ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

MSc. THESIS

TITLE: REFERENCE VALUE DETERMINATION OF $CD4^+$ T-LYMPHOCYTE IN HIV SERO-NEGATIVE ETHIOPIAN PREGNANT WOMEN

BY

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Abbreviations

Abs count	Absolute count
ANC	Antenatal care
ART	Antiretroviral therapy
BD	Becton Dickinson Bioscience
CD	Cluster of differentiation
CDC	Center for disease control and prevention
FACS	Fluorescence activated cell sorter
FALS	Flouresence activated light source
FITC	Fluorescein Isothiocynate
FSC	Forward scattered
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
IgM	Immunoglobulin M
IL	Interleukin
INF	Interferon
IQR	Inter quartile range
K_2EDTA	Di-potasium ethylenediamine tetracetic acid
LASER	Light amplified by stimulated emission of radiation
LP	long pass filiter
MAB	Monoclonal antibody
MHC	Major histocompatibility complex.
MOH	Ministry of health
MTCT	Mother-to-child transmission of HIV
NC	Nitrocellulose
PE	Pycoerythrin
PerCP	Peridinin chlorophyll protein
PMT	Photomultiplier tube
PMTCT	Prevention of mother-to-child transmission of HIV
RPR	Rapid plasma reagin

Sp	Short pass filter
SSC	Side scattered light
TCR	T-cell receptor
TNF	Tumor necrosis factor
WHO	World health organization

Abstract

In Ethiopia, reference intervals in clinical laboratories are commonly based on results obtained for western populations or from test kit manufacturers. The present cross sectional study aimed to establish reference interval for $CD4^+$ T lymphocyte counts, in HIV sero-negative pregnant women attending antenatal care clinic at Yekatit 12 hospital.

Flow cytometric analysis was performed to determine $CD4^+$ and total T lymphocyte counts. Accordingly gestational ages between 4 and 38weeks (n=120) were used. Reference values obtained were 310-1185 cells/µl for Absolute $CD4^+$ T cells count, 17.9-54.0% for $CD4^+$ T cells percentage, 690-2540 cells/µl for absolute $CD3^+$ T cells count, 55-84% for $CD3^+$ T cells percentage, 952-2985 cells/ µl for absolute $CD45^+$ lymphocyte count.

The data revealed that $CD4^+$ T cell percentage was stable throughout the pregnancy period while absolute $CD4^+$ T cell count varies between trimesters indicating that $CD4^+$ T cell percentage could be more reliable in the management of HIV infected pregnant women than the absolute value. In conclusion, T lymphocyte populations in pregnant women were different from non pregnant women. The clinical implication of these differences is the initiation of antiretroviral therapy (ART) in HIV infected pregnant women.

1. Introduction

A measured or observed laboratory test result from a person is compared with a reference interval for the purpose of making a medical diagnosis, therapeutic management decision, or other physiological assessment. The interpretation of clinical laboratory data is, therefore, a comparative decision-making process. For this decision-making processes, reference values are needed for all tests in the laboratory, and the provision of reliable reference intervals is an important task for clinical laboratories and diagnostic test manufacturers [1, 2].

Reference intervals should be determined in a systematic and scientific manner that provides an acceptable degree of confidence for the clinical decision-making process. This includes a consideration of the significant factors and variables introduced by the specific individual's reference sample or by the analytical process it self. Understanding the process used to establish a reference interval yields a better understanding of the limitations of the defined reference interval. Most commonly, reference intervals are derived from samples of assumed healthy individuals and are defined as the central 95% of results tested under defined conditions [3, 4, 5].

The definitions, principles, and procedures for the determination and use of reference values were developed by the Expert Panel on Theory of Reference Values (EPTRV) of the International Federation of Clinical Chemistry (IFCC) and the Standing Committee on Reference Values of the International Council for Standardization in Hematology (ICSH) [1].
Reference values greatly depend on the type of instrument, analytical method (test principle), reagents used, the type of population being served and the over all quality of results that are generated by the laboratory where the tests were done. Quality assurance practices have an impact on the comparability of results from different laboratories found at different levels and on the usability of the established reference values [6, 7].

All approaches to establish reference interval require large number of individuals. Reed et al suggested that a minimum of 120 observations be secured [8]. This sample size (n=120) assumes that no observations were deleted from the reference set. If aberrant or outlying observations were deleted, then additional subjects should be selected until at least 120 acceptable reference values are obtained for each determination of a reference interval [8, 9].

Reference intervals can be established for different groups by partitioning the values to account for differences between groups of individuals. Partitioning is necessary when data show that results are significantly different between particular sub groups. There are biological reasons for this, such as ethnicity, physiological state including pregnancy or dietary habits, and also methodological reasons [5, 10, 11].

In Ethiopia, a country with highly diversified geography and ethnicity, there are no nationally established reference values. Like most developing countries reference intervals in clinical laboratories are commonly using values obtained from that of western populations or from test kit manufacturers. However, Ethio-Netherlands AIDS Research Project established reference intervals for certain Immuno-hematological parameters [14, 65]. Moreover, values for some hematological parameters were determined [15, 16]. It is well documented that Physiological conditions as in pregnancy affect reference values [10-12]. None of the aforementioned studies in Ethiopia which were trying to establish reference values address pregnant women.

$CD4^+$ T lymphocyte count is an important tool in the evaluation of the immunological status of a human subject, especially of value in the management of human immune deficiency virus (HIV) disease. They are used for monitoring HIV infection progression, for the initiation of prophylactic treatment to opportunistic infections and to monitor responses to antiretroviral therapy in HIV infected individuals [17], Establishing reference intervals of $CD4^+$ T cell counts in healthy pregnant women population will help in the management of HIV-infected pregnant women and enlighten the national anti retroviral treatment (ART) treatment program of any emerging biological, physiological and gender-related differences and trends in $CD4^+$ T cell values. In addition, setting treatment criteria based on group specific data will help to ensure that HIV infected pregnant patients receive proper management of opportunistic infections and antiretroviral therapy at the appropriate time during the course of the disease. In so doing, the data will contribute to the on going effort of scaling up of Prevention of mother to child transmission (PMTCT) of HIV programs. The aim of this study is, therefore, to determine reference value of $CD4^+$ T-lymphocyte for HIV negative pregnant women.

1.1 Selecting reference individual

Health is a relative condition lacking a universal definition. Defining what is to be considered healthy becomes the initial problem in any study, and establishing the criteria used to exclude non healthy subjects is the first step in selecting reference individuals. Frequently, it can only be determined that a particular individual is apparently "disease free" i.e., does not have a specific recognizable medical condition that might affect the reference interval study. In some cases, individuals with minor illnesses or "unrelated" conditions may be used as reference individuals. However, it is often difficult to estimate the potential physiological and pharmacological influences in the subjects, and appropriate caution is required [4, 18]. The health status of the reference individuals is not expected to be free of any clinical and laboratory issues. However, if these assessments are performed, they will, strengthen the reliability of the reference interval determination. All criteria and assessments used should be documented so that others can evaluate the health status of the reference sample group. Reference individuals used for the determination of a health-associated reference interval do not have to be young healthy adults but should closely resemble the patient population in the specific hospital or practice [18, 19].

1.2 Sample handling and analysis

Analytical results from reference populations are affected by pre-analytical and analytical variables. Therefore, all these variables must be considered and controlled consistently during determination of reference intervals [20-23]. In addition, it is important that reference subjects and samples be handled in an approved manner and in exactly the same manner as patients and patient samples will be handled in the actual clinical analysis situation. All the pre-analytical variables like time of blood collection, volume of sample, type of anticoagulant used and sample transportation, must be carefully considered. The validity of information provided by the laboratory is critical; thus, the methods chosen for specimen analysis must be described in detail, clearly stating accuracy, precision, minimum detection limit, linearity, and recovery and interference characteristics [20, 21].

