in both groups were similar at baseline. The median effector $CD8^+$ T-cells in the placebo group was 598 (IQR, 252-978) and 589 (IQR, 394-1060) in the active arm. Effector $CD8^+$ T cells after 12 weeks were similar in the arms. The median number in the placebo group was 412 (IQR, 185-765) and 526 (IQR, 205-968) in the active group. The median number in the placebo group was 24 (IQR, 4-128) and 63 (IQR, 13-249) in the active group. Although there was an increase in the number of proliferating and activated $CD8^+$ T cells by week 12, the increase was proportionate in both groups. The median number for proliferating and activated $CD8^+$ T cells in the placebo group was 82 (IQR, 39-158) and 104 (IQR, 44-224) in the active group. After 12 weeks of receiving ART and nutritional support, there was no significant difference between the two arms. The placebo arm had a median number of 354 (IQR, 189-532) and 384 (IQR, 240-663) in the active group. Proliferating $CD8^+$ T cells were comparable after 3 months of receiving ART and the nutritional supplementation in the placebo (median 736) and active (median 853) group. In other words there was no significant difference between the numbers of $CD8^+$ T cells in both arms.

In Summary,

Of all the T cell subsets analysed, only the increase in $CD4^+$ of recent thymic emigrants was significantly different in the participants allocated to receive LNS-VM compared to LNS.

Table 6-9: The Delta Changes in T Cell Subset Markers in Patients Receiving Supplements with (LNS-VM) or without (LNS) Additional Vitamins and Minerals after 12 weeks.

	CD4			CD8		
	Change in LNS-VM group (N=45)	**Change in LNS group (N=43)**		**Change in LNS-VM group (N=45)**	**Change in LNS group (N=43)**	
Variable	**Median (IQR)**	**Median (IQR)**	***P* value**	**Median (IQR)**	**Median (IQR)**	***P* value**
Total count	126 (82, 210)	122 (81, 204)	0.98	-27 (-284, 173)	7 (-300, 213)	0.83
Thymic emigrants	8.5 (1.5, 41)	29 (7, 61)	0.01	144 (-27, 306)	87 (20, 221)	0.88
Senescent	-1 (-11, 8)	-0.5 (-10, 11)	0.93	44 (-112, 191)	10 (-90, 194)	0.81
Naive	2 (-2, 28)	7 (0, 30)	0.27	46 (0, 221)	32 (-19, 380)	0.90
Central memory	5 (-2, 27)	6.5 (1, 25)	0.40	11 (0, 66)	20 (0, 79)	0.34
Effector memory	91 (39, 145)	64 (25, 169)	0.45	-71 (-141, 30)	-65 (228,57)	0.81
Effector	7 (-1, 32)	3 (-10, 30)	0.27	-130 (-299, 72)	-78 (-542, 133)	0.80
Activated	4 (0, 16)	4 (-1, 17)	0.73	194 (-31, 431)	196 (52, 371)	0.94
Proliferation	50 (-30, 126)	62 (-6, 106)	0.69	377 (-98, 877)	350 (25, 782)	0.81
Proliferating & activated	1 (-2, 8)	2 (-1, 10)	0.48	25 (-16, 174)	41 (-16, 123)	0.72
Gut homing	-39 (-78, -22)	-64 (-102, -27)	0.33	-51 (-186, 55)	-65 (-273, 62)	0.93
Gut homing & activated	-8 (-15, -2)	-11 (-20, 0)	0.62	0 (-4, 4)	0 (-6, 3)	0.90

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

6.5 Selenium Dose Response (*in-vitro*) Study Results

Table 6-10: Baseline Characteristics for the Participants

Variable	Females N=17	Males N=34	*P* value
Age (Years)	34 (27, 45)	23 (28, 34)	0.01
Absolute CD4 count (Cells/µL)	1042 (864, 1270)	671 (545, 899)	0.003
Absolute CD8 count (Cells/µL)	1531 (1248, 1740)	1346 (1101, 1801)	0.42
Hb (g/dl)	13.3 (11.3, 14)	15.6 (14.8, 16.6)	0.0001
BMI (Kg/m^2)	25.03 (23.3, 28.88)	23.18 (21.18, 25.5)	0.07
Grip Strength (Kg)	25.9 (23.8, 27.5)	44.7 (39.9, 47.7)	0.0001
Plasma Se (µmol/L)	1.33 (1.06, 1.50)	1.38 (0.78, 1.67)	0.64
Education University College Secondary Primary	 8 (16%) 6 (12) 2 (4%) 1 (2%)	 22 (43%) 6 (12%) 6 (12%) 0	0.21

Occupation Employed Students Business Unemployed	 11 (22%) 4 (8%) 1 (2%) 1 (2%)	 23 (45%) 11 (22%) 0 0	0.22
Marital status Married Single Divorce	 10 (20%) 6 (12%) 1 (2%)	 17 (33.33%) 17 (33.33%) 0	0.23

1 Mann–Whitney U-tests were used for non-parametric variables and Pearson chi square for categorical variables. Continuous characteristics are expressed as median (interquartile range) and dichotomous characteristics are expressed as n (%). g/dl=grams per decilitre; Hb= haemoglobin; BMI= body mass index

Although females appeared to have sufficient Se levels, of all the females (17), four (24 %) were deficient. Whilst of the thirty-four males, 10 (29%) were deficient. Individual surface markers were explored by sex at baseline and at 3 different concentrations of Se.

Table 6-11: Baseline CD4 and CD8 T Cell Subsets Between Females and Males

	CD4			CD8		
Variable (%)	**Females (N= 17)**	**Males (N= 34)**	***P* value**	**Females (N=17)**	**Males (N= 34)**	***P* value**
Thymic	73 (61, 90)	85 (60, 95)	0.22	9 (7, 26)	15 (7, 33)	0.36
Naive	80 (68, 93)	87 (80, 91)	0.20	17 (9, 20)	22 (11, 43)	0.09
Central Memory	6 (1, 8)	6 (2, 12)	0.25	10 (6, 13)	14 (10, 30)	0.03
Effector memory	0 (0, 1)	0 (0, 0)	0.02	30 (25, 38)	27 (5, 31)	0.14
Effector	5 (1, 30)	2 (0, 12)	0.03	43 (39, 51)	38 (8, 47)	0.03
Homing	41 (31, 47)	35 (30, 40)	0.28	41 (32, 46)	47 (41, 59)	0.04
Homing activated	24 (17, 28)	25 (20, 29)	0.68	0 (0, 0)	0 (0, 0)	0.22
Activated	1 (0, 2)	1 (0, 2)	0.29	0 (0, 0)	0 (0, 0)	0.73
Proliferating	0 (0, 1)	1 (0, 1)	0.98	2 (1, 3)	2 (1, 3)	0.89
Proliferating activated	2 (1, 3)	2 (1, 3)	0.65	1 (0, 1)	1 (0, 1)	0.06
Senescent 1	2 (1, 5)	3 (2, 7)	0.46	25 (11, 33)	22 (12, 30)	0.82
Senescent 2	1 (0, 2)	1 (0, 3)	0.87	19 (2, 26)	14 (5, 25)	0.55

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

Table 6-12: CD4 and CD8 T Cell Response to 180 µmol/L Selenium Concentration Between Females and Males

	CD4			CD8		
Variable (%)	**Females (N= 17)**	**Males (N= 34)**	***P* value**	**Females (N=17)**	**Males (N= 34)**	***P* value**
Thymic	40 (24, 82)	85 (60, 95)	0.22	21 (10, 86)	25 (5, 83)	0.58
Naive	75 (60, 82)	74 (50, 92)	0.67	43 (18, 67)	81 (46, 93)	0.02
Central memory	18 (11, 25)	14 (6, 33)	0.61	21 (4, 45)	3 (0, 9)	0.01
Effector memory	0 (0, 3)	0 (0, 2)	0.86	2 (0, 19)	0 (0, 3)	0. 23
Effector	0 (4, 15)	0 (0, 2)	0.04	6 (0, 25)	0 (0, 7)	0.09
Homing	75 (57, 85)	75 (65, 88)	0.07	28 (4, 68)	57 (17, 99)	0.06
Homing activated	57 (17, 88)	57 (39, 100)	0.53	0 (0, 0)	0 (0, 55)	0.83
Activated	0 (0, 3)	1 (0, 2)	0.29	0 (0, 0)	0 (0, 0)	0.63
Proliferating	0 (0, 0)	0 (0, 0)	0.58	0 (0, 0)	0 (0, 0)	0.18
Proliferating activated	0 (0, 0)	0 (0, 0)	-	0 (0, 0)	0 (0, 0)	-
Senescent 1	0 (0, 23)	16 (0, 51)	0.08	20 (0, 29)	31 (0, 60)	0.10
Senescent 2	0 (0, 0)	0 (0, 1)	0.01	0 (0, 5)	0 (0, 11)	0.40

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

Table 6-13: CD4 and CD8 T Cell Response to 200 µmol/L Selenium Concentration Between Females and Males

	CD4			CD8		
Variable (%)	**Females (N= 17)**	**Males (N= 34)**	***P* value**	**Females (N=17)**	**Males (N= 34)**	***P* value**
Thymic	75 (64, 94)	78 (47, 91)	0.67	0 (0, 4)	0 (0, 0)	0.08
Naive	90 (83, 93)	87 (80, 95)	0.84	24 (18, 67)	60 (20, 88)	0.09
Central memory	1 (0, 11)	6 (0, 13)	0.53	33 (9, 44)	10 (0, 46)	0.36
Effector memory	0 (0, 0)	0 (0, 1)	0.71	14 (0, 32)	0 (2, 14)	0.11
Effector	7 (0, 10)	3 (0, 9)	0.40	9 (3, 21)	5 (0, 18)	0.59
Homing	71 (51, 82)	79 (60, 100)	0.93	57 (6, 77)	71 (11, 100)	0.14
Homing activated	35 (28, 80)	42 (31, 57)	0.03	8 (0, 12)	9 (3, 57)	0.22
Activated	0 (0, 3)	0 (0, 5)	0.84	0 (0, 0)	0 (0, 0)	0. 49
Proliferating	0 (0, 0)	0 (0, 0)	0.70	0 (0, 0)	0 (0, 0)	0. 27
Proliferating activated	0 (0, 0)	0 (0, 0)	-	0 (0, 0)	0 (0, 0)	0.22
Senescent 1	16 (0, 63)	43 (16, 76)	0.11	25 (0, 48)	38 (0, 70)	0.24
Senescent 2	0 (0, 0)	0 (0, 2)	0.11	1 (0, 3)	0 (0, 23)	0.59

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

Table 6-14: CD4 and CD8 T Cell Response to 220 µmol/L Selenium Concentration Between Females and Males

	CD4			CD8		
Variable (%)	**Females (N= 17)**	**Males (N= 34)**	***P* value**	**Females (N=17)**	**Males (N= 34)**	***P* value**
Thymic	66 (43, 92)	65 (52, 84)	0.67	2 (0, 6)	2 (0, 9)	0.75
Naive	85 (50, 96)	80 (66, 95)	0.84	36 (0, 50)	61 (0, 93)	0.07
Central memory	0 (0, 11)	8 (2, 14)	0.09	0 (10, 25)	5 (0, 12)	0.38
Effector memory	0 (0, 6)	0 (0, 5)	0.76	6 (0, 14)	0 (0, 12)	0.60
Effector	6 (0, 15)	4 (0, 17)	0.81	14 (0, 29)	0 (0, 12)	0.19
Homing	78 (71, 94)	83 (42, 100)	0.92	23 (8, 84)	64 (19, 100)	0.03
Homing activated	54 (22, 95)	80 (71, 86)	0.05	0 (0, 19)	33 (8, 62)	0.02
Activated	0 (0, 6)	0 (0, 1)	0.62	0 (0, 0)	0 (0, 0)	0.15
Proliferating	0 (0, 0)	0 (0, 0)	0.50	0 (0, 0)	0 (0, 0)	0.21
Proliferating activated	0 (0, 0)	0 (0, 0)	0.49	0 (0, 0)	0 (0, 0)	0.94
Senescent 1	30 (0, 37)	38 (10, 61)	0.15	40 (0, 50)	41 (19, 64)	0.48
Senescent 2	0 (0, 0)	0 (0, 1)	0.01	6 (0, 10)	9 (0, 22)	0.14

Mann–Whitney U-tests were used for non-parametric variables and continuous characteristics are expressed as median (interquartile range)

There were some significant differences observed for some markers when CD4 and CD8 surface markers were analyzed using Friedman test, (Figures 6-10 to 6-33). In that, some marker expression markedly increased (CD4 gut homing cells) whilst others decreased (CD4 proliferating cells).

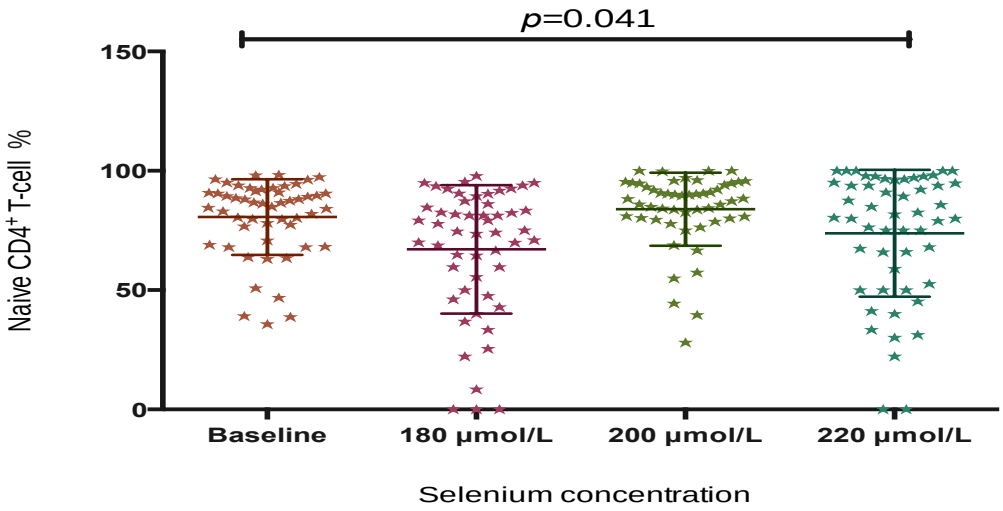


Figure 6-10 shows median naive marker expression on $CD4^+$ T cells at different concentrations of selenium *in vitro*. There was an up and down change observed with the change in concentration in naive markers expressed. Overall, using Friedman test, there was a significant change with a p value of 0.041.

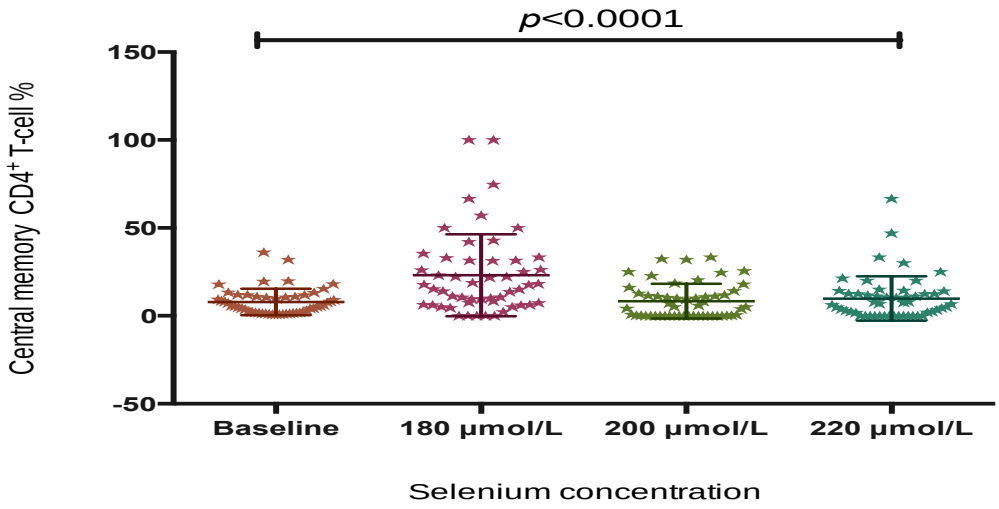


Figure 6-11 shows median central memory marker expression on $CD4^+$ T cells at different concentrations. There was a significant increase in central memory marker expression on the $CD4^+$ T cells at 180 µmol/L. However when all concentrations were compared using Friedman test, overall there was a significant difference ($p<0.0001$).

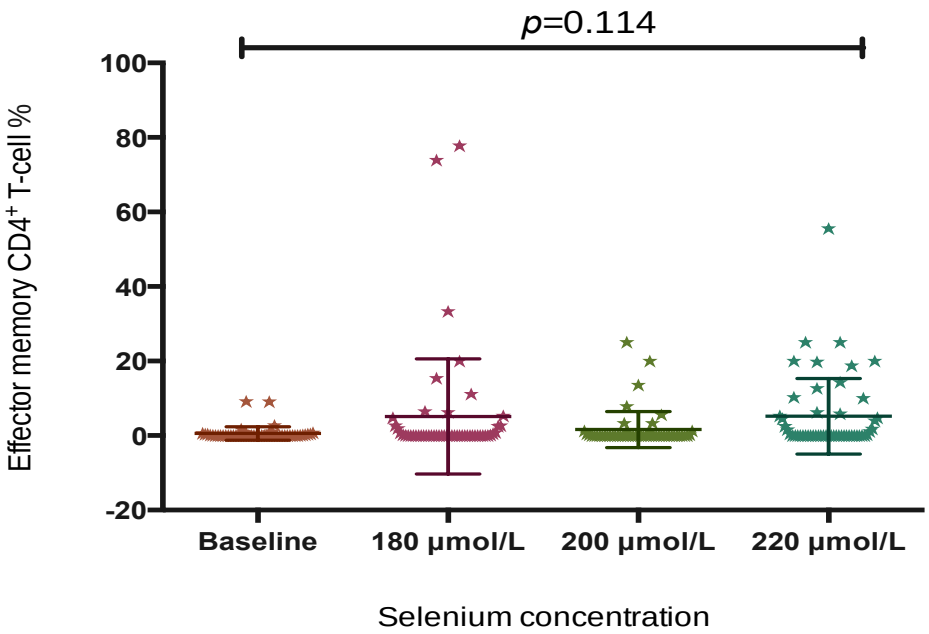


Figure 6-12 shows median effector memory marker expression on $CD4^+$ T cells at different concentrations. There were no observed significant differences in effector memory marker expression on the $CD4^+$ T cells at any of the concentrations compared to the baseline marker profile using Friedman test, p=0. 114.

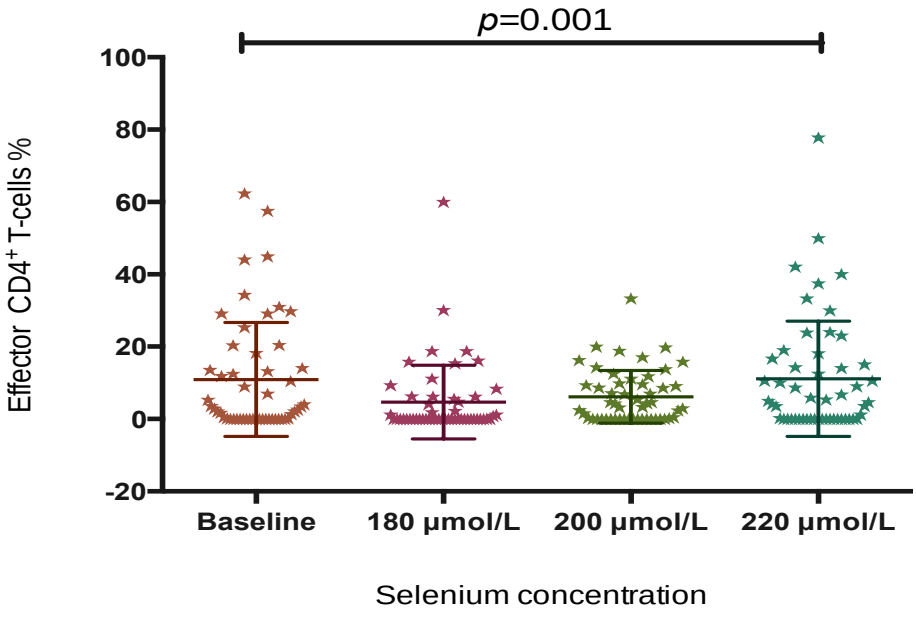


Figure 6-13 shows median effector marker expression on $CD4^+$ T cells at different concentrations. There was a significant downward change in effector marker expression on the $CD4^+$ T cells at 180 µmol/L and 200 µmol/L. However the markers increased to baseline levels at the highest concentration 220 µmol/L. However, overall after analysing all different concentrations together using Friedman test, there was a significant difference (p=0.001).

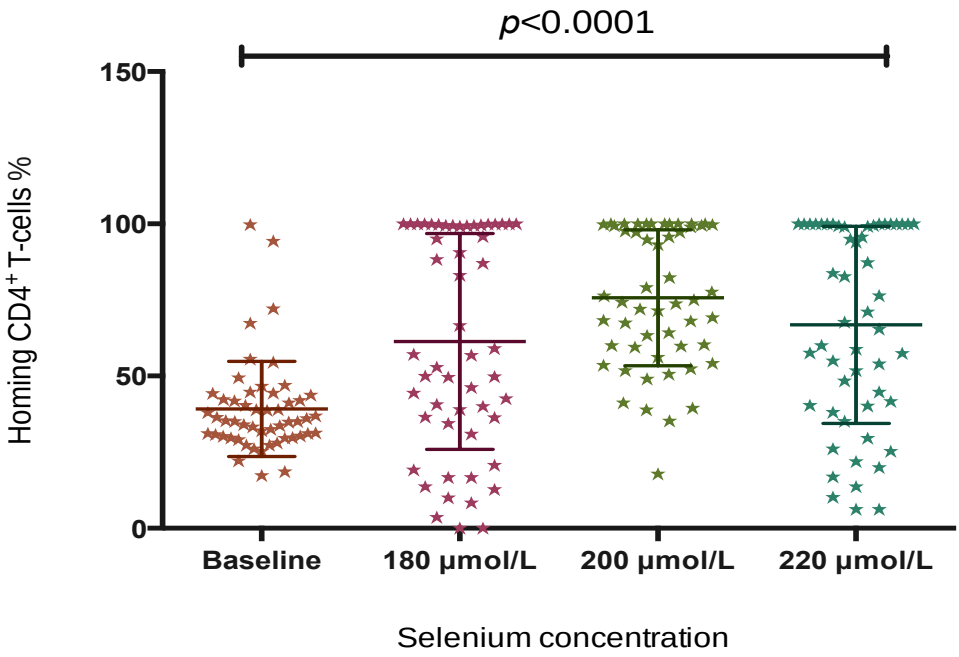


Figure 6-14 shows median homing marker expression on $CD4^+$ T cells at different concentrations. Although generally there was a significant increase in homing marker expression on the $CD4^+$ T cells at all the concentrations when compared using the Friedman test (p=<0.0001), 200 µmol/L showed the highest increase.

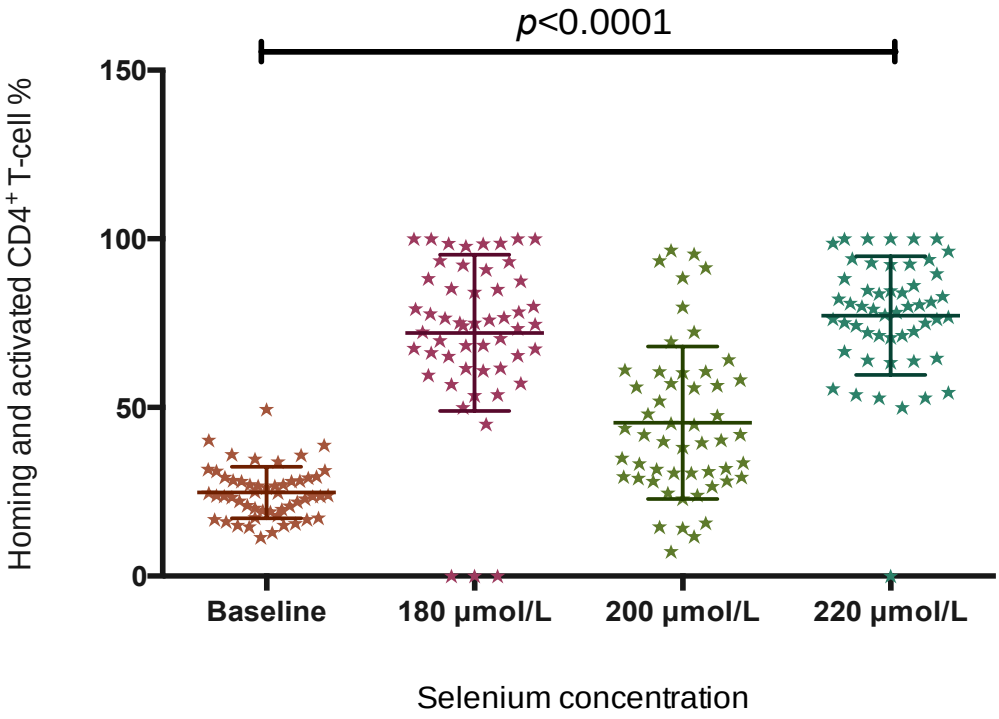


Figure 6-15 shows median homing and activated marker expression on $CD4^+$ T cells at different concentrations. Although there was a significant (p<0.0001) change, there was an observed up and down marker expression with change in concentration.