1.3 CD4+ T lymphocytes

The cellular and humoral immune systems are mediated by distinct lymphocyte classes or subsets including T-lymphocytes, B- lymphocytes, and natural killer (NK) cells. T lymphocytes

can be subdivided into two populations $CD4^+$ T lymphocyte (T helper cells) and $CD8^+$ T lymphocyte (Cytotoxic T cells) according to their expression of CD4 or CD8 membrane molecules. $CD4^+$ T lymphocyte is a subset of T lymphocytes expressing the CD4 molecule on the cell surface. CD4 is a 55-kDa monomeric membrane glycoprotein that contains an extra cellular region, composed of 370 amino acids; a hydrophobic transmembrane region consist of 25 amino acids, and 38 amino acids long cytoplasmic tail which contains three serine residues that can be phosphorylated [24, 25]. Within the extracellular part of CD4, four regions D1-D4 have been characterized that represent immunoglobulin-like domains (Figure 1.1).

Residues within the V2 region of CD4 (amino acids 40-55) are important parts of the CD4 where its natural ligands, HLA class II molecules bind during interaction. This part is important for the bonding of gp120 of HIV to CD4 molecule [26].

$CD4^+$ T-lymphocyte recognize antigen that is combined with class II MHC molecules (Figure 1.2 and 1.3) and function largely as helper cells, whereas $CD8^+$ T-lymphocytes recognize antigen that is combined with class I MHC molecules and function largely as cytotoxic cells [25].

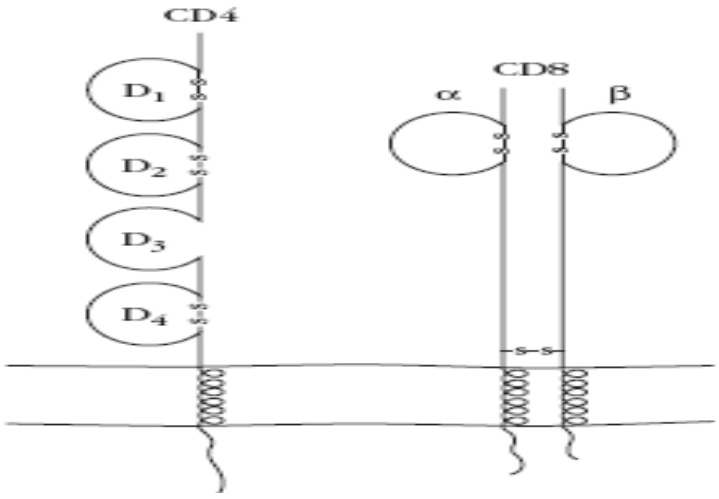


Figure 1.1 *Two dimensional structure of CD4 and CD8 molecules [From Immunology Cubey, 5th edition, 2005]*

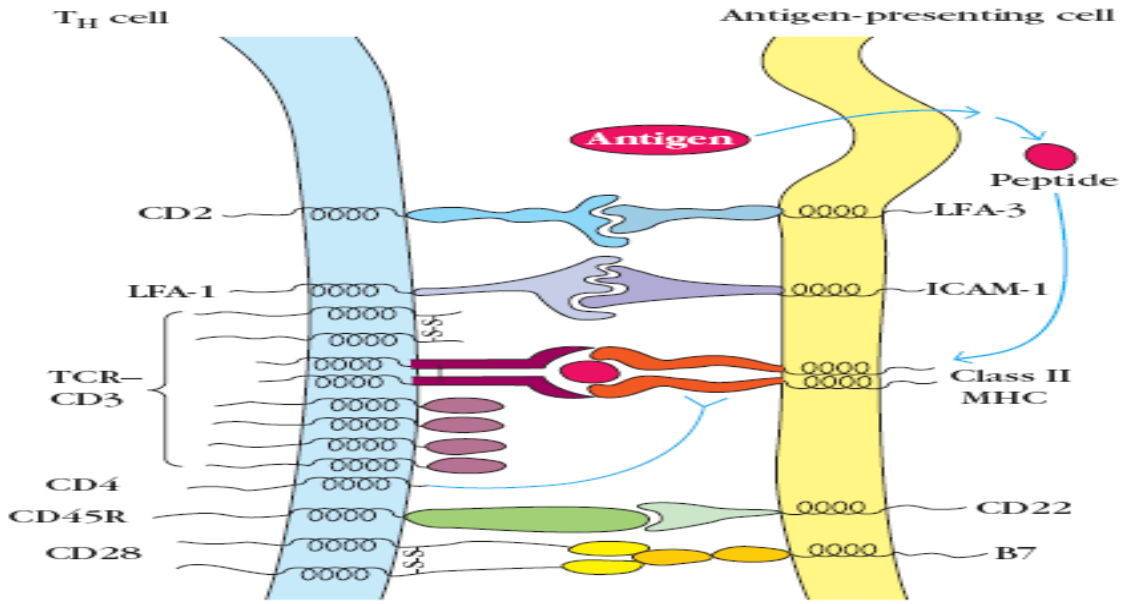


Figure 1.2 *Schematic diagram of the interactions between the T-cell receptor and the peptide-MHC complex and of various accessory molecules [From Immunology cubey, 5th edition, 2005]*

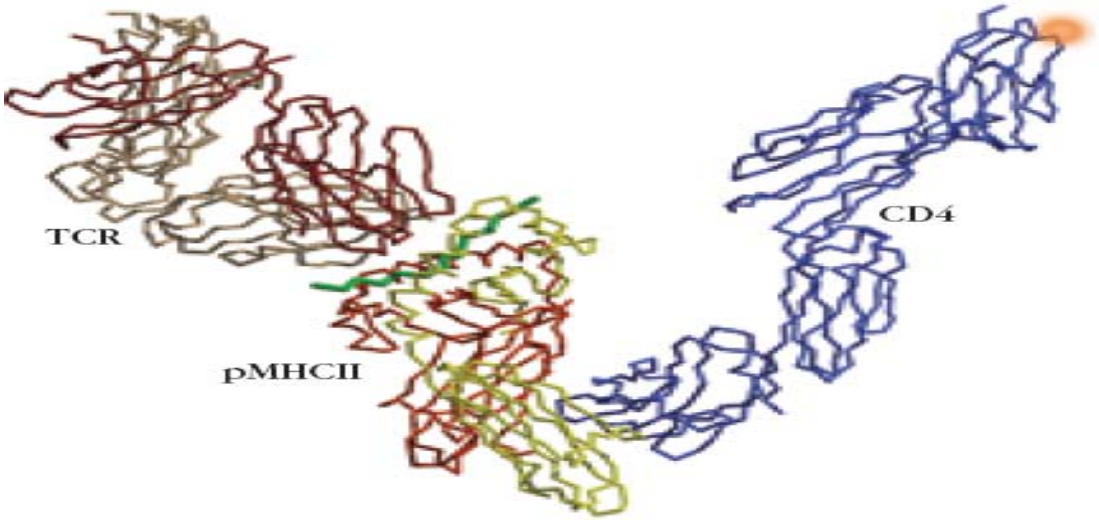


Figure 1.3 *Interaction of CD4 molecule with the class II MHC peptide complex (pMHCII) [From Immunology cubey, 5th edition, 2005]*

$CD4^+$ T lymphocytes, after interacting with an antigen–MHC class II molecule complex, the cells are activated and become a vital source of cytokines (IL-4, 5, 6, 9, 10, 13, IFN γ, TNF α) required for migration of leukocyte to the site of infection, differentiation of natural killer (NK) and γδ T cells, which possess a natural antiviral cytotoxic activity and may help in eliminating virus-infected cells [26,27], for maturation of B-lymphocytes into plasmacytes, which can produce neutralizing antibodies, and for differentiation of CD8+ T-lymphocytes into virus-specific cytotoxic T lymphocytes [28].

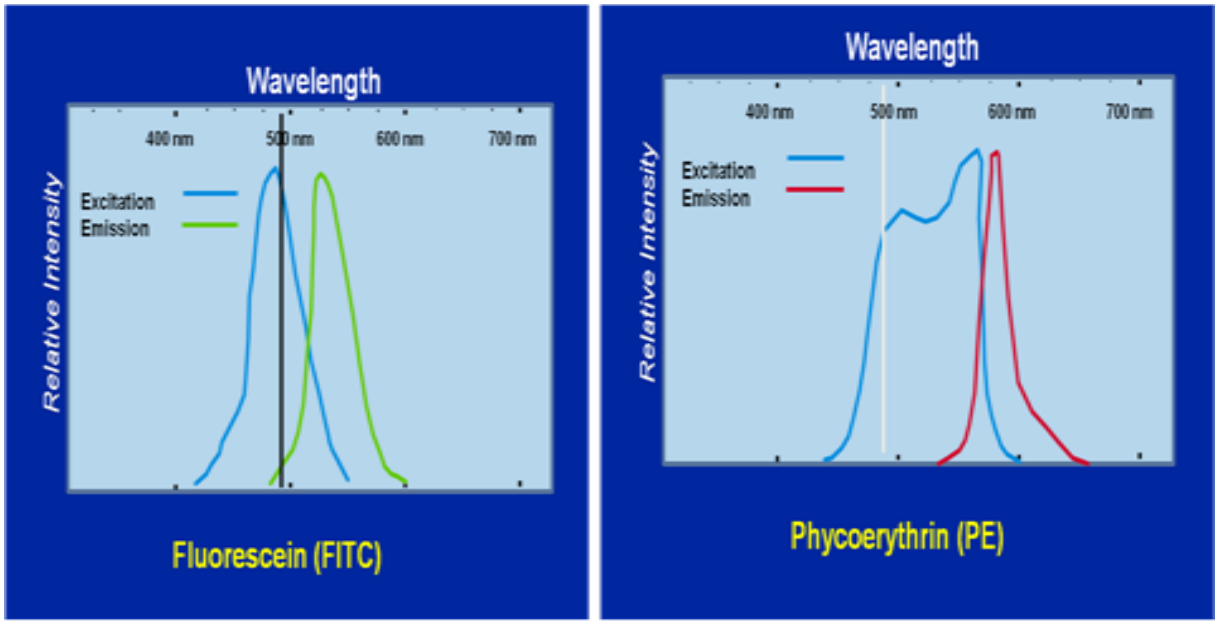
1.3.1 CD4+ T cells Immunophenotyping

T-Cells express proteins (antigens) on their surface which is unique to that cell. These surface markers on the cells called cluster of differentiations (CD) are usually functional molecules reflecting the state of cellular differentiation. There are now over 300 CD specificities assigned.[24] When enumerating cells in suspensions such as in blood or other fluids, these surface antigens can be detected by the use of labeled antibodies. Antibodies (immunoglobulins) are proteins that bind to epitops of cell surface antigen specifically.

Immunophenotyping is a technique which utilizes immunologic methods (eg. Antibodies) to determine type or function of cells by appearance. Fluorochrome labeled monoclonal antibodies are used to immunophenotype any lymphocyte subset (monoclonal means same clone) [25, 32].

By tagging each antibody with a colored fluorochrome it is easy to distinguish the cell type and quantity of antigens expressed by each cell. It is possible to stain single cell with several different fluorochromes and analyze the cells in individual droplets as they flow past a laser beam in a flow cytometer [33].

Fluorescence is the property of a substance that results in a higher wavelength of light being emitted when the substance is struck by a lower wavelength of light. In flow cytometry, cells are labeled with one or more fluorescent molecules. These molecules called fluorochromes are fluorescent proteins from algae, [35] which fluoresce (emit light) at higher wavelength when excited by a laser at a lower wavelength. Different fluorochromes emit different wavelengths of light when struck by their “excitation wavelengths”. Each has different emission spectra (Figure 1.4a and b) and so their emissions can be individually detected using appropriate filters.



(a) (b)

***Figure** 1.4.a) Excitation (Blue) and Emission (Green) spectra for Fluorescein Isothiocynate (FITC). b. Excitation (Blue) and Emission (Red) for Pycoerythrin (PE)*

As each cell, tagged with a fluorochrome labeled antibody, enters the laser light outer orbital electrons in the fluorochrome are excited at a specific excitation wavelength to greater and more unstable energy levels. As it transitions the width of the laser beam maximum peak fluorescence is achieved within approximately 10nsec.

As the excited outer orbital electrons return to their more stable ground state and emit a photon of light at a longer wavelength than that at which they were excited. Photomultiplier tubes (PMT's) detect these faint fluorescent signals and change discrete packets of light called photons (hv) into electrons and amplify them by producing as much as 10 million electrons for every photon captured to be quantified by electronic section of the flow cytometer [33]. Thus cells can be quantified depending on the surface marker they expressed using antibodies against the surface markers conjugated with fluorochromes.

For example, helper T-lymphocytes are CD3, CD4 positive, whereas cytotoxic T- lymphocytes are CD3, CD8 positive. B- Lymphocytes are CD19 positive, and Natural killer cells (NK) cells are CD3 negative and CD16 and CD56 positive [24].

Although there is a relatively fixed number and proportion of these lymphocyte subsets in normal individuals, the absolute number and proportion is altered in various conditions:

Malnutrition has an effect on hematopoietic dysfunction regarding the rate of proliferation, amount and rate of protein synthesis that result in a significant reduction in CMI indicted by a reduced number of $CD4^+$ T helper cells and lower $CD4^+/CD8^+$ ratio; Steroid treatment cause decreased T cells number, with in the sub set $CD4^+$ T cells are more depleted; Immune activation due to recent illness or chronic infection also affects the number of lymphocyte population [29-31].

In immunodeficiency virus (HIV) infection, the number of $CD4^+$ T cells, the percentage of $CD4^+$ T cells in relation to other lymphocytes and the proportion of $CD4^+$ T cells relative to $CD8^+$ T lymphocytes vary based on the stage of disease and therapeutic response. Thus, lymphocyte subset analysis can provide information regarding the immune status of the patient, can assist in monitoring therapy, and can help characterize congenital and acquired immune deficiencies [34, 36, 37].

1.4 HIV infection and $CD4^+$ T Lymphocytes

The hallmark of HIV disease is a profound immunodeficiency resulting primarily from a progressive quantitative and qualitative deficiency of the $CD4^+$ T lymphocytes.

The reduction of $CD4^+$ T-cell population reflects the sum of shifts between production, proliferation, differentiation, and death of thymic emigrants, naive, effector, and memory T cells [38]. Although a vast majority of the gut associated short-lived $CCR5^+CD4^+$-activated T cells are depleted during acute HIV infection, [39,40] a commonly observed immune change is the reduction of the naive T-cell compartment within blood as disease progresses[41].

The exact mechanisms underlying this change is likely the result of a combination of direct and indirect cytopathogenicity, disorganization of lymphoid organs, heightened immune activation,

and/or loss of thymic function [42,43]. The decline in these lymphocyte subsets may be gradual or abrupt, the latter usually reflecting a significant spike in the level of plasma viremia. The disappearance of CD4+ T-lymphocytes is responsible for the lack of control of HIV replication and the development of severe Immune deficiency [24].

The slope of $CD4^+$ T cells decline is usually a good predictor of the pattern of the clinical course and the development of advanced disease [25, 36].

1.4.1 CD4+ T lymphocyte count and HIV monitoring

In HIV infection, the $CD4^+$ T-cell count is the most commonly utilized laboratory measure for clinical prognosis, therapeutic monitoring, and entry criteria for clinical trials [17, 37]. Absolute and percent $CD4^+$ T count are strong predictor of HIV-1/2 disease progression, independent of the HIV RNA plasma viral load, and the parameters to use in association with the World Health Organization (WHO) clinical staging system to decide when to initiate highly active antiretroviral therapy (HAART) in resource limited settings. According to the 2003 and 2006 WHO guidelines, HIV-infected adults are eligible for HAART if they meet one of the following conditions: [44, 45]

- WHO clinical stage 4 irrespective of CD4 count
- CD4 count $\leq 200/mm^3$ irrespective of WHO clinical stage
- WHO stage 3 and CD4 count between 200 and $350/mm^3$

These criteria apply to all HIV-infected adults, including pregnant women, as suggested by WHO 2004 and Ethiopian Ministry of Health (MOH) 2007 guidelines for the prevention of mother-to-child transmission (PMTCT) of HIV [47]. Though $CD4^+$ T-cell count is a versatile test, variability reported among healthy individuals due to biological parameters like age, gender, race, pregnancy, diurnal variation, habit and methodological differences used to enumeration [13, 48-52].