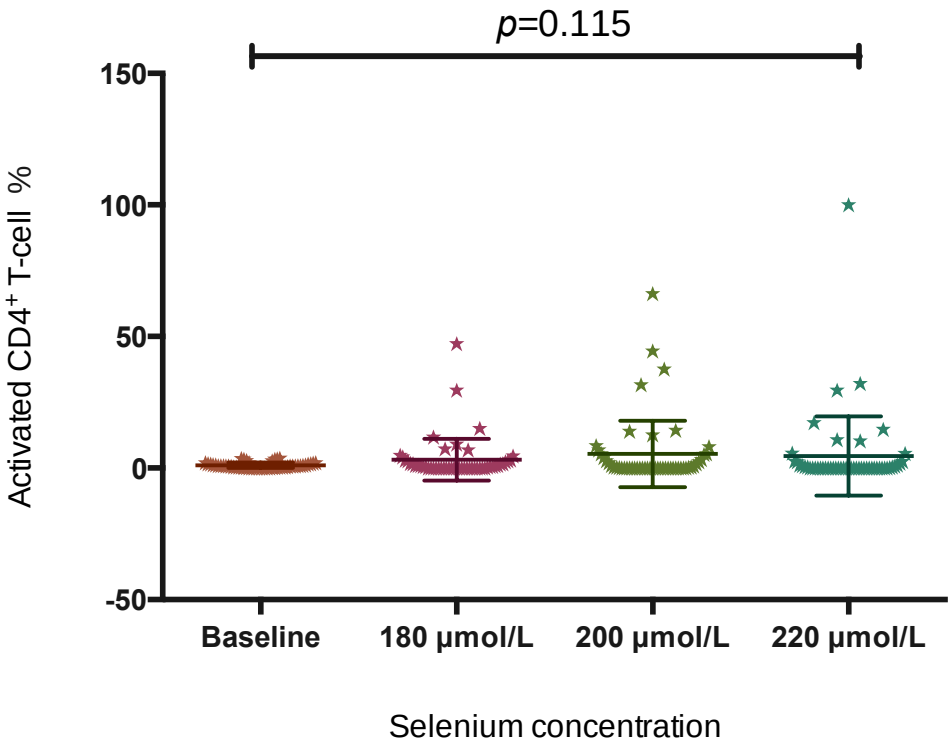


Figure 6-16 shows median activated marker expression on $CD4^+$ T cells at different concentrations. There was no significant difference in marker expression at all the concentrations compared to baseline marker profile (p=0.115) when analysed using Friedman test.

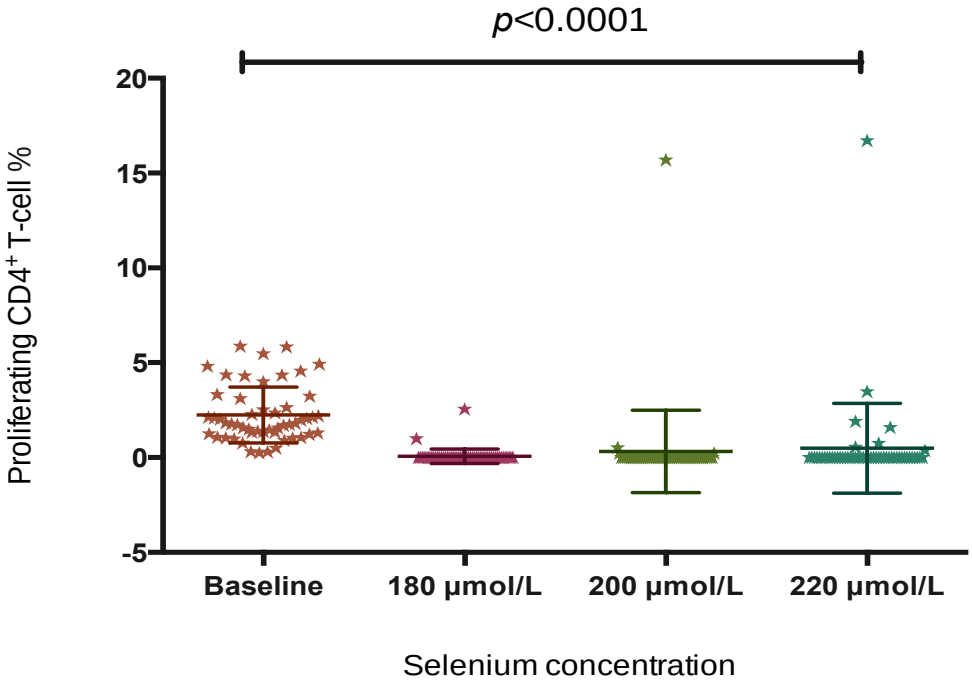


Figure 6-17 shows median proliferating marker expression on $CD4^+$ T cells at different concentrations. Overall after analyzing responses at all three concentrations together using Friedman test, there was a significant decrease (p<0.0001) in proliferating marker expression on the $CD4^+$ T cells.

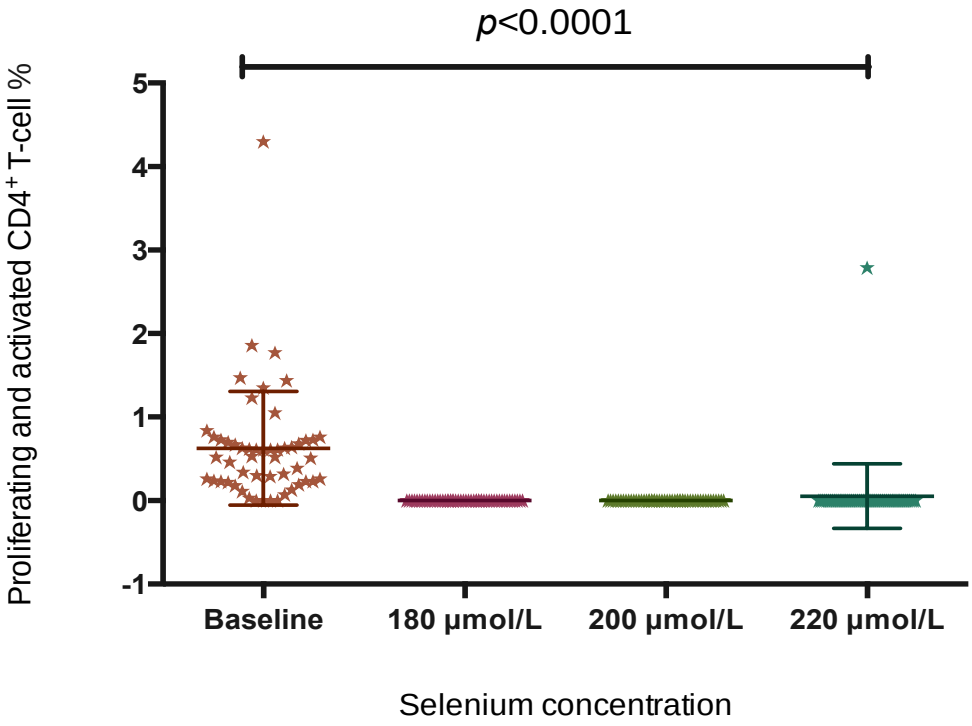


Figure 6-18 shows median number of proliferating and activated marker expression on $CD4^+$ T cells at different concentrations. Overall after analyzing responses at all three concentrations together using Friedman test, there was a significant decrease ($p<0.0001$) in proliferating and activated marker expression on the $CD4^+$ T cells.

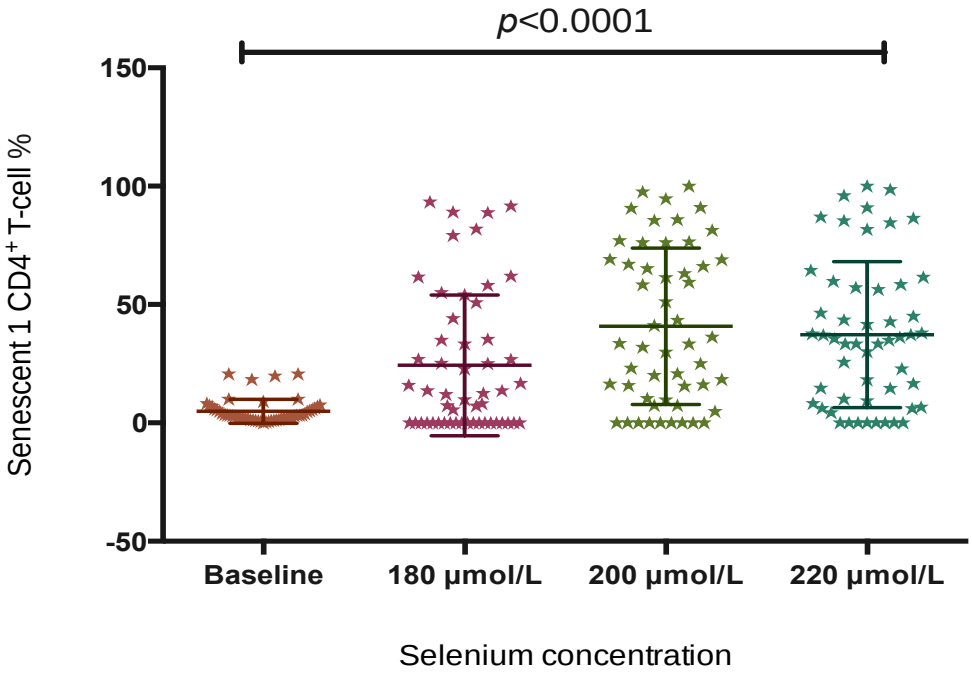


Figure 6-19 shows the median senescent 1 marker expression on $CD4^+$ T cells at different concentrations. Compared to baseline marker profile, there was a significant increase ($p<0.0001$) in senescent marker expression on the $CD4^+$ T cells with an increase in selenium concentration.

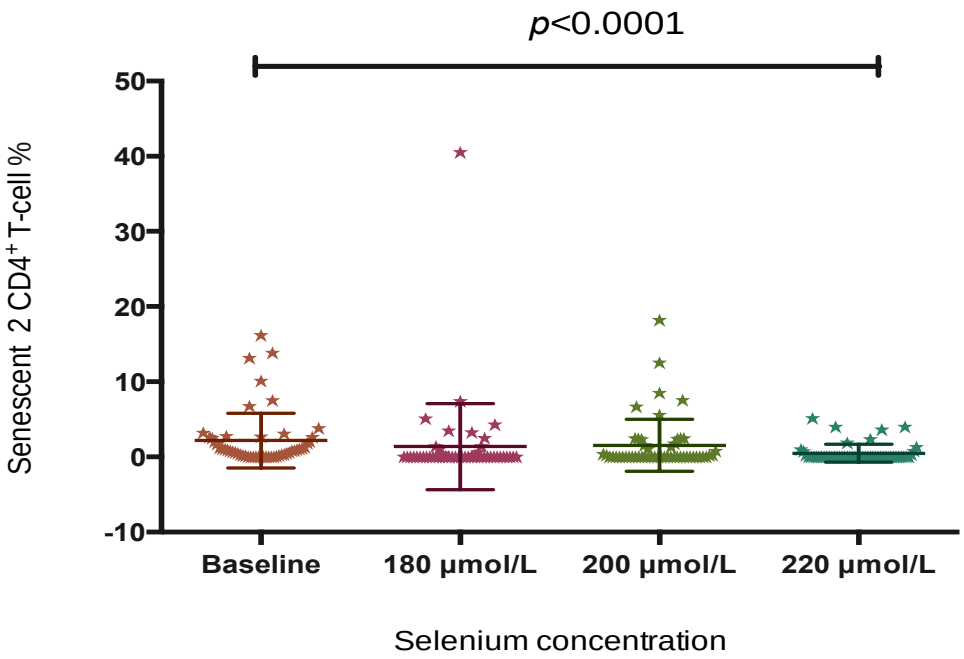


Figure 6-20 shows the median number of senescent 2 marker expression on $CD4^+$ T cells at different concentrations. Compared to baseline marker profile, senescent 2 $CD4^+$ T-cells seemed to decrease with an increase in selenium concentration. After analyzing responses at all three concentrations together using Friedman test, there was a significant decrease ($p<0.0001$) in senescent 2-marker expression on the $CD4^+$ T cells.

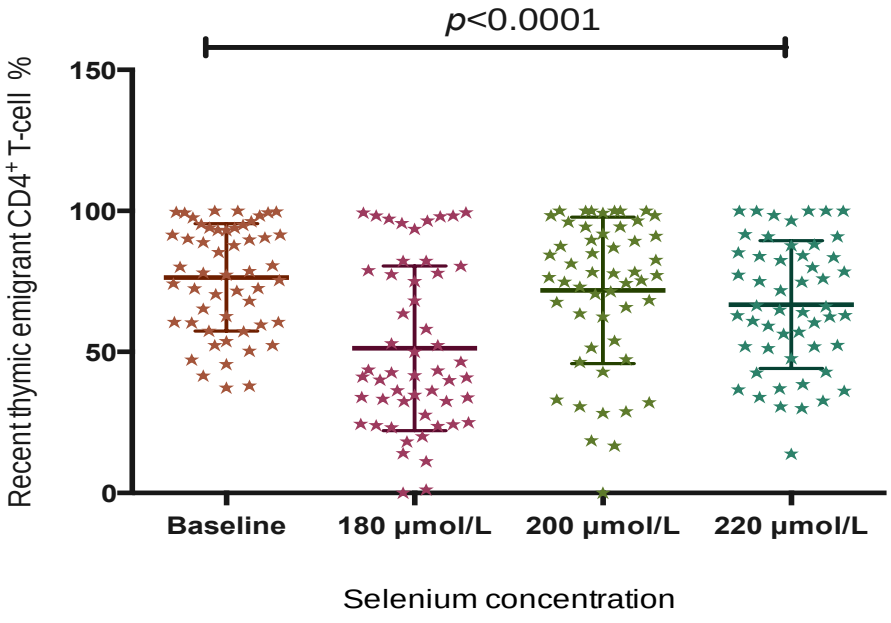


Figure 6-21 shows the median number of recent thymic marker expression on $CD4^+$ T cells at different concentrations. Although a significant ($p<0.0001$) change was observed, there was an observed up and down marker expression with change in concentration.

We found that the Se concentration levels were consistent at different concentrations in all CD4 subset with an exception of effector memory (Figure 6-12) and activated $CD4^+$ T cells (Figure 6-16). Cells expressing senescent 1 surface marker were consistently increased at almost all the concentrations in the CD4s whereas cells expressing senescent 2 markers consistently decreased at all concentrations.

Likewise, the dynamics of individual cell populations expressed on the $CD8^+$ T cells at different Se concentrations were examined and results presented in Figures 6-22 to 6-33.

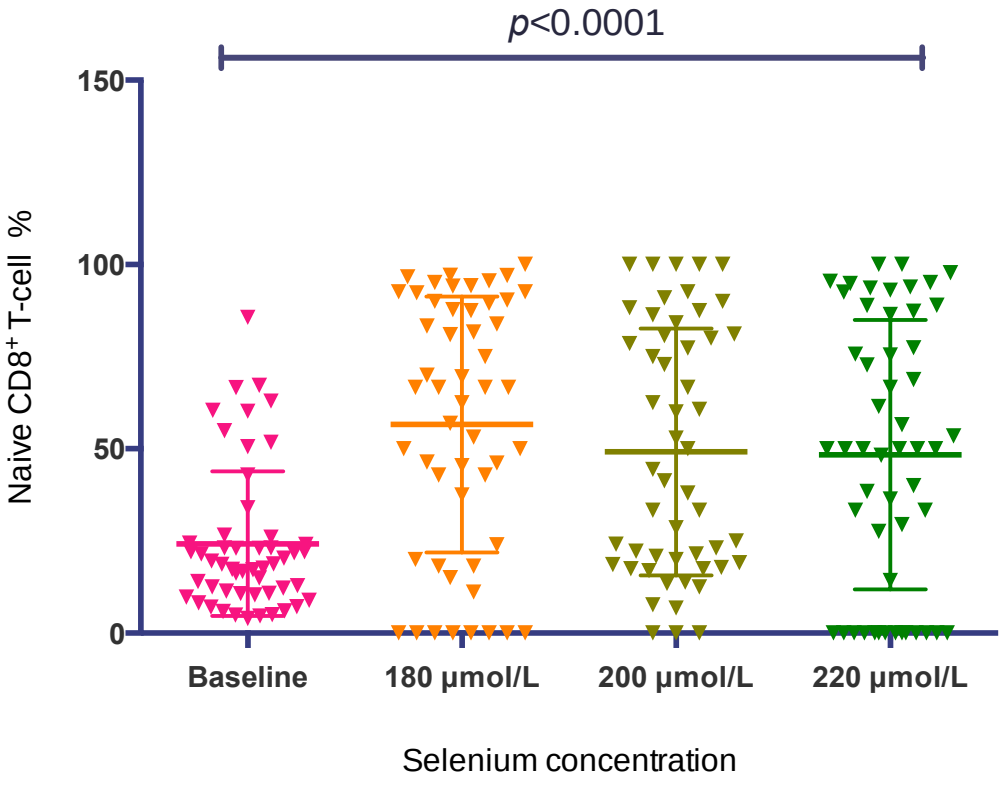


Figure 6-22 shows changes in median naive marker expression at different selenium concentrations in the $CD8^+$ T cells. There was a significant increase in naive marker expression as the selenium concentration was increased compared to markers expressed at baseline (P<0.0001)

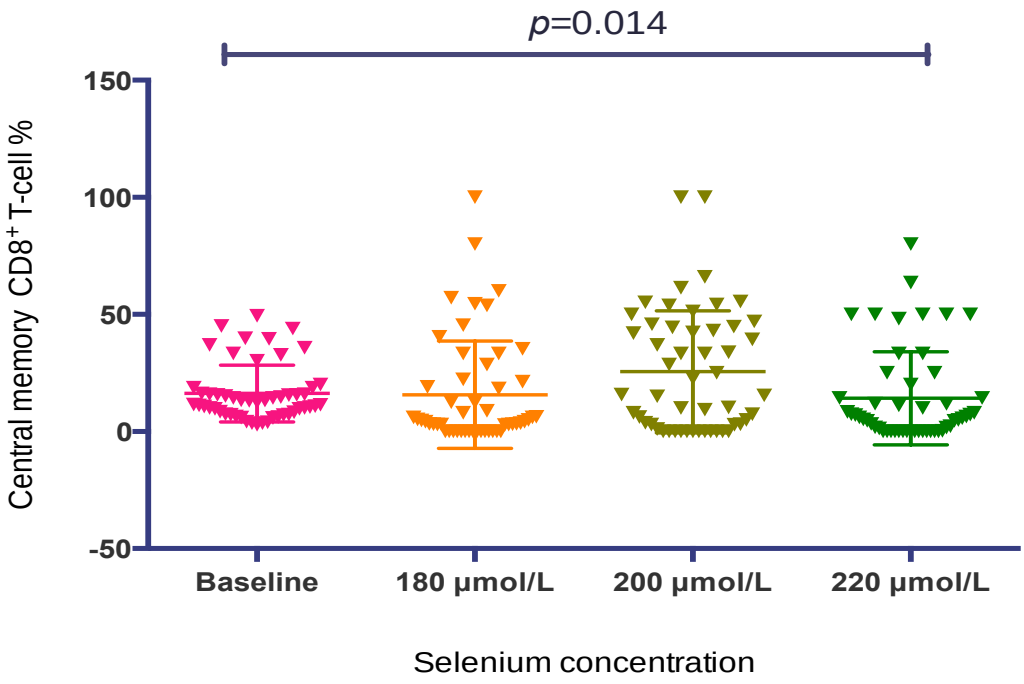


Figure 6-23 presents the differences in central memory markers expressed on the $CD8^+$ T cells during incubations with different concentrations of selenium. Friedman test showed a significant difference after selenium increase compared to markers expressed at baseline (p=0.014).

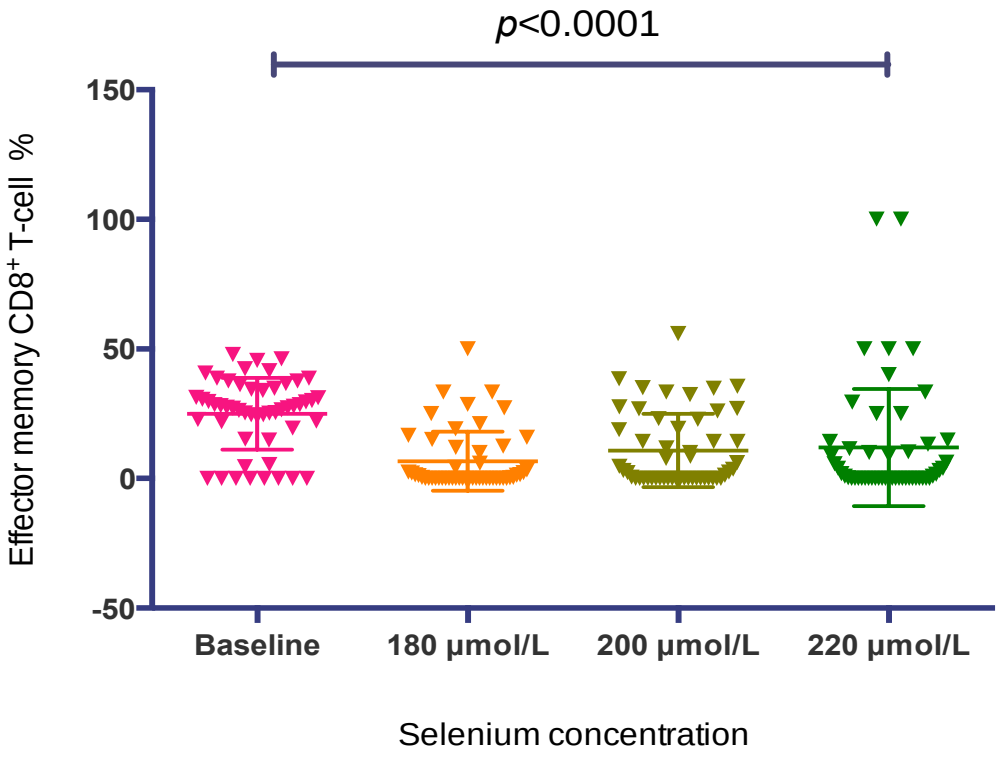


Figure 6-24 shows the median number of effector memory markers expressed on $CD8^+$ T cells. Using Friedman test, there was an observed significant decreased with an increase in concentration compared to markers expressed at baseline.

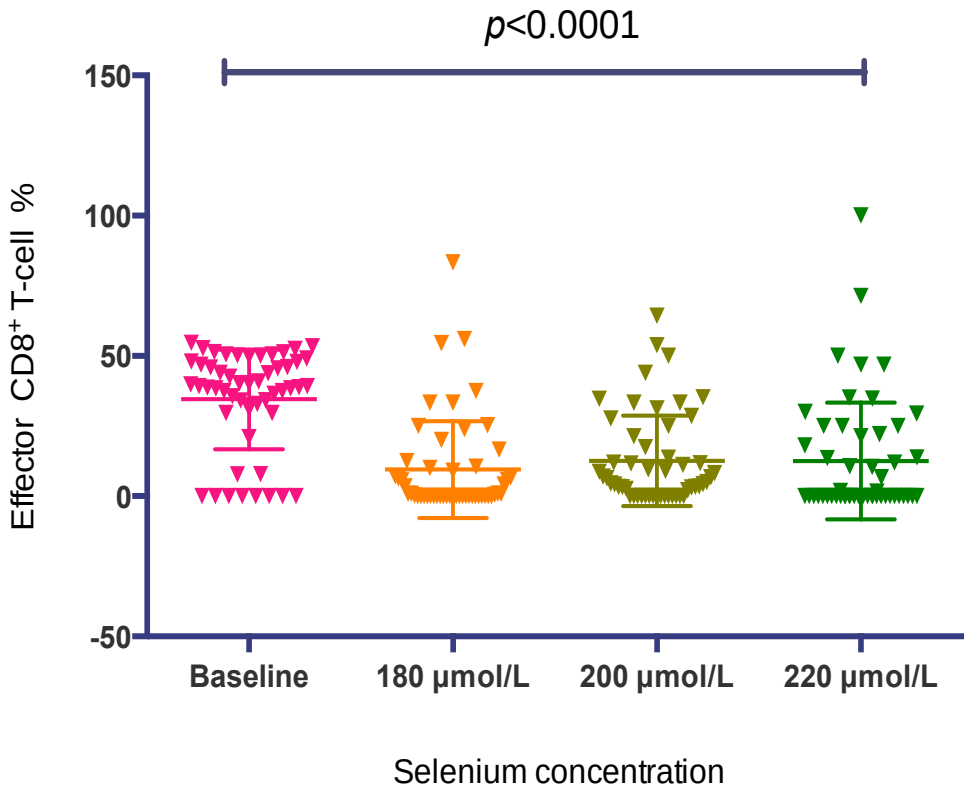


Figure 6-25 similar to effector memory marker expression**,** using Friedman test we found a significant decrease (p<0.0001) in the effector cells with an increase in concentration compared to markers expressed at baseline.