However, the eligibility criteria to initiate HAART do not take into account the special conditions that occur in pregnancy. HIV positive pregnant women who do not meet these criteria should receive short-course antiretroviral prophylaxis for PMTCT [42].

According to different researches conducted in different countries $CD4^+$ T-lymphocyte absolute count has been reported to be slightly modified during pregnancy irrespective of HIV infection [53-57]. This is due to a physiological hemodilution which is likely due to greatly increases in aldosterone and estrogens levels in pregnancy, increased fluid retention by the kidneys and expansion of plasma volume [58]. Because of such physiological conditions $CD4^+$ T lymphocyte count may not accurately indicate the need for initiation of HAART in HIV sero-positive pregnant women.

Although its efficacy for PMTCT is very high, the use of HAART during pregnancy has been associated with increased rates of preterm delivery [59,60], low birth weight and teratogenic effect on the fetus in the first trimester of pregnancy, reduced CD4+T cell counts in the first year of infant's life, and with reduced CD8+T cell counts until at least 8 years of age and risk of drug resistance [61-63] Furthermore, nevirapine (NVP)-containing HAART regimens are no longer recommended for women with CD4 count >250/mm^3 considering the elevated risk of hepatotoxicity [64]. Thus, important treatment decisions may be taken during pregnancy, with potential consequences for the mother and her child.

Because of the risk benefit concerns, accurate targeting of the time to initiate HAART is important. Therefore; it is important to know the base-line levels of $CD4^+$ T-lymphocyte count in normal pregnant women before using this level as prognostic markers.

1.5 Significance of the study

Establishing local group specific reference intervals for absolute and percent $CD4^+$ T cell counts in the healthy Ethiopian pregnant women population may help for proper management of opportunistic infections and initiation of antiretroviral therapy at appropriate time during the course of the disease in HIV infected pregnant women. Moreover, the information helps to strengthen the national PMTCT and ART programs.

1.6 Objective

1.6.1 General Objective

- To establish reference interval of total and CD4+ T-lymphocyte in HIV sero-negative Ethiopian pregnant women.

1.6.2 Specific objectives

- To establish reference interval for absolute and percentage $CD4^+$ T-lymphocytes.
- To compare the absolute and percent $CD4^+$ T-lymphocytes, $CD3^+$ T-lymphocytes, and $CD45^+$ lymphocyte values by trimester.
- To evaluate the comparability of established reference values with the currently used reference value.

1.7 Hypothesis of the study

The reference values for lymphocyte sub populations in Ethiopian pregnant women are similar to the reference values given by the test kit manufacturers and the reference values established for Ethiopian non pregnant women.

2. Materials and Methods

2.1 Study design and study area

This study was a prospective study of healthy HIV sero-negative pregnant women attending antenatal care (ANC) at Yekatit 12 hospital, Addis Ababa.

Yekatit 12 hospital is a general hospital with six departments including gynecology and obstetrics. It is also one of the centers for ART and PMTCT programs lunched by the Ethiopian MOH.

2.2 Sampling and sample size

A minimum sample size of 120 is recommended for establishing reference values [8, 9]. A total of 131 pregnant women were willing to participate in the study. One hundred twenty of them fulfilling the inclusion criteria and who were willing to participate were enrolled in this study.

2.2.1 Recruitment of study participants

The study enrolled 120 HIV sero-negative pregnant women attending ANC at Yekatit 12 hospital between July and September 2009. All participants were informed about the study and requested to participate. They went through HIV counseling, responded for the questionnaire (annex III), and blood sample were collected for Hemoglobin (Hb), syphilis, HIV testing and FACS analysis.

Inclusion criteria

The following inclusion criteria used in the study

- Pregnant women
- Willing to participate in the study
- HIV negative
- Rapid plasma reagin (RPR) negative
- Did not have sign of infection during the last one month

Exclusion criteria

The following exclusion criteria were used in the study.

- Severe anemia (hemoglobin less than 6.0g/dL)
- Rapid plasma reagin (RPR) positives
- HIV positives

- Having fever, cough, cold, in the last one month.

The above laboratory tests:-hemoglobin, RPR test and HIV antibody test were used to assess hematopoietic dysfunction due to malnutrition, bacterial infection, and HIV infection respectively. These tests were selected because of their availability.

2.3 Specimen Collection and storage

To avoid any error in $CD4^+$ T lymphocyte subset counts due to diurnal variation [48, 51] the time of blood collection was in the morning from 09.00 AM-12.00 AM. After a pretest counseling about 8 ml venous blood was collected aseptically from each study participant into two tubes; 4ml blood was transferred into K_2EDTA anti-coagulated tubes from Beckton Dikinson, USA and another 4 ml of blood was collected in plain tubes for serum separation. Each tube was labeled with the Study participant's laboratory Serial number.

After complete clot retraction was formed, the serum was separated from the plain tube with centrifugation at 3500 rpm for 5 minute. Then the serum was tested for syphilis using RPR and for HIV antibody using KHB (from KHB, Shanghai, China) which is the rapid immuno-chromatographic test currently used as a first line screening test in the Ethiopian national HIV testing centers.

The blood in k_2EDTA tube from HIV sero-negative reference individuals was transported at room temperature within same day to Addis Ababa regional research and public health laboratory and lymphocyte subset enumeration was performed using the fluorescence activated cell sorter (FACS calibur^{TM}, Becton Dickinson Bioscience, USA).

2.4 Hemoglobin determination

2.4.1 Principle:

When the blood mixed with Drabkin's solution (ferric cyanide), Hemoglobin is oxidized to stable brown cyanmethemoglobin. The intensity of the brown color was measured at 540nm. The absorbance of cyanmethemoglobin directly proportional to the concentration of Hemoglobin in the blood (Fig 2.1).

Reaction:

Hgb (Fe++) + ferricyanide → methemoglobin (Fe+++) + KCN

↓

Cyanmethemoglobin
(stable pigment)
Read at 540 nm.

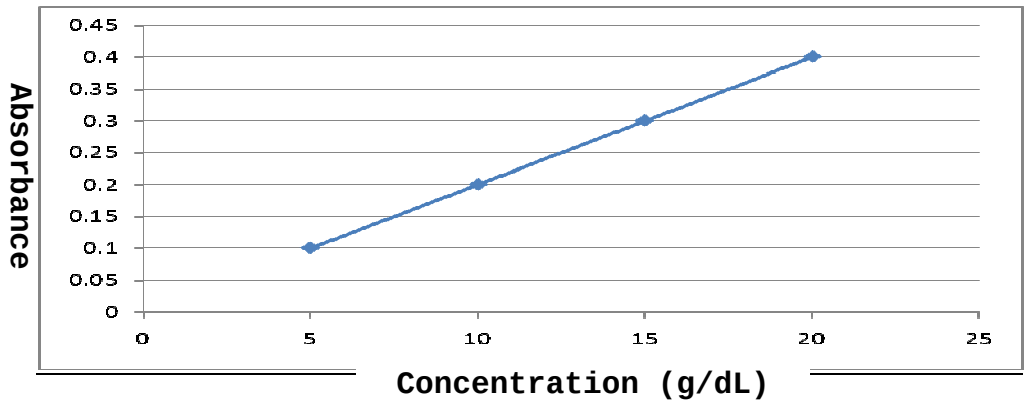


Figure 2.1. *Shows the concentration versus absorbance of hemoglobin*

2.4.2 Materials: Drabkin's ($NaHCO_3$, KCN, and $KFe(CN)_4$)

2.4.3 Procedure

- Twenty microliter of well mixed blood was mixed with 1ml of Drabkin's solution
- After three minute the optical density read at 540 nm
- The result is displayed on the screen as g/dL

2.5 Test for syphilis infection

2.5.1 Principle

The serum contains IgM (anticardiolipin) antibody. It reacts with cardiolipin antigen and forms aggregates. The carbon suspended in the reagent trapped in the aggregate forming a black spot (Fig 2.2a).