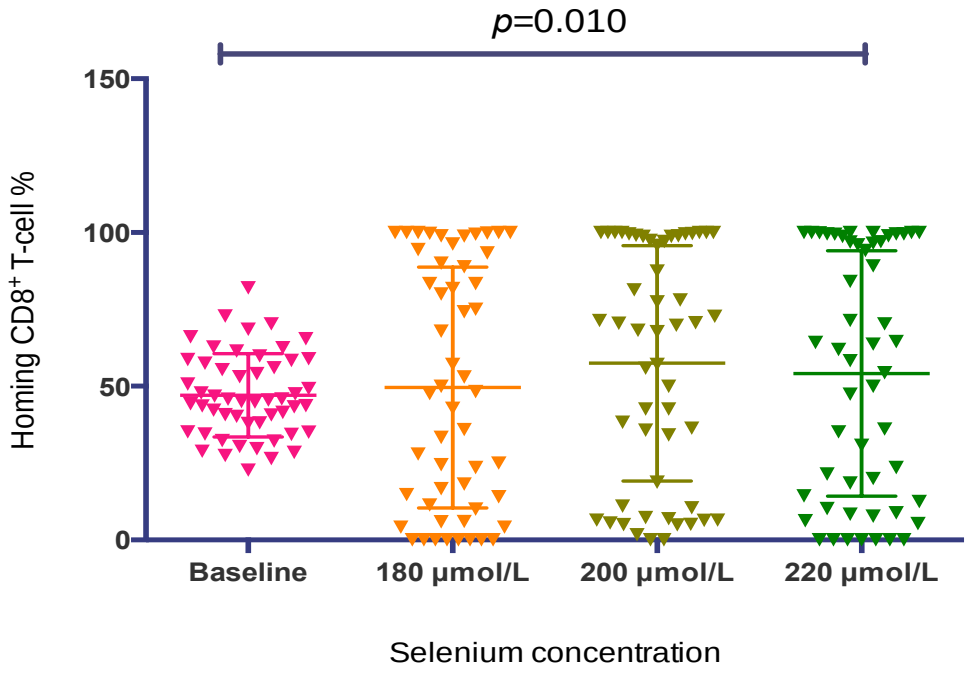


Figure 6-26 shows baseline homing surface marker expression on $CD8^+$ T cells and increases as the concentration of selenium was increased. Using the Friedman test, we found a significant difference (p=0.010) when surface marker expression was analysed for all concentrations.

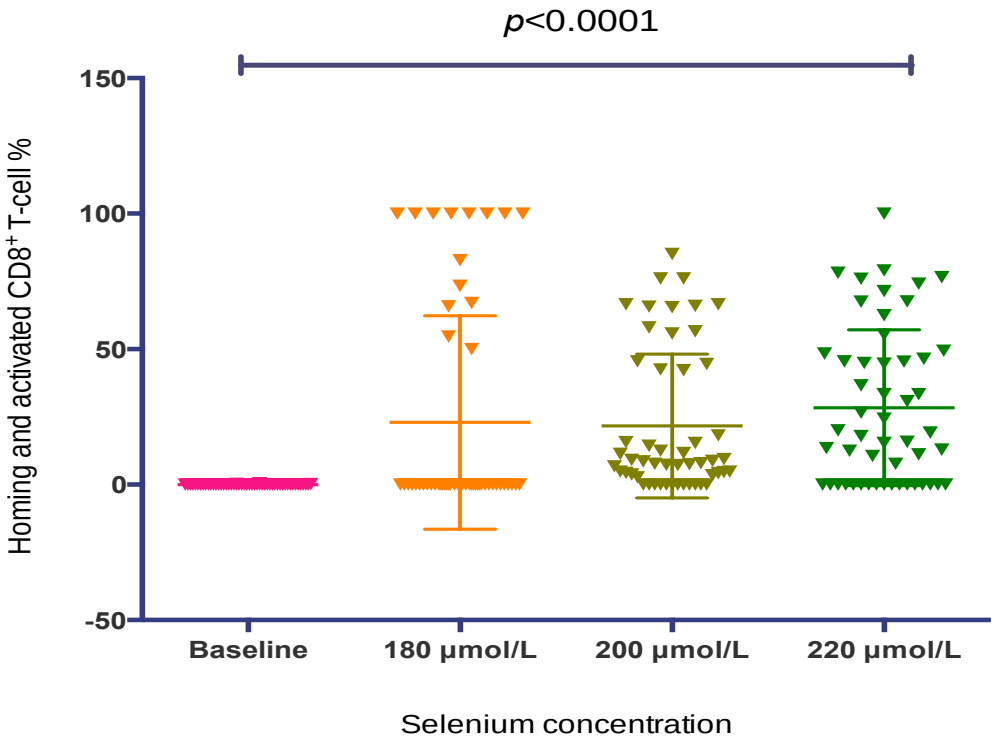


Figure 6-27 shows the median number of homing and activated surface markers expressed at baseline and changes after selenium was increased. The number of homing and activated markers expressed significantly increased with the increase in selenium concentration. Using the Friedman test, the p value was less than 0.0001.

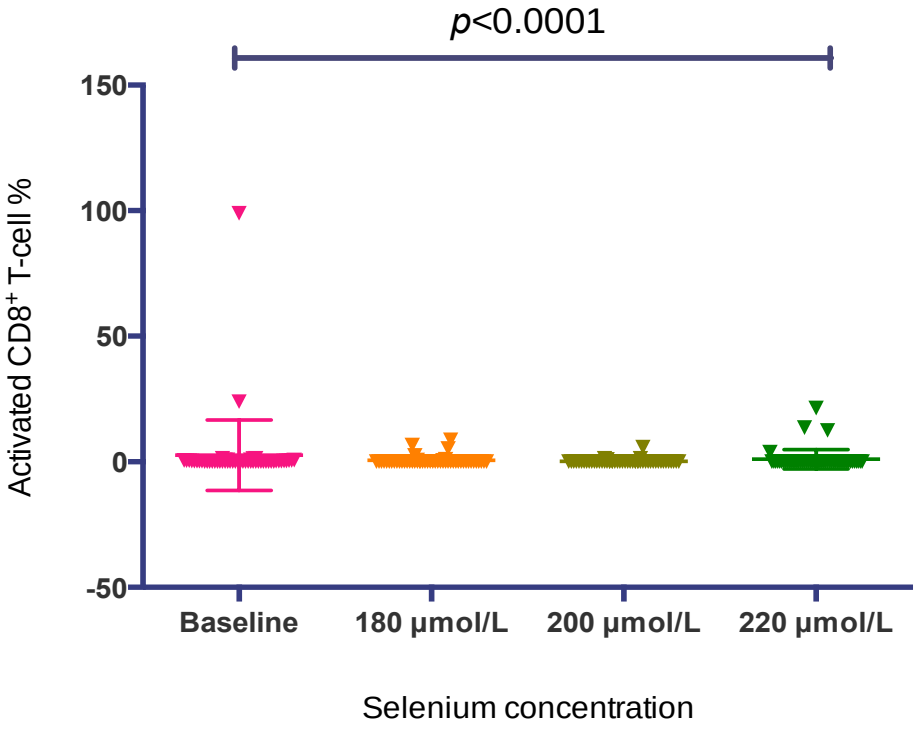
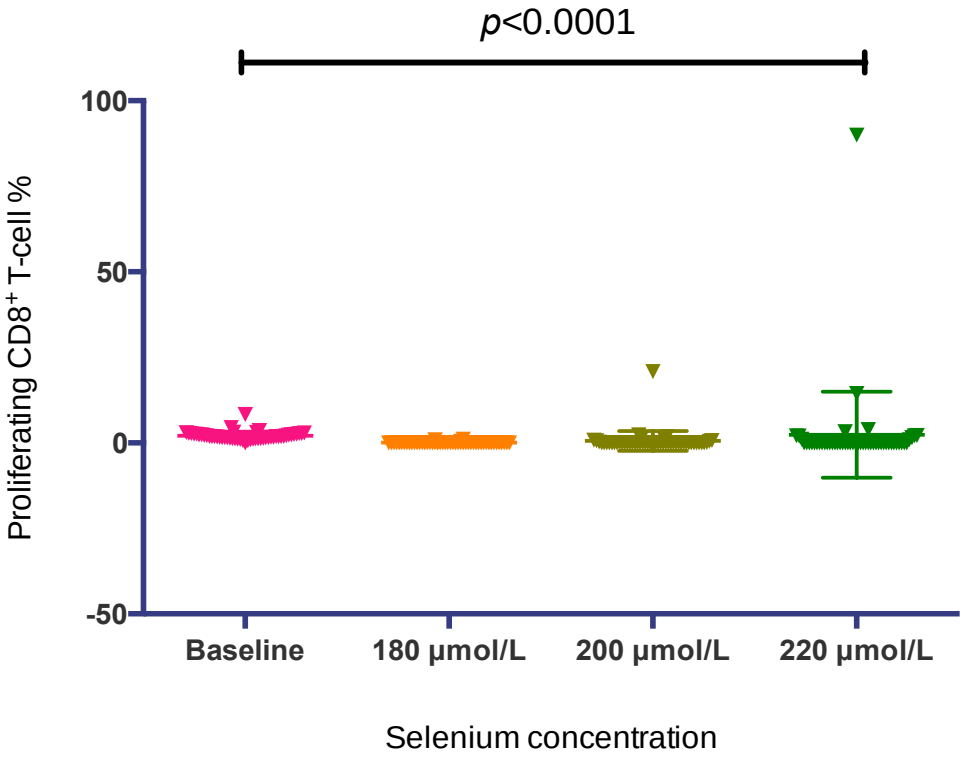


Figure 6-28 compared to baseline activated surface markers, as selenium concentration increased the number of activated surface markers expressed on the $CD8^+$ T cells completely disappeared ($p<0.0001$).



Similar to Figure 6-28, proliferating surface markers on the $CD8^+$ T cells completely disappeared as selenium concentration increased ($p<0.0001$) Figure 6-29.

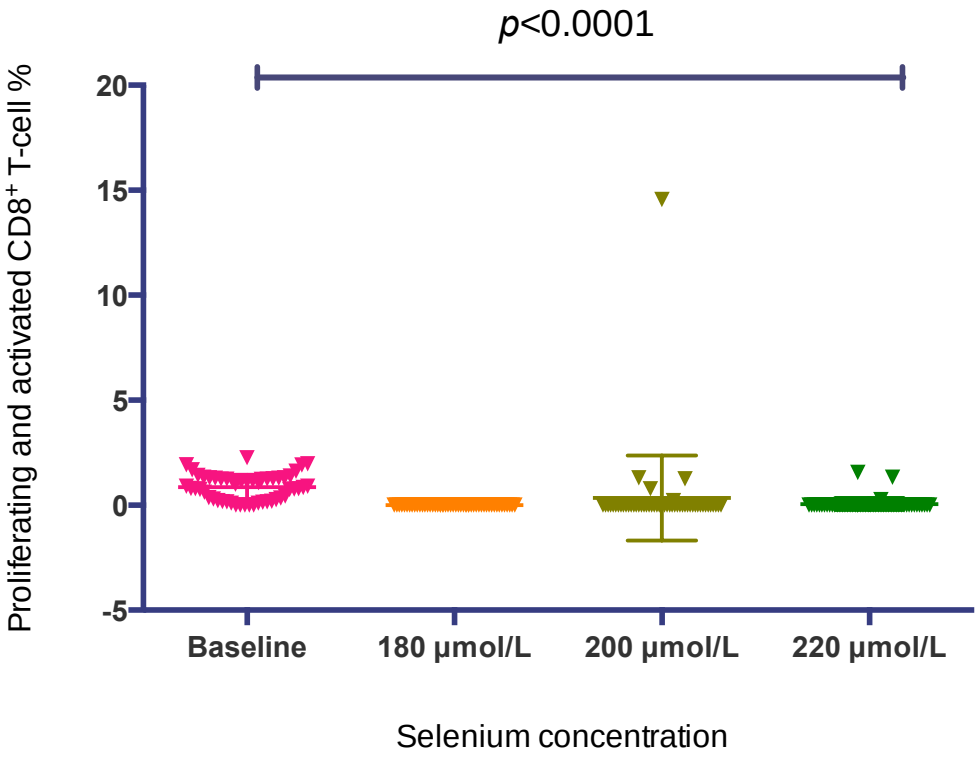


Figure 6-30 shows the decrease in proliferating and activated surface markers expressed on the $CD8^+$ T cells. There was an observed significant decrease compared to baseline marker expression with an increase in selenium concentration ($p<0.0001$)

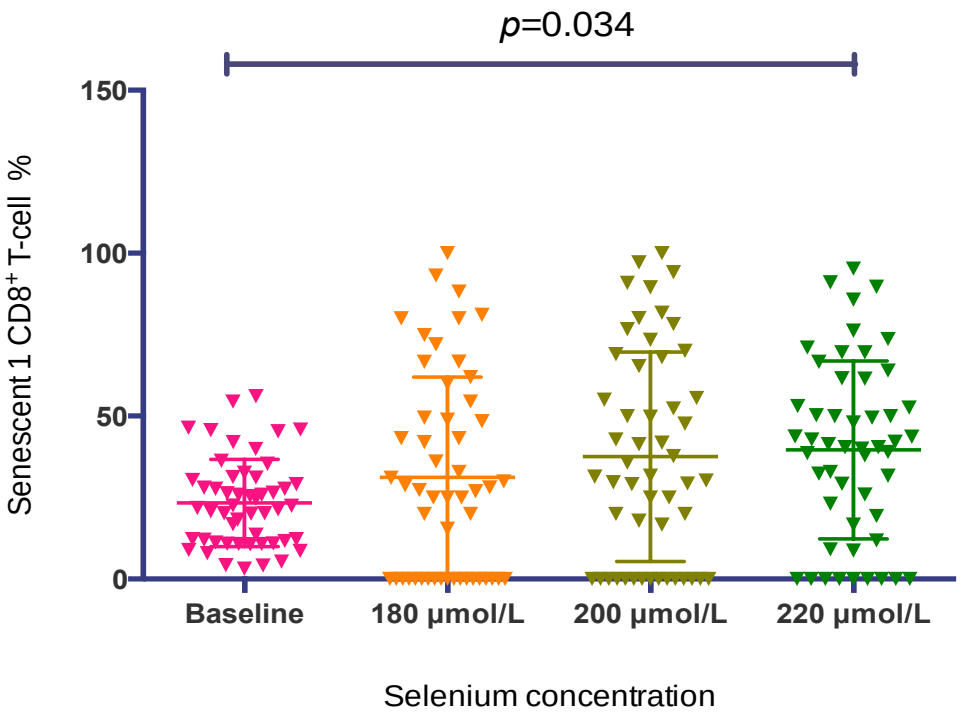
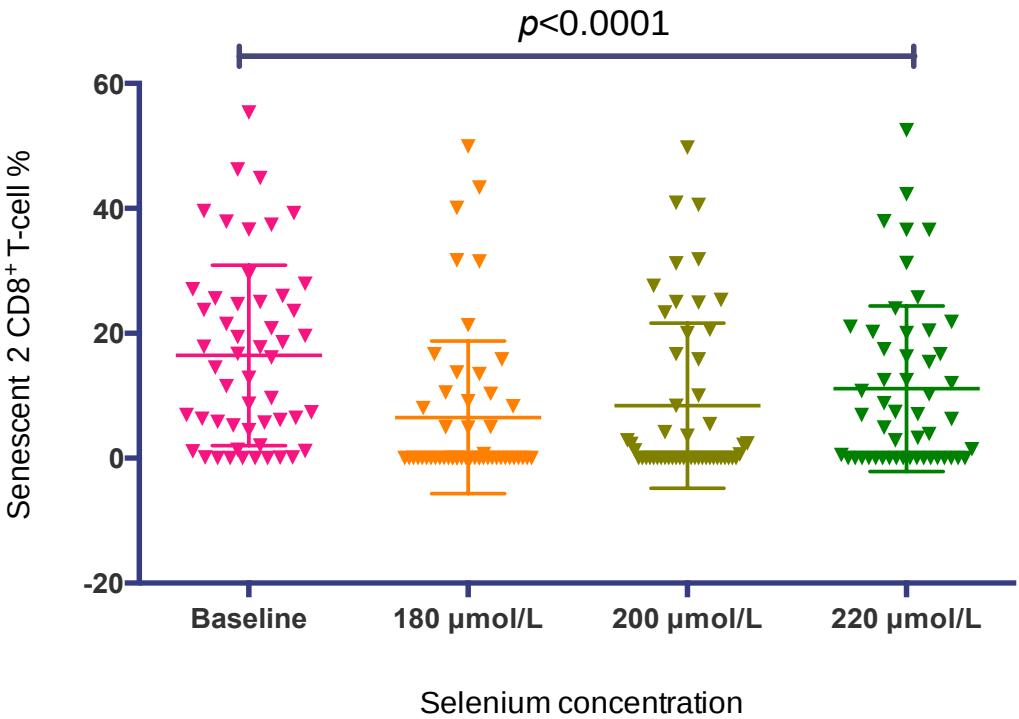
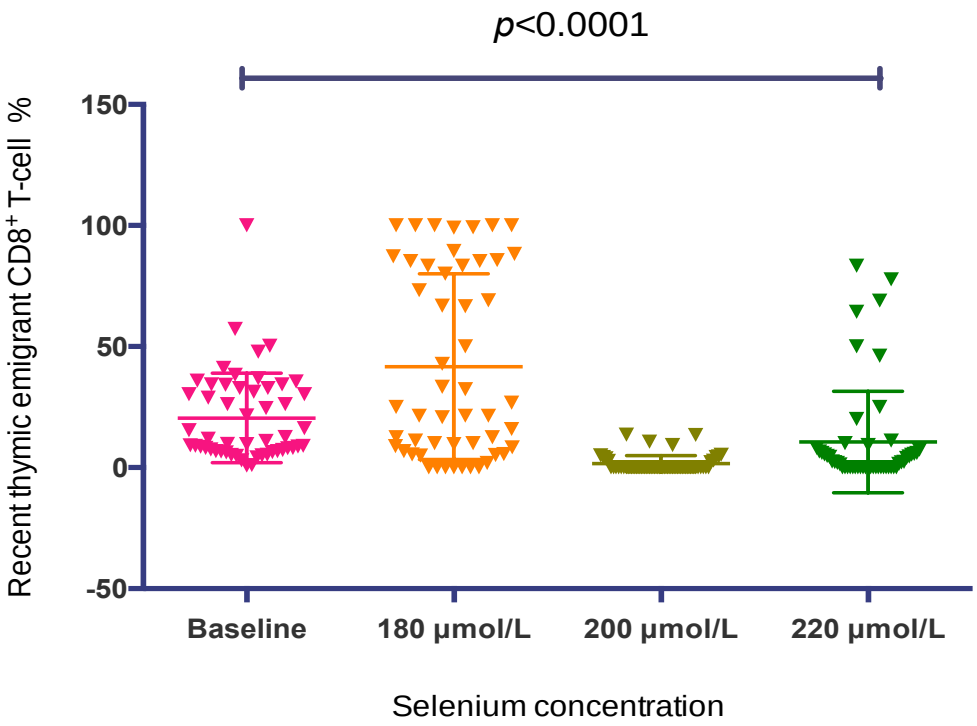


Figure 6-31 shows the median senescent 1 marker expression on $CD8^+$ T cells at different concentrations. Compared to baseline marker profile, senescent 1 $CD8^+$ T cells increased with an increase in selenium concentration (p=0.034).



Contrary to Figure 6-31, in Figure 6-32 the median senescent 2 marker expression on $CD8^+$ T cells decreased with an increase in selenium concentration. When responses at all three concentrations were analyzed using Friedman test, there was a significant decrease (p<0.0001) in senescent marker expression on the $CD8^+$ T cells.



In Figure 6-33 using the Friedman test, recent thymic emigrant surface marker expression was highest at 180 µmol/L and lowest at the remaining selenium concentrations ($p<0.0001$).

In the CD8 population, there was either a consistent increase from baseline to 220 µmol/L or a consistent decrease, or an up and down pattern or a down and up surface marker expression (inconsistent response; up at one concentration and down at the next).

Further analysis of these responses in relation to baseline Se concentration, as a marker of Se status shows that there were significant differences in gut homing responses in CD4 cells (Figure 6-34).

Figure 6-34 shows that there were more gut homing markers expressed on CD4 cells in the deficient group compared to the non-deficient group. Using Wilcoxon rank-sum test the p value was 0.01.

Figure 6-35 shows that expression of homing and activated markers on the CD4 cells in both the deficient and non-deficient individuals was comparable. Using Wilcoxon rank-sum test, the p value was found to be insignificant (p=0.46) between the two groups.

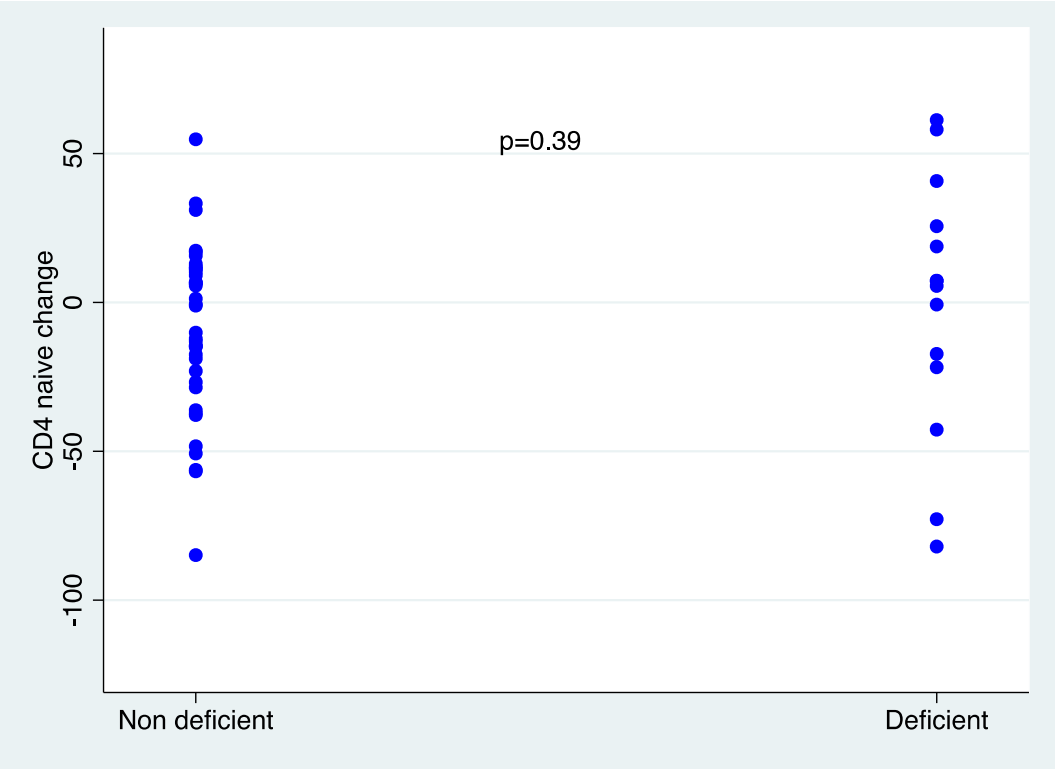


Figure 6-36 shows that expression of naive markers on the CD4 cells in both the deficient and non-deficient individuals was comparable. Using Wilcoxon rank-sum test, the p value was found to be insignificant (p=0.39) between the two groups.

Figure 6-37 shows that there was no significant difference in senescent 1 marker expression on CD4 cells of the deficient and non-deficient groups. Using Wilcoxon rank-sum test, the p value was calculated as p=0.89.