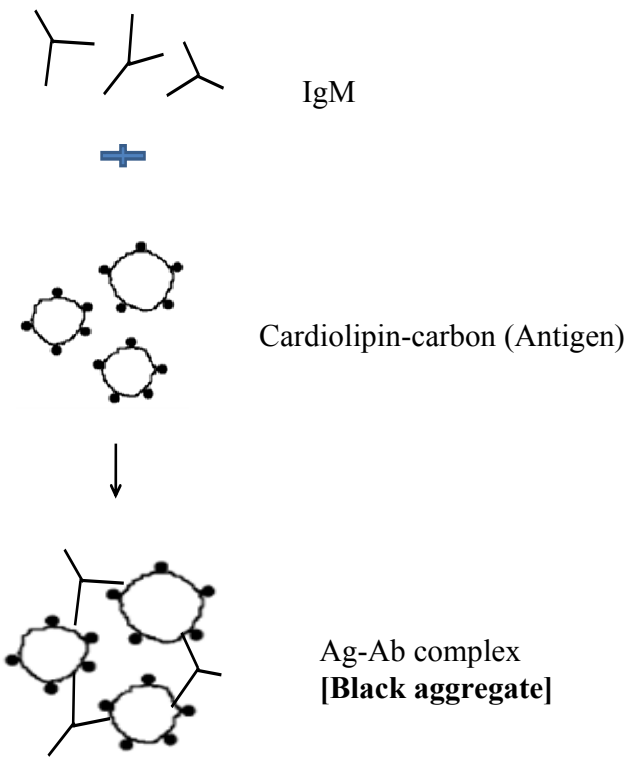
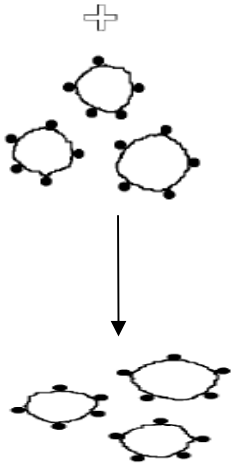


Figure 2.2a. *Positive syphilis test reaction*

No IgM in the sample



No immune complex is formed

[No black aggregate]

Figure 2.2b. *Negative syphilis test reaction*

2.5.2 Materials: carbon particles coated with cardiolipin

2.5.3 Procedure

- The control sera and RPR reagent was brought to room temperature.
- One drop of each of the test and control sera was dispensed on to the test plates and spread carefully in the individual wells.
- One drop of RPR antigen was added to each well of the test plates. The test plates were rotated for 8 minutes at 100rpm.
- Compared the reactions of the test sera with those of the controls for flocculation.

Result:

Positive - visible black aggregate

Negative - no visible black aggregate

2.6 Immunochromatofocusing for HIV

2.6.1 Principle

Fig 2.3a displays the principle of chromatofocusing Antigen-1(gp160) reacts with antibody (Ab-1), if any, in serum. The complex and the serum diffuse to test spot. There Ab1~Ag1 complex reacts with Antigen-2 and form complex-2 a reddish purple product. It indicates Positive sample. The serum continues to diffuse to control spot. Here it reacts with Ab-2 forming Ag-1~Ab-2 complex, a reddish purple complex-3. Equivalent test with negative samples gives complexes given in (Fig 2.3b).

i. At the sample spot

Ag1 on NC paper at the sample spot

+

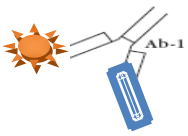
Ab1 from positive sample

Ag1~Ab1 complex at sample site,complex-1
[No color product]

+

ii. At the test spot

Ag2 on NC paper at the test spot



Ag1~Ab1~Ag2 complex ,complex-2 at test site
[Reddish purple product]

iii. At control spot

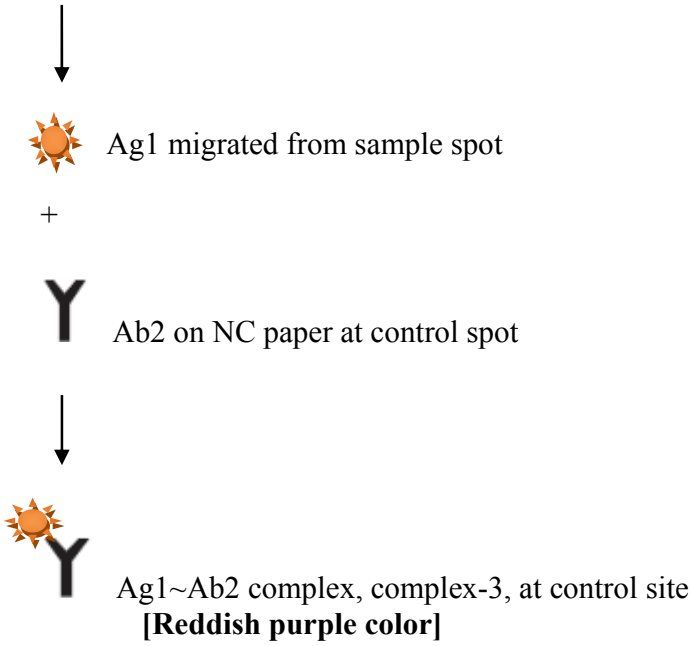
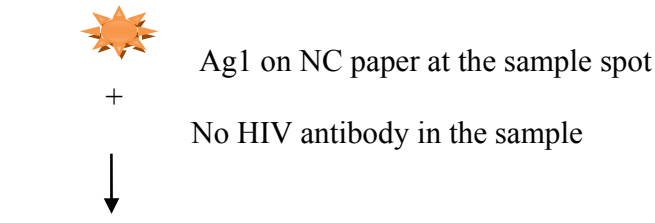


Figure 2.3a. *Indicates reaction of positive sample*

---

i. At Sample spot

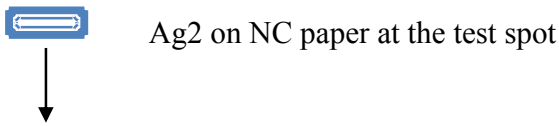


No reaction at sample site
[No color product]

+

ii. At test spot

No Ab1 in the serum



No reaction at test site
[No color product]

iii. At control spot

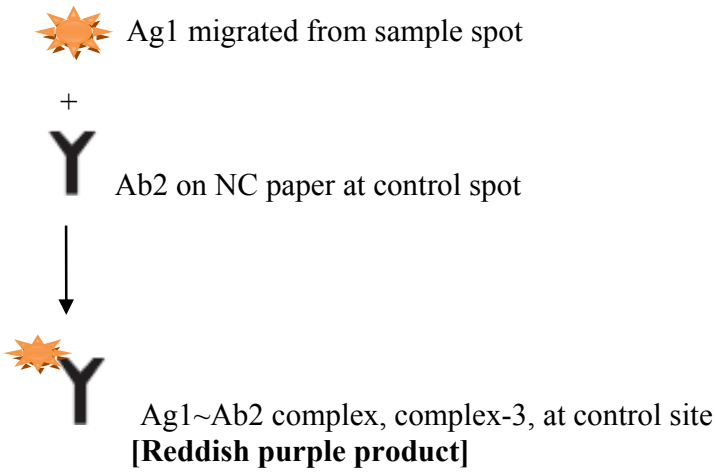


Figure 2.3b. *Shows reaction of negative sample*

2.6.2 Procedure

- 40µl of sample added to the sample spot
- 40µl of diluent is added
- Observe the result after half hour

Result:

Positive = Two red purple color bands (Fig 2.3a)

Negative = One red purple color band is seen at control spot (Fig 2.3b)

2.7 Flow cytometry

2.7.1 Principle

White blood cells count occurs in the following stages:

- Elimination of interfering cells(RBC)
- Recognition of white blood cell sub population
- Cell counting and analysis

A. Elimination of red blood cells

RBCs out number WBCs five hundred to one. They are eliminated by hemolysis. In this particular case diethylene glycol and formaldehyde fulfills this job.

B. Recognition of white blood cells subpopulations

This is achieved by flourochrome tagged monoclonal antibody. The reaction occurs as the cells are diluted. It is followed by bombardment of the cells by 488 nm LASER beam. In turn the three sub groups emit their characteristic light (Table 2.1) to be detected and counted by a specific photomultiplier.

In flow cytometry each wave length is recognized by unique photomultiplier, i.e. it is multichannel equipment (Fig 2.5). The collected information is processed by comparing it with emission from beads with identical wave length. The data is calculated and displayed automatically as indicated below.

Cells	Absolute count	Percentage (%)
$CD3^+$ lymphocytes(total T cells)	✓	✓
$CD4^+$ lymphocytes(helper T cells)	✓	✓
$CD45^+$ lymphocytes(total T and B lymphocytes)	✓	100

Calculation: The machine uses the following formula to determine absolute count (cells/μL).

$$\frac{\text{Observed count of interest population}}{\text{Observed reference bead count}} \times \frac{\text{reference bead count}}{\text{sample volume (μL)}}$$

Table 2.1 *Cells recognized by flurochrome tagged monoclonal antibody and their emission spectra*

Cells recognized	***Monoclonal antibodies***	***Label***	***Emission Spectra***
$CD3^+$ lymphocytes	Anti CD3	Fluorescein isothiocyanate (FITC)	530nm
$CD4^+$ lymphocytes	Anti CD4	Phycoerythrin (PE)	580nm
$CD45^+$ lymphocyte	Anti CD45	Peridinin chlorophyll protein (PerCp)	670nm

2.7.2 Materials

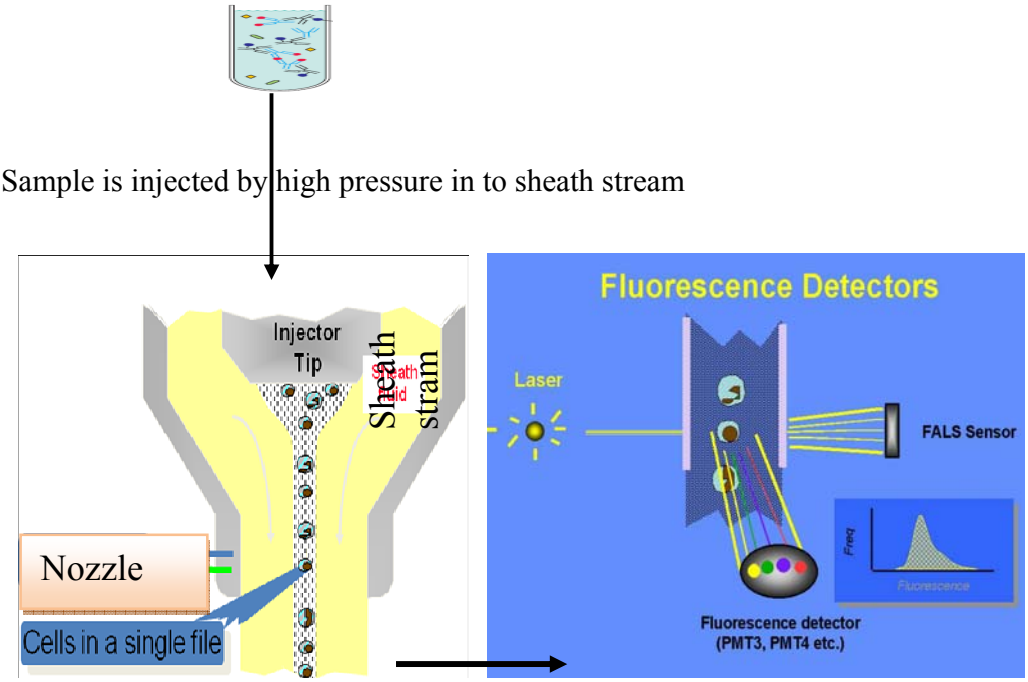
- Tubes that have control beads
- Flourochrome tagged monoclonal antibody
- Diethylene glycol and formaldehyde (Hemolser)
- Pipette tips
- Micropipettes

2.7.3 Procedure (see table 2.2)

Table 2.2 *Assay*

Additives	**Volume**
Fluorescent tagged monoclonal antibody	20µL
Sample	50µL
Mix and incubate at room temperature for 15 minute	
Hemolyser	450µL
Mix and incubate at room temperature for 15 minute	
Collect the data at three different wave length	

I. Sample is injected by high pressure in to sheath stream



II. Counting lymphocytes by comparing their emission with that of beads

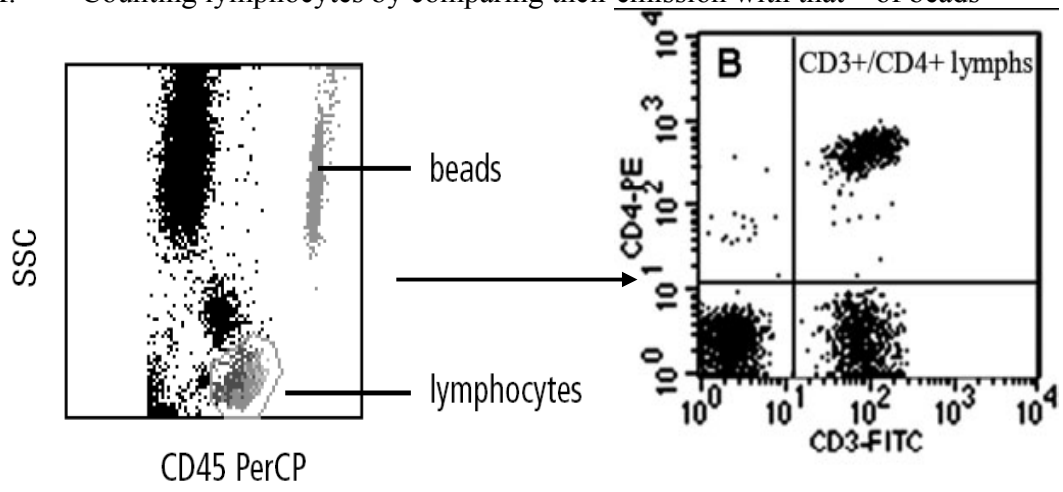


Figure 2.4 *Fluorescence activated light sorting*

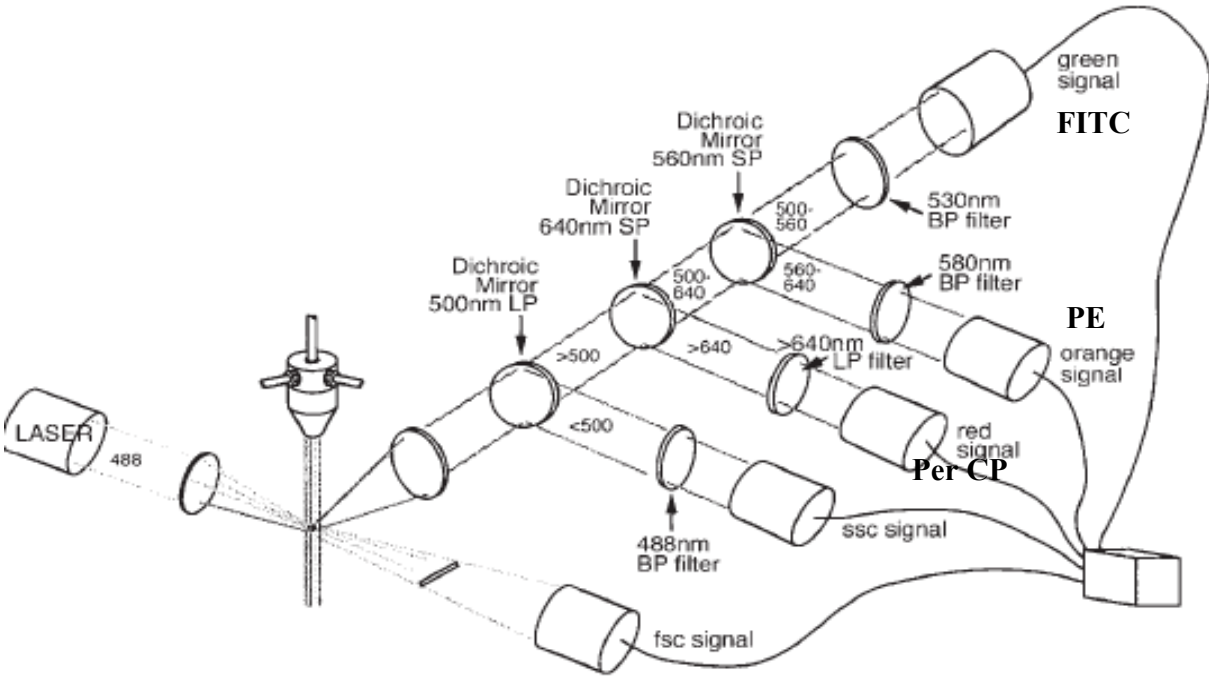


***Figure 2.5**. Schematic diagram of flow cytometer that shows excitation and emission spectra of flourochromes (FITC, PE and PerCP).*