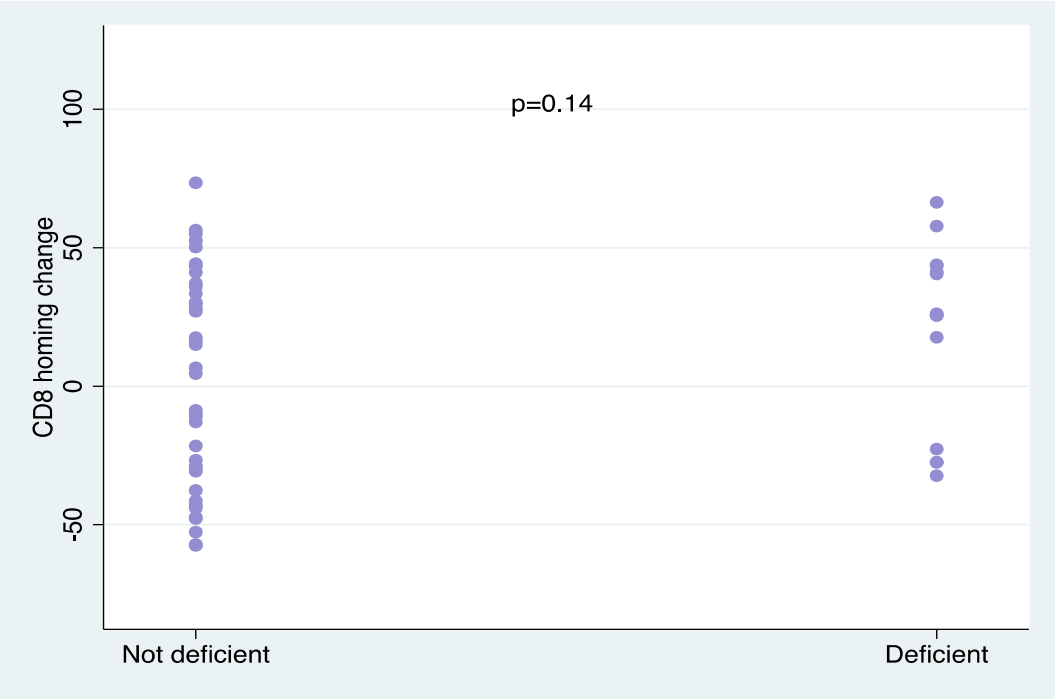


Figure 6-38 shows gut homing marker expression on CD8 cells in the deficient and non-deficient individuals. Gut homing marker expression was comparable between the two groups. Using Wilcoxon rank-sum test, the p value was calculated as *p*=0.14.

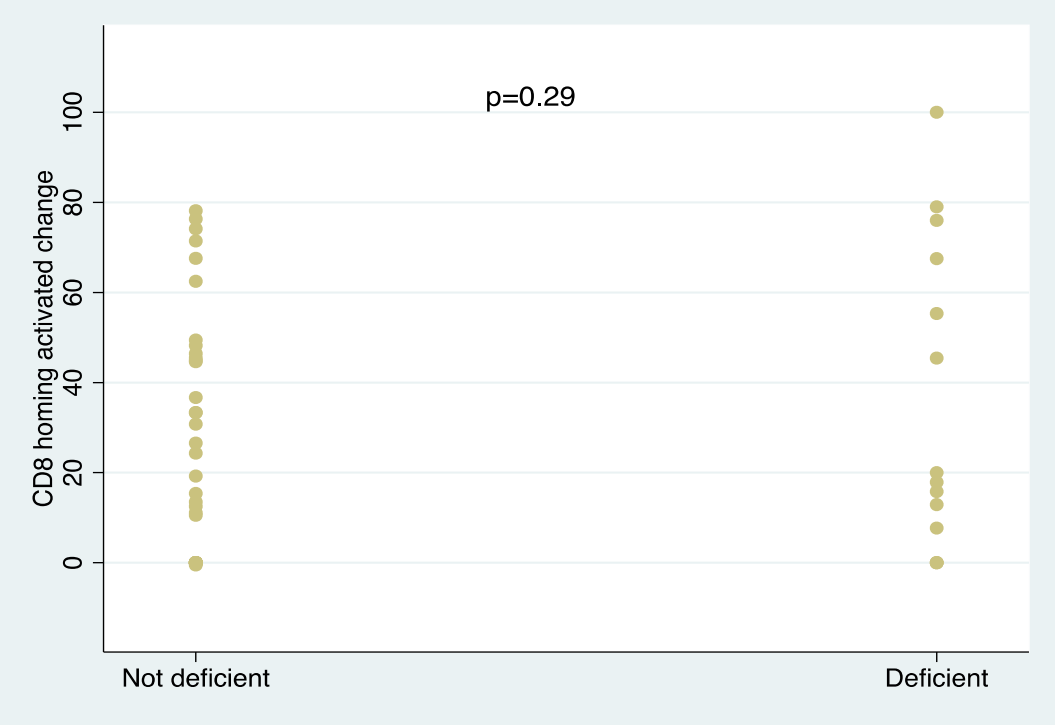


Figure 6-39 shows homing and activated markers CD8 cells in deficient and non-deficient groups. There was significant difference (p=0.29) found between the two groups using Wilcoxon rank-sum test.

Figure 6-40 shows that expression of naive markers on CD8 cells in deficient and non-deficient individuals was comparable. Using Wilcoxon rank-sum test, the p value was p=0.83.

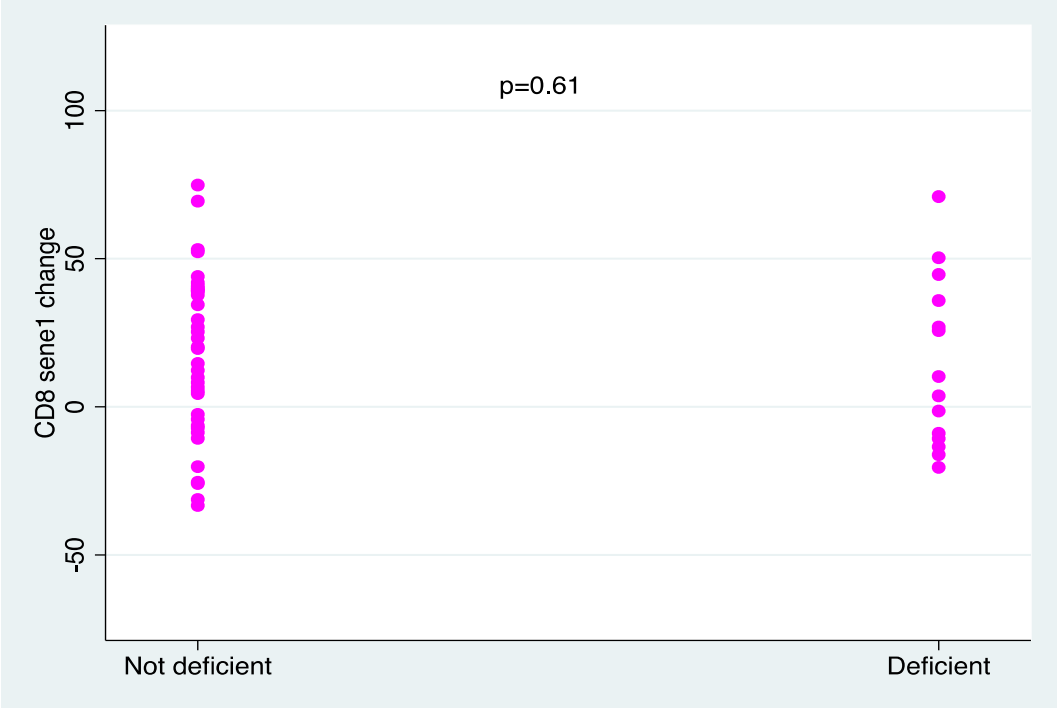


Figure 6-41 shows senescent 1 surface marker expression on CD8 cells in deficient and non-deficient individuals. In both groups, senescent 1 surface maker expressions

on $CD8^+$ cells were comparable. Using Wilcoxon rank-sum test, the p value was p=0.61.

In Summary;

Most of the CD4 and CD8 T cell subsets showed significant responses to ambient Se in vitro (Table 10-7 and Table 10-8). Further, baseline Se and both absolute CD4 and CD8 count were not correlated (CD4 rho=0.08, P=0.58 and CD8 rho= -0.02, P=0.87). There were some significant differences in CD4 and CD8 T cell subsets between the males and females, with significant increases seen in males. It was assumed that this difference might be Se dependent since there were more Se deficient males than females. Overall, no correlation was found between baseline Se and surface marker expression, but hut homing response was greater in Se deficient individuals.

6.6 Results for a Survey of Treated HIV-infected Patients and HIV- Adults

In view of the response of CD4 and CD8 response to Se *in-vitro*, a survey was undertaken. The objective of this survey was to ascertain whether T cell subset numbers were restored completely to normal after 12 weeks of ART. In this analysis, all the HIV negative adults in the cross section study were enrolled. HIV-infected adults were randomly selected from the 88 that had completed 12 weeks of ART and nutritional support in the NUSTART trial. Random selection was done using the "=RAND (1-88)" commands in excel. This yielded a ratio of 1:1 and the results for each CD4 and CD8 subset studied are presented figures below.

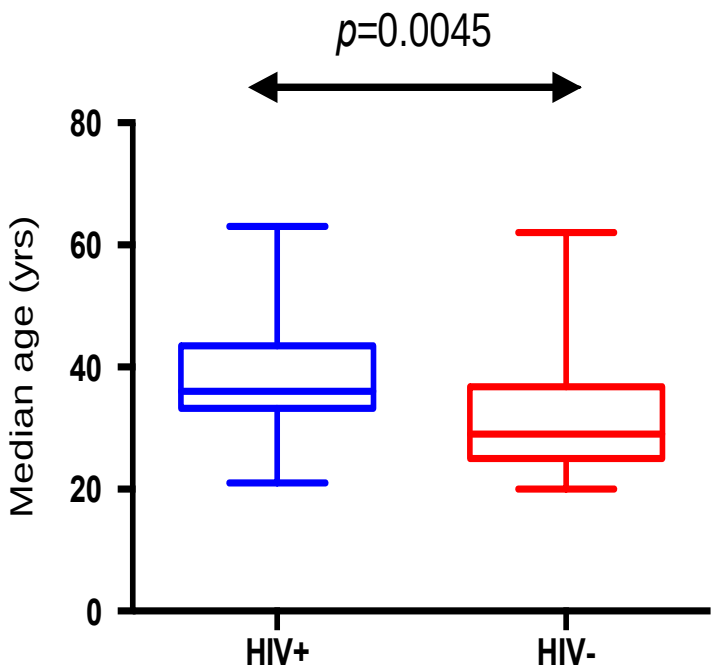


Figure 6-42 shows the mean age between the HIV+ and HIV- individuals. The HIV+ individuals were older (median 36 IQR, 34-43) than the HIV- individuals (median 29 IQR, 25-37). The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined.

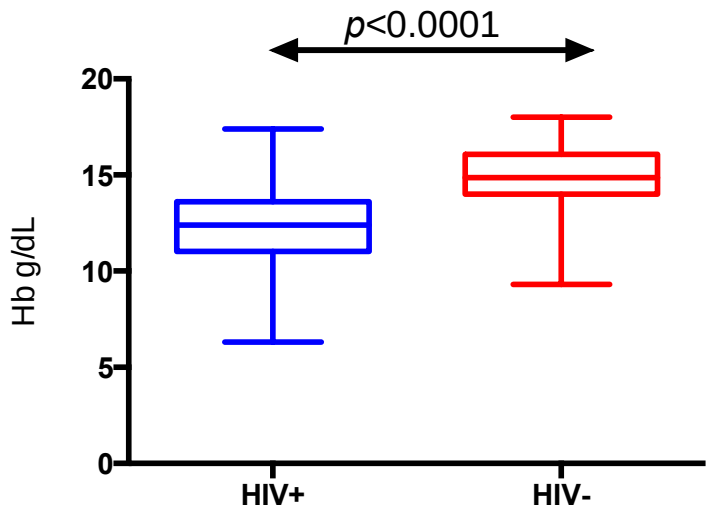


Figure 6-43 shows the median hemoglobin between the HIV+ and the HIV- participants. The median hemoglobin (13 IQR, 11-13.6) level for HIV+ individuals after 3 months of taking ART plus nutritional support was significantly lower than in the HIV- individuals, (14.85 IQR, 14-16.05). The p value was determined using the two-sample Wilcoxon rank-sum (Mann-Whitney) test.

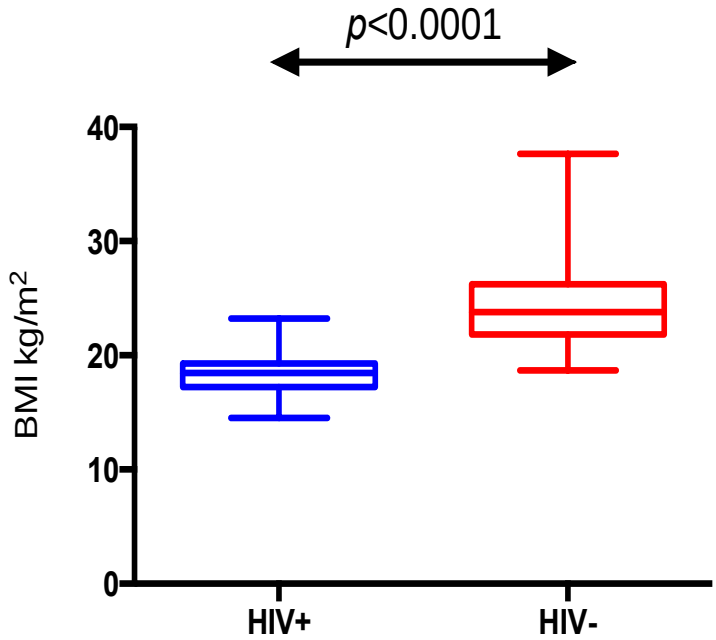


Figure 6-44 shows the differences in BMI for the HIV+ and HIV- participants. HIV+ participants had a significantly lower 18.4 (IQR, 17.3-19.45) BMI than the HIV- participants 23.78 (IQR, 221.83-26.10) after 3 months of receiving ART. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined.

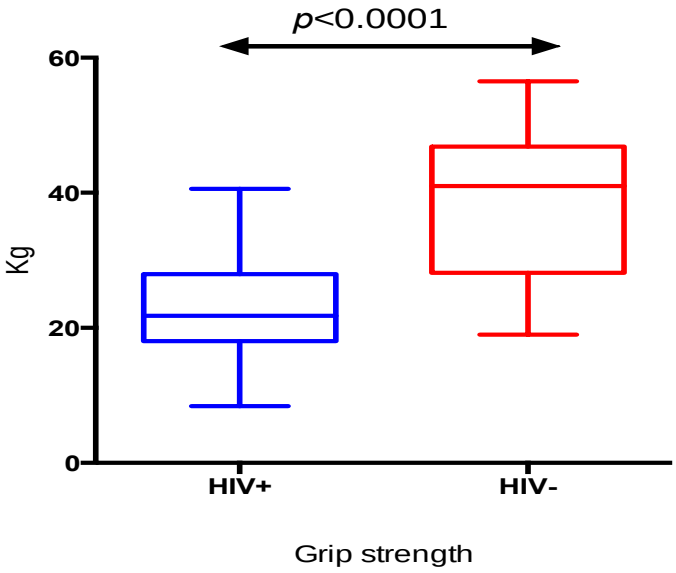


Figure 6-45 shows the grip strength results for the HIV+ and HIV- participants. The HIV+ participants had lower grip strength (22.2 IQR, 17.9-28.2) than the HIV- participants (41 IQR, 28.85-46.8) after 3 months of ART and nutritional support. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

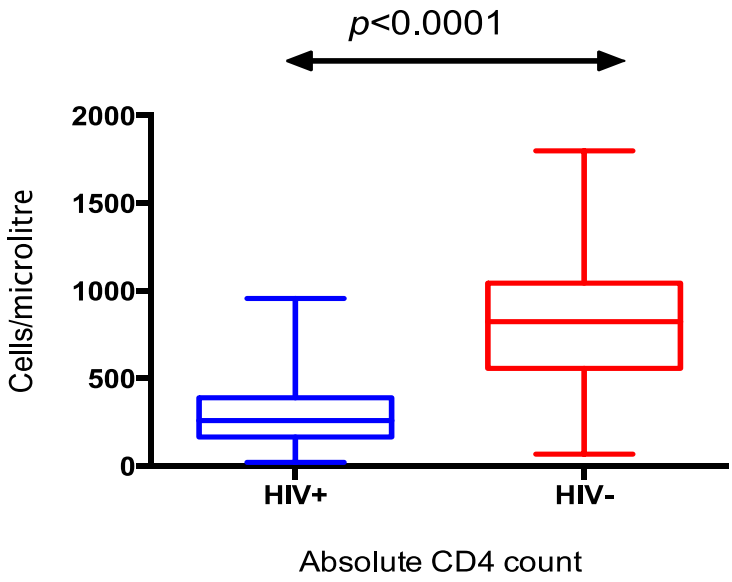


Figure 6-46 shows the median absolute CD4 count between the HIV+ and the HIV- participants. HIV+ participants had a lower (287 IQR, 191-387) absolute CD4 count compared to the HIV- participants (824 IQR, 558-1044) after 3 months of ART. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

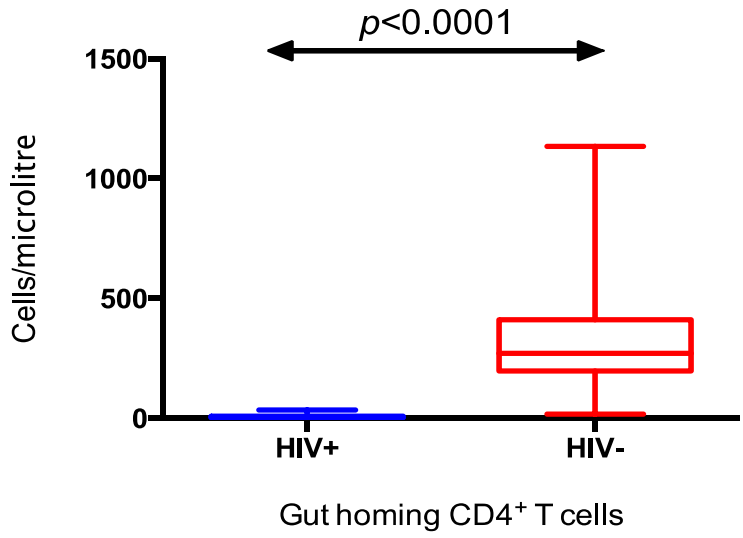


Figure 6-47 shows the expression of gut homing markers between the HIV+ and the HIV- participants. Gut homing $CD4^+$ T cells were few in the HIV+ (4 IQR, 2-8) after 3 months of ART, compared to the HIV- participants (271 IQR, 198-408). The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined by this test.

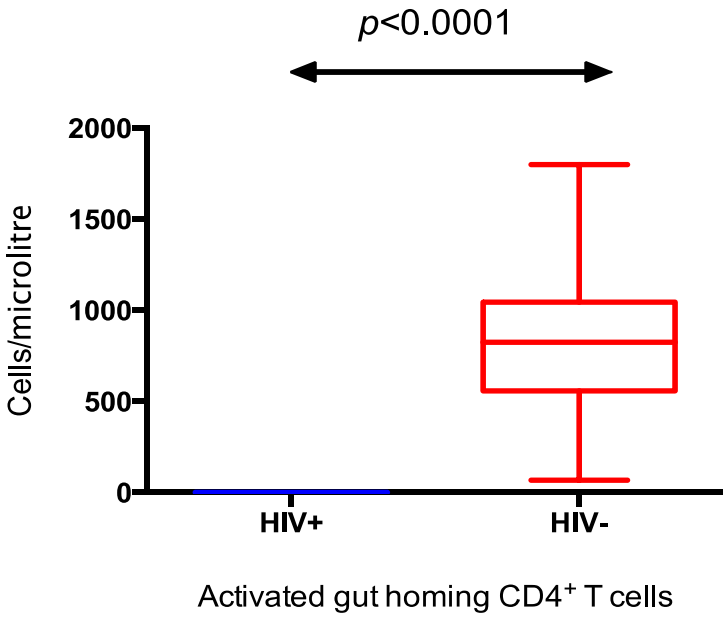


Figure 6-48 shows the number of activated gut homing $CD4^+$ T-cells between the HIV+ and the HIV- participants. Marker expression was much higher in the HIV- (170 IQR, 128-269) than the HIV+ (0 IQR, 0-1) after 3 months of receiving ART. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined.

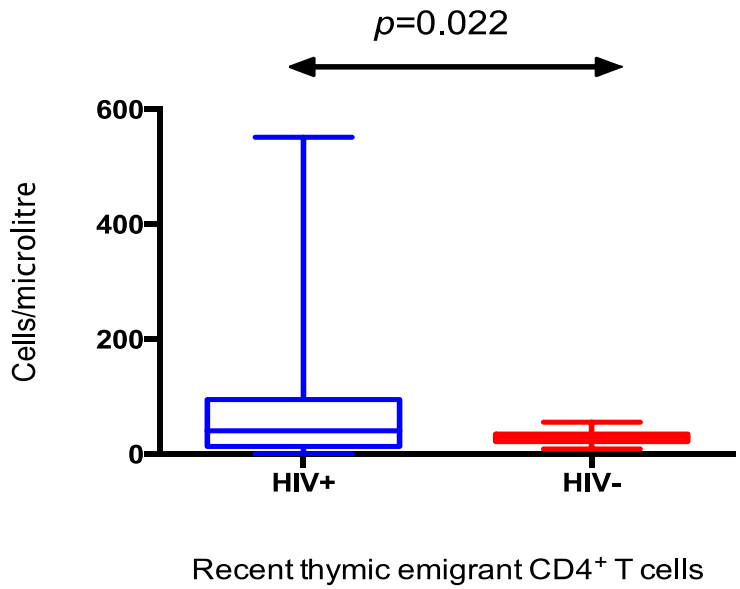


Figure 6-49 shows the number of recent thymic emigrants between the HIV+ and the HIV- participants. There were more recent thymic emigrant $CD4^+$ T cells in the HIV+ (40 IQR, 14-94) than the HIV- participants (30 IQR, 22-35) after 3 months of ART. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined.

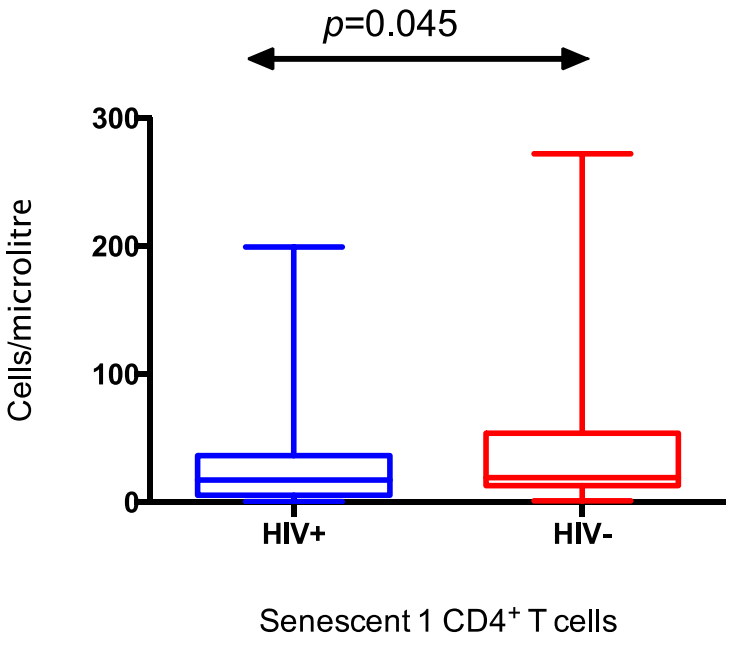


Figure 6-50 shows the number of senescent 1 $CD4^+$ T cells between the HIV+ and the HIV- participants. The numbers of senescent 1 CD4+ T cells were slightly lower in the HIV+ (17 IQR, 5-36) compared to the HIV- participants (19 IQR 13-53). Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

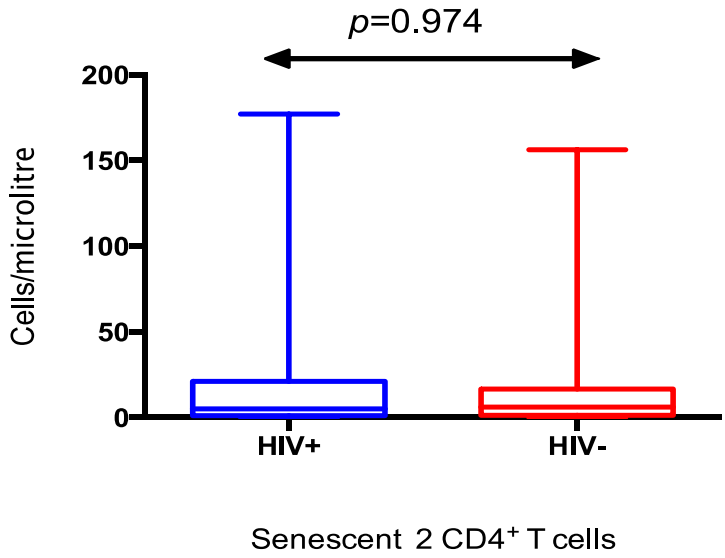


Figure 6-51 shows the number of senescent 2 $CD4^+$ T cells between the HIV+ and HIV- participants. Senescent 2 $CD4^+$ T cells were comparable in both the HIV+ (5 IQR, 1-21) and HIV- participants (6 IQR, 2-17) after 3 months of ART. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

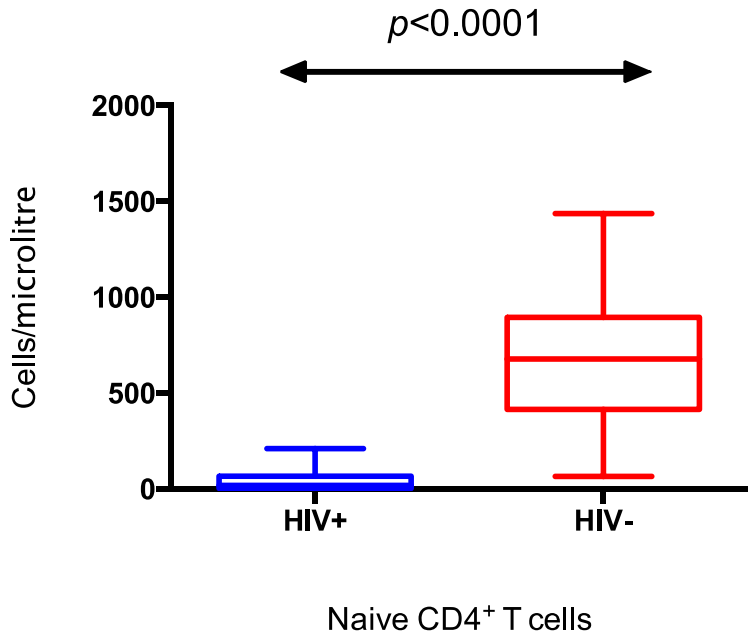


Figure 6-52 shows the distribution of naive $CD4^+$ T cells between the HIV+ and HIV- participants. The number of naive $CD4^+$ T cells after 3 months of ART was not comparable between the HIV+ (17 IQR 3-65) and the HIV- (677 IQR, 418-885) participants. The comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test. The p value was determined using this test.