2.8 Statistical analysis

Data were entered and analyzed with the Excel data sheet and STATA.9 (STATA version 9, Stata Corporation, College Station, Texas, USA). Mean, median, and standard deviation were calculated for each T-lymphocytic parameter. The 95 percentile reference ranges were determined by using 2.5^{th} and 97.5^{th} percentiles. The non parametric Wilcoxon rank-sum test (Mann-Whitney test) was used to compare the distribution of T-lymphocytic parameters between trimesters. Correlation analysis was performed to see associations between age, gestational age and T cell subsets. P value <0.05 was considered as statistically significant.

3. Result

3.1 Demographic characteristics

In this study 120 HIV sero-negative pregnant women attending antenatal care service at Yekatit 12 hospital were involved; though a total of 131 voluntary individuals were agreed to participate in the study, eleven (8.4%) of them excluded from the study. Three of them were HIV positive, six of them had sign of infection during the study period and two of them had incomplete demographic data. All the participants were residents of Addis Ababa, and from different ethnic and religion groups; Amhara 65 (54.17 %), Oromo 25 (20.83%), South (Gurage, Welayta, Dorze, Kenbata) 22 (18.33%), and Tigray 8(6.67%). Ninety (75%) were Orthodox Christians, 16 Muslims (13.33%) and 14 (11.67%) were Protestants. Most of them were married, 117(95%) and 3 (2.5%) were unmarried. The age ranges between 18 and 36 years with mean ± SD and median of 25.44 ± 4.93 and 25 years [IQR, 21 to 28 years], respectively. The majority of subjects were in the age groups <25 (49.17%), followed by 25-34 years (42.5%) ≥35years (8.33%) (Fig 3.1). The mean ± SD and the median height were 160.01± 4.71 cm and 159 cm [IQR 153 to 170cm], respectively (Table 3.1). None of them were smokers.

Table 3.1: *Age, height, weight, gestational age description of the study participants (n=120).*

Parameters	***Mean(SD)***	***Median***	***IQR***
Age(year)	25.44 (4.93)	25	18- 37
Height(cm)	160.01 (4.71)	159	152- 175
Weight(kg)	58.05 (6.59)	58	41- 85
Gestational age (wks)	20.93 (7.17)	21	4- 37

Among the participants 24(20%) of them were at first trimester (≤14weeks); 84(70%) in the second trimester (15-28 weeks) and 12(10.0%) in the third trimester (>28weeks) (Table 3.2).

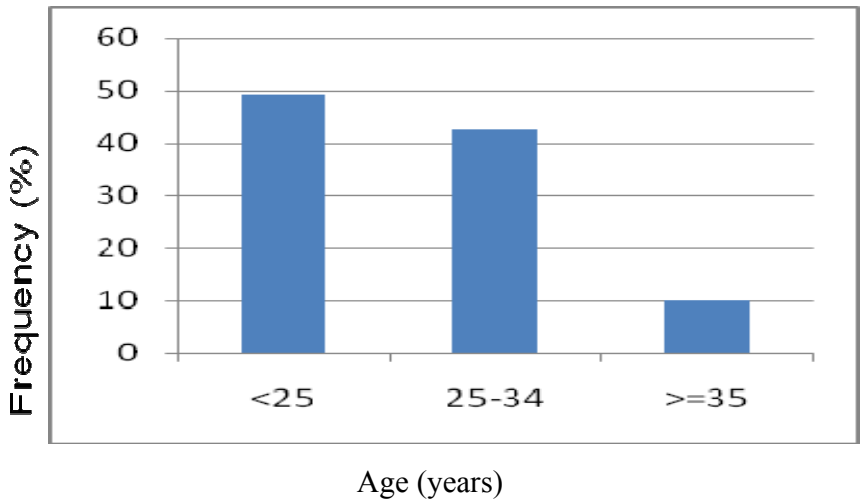


Figure 3.1 *Age distribution of the study Participants*

All the subjects included in the study were free of recognizable infection and had no any clinical symptoms of infection in the last one month, based on the standard stated in the study (Annex III).

3.2 Laboratory variables

The laboratory analysis was performed at Addis Ababa Health Bureau Regional Research and Public Health Laboratory located in Cherkos sub-city.

3.2.1 Absolute CD4+ T-lymphocyte count

The mean and median absolute CD4+ T-lymphocyte count was 671 ± 225, 632.5 cells/µl [IQR: 503-824 cell/µl], respectively. The 2.5th and 97.5th percentile distribution of CD4+ T cells was 309.9 and 1185.4 cells/µl, respectively. The CD4+ T cell counts were divided into three categories (category I: less than 200, category II: 200-499, category III :≥ 500 cells/µl. There was no observation in category I. The data showed that 11.76 and 88.24% of the first, 31.25 and 68.75% of the second and 8.7 and 91.30% of third trimester were in category II and III respectively.

There was an inverse correlation between age advancement and absolute $CD4^+$ T cell count; however, the age dependent change had marginal statistical significance (Fig 3.2).