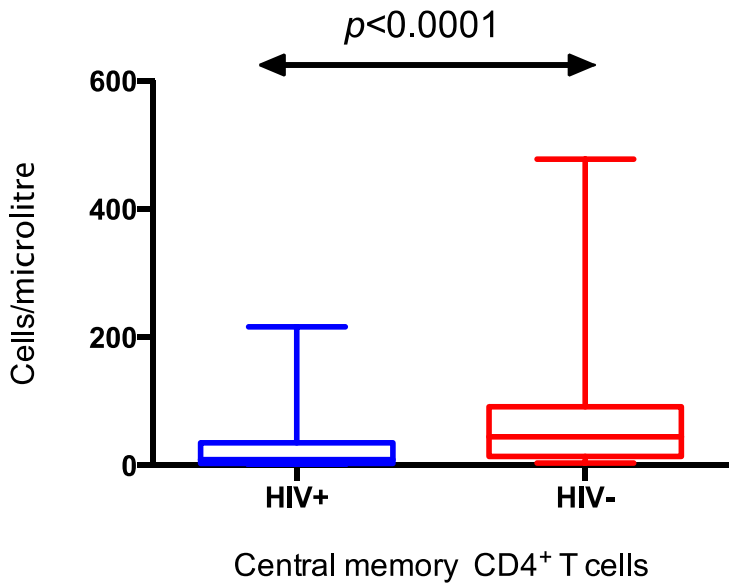


Figure 6-53 shows the number of $CD4^+$ central memory T cells between the HIV+ and the HIV- participants. HIV+ had fewer central memory cells (9 IQR, 3-35) than in the HIV- (45 IQR, 14-91) after 3 months of taking ART. The comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test. The p value was also determined this test.

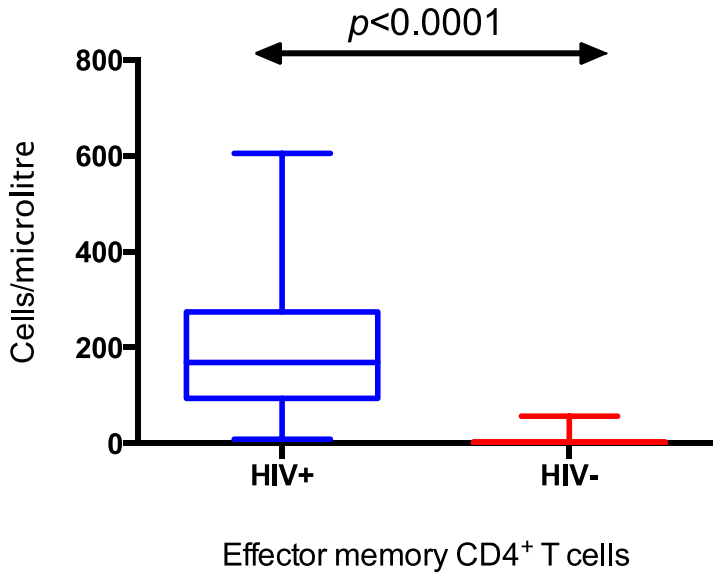
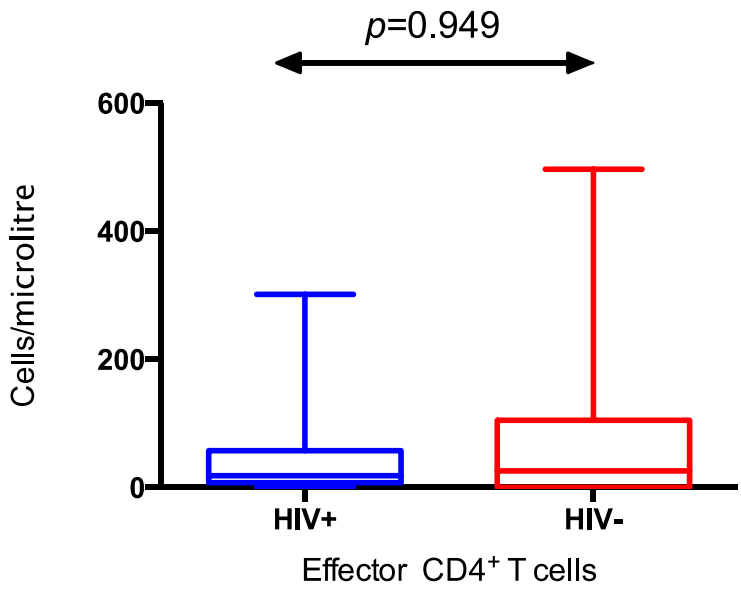


Figure 6-54 shows the distribution of effector memory $CD4^+$ T cells between the HIV+ and HIV- participants. There were more effector memory $CD4^+$ T cells (169 IQR, 94-274) in the HIV+ than the HIV- participants (0 IQR, 0-3) after 3 months of taking ART. These comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined by this test.



In **Figure 6-55** show the number of effector $CD4^+$ T cells between the HIV+ and the HIV- participants. The numbers of effector $CD4^+$ T cells were comparable in the HIV+ (18 IQR, 7-57) and the HIV- participants (26 IQR, 0-103) after 3 months of taking ART. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

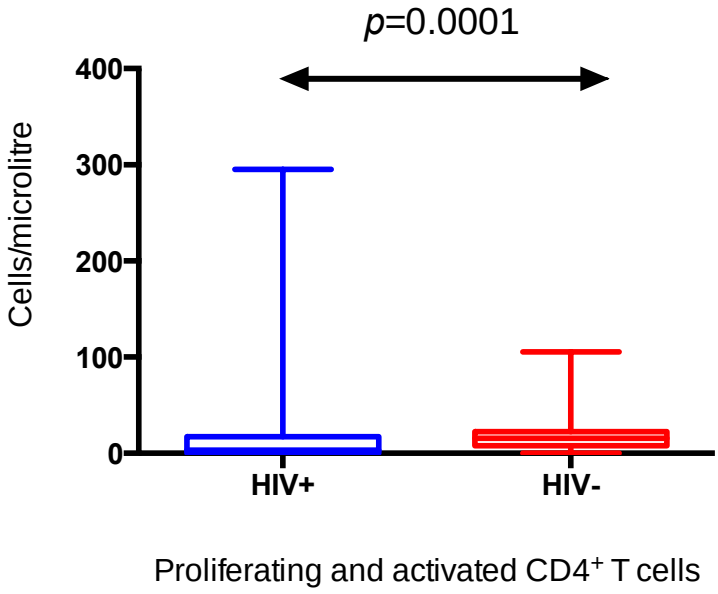


Figure 6-56 shows the number of proliferating and activated $CD4^+$ T cells between the HIV+ and HIV-. There were few proliferating activated $CD4^+$ T cells in the HIV+ (3 IQR, 0-17) after 3 months of ART compared to HIV- (16 IQR, 8-22) in the healthy population. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test the p value was determined.

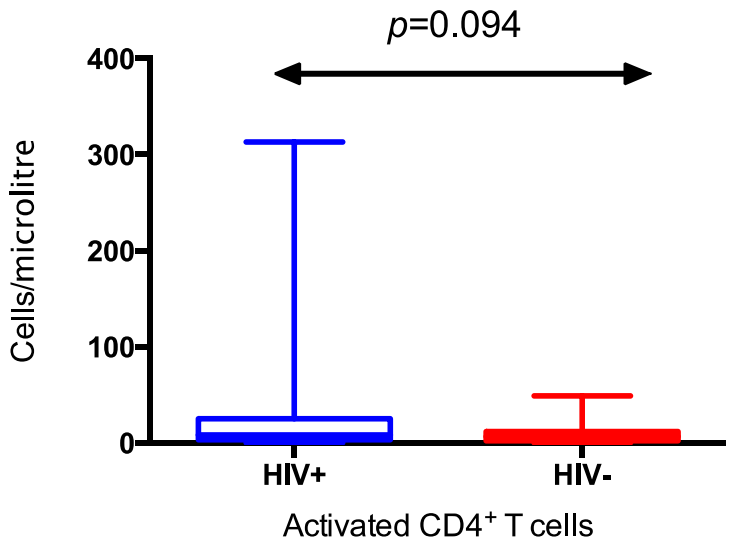


Figure 6-57 shows the numbers of activated $CD4^+$ T between the HIV+ and the HIV- participants. The number of activated $CD4^+$ T cells in both the HIV+ (8 IQR, 3-25) and the HIV- (6 IQR, 2-12) were comparable at 3 months post ART. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test with the p value determined by this test.

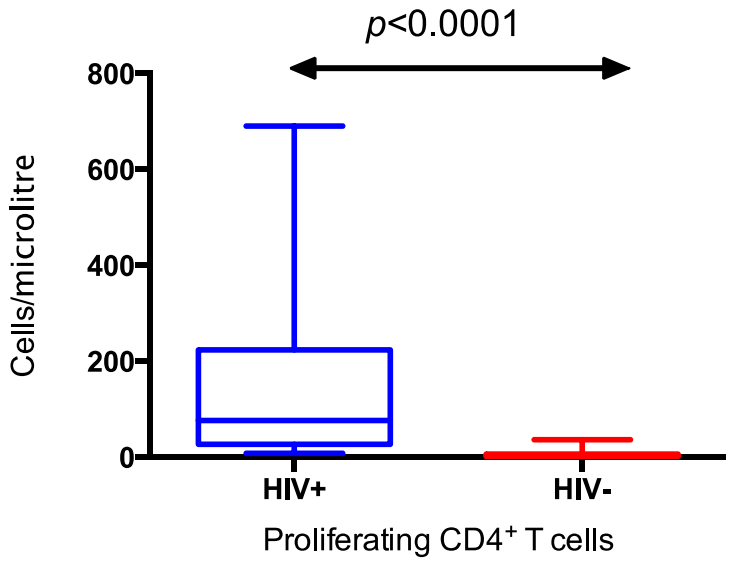


Figure 6-58 shows the numbers of proliferating $CD4^+$ T cells between the HIV+ and the HIV- participants. The HIV+ had more proliferating $CD4^+$ T cells (77 IQR, 27-218) than the HIV- participants (4 IQR, 2-6) after 3 months of ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was also determined using this test.

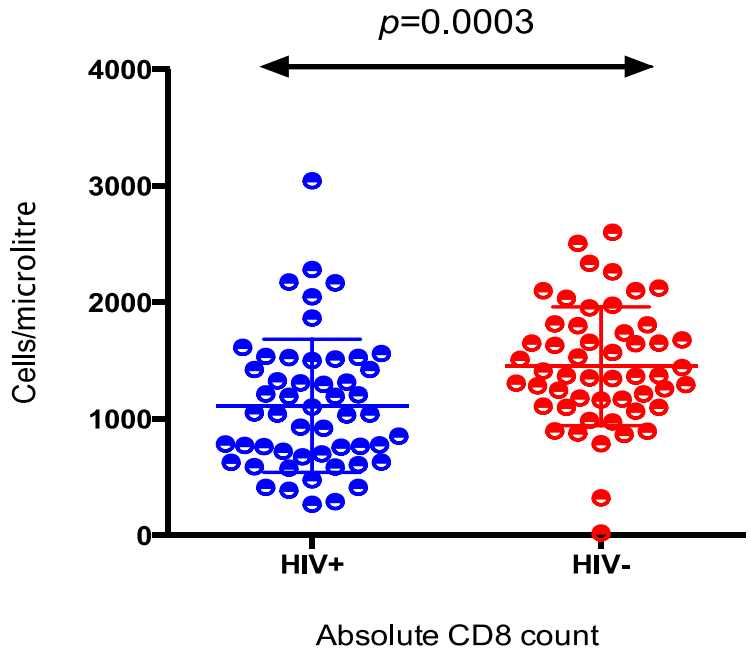


Figure 6-59 shows the number of absolute $CD8^+$ T cells between the HIV+ and the HIV- participants. Absolute $CD8^+$ T cell counts in the HIV+ (1042 IQR, 688-1464) were lower after 3 months of ART than in the HIV- participants (1371 IQR, 1136-1771). Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined using this test.

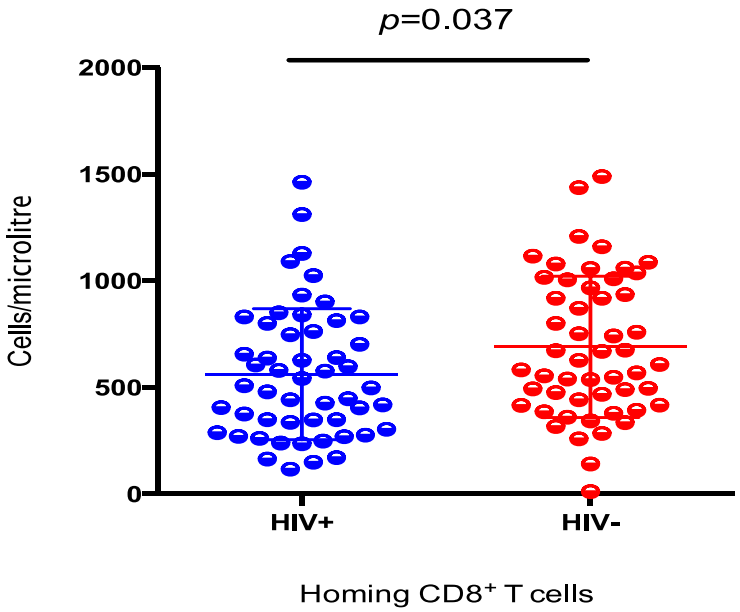
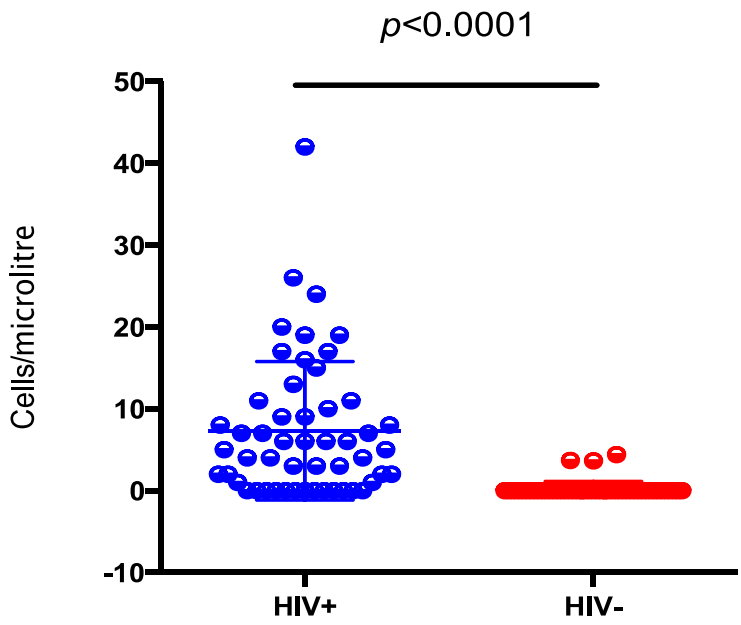
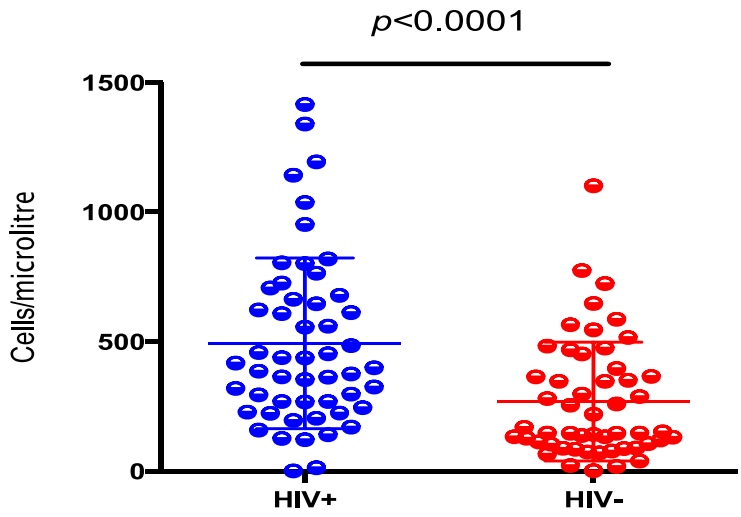


Figure 6-60 shows the number of homing $CD8^+$ T cells between the HIV+ and the HIV- participants. Gut homing $CD8^+$ T cells were few in the HIV+ (median 503 IQR, 319-781) after 3 months of ART compared to the HIV- participants (median 617 IQR, 429-985). Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined.



Activated gut homing and CD8⁺ T cells

Figure 6-61 shows the numbers of activated gut homing $CD8^+$ T cells between the HIV+ and the HIV- participants. There were more homing and activated $CD8^+$ T cells in the HIV+ (5 IQR, 1-11) than HIV- participants (0 IQR, 0-0) after 3 months of ART. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined by this test.



Recent thymic emigrant CD8⁺ T cells

Figure 6-62 shows the distribution of recent thymic emigrant $CD8^+$ T cells between the HIV+ and the HIV- participants. Recent thymic emigrants were higher in the HIV+ (median 408 IQR, 251-671) than the HIV- participants (149 IQR, 105-381) after 3 months of ART. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test the p value was determined.

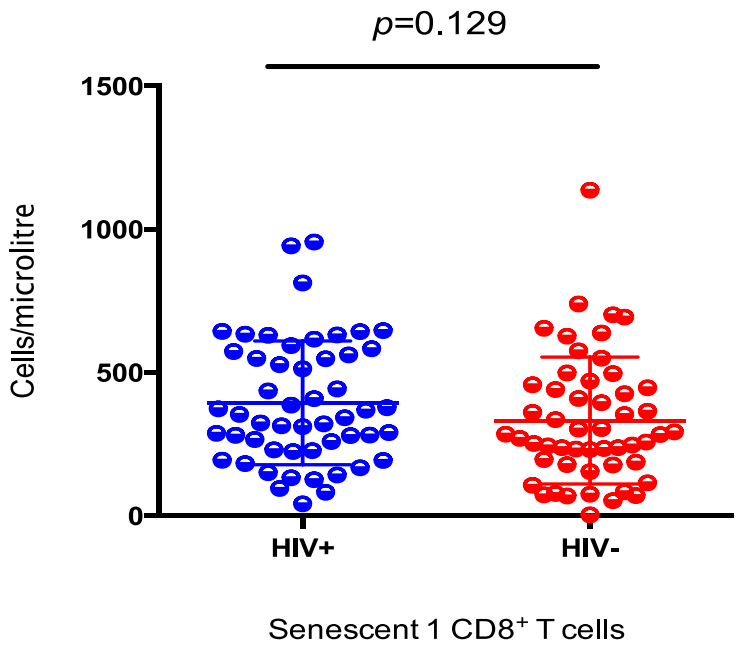


Figure 6-63 shows the numbers of senescent 1 $CD8^+$ T cells between the HIV+ and the HIV- participants. In both the HIV+ (median 348 IQR, 229-567) and the HIV- (median 283 IQR, 182-452) participants, the numbers of senescent 1 $CD8^+$ T cells were comparable after 3 months of ART. Comparisons were made using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value was determined using this test.

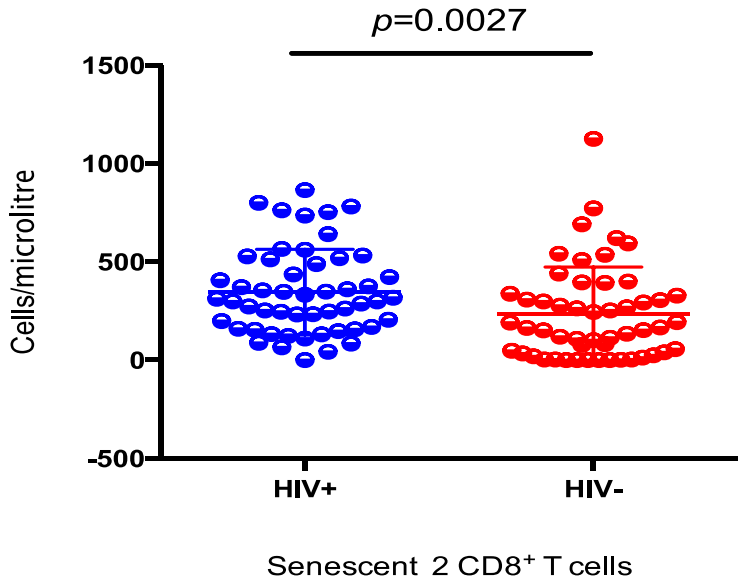


Figure 6-64 shows the numbers of senescent 2 $CD8^+$ T cells between the HIV+ and the HIV- participants. There were more senescent 2 $CD8^+$ T cells in the HIV+ (median 305 IQR, 164-500) than the HIV- participants (median 164 IQR, 36-332) at 3 months post ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test the p value was determined.

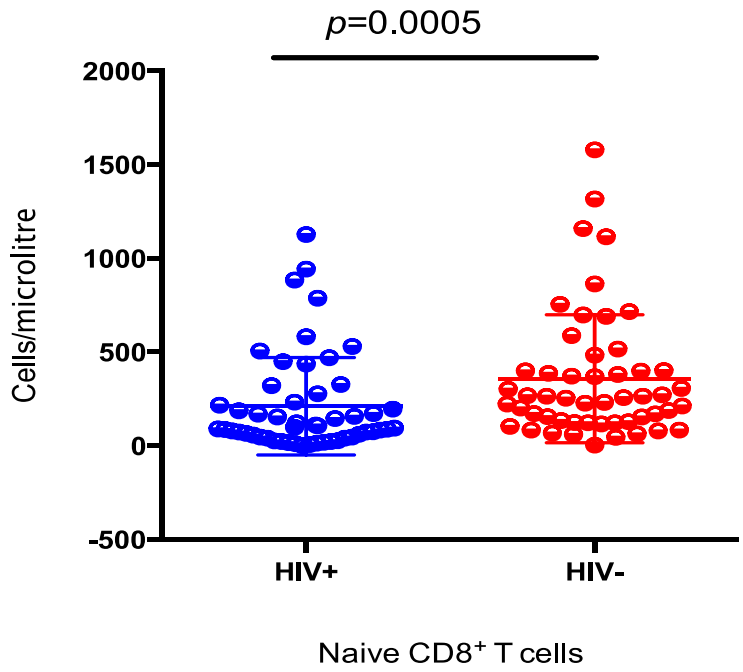


Figure 6-65 shows the differences in the numbers of naive $CD8^+$ T cells between the HIV+ and the HIV- participants. There were fewer naive $CD8^+$ T cells in the HIV+ (median 97 IQR, 47-255) than the HIV- participants (median 255 IQR, 127-401) after 3 months of ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test the p value was determined.

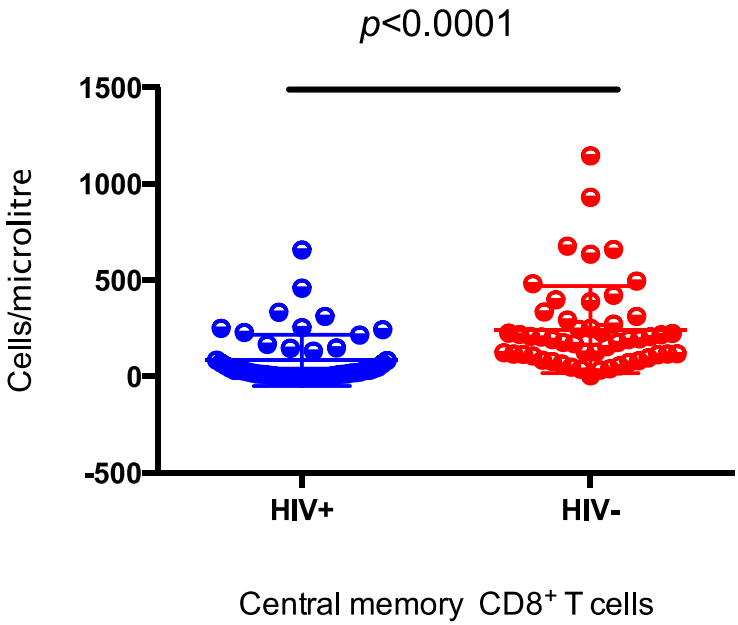


Figure 6-66 shows the numbers of central memory $CD8^+$ T cells between the HIV+ and the HIV- participants. There were few central memory $CD8^+$ T cells in the HIV+ (median 25 IQR, 4-108) compared to the HIV- participants (median 191 IQR, 109-282) by 3 months of ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test the p value was determined.

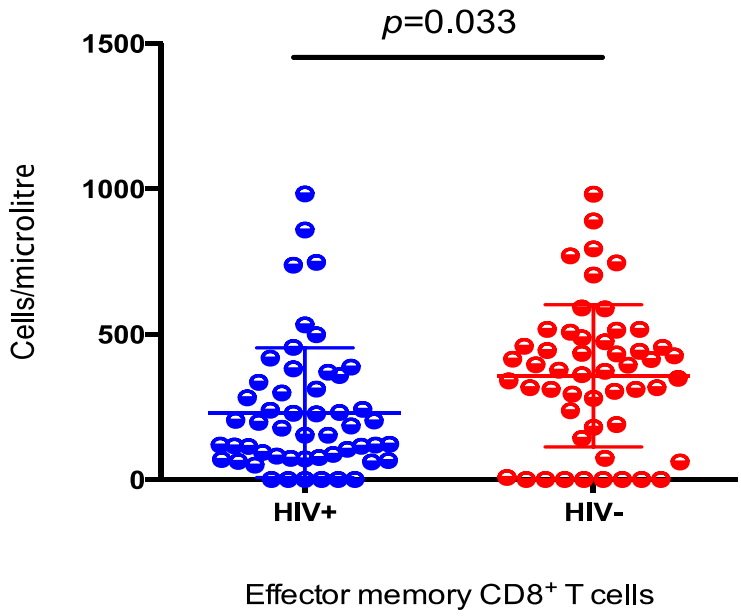


Figure 6-67 shows the effector memory $CD8^+$ T cells between the HIV+ and the HIV- participants. There were few effector memory $CD8^+$ T cells in the HIV+ (median 165 IQR, 75-323) compared to the HIV- participants (median 374 IQR, 185-481) after 3 months of receiving ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined using this test.

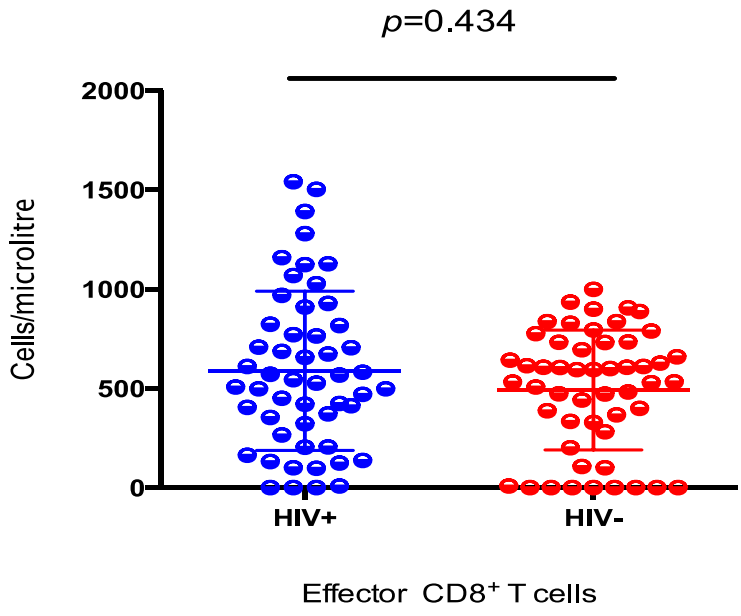


Figure 6-68 shows the number of effector $CD8^+$ T cells for the HIV+ and HIV- participants. In both HIV+ (median 535 IQR, 294-820) and the HIV- participants (median 564 IQR, 305-732), the numbers of effector $CD8^+$ T cells were comparable. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined using this test.

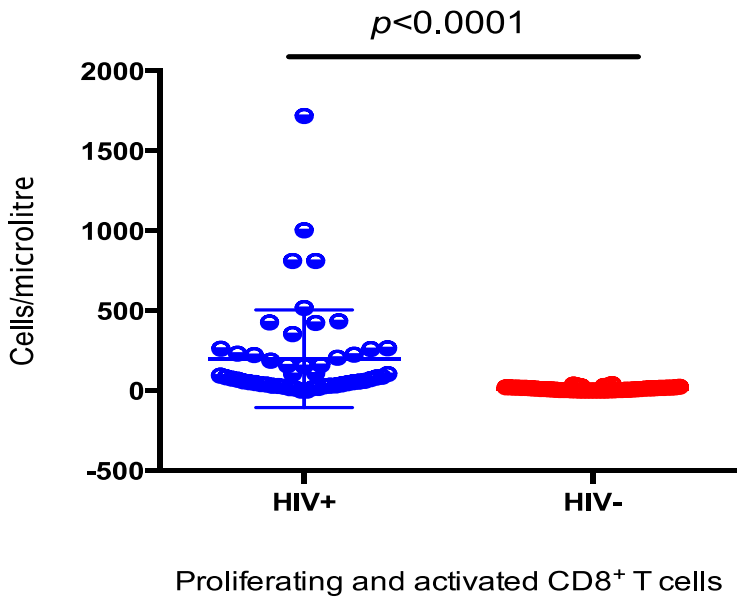


Figure 6-69 shows the number of proliferating and activated $CD8^+$ T cells between the HIV+ and HIV- participants. By 3 months of ART, HIV+ had significantly more proliferating and activated $CD8^+$ T cells (median 83 IQR, 39-228), than the HIV- (median 10 IQR, 4-19) participants. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value determined using this test.

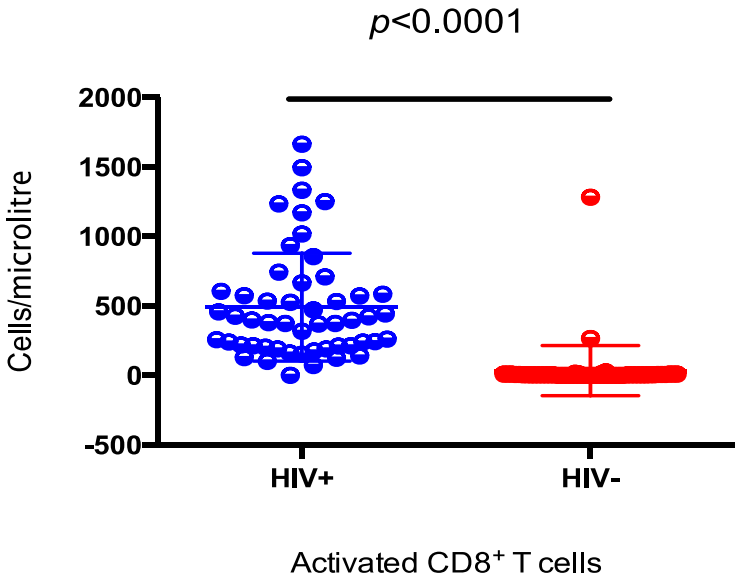


Figure 6-70 shows the diffrences in the activated $CD8^+$ T cells between the HIV+ and HIV- participants. Activated $CD8^+$ T cells were significantly higher in the HIV+ (median 387 IQR, 211-593) than the HIV- participants (median 2 IQR, 0-6) at 3 months post ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value ($p<0.0001$) determined using this test.

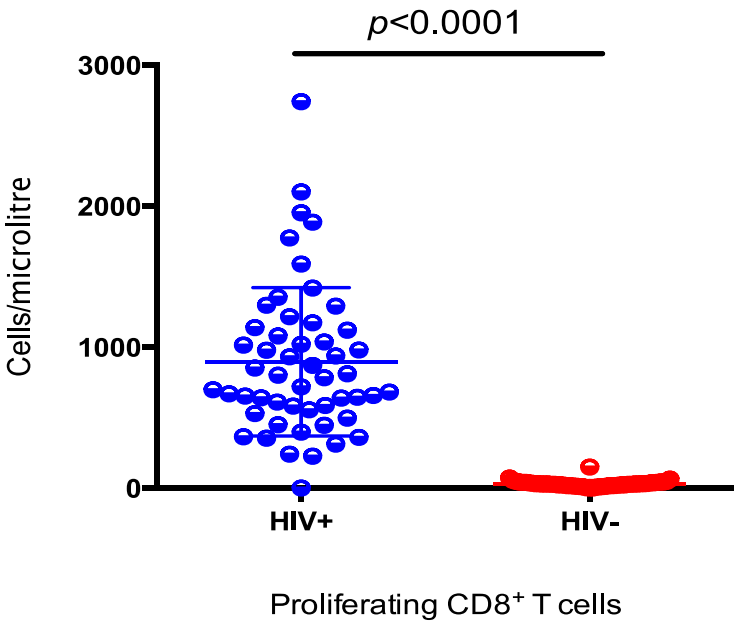


Figure 6-71 shows the differences between the HIV+ and the HIV- participants in the proliferating $CD8^+$ T cells. Proliferating $CD8^+$ T cells were significantly increased in the HIV+ (median 792 IQR, 568-1130) than the HIV- participants (median 28 IQR, 17-38) at 3 months post ART. Comparisons were done using the two-sample Wilcoxon rank-sum (Mann-Whitney) test and the p value ($p<0.0001$) determined using this test.

Chapter 7: Discussion

7.1 What is the Distribution of CD4 and CD8 T Cell Subsets in HIV-negative Adults?

In order to come up with a reference group, a six colour FACSVerse was used to characterise the $CD4^+$ and $CD8^+$ T cells in HIV-negative adults in a Zambian population. When data was stratified by sex (male and female), the mean (SD) $CD4^+$ cell counts was 828 (336) cells/μL, which is higher than the values reported for healthy adult Ethiopians (Tsegaye et al., 1999), Chinese (Kam et al., 1996), and Swiss (Bisset et al., 2004). The values were comparable to those in Tanzanians (Urassa et al., 2004) and Indians (Uppal et al., 2003) and markedly lower than those reported for Ugandans (Tugume et al., 1996), Kuwaitis (Kaaba et al., 2002), and Dutch (Tsegaye et al., 1999).

Furthermore, results showed that in healthy adults majority of the $CD4^+$ T cells were naïve. This finding was similar to findings from a study conducted in a similar cohort in san Francisco U.S.A (Roederer et al., 1995). Naïve T cell subsets are responsible for mounting immune responses to newly encountered antigens. Studies also show that naïve cells have a better capacity for proliferation in response to mitogenic stimuli (Sanders et al., 1998). A study conducted in healthy Ethiopians and Dutch showed lower numbers of naïve $CD4^+$ T cells (Massele et al., 1999) compared to what was found in the Zambian cohort. It is postulated that the difference could be as a result of differences in average ranges for $CD4^+$ T cells in the different cohorts. Additionally, high naïve $CD4^+$ T cells in black Africans could mean having an increased ability to build effective immune responses to newly encountered infections. Similarly, there were very few circulating effector memory and effector

$CD4^+$ T cells in the healthy participants. When naïve $CD4^+$ T cells are stimulated by their cognate antigen presented by competent antigen-presenting cells they differentiate into specialized effector and/or memory cells. Effector T cells induce cytolysis of infected cells or activate other lymphocytes and immune effector cells, whereas memory T cells maintain the capacity to respond rapidly to previously encountered antigens. In the healthy volunteers, however, there were very few circulating effector and or memory $CD4^+$ T cells in the blood.

For people living with HIV, It has been reported that naïve $CD4^+$ T cells are progressively depleted during infection because of their frequent activation and differentiation into memory cells (Bandera et al., 2010). A study conducted in the U.S (Minneapolis) by Schacker and colleagues found that patients with relatively high numbers of naïve $CD4^+$ T cells were less likely to have failure of immune reconstitution with ART and had lower risk of opportunistic infections and mortality (Schacker et al., 2010). By contrast, low pre-ART naïve $CD4^+$ T cell frequencies predicted poor ART-mediated $CD4^+$ T cell recovery in other studies (Notermans et al., 1999; Gandi et al., 2006). Additionally, Zhang and colleagues recently showed that increases in naïve $CD4^+$ T cells following ART initiation independently predicted subsequent $CD4^+$ T cell increases (Zhang et al., 2013).

Further to this, proliferating, proliferating and activated, activated and both senescent 1 and 2 $CD4^+$ T cells in the healthy volunteers were very few. Since activation is triggered by the presence of antigen and is followed by proliferation, an indicator of cell turnover or increase in cell destruction, having minimal cells activated, proliferating and proliferating and activated may indicate absence of disease in the healthy population. Additionally, having few cells activated and proliferating could

mean a good T cell activation regulation (Chattopadhyay and Roederer, 2011). On the contrary, other studies have shown that $CD4^+$ proliferation correlates with the amount of antigen present, e.g. HIV (Koopman et al., 2009). Thus, increase in proliferation means an increase in viral load and this is because proliferating ($Ki67^+$) $CD4^+$ T cells can efficiently support virus replication (Fleury et al., 2000; Koopman et al., 2009) and lead to more rapid progression of the disease (Ganesan et al., 2010).

According to literature, most of the lymphocytes are in lymphoid tissues with the lymph nodes harbouring the majority of the lymphocytes followed by the spleen (Ganusov and De Boer, 2007). Similarly, we also found a higher number of $CD4^+$ T cells expressing the gut homing marker (about 35%) and activated gut homing (23%) in the healthy adults. Gut homing cells are cells that are channelled to gut associated lymphoid tissues especially when there is an injury to the gut. Having a representative number of these cells in the peripheral blood could mean that priming of $CD4^+$ T cells is by the intestinal dendritic cells (Johansson-Lindbom et al., 2003).

There was a different trend, however, in the $CD8^+$ T cell distribution for the healthy population in that majority of the $CD8^+$ T cells were expressing gut homing markers. Nevertheless, because of their natural migratory ability, lymphocytes, although present in the peripheral blood, are capable of returning to the gut associated lymphoid tissue when there is need (Shacklett et al., 2003). Contrary to the $CD4^+$ T cell population, there were more effector and effector memory $CD8^+$ T cells in the healthy adults. These are a population of cells that provide acute defense to the host and durable immunity (Ahmed and Gray, 1996).

During infection, persistent T cell activation drives proliferation that results in the accumulation of senescent, antigen-experienced memory T cells with reduced expression of CD28 and increased expression of CD57 (Focosi et al., 2010).

Similarly, we also found a higher number of senescent $CD8^+$ T cells in the HIV-negative cohorts. Expression of CD57 has been linked to greater resistance to apoptosis in $CD8^+$ T lymphocytes especially during HIV-infection, facilitating accumulation (Petrovas et al., 2009). Since $CD8^+$ T cells are crucial to the recognition and clearance of virus-infected cells (Carmichael et al.,1993; Kaech and Amed 2001), a high number of senescent $CD8^+$ T cells may underlie the inability of T cell immunity to suppress virus adequately in an event of infection in the healthy population. In addition, for the healthy population, having senescent cells could also mean the presence of an insignificant activation, although the numbers of proliferating, proliferating and activated and activated $CD8^+$ T cells were very low.

Finally, contrary to what has been reported by Aina and colleagues (2005), we found higher $CD4^+$ count in the female Zambians similar to an Ethiopian study (Lee et al., 1996) $CD4^+$ cell counts were they reported to be significantly higher in women than in men, as did studies in Indian (Uppal et al., 2003) and in Uganda (Tugume et al., 1996). It is not clear whether these are true variations across countries in the relationship between gender and $CD4^+$ cell counts or these results are due to confounding factors such as the differences in study participants (Pregnant women in the case of a study by Aina and colleagues).

In summary, in healthy population, the majority of circulating $CD4^+$ T cells were naïve. The majority of circulating $CD8^+$ T cells expressed gut homing markers. Although there are many $CD4^+$ recent thymic emigrants in this population (about 80%), $CD8^+$ recent thymic emigrants were lower (about 10%).

$CD8^+$ senescent T cells were more common in the healthy population than senescent $CD4^+$ T cells. This might be because of repeated antigenic stimulation, which is associated with a progressive decline in the CD28. This loss is at an accelerated rate

in the $CD8^+$ T cells compared to the $CD4^+$ T cells (Valenzuela and Effros, 2002). Finally, there were significant differences in absolute CD4 count between females and males and some of the subsets. The difference could be as a result of many factors, among them; demographic, environmental, immunological and genetic.

The major strength of this study is the setting of provisional reference parameters for the CD4 and CD8 T cell subsets for the HIV negative population. The limitations however are that, the parameters used to describe the healthy volunteers were not exhaustive and as such, there could be other ailments e.g. CMV which could have affected the CD4 and CD8 results. Furthermore, the sample size was small and samples were drawn from the same area.

After describing the distribution of $CD4^+$ and $CD8^+$ T cell subsets in the HIV-negative population (reference group), the second step was to determine the prognostic value of T cell subsets in the HIV-infected adults initiating ART.

The third step was to explore the effect of lipid based nutritional supplements with extract vitamins and minerals on the reconstitution pattern of T cell subsets in the HIV-infected population after receiving ART plus nutritional supplements.

7.2 What are the Immunological Risk Factors Associated with Death Within 12 Weeks of Initiating ART and Do CD4 or CD8 T Cell Subsets Respond to Extra Vitamins and Minerals *in vivo*?

Although there is vast literature showing an increase in CD4 count after ART and nutritional support **(section 3.12),** the magnitude of increase observed in the NUSTART trial participants was higher than what has been reported previously. Therefore, it is envisaged that analysis of the effects of nutritional supplementation on T cell subsets would provide much detail in the causes of sharp rises in CD4 counts.

Consistent with previous reports from sub-Saharan Africa (Stringer et al., 2006; Verguest et al., 2013), we found high mortality rates (20%) within 12 weeks of initiating ART among a cohort of malnourished Zambian adults with advanced stages of HIV-infection (Filteau et al., 2015). We explored T cell subsets as predictors of mortality and found that they predicted death and displaced total $CD4^+$ count in multivariate models. The nutritional intervention we trialled did not affect these subsets, so we have not yet found a tool for manipulating T cell subsets or ameliorating the impact of under nutrition on mortality during ART initiation (Filteau et al., 2015). In our analysis, a high number of naïve $CD4^+$ T cells and a high number of senescent $CD8^+$ T cells at ART initiation were associated with higher mortality rates, whilst a high number of proliferating $CD4^+$ or $CD8^+$ T cells were associated with survival meaning the body's natural way of responding to antigens was not lost.

We found a consistent effect of proliferating $CD4^+$ and $CD8^+$ T cells on mortality, such that higher levels of proliferation were associated with protection against death.

Other studies have shown that $CD4^+$ proliferation positively correlates with viral load; it has been suggested that this is because proliferating ($Ki67^+$) $CD4^+$ T cells can efficiently support virus replication (Fluery et al., 2000; Koopman et al., 2009) and lead to more rapid progression to AIDS (Ganesan et al., 2010). In our cohort of malnourished adults, however, the effect was opposite. Importantly, our cohort was moderately or severely malnourished, and this may be a critical point of difference. We speculate that T cell proliferative ability reflects a greater ability to respond to co-infections (e.g. Tuberculosis), which are the most frequent cause of mortality after ART initiation (Walker et al., 2012). By contrast, a low proportion of proliferating cells in those who subsequently died may signify immune exhaustion, which is known to affect proliferative capacity and may be a pre-terminal event in a malnourished individual. Although we did not evaluate cellular exhaustion markers, other studies have shown that elevated T cell expression of PD-1 (Zhang et al., 2007), CTLA-4 (Kaufmann et al., 2007) and TIM-3 (Jones et al., 2008) in advanced HIV disease, which correlate inversely with cellular proliferative capacity.

Our results showed that higher numbers of senescent $CD8^+$ T cells predict mortality. During HIV-infection persistent T cell activation drives proliferation that results in the accumulation of senescent, antigen-experienced memory T cells with reduced expression of CD28 and increased expression of CD57 (Focosi et al., 2010). Expression of CD57 has been linked to greater resistance to apoptosis in $CD8^+$ T lymphocytes during HIV-infection, facilitating accumulation (Petrovas et al., 2009). Since $CD8^+$ T cells are crucial to the recognition and clearance of virus-infected cells (Carmichael et al.,1993; Kaech et al., 2001), a high number of senescent $CD8^+$ T cells may underlie the inability of T cell immunity to suppress virus adequately.

It has been reported that naïve $CD4^+$ T cells are progressively depleted during HIV-

infection because of their frequent activation and differentiation into memory cells (Bandera et al., 2010). A study by Schacker and colleagues found that patients with relatively high numbers of naïve $CD4^+$ T cells were less likely to have failure of immune reconstitution with ART and had lower risk of opportunistic infections and mortality (Schacker et al., 2010). By contrast, low pre-ART naïve $CD4^+$ T cell frequencies predicted poor ART-mediated $CD4^+$ T cell recovery in other studies (Notermans et al., 1999 Gandi et al., 2006). Additionally, Zhang and colleagues recently showed that increases in naive $CD4^+$ T cells following ART initiation independently predicted subsequent $CD4^+$ T cell increases (Zhang et al., 2013). However, our data show that having more naive cells at baseline increased the likelihood of death during ART in this Zambian cohort with advanced disease. We speculate that predominance of naïve cells at baseline may reflect a permanent loss of important memory cell clones that cannot be reconstituted despite effective ART. Again, this might be critically dependent on nutritional status (if severe malnutrition imposes a replication constraint on proliferating cell lineages) and/or in a setting where opportunistic infections are highly prevalent. Further studies in different patient groups may clarify these differences.

Although there was a small but significantly greater rise in total $CD4^+$ count in the LNS-VM vs. LNS group in the main NUSTART trial (difference +25 cells/μL; P=0.02) (Filteau et al., 2015), we found no major differences among T cell subsets in this sub-study. The only exception was the population of recent thymic emigrants, which increased more in the LNS group than in the LNS-VM group. It is speculated that although there may be a constraint on proliferation imposed by severe

malnutrition, the LNS alone might be sufficient to permit restoration of thymic output.

This study had several strengths and some limitations. The major strength of this study is the detailed immunophenotyping that was conducted in malnourished HIV-infected adults with advanced disease, which allowed us to address questions related to early mortality, together with the randomized trial design, which allowed us to evaluate the impact of a nutritional intervention on lymphocyte subsets. Study limitations included lack of viral loads, which are not generally available in sub-Saharan Africa for monitoring ART response, and the small sample size at 12 weeks, due to loss of follow-up. Because the participants in this study had advanced disease (median CD4 at ART initiation 98 cells/μL) and malnutrition, our findings may not be generalizable to the larger population of HIV-infected adults initiating ART in sub-Saharan Africa, although it is worth noting that the mean CD4 count at ART initiation in sub-Saharan African programme in 2002 was 152 cells/μL and has not increased significantly since then, meaning that many patients still start ART in the setting of advanced immunodeficiency (Siedner et al., 2015).

In this report of a sub-study of a large clinical trial in adults initiating ART, there were no substantial differences in the effects of a nutritional intervention on specific T cell subsets, but it was found that baseline numbers of proliferating $CD4^+$ and $CD8^+$ cells, naive $CD4^+$ cells, and senescent $CD8^+$ cells at ART initiation impacted early mortality on ART. These findings provide insights into the immune dysfunction of AIDS in the context of malnutrition and suggest that baseline immune abnormalities in this population render individuals susceptible to co-infections, which are associated with very high mortality despite ART initiation. Whether patients would benefit from adjunctive infection control interventions at initiation of

anti-retroviral therapy (such as additional antiretrovirals and a package of anti-microbial drugs) in addition to a nutritional intervention, is currently being explored in Sub-Saharan Africa (REALITY trial; ISRCTN43622374). These findings also argue strongly for earlier initiation of antiretroviral therapy in sub-Saharan Africa (Siedner et al., 2015), because the immune perturbations that characterize advanced immunodeficiency are difficult to reverse.

Although the effects of the nutrients on T cell subsets was not as expected, there is vast amount of literature that shows the key role that certain micronutrients play in boosting immune cells. Therefore an *in vitro* study of the effects of specific micronutrients on T cells subsets was explored. In particular, an *in vitro* study of Se one of the micronutrient present in the bulk of the micronutrients NUSTART trial participants were receiving would be important. Se is preferred because of its implication as an immune booster.

7.3 What is the Effect of Selenium on T Cell Subsets *In Vitro?*

Similar to what has been reported elsewhere (Dickson and Tomlinson, 1967), plasma Se for most of the healthy Zambian adult population was within the reported reference range (1.37; 0.86, 1.53 μmol/L), but about one quarter of healthy adults were deficient in Se. It is supposed that although the African culture of eating unprocessed foods is being eroded, there is still a considerable amount of Se present in the foods available on the market. However, we speculate that since poverty levels might be escalating in Sub-Saharan Africa, together with climatic changes and the burden of epidemic and endemic disease (e.g. HIV), the Se status of our population may change in the future. This study found that Se concentration in the culture medium increased marker expression on T cell subsets. Importantly, this effect was most pronounced in individuals with baseline Se levels consistent with deficiency. This suggests that deficiency has a real and important effect on T cell phenotype as reported elsewhere (Look et al., 1997).

A study by Broome and colleagues demonstrated that T cell proliferation increased with an increase in Se. Contrary to these findings, this study found that as the concentration of Se was increased, both $CD4^+$ and $CD8^+$ T cell proliferation was reduced. Thus, it was postulated that the increase in the concentration could have been toxic to the cells. On the contrary however, one of the significant and unexpected finding was the consistent increase in the gut homing, homing and activated $CD4^+$ T cells and gut homing and homing and activated $CD8^+$ T cells. These results seem to suggest that an increase in ambient Se concentration positively affected gut homing $CD4^+$ and $CD8^+$ T cells and negatively affected the proliferating cells. These results could mean that T cell subsets respond differently at different Se concentrations and finding an ideal concentration for each subset require further

research. A study conducted in HIV-infected and HIV-negative adults with pulmonary tuberculosis reported that wasting increased as Se levels decreased (van Lettow et al., 2004). This is because Se plays an important role in the selenoenzyme glutathione peroxidase that protects cells against free radical damage and oxidative stress (van Lettow et al., 2004).

In another study, Se increased $CD4^+$ T cell activation, proliferation and differentiation (Hoffmann et al., 2010). In our study $CD4^+$ T cell activation and proliferation was decreased and similar to what has been reported by Hoffmann and colleagues, $CD4^+$ T cell differentiation was increased.

A study by wood and colleagues demonstrated that supplementation with 400 µg Se per day (as Se yeast), increased the total T cell count significantly by 27% due largely to the increase in subsets of $CD4^+$ T cells (Woods et al., 2000). This study however does not indicate which subsets in particular were being altered. Several studies have reported a positive correlation between low serum or plasma Se and absolute CD4 count (Look et al., 1997; Drain et al., 2006; Hurwitz et al., 2007; Kamwesiga et al., 2015). Nonetheless, our study found that both the absolute CD4 and CD8 cells were negatively correlated with plasma Se in our population at baseline. It is speculated that this finding is odd and therefore difficult to explain.

There is evidence to the effect that inadequate Se intake can result in increased levels of reactive oxygen species (ROS) or oxidative stress, especially in individuals deficient in antioxidants like vitamins E and C (Steinbrenner et al., 2009). It has also been established that ROS related oxidative stress induces cell cycle arrest, senescence, apoptosis and/or necrotic cell death (Day et al., 2006; Stone et al., 2006). Nevertheless, in this study, there was a consistent increase in both the CD4 and CD8

senescent 1 marker expression as the concentration of Se increased. This finding may mean that at higher concentrations, Se becomes toxic to certain cells. However, when we baseline number of senescent 1 CD4 cells were compared to baseline plasma Se, a weak correlation (rho=0.29) was found though the *p* value was significant (*p*=0.04). On the contrary, both the CD4 and CD8s senescent 2 cells disappeared as Se concentration was increased. It was postulated that increases in Se concentration enhanced elimination of senescent 2 cells from the cell cycle and ultimately death.

One of the major strength of this study was the detailed analysis of about eighteen makers expressed on T lymphocytes and their response to Se. However, these results cannot be generalized to the HIV-infected population since the study was undertaken in an HIV sero negative population.

Finally, although there was a trend in naïve CD8 to increase with the increase in Se, this was not very pronounced in the CD4 population. Huang and colleagues report that higher levels of dietary Se enhanced activation signals in naïve T cells during TCR-stimulation because higher Se increases free thiols (Huang et al., 2012).

The limitations for this study are that the effects on gut homing cells were shown in peripheral blood only and it is not known what happened in the gut mucosal compartment. Additionally, we did not study mRNA of Se dependent enzymes or proteins or their transcription factors so we do not know how the *in vitro* effects were mediated.

In conclusion, this data suggest that Se has rapid effects on T cell subset marker expression. These effects appear to be direct, although the possibility that other cell lineages (neutrophils, monocytes, eosinophils) might contribute indirect effects through cytokines or chemokines cannot be excluded.

7.4 Are CD4 and CD8 T cell subsets in HIV+ adults comparable to HIV- adults after 3 months of ART plus nutritional support?

In both the CD4 and CD8 population, results showed that after 3 months of taking ART plus nutritional support (NUSTART trial participants), most of the markers were still below what existed in the healthy population.

A review of literature that address changes in CD4 count after ART and nutritional support report minimal increases in CD4 count for HIV-positive women from pre ART initiation at 6, 12, and 24 months post initiation as 71, 89 and 153 cells/μL (Kiefer et al., 2011). Another study conducted in HIV-infected Ethiopian adults showed that there was a small increase observed following ART plus nutritional support (25 cells/μL), a borderline significance (Olsen et al., 2014). In a study of 40 HIV-infected patients in the United States, the absolute CD4 count increased by an average of 24% versus 0% change in the placebo group. This study concluded that micronutrient supplementation significantly improves CD4 cell count reconstitution in HIV-infected patients taking HAART (Kaiser et al., 2006). Furthermore, evidence from a Kenyan study showed that micronutrient supplementation when starting ART resulted in higher $CD4^+$ (+23 cells/ml, P=0.03) and $CD8^+$ (+74 cells/ml, p=0.005) counts (McClelland et al., 2004). Hurwitz and colleagues (Miami USA), also reported an increase in CD4 count of 27.9 ± 150.2 cells/μL following micronutrient supplementation with selenium (Hurwitz et al., 2007).

In a recent study by Filteau and team conducted in Zambia, it was been demonstrated that following ART with a bulk of micronutrients with vitamins and minerals, there was a significant change in mean CD4 count at 12 weeks post-ART was 25 cells/μL (95% CI, 4–46) (Filteau et al., 2015). Although all these studies showed an

improvement in total CD4, our study revealed that $CD4^+$ and $CD8^+$ T cell subsets do not reconstitute to levels of healthy controls. We assumed that this could be because we enrolled participants who were very malnourished with CD4 counts of less than a 100 cells/μL for some of them.

The number of naïve $CD4^+$ T cells in the HIV-infected cohorts after treatment was low. It is assumed that the loss of naïve T cells has important consequences for the development of immune responses particularly in HIV-infected individuals. As the naïve subsets disappear, there will be a progressive inability to mount responses to novel antigens, which might result in high susceptibility to opportunistic infections (Janeway et al., 2001). Additionally, this loss compromises the ability to deal with the constantly mutating virus itself (Janeway et al., 2001). The impairment of immune function due to the loss of naïve T cells from HIV-infected adults and children also has important consequences for therapeutic strategies for AIDS (Roederer et al. 1995). Since responses to novel antigens arise (usually) from the naïve compartment, the magnitude or effectiveness of such responses is necessarily dependent on the availability of naive T cells. Therefore, an HIV-infected individual with no naïve T cells is likely to fail to respond to any primary immunization involving T cells.

The major limitation for this study is that these patients were not screened for all opportunistic infections, with that could have confounded the response to treatment. Furthermore, since these patients were very sick, malnourished and did not undergo endoscopy, chances are that they could have had HIV enteropathy that could have affected the absorption of supplements.

In summary, the survey revealed that functional recovery might be slow in malnourished HIV-infected individuals.

Chapter 8: Conclusions and Future Work

8.1 Overall Findings

This study was designed to explore CD4 and CD8 changes during HIV-infection, how they predict mortality and their response to micronutrients both *in vivo* and *in vitro.* Some prominent findings during this work are that naïve and gut homing CD4 T cell subsets are profoundly depleted in malnourished Zambian adults initiating ART, T cell subsets do not reconstitute well during the first 3 months of ART even when additional vitamins and minerals are given, and finally that Se enhances gut homing marker expression on T cells *in vitro.*

8.2 Significance of the Research

Throughout the thesis, it has been argued that T cell subsets are more informative compared to absolute values, both in terms of understanding their prognostic value and searching for evidence of their response to nutritional interventions.

The predictive value of the proliferation marker Ki67 underlines the importance of T cell dynamics for outcome. The predictive value of T cell subsets for mortality could suggest new possibilities for intervention in order to reduce the risk of early mortality. The data from the clinical trial do not confirm that provision of additional bulk minerals and micronutrients have a significant impact on T cell subsets; and the results of the published clinical trial shows that there was no effect on mortality. However, both study arms were given a lipid-based nutritional supplement and it is possible that patients in both trial arms benefited. In Zambia it is policy to give minimal micronutrients to HIV-infected individuals, but it may be that this is insufficient. Unfortunately, it is unlikely that a placebo-controlled trial will ever be

carried out because of ethical concerns about giving no nutrients to seriously malnourished people. It would be sensible for the government to strategise on how to ensure that this population has an adequate supply of food during the first three months of commencing treatment. Additionally, it has been shown in this study that T cell subsets respond well to selenium in vitro. Further work needs to be done to see if this can be translated into a benefit in patients, and perhaps there is an optimal micronutrient combination, which would be useful for patients initiating ART.

The *in vitro* effect of selenium raises another important question. If selenium can increase expression of gut homing receptors on T cells in vitro, why does a micronutrient supplement not achieve this in patients? There are several possible explanations, including poor absorption of selenium, competition for absorption between micronutrients (iron and zinc are absorbed by a similar range of ion transporters at supra-physiological levels), or some other aspect of T cell regulation, which is not yet understood.

In conclusion, I have found that Se can alter gut homing receptor profile on T cells in vitro, but we have no evidence of a direct effect in patients initiating ART. In univariate, but not multivariate analysis, gut homing predicted death over the first 3 months of ART treatment. Further work is needed to dissect out the complex relationships between nutrients and immune responses, in the hope of improving outcome.

8.3 Contribution of knowledge to Science

This research has demonstrated the prognostic values of CD4 and CD8 T cell proliferation markers and also shown for the first time that some T cell subsets reconstitute slowly during ART initiation in malnourished AIDS patients. Furthermore, it has been demonstrated that selenium can achieve effects *in vitro,* which are not seen *in vivo.*

8.4 Application of Results in Public Health

These results may help design targeted nutritional interventions that could enhance T cell proliferation in malnourished HIV-infected adults.

8.5 Future Work

There is urgent need to further look into why giving multiple nutrients did not improve T cell subset response *in vivo,* and how to overcome that barrier to reconstitution. There is also need to understand the mechanism of selenium effect, and whether other nutrients interfere with it.

micronutrient combinations is key since zinc and iron are known to compete for the same ion transporters.

APPENDIX A Publications from the PhD study

APPENDIX B: **Awards won during the PhD work**

Was awarded the first prize for best oral and poster presenter during the UNZA seminar week presentations by the director of research and postgraduate studies' office (DGRS).

THE UNIVERSITY OF ZAMBIA

DIRECTORATE OF RESEARCH AND GRADUATE STUDIES

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E-mail drgs@unza.zm

P O Box 32379
Lusaka, Zambia

15th July 2015

Ms. Caroline. Cleopatra. Chisenga (512809003)
C/O School of Medicine
Department of Internal Medicine
P.O. Box 32379
LUSAKA

Dear Ms. Chisenga,

LETTER OF COMMENDATION

On behalf of the Directorate of Research and Graduate Studies, am pleased to congratulate you for being awarded a prize as First Best Oral Presenter (Biomedical and Agriculture Sciences Category) at the Postgraduate Seminar Week held from Monday 7th July to Friday 11th July, 2014, at the University of Zambia.

You should keep up the good work.

Dr. J. Simwinga
ACTING DIRECTOR

Cc Dean, School of Medicine
Assistant Dean (PG), School of Medicine
Head, Department of Internal Medicine
Assistant Registrar- Graduate Studies

APPENDIX **C NUTRITIONAL SUPPORT FOR AFRICAN ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects on T cell subsets**

Information sheet (to be kept by participant)

We have invited you to participate in a study that is designed to establish the pattern of distribution for CD4 T cell subsets in healthy volunteers.
Recent results of HIV-infected adults receiving antiretroviral therapy (ART) plus nutritional supplementation have shown an improvement in total CD4 count and T cell subsets by 12 weeks. However, we don't have information on the pattern of distribution for CD4 T cell subsets in healthy individuals.

Therefore, in this study we want to find out what the pattern of distribution of CD4 T cell subsets is like in a healthy population. Results of this study will be used to determine whether ART plus nutritional supplementation improves CD4 T cell subsets to clinically significant levels.

What are we asking you to do?
If you are willing to participate in this study, we will collect 10mls of blood (one tablespoon). During blood collection, you may experience a little discomfort (equivalent to a mosquito bite) at the time of pricking into the vein on your arm. However, this pain will quickly disappear or disappear soon after. The whole process will require a maximum of 30minutes of your time. An HIV test will be done and should you wish to know your results, you will have to undergo counseling with our nurse counselor. This will take an extra 45 minutes for both pre and post test.

What do we do with the samples we collect?
The blood samples that we will collect from you will be used for testing HIV, full blood count, CD4 count and for the analysis of CD4 T cell subset. No blood samples will be stored for any future work, as this analysis requires fresh blood.

What are the possible benefits to me?
Should we find anything that may require medical attention, this will be facilitated.

What are the possible disadvantages to me?
The only foreseeable disadvantage is the inconvenience of spending 30 minutes at our lab for blood collection. You may also experience a little discomfort at the site of a needle prick on your arm.

Confidentiality
Your details will be recorded on a paper form that will be locked away in our office within UTH. Furthermore, your details will be entered on a computer in a coded form and your name will not be included, only your enrolment number will be used. Any

information and results obtained will remain absolutely confidential, and other family members and work colleagues will not be granted access to this information.

The study is voluntary

You do not have to participate in this study if you do not wish to, and even if you agree, you are free to change your mind at a later stage. The Research Ethics Committee of the University of Zambia has approved this study and their contact details are given below.

Contact details of the Principal investigator: Mrs. Caroline C. Chisenga, Tropical Gastroenterology & Nutrition Group (TROPGAN), Department of Internal Medicine, University of Zambia School of Medicine, UTH, Nationalist Rd P.O. BOX 50398, Lusaka, Zambia (Phone 0953591762)

Contact details of Research Ethics Committee: The Chairperson, REC office, Department of Anatomy, Ridgeway Campus, Nationalist Rd, Lusaka (Phone 0211 256067)

APPENDIX D

NUTRITIONAL SUPPORT FOR AFRICAN ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects on T cell subsets

Consent record sheet (to be kept by study team)

I confirm that I have understood the information I have been given about the study. I agree to participate in the study. I confirm that I am joining the study of my free will and that I can withdraw at any time without affecting the care available to me. I understand what will be required of me.

Name:

Signed (or thumbprint)

Date:

Signature (or thumbprint) of witness

Name:

Date:

I confirm that I have explained the information fully and answered any questions

Signed for the study team

Name:

Date:

APPENDIX E

NUTRITIONAL SUPPORT FOR AFRICAN ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)-Effects on T cell subsets

Questionnaire Study ID NO._____

1. Date:

2. Age
(years)_____

3. Sex
Male
Female

4. Religion
None
Christian
Muslim
Hindu

Other_____

5. Occupation
Employed
Student

Other_____

6. Highest education level completed
No education
Primary
Secondary
College
University

7. Marital status
Married
Widow/widower
Divorced
Single-never married

Other_____

General health and well being

1. Have you been sick and or admitted to the hospital for at least overnight in the last three months? Yes /No

    a. If yes, for how long _____(Days)

    b. What was the diagnosis (Problem)?_____

2. Currently are you on any medication? Yes/No

2. If yes, indicate the type of medication
    a. Antibiotic(s)
    b. Other (specify)_____

Blood Sample

1. Time of phlebotomy (24 hour clock)
    _____:_____

2. Problems with blood sampling if any
    _____

3. Time received in the lab (24 hour clock) _____:_____

General Knowledge Test

1. Have you ever heard of CD4 count testing? Yes/No

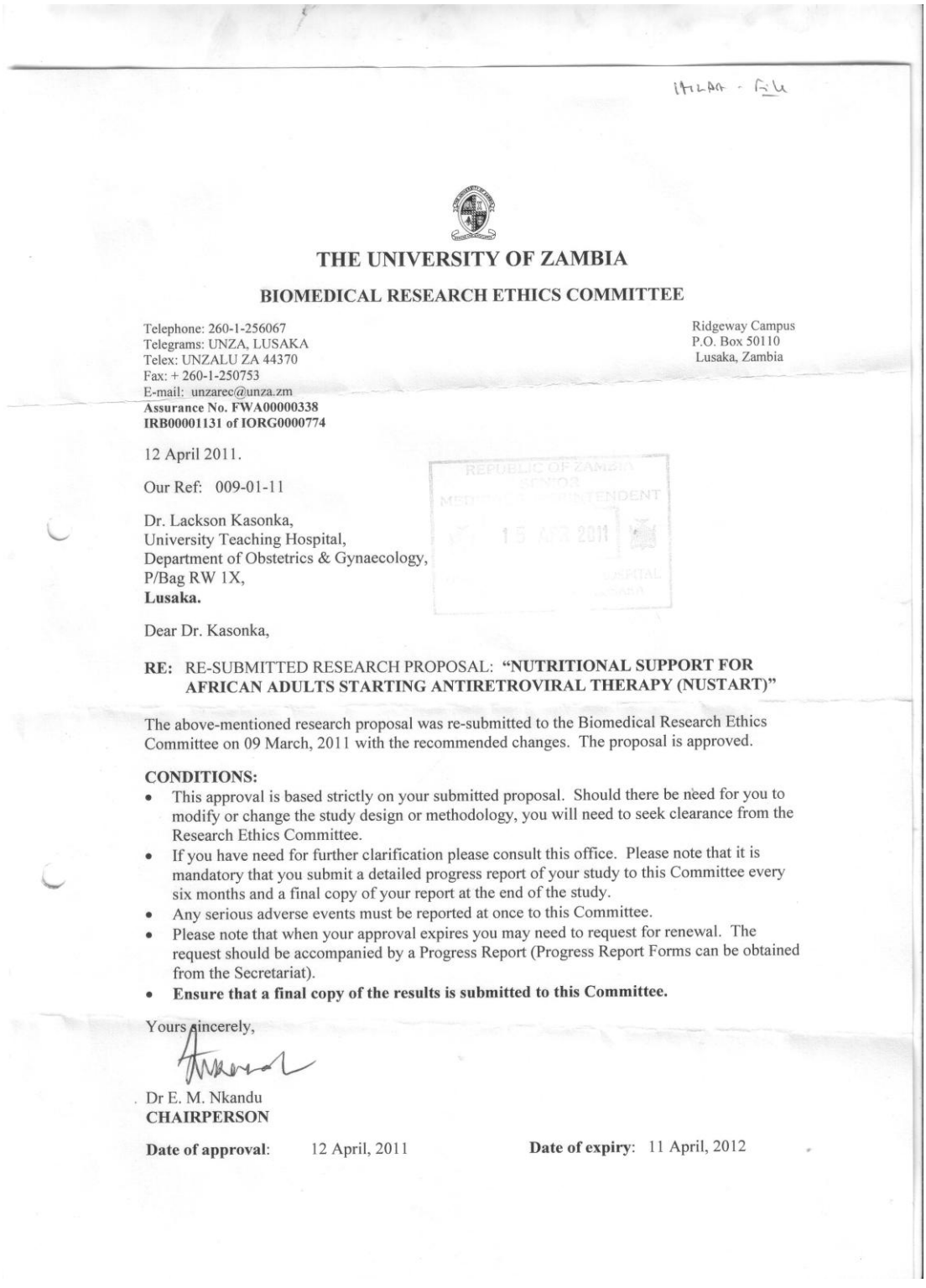
2. Do you know what CD4 T cell subsets are? Yes/No

Measure and record height in cm to the nearest 0.1cm
_____cm

Measure and record weight in kilograms to the nearest 0.1 kg
_____kg

Grip strength (kg to the nearest 0.1) _____kg

APPENDIX F



THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

12 April 2011.

Our Ref: 009-01-11

Dr. Lackson Kasonka,
University Teaching Hospital,
Department of Obstetrics & Gynaecology,
P/Bag RW 1X,
Lusaka.

Dear Dr. Kasonka,

RE: RE-SUBMITTED RESEARCH PROPOSAL: **"NUTRITIONAL SUPPORT FOR AFRICAN ADULTS STARTING ANTIRETROVIRAL THERAPY (NUSTART)"**

The above-mentioned research proposal was re-submitted to the Biomedical Research Ethics Committee on 09 March, 2011 with the recommended changes. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr E. M. Nkandu
CHAIRPERSON

Date of approval: 12 April, 2011 **Date of expiry**: 11 April, 2012

APPENDIX G

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **5876**

Name of Principal Investigator	**Suzanne Filteau**
Faculty	**Epidemiology and Population Health**
Head of Faculty	**Professor Laura Rodrigues**

Title: **Nutritional support for African adults starting antiretroviral therapy (NUSTART)**

This application is approved by the Committee.

Chair of the Ethics Committee

Date .....10 January 2011 .....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

APPENDIX H

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

25th November, 2013.

Your Ref: 009-01-11.

Ms. Caroline C. Chisenga,
University Teaching Hospital,
Department of Internal Medicine,
Tropical Gastroenterology,
P/Bag RW 1X,
Lusaka.

Dear Ms. Chisenga,

RE: RE-SUBMITTED RESEARCH PROPOSAL: **"THE EFFECT OF NUTRIENT SUPPLEMENTATION ON T-CELL RECONSTITUTION IN HIV INFECTED ADULTS STARTING ANTIRETROVIRAL THERAPY" (REF. NO. 009-01-11) (SUB-STUDY)**

The above mentioned research proposal was re-submitted to the Biomedical Research Ethics Committee with recommended changes on 25th November, 2013. The proposal is approved.

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit a detailed progress report of your study to this Committee every six months and a final copy of your report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- Please note that when your approval expires you may need to request for renewal. The request should be accompanied by a Progress Report (Progress Report Forms can be obtained from the Secretariat).
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C Munthali
CHAIRPERSON

Date of approval: 25th November, 2013. **Date of expiry**: 24th November, 2014.

APPENDIX I

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

17th March, 2015.

Your Ref: 009-01-11.

Ms. Caroline C. Chisenga,
University Teaching Hospital,
Department of Internal Medicine,
Tropical Gastroenterology,
P/Bag RW 1X,
Lusaka.

Dear Ms. Chisenga,

RE: **PROTOCOL AND INFORMATION SHEET AMENDMENT: "THE EFFECT OF NUTRIENT SUPPLEMENTATION ON T-CELL RECONSTITUTION IN HIV-INFECTED ADULTS ON ART AT THE UNIVERSITY TEACHING HOSPITAL LUSAKA, ZAMBIA" (REF. No. 009-01-11)**

We acknowledge receipt of your request of approval of amendment in the protocol and Information Sheet.

The amendment was reviewed and approved and follows:

(1) Protocol page 33 to read: In order to establish the baseline selenium level in healthy volunteers before incubating samples with selenium, blood will be spun down in order to collect serum or plasma and analyzed.
(2) Information Sheet page 53: Samples collected will include "baseline selenium testing."

Yours sincerely,

M.M.Mbewe (mrs)
CHAIRPERSON

APPENDIX J Laboratory protocol details.

FACSVerse principle

This is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them through an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second.

Principles of panel design

a) Matching of fluorochromes was done by brightness and smeared antigens. Monoclonal antibodies (mAbs) that were used for staining were as follows: allophycocynanin-H7 (APC-H7) labelled CD3, allophycocyanin (APC) labelled CD27 or CD57 or HLA-DR or integrin β7, phycoerythrin (PE) labelled CCR7 or CD28 or CD31, peridinin chlorophyll protein (PerCP) labelled CD4 or 7AAD, and fluorescein isothiocyanate (FITC)-labelled CD28 or CD4 or Ki67 or α4, and phycoerythrin cyanine dye (PE-CY7) labelled CD25 or CD45RA or CD27.

Panel design

Fluorochrome	**APC-H7**	**PerCP**	**FITC**	**APC**	**PE-Cy7**	**PE**
Homing Activated	CD3	CD4	α4	β7	CD25	
Thymic	CD3	CD4	Ki67	HLA-DR	CD45RA	CD31
Senescence	CD3	7AAD	CD4	CD57	CD27	CD28
Differentiation	CD3	CD4	CD28	CD27	CD45RA	CCR7

b) The potential for spectra overlap was minimized.

c) Tandem dyes were used with consideration of their technical limitations.

Compensation requirements for tandem dye conjugates can vary, even between two experiments with the same antibody. It requires compensation that is: lot specific, experiment specific, and label specific. Certain tandem dye conjugates (APC-Cy7, PE-Cy7) can degrade with exposure to light, elevated temperature, or fixation. As such, exposure to these conditions was minimized.

d) CS&T beads were used for quality control performance.

e) Fluorescence Minus One (FMO) contains all the lineage markers except the one of interest. FMO controls were used to determine the gating boundaries and to increase the accuracy by delineating positively vs negatively stained cells as follows;

FMO for homing panel

Antigen (FMO)	**APC-H7**	**PerCP**	**FITC**	**APC**	**PE-Cy7**
CD3	-	CD4	α4	β7	CD25
CD4	CD3	-	α4	β7	CD25
α4	CD3	CD4	-	β7	CD25
β7	CD3	CD4	α4	-	CD25
CD25	CD3	CD4	α4	β7	-

FMO for thymic panel

Antigen (FMO)	**APC-H7**	**PerCP**	**FITC**	**APC**	**PE-Cy7**	**PE**
CD3	-	CD4	Ki67	HLA-DR	CD45RA	CD31
CD4	CD3	-	Ki67	HLA-DR	CD45RA	CD31
Ki67	CD3	CD4	-	HLA-DR	CD45RA	CD31
HLA-DR	CD3	CD4	Ki67	-	CD45RA	CD31
CD45RA	CD3	CD4	Ki67	HLA-DR	-	CD31

CD31	CD3	CD4	Ki67	HLA-DR	CD45RA	-

FMO for senescent panel

Antigen (FMO)	**APC-H7**	**PerCP**	**FITC**	**APC**	**PE-Cy7**	**PE**
CD3	-	7AAD	CD4	CD57	CD27	CD28
7AAD	CD3	-	CD4	CD57	CD27	CD28
CD4	CD3	7AAD	-	CD57	CD27	CD28
CD57	CD3	7AAD	CD4	-	CD27	CD28
CD27	CD3	7AAD	CD4	CD57	-	CD28
CD28	CD3	7AAD	CD4	CD57	CD27	-

FMO for differentiation panel

Antigen (FMO)	**APC-H7**	**PerCP**	**FITC**	**APC**	**PE-Cy7**	**PE**
CD3	-	CD4	CD28	CD27	CD45RA	CCR7
CD4	CD3	-	CD28	CD27	CD45RA	CCR7
CD28	CD3	CD4	-	CD27	CD45RA	CCR7
CD27	CD3	CD4	CD28	-	CD45RA	CCR7
CD45RA	CD3	CD4	CD28	CD27	-	CCR7
CCR7	CD3	CD4	CD28	CD27	CD45RA	-

f) Antibody titration protocol

1. In the first tube 20 μL recommended volume of antibody was added
2. In the second tube, 10 μL
3. Third tube 5 μL
4. Fourth tube 2.5 μL

5. Fifth tube 1 μL
6. Sixth tube 0.5 μL
7. After adding the antibody, 100 μL whole blood was added to all the tubes.
8. The first tube had a volume of 120 μL. The other tubes were brought up to 120 μLwith PBS.
9. This was followed by incubation and lysing as per the staining protocol.
10. The FACSVerse and gating protocol were set up as required.
11. The histogram for the CD4 and or CD8 FITC data were drawn.
12. Data were acquired using the optimized voltage settings. About 50,000 to 100,000 events were acquired.
13. Since these were not single stains, compensation was needed.
14. On the 20 μL tube, $CD8^-$ population and $CD8^+$ population on the histogram were gated.
15. Statistics for these gates were displayed.
16. Statistics were then exported to excel and the ratio between $CD8^+$ and $CD8^-$ cell populations calculated.

The table below shows the final working volumes of all antibodies after titration. **Note** that all the antibodies were titrated as recommended by the manufacturers to suit the variances in investigations, and the concentrations picked gave the optimal results.

Antibody titration and final working volume

Antibody	**Fluorochrome**	**Recommended volume**	**Final volume titrated**
CD3	APC-H7	20 μL	1 μL
CD4	Per CP	20 μL	1 μL
CD8	Per CP	20 μL	1 μL
7AAD	Per CP	5 μL	1 μL
α4	FITC	5 μL	2.5 μL
CD4	FITC	20 μL	1 μL
β7	APC	20 μL	1 μL
Ki67	FITC	20 μL	1 μL
CD28	FITC	20 μL	1 μL
HLA-DR	APC	20 μL	1 μL
CD57	APC	5 μL	1 μL
CD27	APC	20 μL	1 μL
CD25	PE-Cy7	5 μL	1 μL
CD45RA	PE-Cy7	5 μL	1 μL
CD28	PE	20 μL	1 μL
CD27	PE-Cy7	5 μL	1 μL
CCR7	PE	20 μL	1 μL
CD31	PE	20 μL	1 μL

Daily clean

This was done every morning before running samples and before shutting down the machine.

1. From the menu bar, the cytometer daily clean was selected. This opened the daily clean dialog.
2. 10% bleach was prepared (10 ml bleach and 90 ml distilled water).
3. The tube containing 10% bleach was placed on the manual port and the process was started. This dialog closed once the daily clean was completed.
4. The tube containing 10% bleach was removed immediately the dialog window closed.
5. A tube containing 50 ml distilled water was placed on the manual port and the process was started.
6. This was followed by purging the sheath filter twice with distilled water (cytometer>fluidics>purge sheath filter).
7. Drain and fill flow cell was done twice using distilled water (cytometer >fluidics>drain and fill flow cell).
8. After the above tasks were done, the FACSVerse was ready for performance QC.

Performance QC for FACSVerse (Daily)

The setup and QC was done daily before samples were run as follows;

1. If using a new box for CS&T beads, bdbiosciences.com website was visited and the CS&T research beads page located.
2. Using the instructions on the website, the appropriate bead lot file corresponding to the current lot of CS&T research beads was downloaded and imported.

3. The CS&T research beads were thoroughly mixed using the vortex.
4. The 12x75-mm capped polystyrene tube was labeled.
5. Using a pipette, 0.5 ml of sheath fluid was drawn into the labeled tube.
6. Following thorough mixing of the CS&T research beads, 2 drops of the beads were put into the labeled tube and mixed with the sheath fluid.
7. The tube was again vortexed before use.
8. Using the setup and QC option on the FACSverse, the setup and QC tasks was selected.
9. The CS&T bead lot was selected.
10. Using the performance QC icon, the QC was started.
11. If the performance QC passed, the machine was ready for sample processing.

Characterization QC for FACSVerse (Monthly)

This was done monthly or as and when there was need to do so. Characterization QC beads were prepared as the performance QC beads. Except for the following;

1. Instead of 0.5 ml sheath fluid, 1.0 ml was used.
2. Four drops of the CS&T research beads were added.
3. On the setup and QC option the setup and QC tasks was selected.
4. The CS&T bead lot was selected.
5. Using the characterization QC icon, the QC was started.

Creating a new experiment for sample acquisition

1. Using the navigation bar, the experiments icon was clicked.
2. The manage experiments tab opened in the experiments workspace.
3. Using the experiments browser panel, the default folder or sub folder was

clicked.

4. Then new experiment was clicked. This opens a new experiment with the new experiment name and creation date displayed.
5. Existing experiments were opened by double clicking.
6. To add tube to an experiment, in the data sources panel, new tube icon was clicked.
7. To acquire data in the experiment, the tube was loaded onto the manual tube port.
8. Using data sources, the pointer was set on the sample tube and acquire clicked.
9. During acquisition, the run pointer turned orange and displayed an activity indicator. Acquisition continued until the stopping rules were satisfied.
10. Following acquisition, the tube icon displayed as a filled tube indicating hat data had been acquired. To go to the next tube, the run pointer was moved to the next tube and clicked.

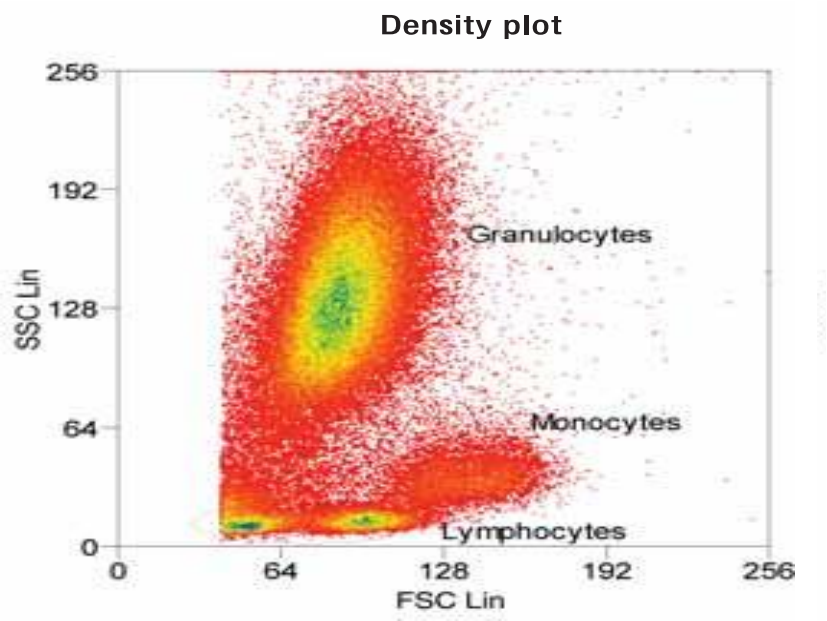
Adjusting data plots

1. Data in a specific tube was previewed by clicking preview.
2. During preview, the pointer runner remained blue whilst displaying the activity indictor.
3. When data was displayed in plots, adjustments were done using the cytometer settings and gates drawn in order to identify the populations of interest.
4. To put all the populations on scale, PMT voltages were adjusted.
5. To enable the data sliders, the PMTV button in the lower-left corner of the plot was clicked.
6. The slider control for each axis was dragged and the PMTV value displayed on

the slider control.

7. Threshold values were adjusted as needed using the checkbox.
8. Heights and width were also adjusted as needed
9. To stop previewing, stop was clicked.

Analysis of a sample by flow cytometry showing three different populations (lymphocytes, monocytes, and granulocytes)



Density plot

Compensation

This ensures that the fluorescence detected in a particular detector derives from the fluorochrome that is being measured.

Briefly, all fluorochromes have excitation and emission spectra. The excitation spectrum is a range of light wavelengths that add energy to a fluorochrome, causing it to emit light in another range of wavelengths, the emission spectrum. Within a flow cytometer, the appropriate ranges of excitation and emission wavelengths are selected

by bandpass filters. However, when emission spectra overlap, fluorescence from more than one fluorochrome may be detected. To correct for this spectral overlap, a process of fluorescence compensation is used and the following were the steps used.

1. BD FACSuite FC beads webpage was looked up (bdbiosciences.com)
2. The appropriate bead lot file was imported into BD FACSuite using the manufactures instructions.
3. The polystyrene tubes were labeled for each fluorochrome in the kit.
4. Bead vials were mixed by gentle vortexing.
5. Two drops of the individual fluorochrome were added to the fluorochrome labeled tube
6. One drop of the FC beads was also added to the bottom of the tube.
7. The tubes were gently vortexed and incubated for 15-30 minutes at room temperature in the dark.
8. To each tube, 0.5 ml bead diluent was added and the tubes gently vortexed before use.
9. An experiment was set up and tubes added for each flourochrome (FITC, PE, PerCP, APC, PerCP-Cy5.5, PE-Cy7, APC-Cy7 and APC-H7).
10. Dot plots of forward scatter-A (FSC-A) versus side scatter-A (SSC-A) were drawn. For every other fluorochrome that was used, dot plots were duplicated.
11. The sample was then previewed, followed by right clicking on the tube and selecting properties.
12. Compensation was selected and values adjusted followed by acquisition.
13. Compensation is correctly set when the median of the negative population is equal to the median of the positive population in the spillover channel.

Compensation for flurochromes without tandems

		Fluorochrome in the tube			
		FITC	PE	PerCP	APC
Filters (voltage)	FITC	100	1.00	1.00	0.86
	PE	8.53	100	0.85	5.00
	PerCP	0.00	1.00	100	9.00
	APC	1.10	10.0	0.75	100

Compensation for fluorochromes with tandems

		Fluorochrome in the tube			
		PerCP-Cy5	PE-Cy7	APC-Cy7	APC-H7
Filters (voltage)	PerCP-Cy5	100	1.00	1.00	0.84
	PE-Cy7	8.45	100	0.80	5.00
	APC-Cy7	0.00	1.00	100	1.00
	APC-H7	1.10	10.0	0.70	100

Whole blood subset staining and testing-lyse wash protocol

1. A new experiment was created with lyse wash settings.
2. EDTA blood tube was inverted to ensure the cells are well mixed.
3. 100 μL of blood was added to the tube and labeled.
4. Titrated volume of antibodies were added to the tube (2 μL each)
5. Samples were then vortexed and incubated for 15 minutes at room temperature (RT) in the dark
6. BD FACSlyse 10X was diluted with distilled water to a 1X working solution

a. This lysing solution was stable for a maximum of 1 week. However, when

preparing new lysing solution a new container was always used.

7. 2 ml of 1X lysing solution was added to the tube
8. Samples were vortexed and incubated for 10 minutes at RT in the dark
9. Samples were then Spun at 1500 r. p. m for 5 minutes
10. The supernatant was discarded, leaving behind about 50 μL
11. To re-suspend the cells, tubes were vortexed once again
12. 2 ml PBS was added and tubes were centrifuged at 1500 r. p. m for 5 minutes at room temperature
13. The supernatant was discarded, leaving behind 50 μL
14. Samples were vortexed and 250 μL PBS added when using immediately, and 250 μL 1X cell fix when leaving overnight
15. Samples were then ready for acquisition


Step 1: sample pipetting & staining with mAbs


Step 2: red blood cell lysis


Step 3: washing of white cells


Step 4: resuspension & second wash


Step 5: centrifugation @ 1500 rpm for 5min


Step 6: sample analysis

FACS intracellular staining protocol

1. Extracellular staining protocol was followed up to the cell fix stage.
2. A 100 μL of cell fix was added to the tubes requiring extracellular staining.
3. Tubes were vortexed and incubated at room temperature for 10 minutes.
4. Cells were washed with 1 ml perm buffer (for permeabilising cells) at 1800 r. p. m. for 5 minutes.
5. The supernatant was then decanted and 1μL intracellular antibody added i.e. Ki67.
6. Sample tubes were vortexed and incubated at 2-8^{0}c in the dark for 30 minutes.
7. Samples were then washed with 1 ml PBS at 1800 r. p. m.
8. The supernatant decanted and 250 μL cell fix or PBS added.
9. Samples were then ready for acquisition.

Lyse no wash protocol for absolute CD4 and CD8 count

1. Add 50 μL of blood to a BD trucount tube
2. Add 5 μL of each flouorochrome and vortex
3. Incubate for 15 minutes in the dark
4. Add 450 μL of 1X lysing solution
5. Incubate for another 15 minutes in the dark
6. Sample is ready for acquisition
7. To gate lymphocytes, SSC vs CD45 was plotted
8. SSC vs CD3 was plotted to get T cells
9. CD4 vs CD8 was used to show data from CD3 population
10. An equation was then setup to do the calculations.

    (CD4 events/ Bead events) x (bead count/50 μL) = CD4 count

Number of CD4 events/ number of CD45 events x 100 = CD4%

CD4 and CD8 antibodies and flourochromes

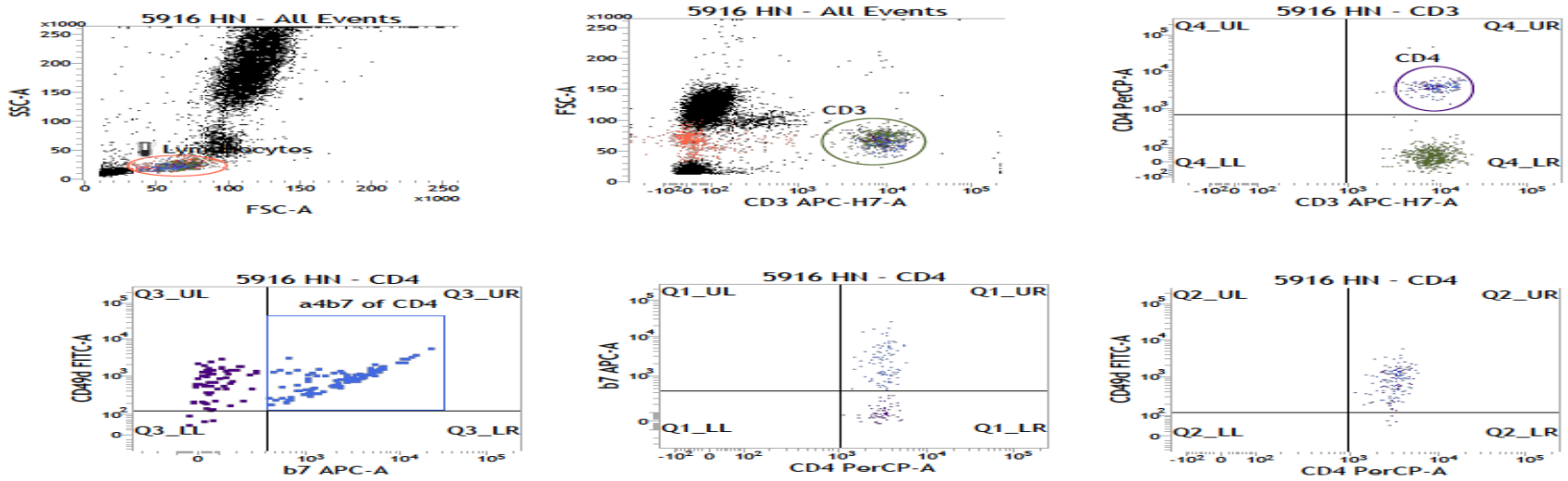
CD45	Per CP CY5.5
CD3	APC-H7
CD4	PE
CD8	FITC

Gating

The data generated via flow-cytometry were plotted in a single dimension, or in two-dimensional dot plots or even in three dimensions. The regions on these plots were sequentially separated, based on fluorescence intensity, by creating a series of subset extractions, termed "gates." The plots were made on logarithmic scales. Because different fluorescent dyes' emission spectra overlap, signals at the detectors were compensated electronically as well as computationally.

In immunofluorescence analysis, quadrants are often drawn on a cytogram and the number of cells in each quadrant recorded. Quadrants were set to delineate the CD4 positive and negative populations.

Data accumulated using the flow cytometer were analyzed using FACSuite software



Statistics

Name	Events	% Parent	% Grandparent	% Total	FSC-A Mean	SSC-A Mean
5916 HN:All Events	10,000	***	***	100.00	96,088	141,204
5916 HN:Lymphocytes	1,124	11.24	***	11.24	66,130	21,799
5916 HN:CD3	670	6.70	***	6.70	67,277	22,111
5916 HN:CD4	151	22.54	1.51	1.51	61,683	19,910
5916 HN:Q1_UL	0	0.00	0.00	0.00	***	***
5916 HN:Q1_UR	90	59.60	13.43	0.90	59,377	19,781
5916 HN:Q1_LL	0	0.00	0.00	0.00	***	***
5916 HN:Q1_LR	61	40.40	9.10	0.61	65,085	20,100
5916 HN:Q2_UL	0	0.00	0.00	0.00	***	***
5916 HN:Q2_UR	147	97.35	21.94	1.47	61,694	20,023
5916 HN:Q2_LL	0	0.00	0.00	0.00	***	***
5916 HN:Q2_LR	4	2.65	0.60	0.04	61,268	15,736
5916 HN:Q3_UL	57	37.75	8.51	0.57	65,353	20,406
5916 HN:Q3_UR	90	59.60	13.43	0.90	59,377	19,781
5916 HN:Q3_LL	4	2.65	0.60	0.04	61,268	15,736
5916 HN:Q3_LR	0	0.00	0.00	0.00	***	***
5916 HN:a4b7 of CD4	90	59.60	13.43	0.90	59,377	19,781
5916 HN:Q4_UL	0	0.00	0.00	0.00	***	***
5916 HN:Q4_UR	156	23.28	1.56	1.56	61,609	23,229
5916 HN:Q4_LL	0	0.00	0.00	0.00	***	***
5916 HN:Q4_LR	514	76.72	5.14	5.14	68,997	21,772

Figure 5-3: Gating strategies

The light scatter (SSC-A versus FSC-A) defines three distinct populations; these are the granulocytes, monocytes and lymphocytes. Similarly, populations of interest were gated accordingly and statistics for each gate calculated.

APPENDIX K**: Laboratory practices and safety procedures**

The following practices were followed for the prevention of laboratory infection and disease, as well as for the reduction of the potential for contamination of experimental material. These practices and procedures provide the foundation for the more restrictive containment of organisms.

1. Personal Hygiene

1. Do not eat, drink, chew gum, use tobacco, apply cosmetics (including chap stick), or handle contact lenses in the laboratory.
2. Do not store food for human consumption in laboratory refrigerators.
3. Wash hands frequently after handling infectious materials, after removing latex/nitrile gloves and protective clothing, and always before leaving the laboratory.
4. Keep hands away from mouth, nose, eyes, face, and hair.
5. Do not remove personal protective equipment (such as cloth lab coats) from the lab.
6. First-aid kit(s) should be available.

2. Laboratory Procedures For Handling Infectious Microorganisms

1. A laboratory biosafety manual was assembled outlining activities and defining standard operating procedures.
2. Training employees and ensuring that all personnel are informed of hazards.
3. Plan and organize materials/equipment before starting work.
4. Keep laboratory doors closed; limit access to lab personnel.

5. When pathogens are used in long-term studies, post a biohazard sign at the laboratory entrance identifying the agents in use and the appropriate emergency contact personnel.
6. Laboratories should have a sink for hand washing, an eyewash station in which the eyewash is tested/flushed weekly, be relatively clutter-free, and be easy to clean.
7. Wear a fully fastened laboratory coat when working with infectious agents. Wear protective gloves whenever handling potentially hazardous materials, including human blood and body fluids. Wear eye protection when working in the BSL2 laboratory when necessary.
8. Remove and leave all protective clothing, including gloves, within the laboratory before exiting. If transport of research materials through public spaces is required, one glove may be removed and ungloved hand used to handle public equipment (door handles, elevator buttons, etc.) and lab coats may be carried.
9. Never mouth-pipette; use mechanical pipetting devices.
10. When practical, perform all aerosol-producing procedures such as shaking, grinding, sonicating, mixing, and blending in a properly operating biological safety cabinet (BSC).
11. Centrifuge materials containing infectious agents in durable, shatter-resistant, closable tubes. Use a centrifuge with sealed heads or screw-capped safety cups. After centrifugation, open the tubes within a BSC.

12. Minimize the use of needles, syringes, razor blades, and other sharps when possible. After use, syringe-needle units must be disposed in a dedicated sharps container without removing or recapping the needles.
13. Cover countertops where hazardous materials are used with plastic-backed disposable paper to absorb spills and dispose of them daily or following a spill.
14. Wipe work surfaces with an appropriate disinfectant according to corresponding IBC protocol after experiments and immediately after spills.
15. Decontaminate all contaminated or potentially contaminated materials by appropriate methods before disposal (See Chapter V of this Manual).
16. Report all accidents and spills to the laboratory supervisor. All laboratory personnel should be familiar with the emergency spill protocol and the location of cleanup equipment. Step-by-step Spill response protocols should be posted in the laboratory.
17. Good housekeeping practices are essential in laboratories engaged in work with infectious microorganisms. Do not forget to routinely decontaminate all shared equipment and equipment in common areas.
18. Be sure to advise custodial staff of hazardous areas and places they are not to enter. Use appropriate biohazard signs.
19. Equipment used with biohazards must be decontaminated prior to repair.

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