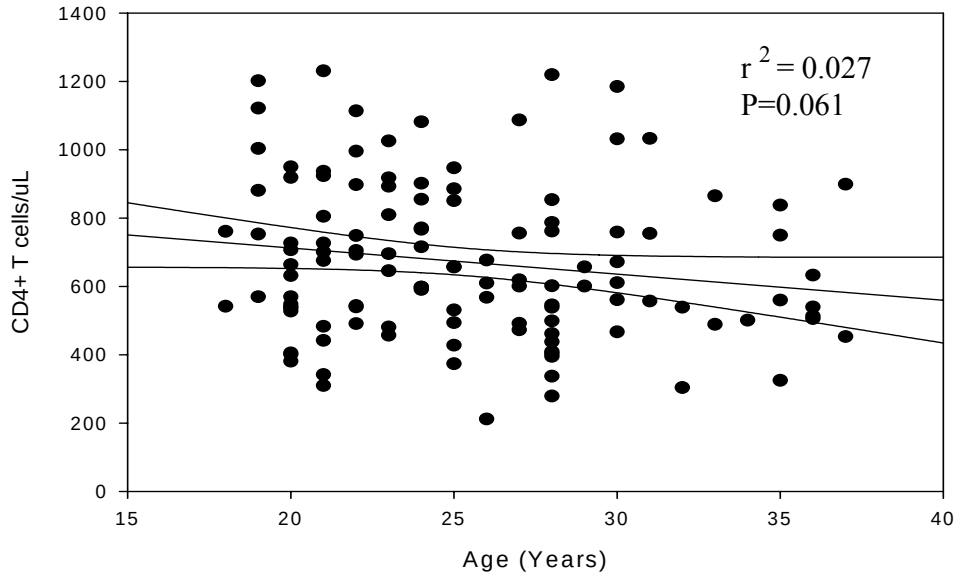


Figure 3.2 *Regression fit of absolute CD4$^+$ T cell versus age*

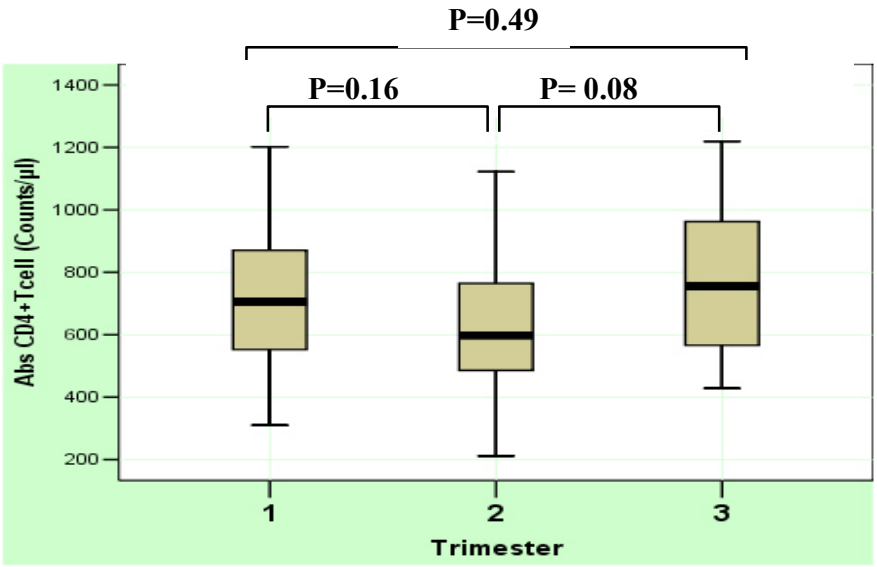


Figure 3.3 *Box plot showing absolute CD4$^+$ T cell counts by Trimester.*

As it is shown in Figure 3.3 and Table 3.2, the median CD4$^+$ T cell count was higher in the third trimester, particularly with marginal significance compared to the second trimester.

Table3.2 *Trimester adjusted mean (SD), median, central 95% distribution of absolute CD4+ T cell count.*

Trimester	*Number of subjects (%)*	*Mean (SD)*	*Median*	*95% Reference range*
First [a]	24 (20%)	715(221)	675	403-1082
Second [b]	86 (70%)	643(216)	599	337-1033
Third [c]	12 (10%)	785(261)	755	428-1220
Total	120(100%)	671(225)	632	310-1185

a compared to b, Mann-Whitney, p.value =NS
b compared to c, Mann-Whitney, p.value =NS
a compared to c, Mann-Whitney, p.value =NS
NS= non significant

3.2.2 CD4$^+$ T-lymphocyte percentage

The mean and median CD4+ T-lymphocyte percentage (CD4+T %) was 40.8% ± 8.7% and 42.5% [IQR: 37-47%], respectively (Table 3.3). The 2.5th and 97.5th percentile distribution of % CD4$^+$ T cells was 17.9% and 54 %, respectively. As shown in the (table 3.3), (Figure3.4) %CD4$^+$ T cells were stable throughout the gestation period and did not have correlation with the age of pregnant women as well (Figure 3.5).

Table 3.3 *Trimester adjusted CD4%; mean (SD), Median, 95% reference interval*

Trimester	*Number of subjects (%)*	*Mean (SD)*	*Median*	*95% Reference range*
First[a]	22 (20%)	41.2 (5.7)	42.5	28.4-48.0
Second[b]	86 (70%)	40.3 (9.6)	42.0	17.1-54.0
Third[c]	12 (10%)	43.1 (6.2)	43.0	32.1-50.7
Total	120(100%)	40.8 (8.7)	42.5	17.9-54.0

a compared to b, Mann-Whitney test, p.value =NS

b compared to c, Mann-Whitney test, p.value =NS

a compared to c, Mann-Whitney test, p.value =NS

NS= non significant

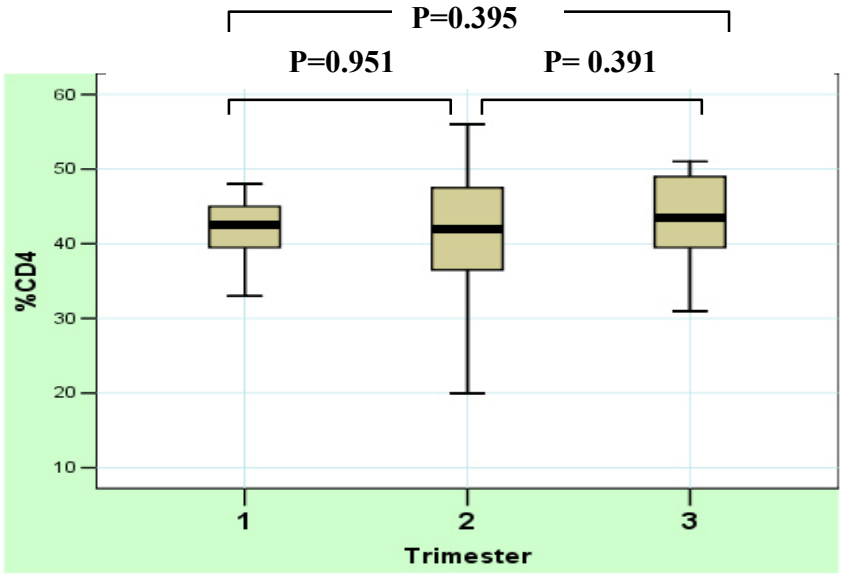


Figure 3.4 *Box plot showing CD4+ T cell percentage by Trimester*

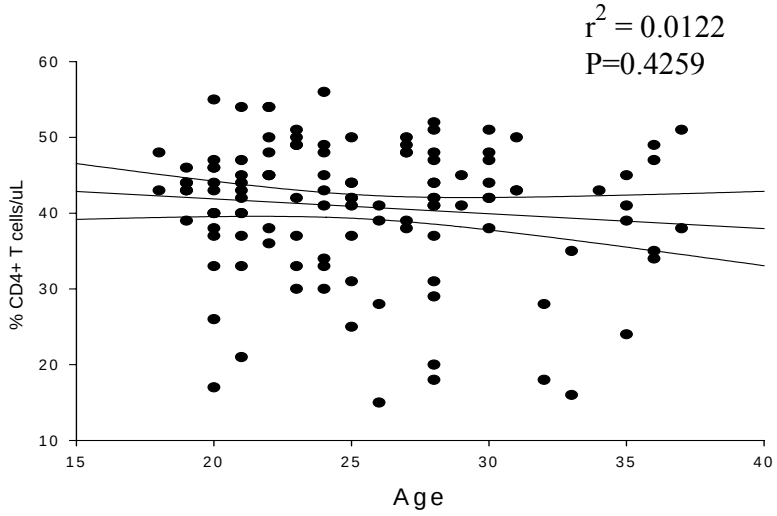


Figure 3.5 *Regression fit of %CD4$^+$ T cells versus age*

3.2.3 Absolute CD3+ T-lymphocyte count

The mean and median of CD3$^+$ T cell count was 1213.6 ± 368.4 and 1165.5 cells/µl [IQR: 916-1480 cells/µl], respectively. The 2.5th and 97.5th percentiles were 663.1 and 2061 cells/µl (Table 3.4). Absolute CD3$^+$ T cell count was slightly negatively correlated with age (r^2=0.02). The absolute T-cell count was compared between trimesters. Although there were no statistically significant differences between the three trimesters, the value in the second trimester was lower than in the other two trimesters.

Table 3.4 *Trimester adjusted absolute CD3+ T-cell count mean (SD), Median, 95% reference interval*

Trimester	***Number of subjects***	***Mean (SD)***	***Median***	***95% Reference range***
First[a]	24(20%)	1222.95 (387.4)	1138	681.0-1955.3
Second[b]	84(70%)	1183.4 (343.7)	1156.5	665.3-1921.3
Third[c]	12(10%)	1413.1 (466.5)	1352	808.1-2189.2
Total	120(100%)	1213.6 (368.4)	1165.5	603.1-2061

a compared to b, Mann-Whitney test, p.value =NS

b compared to c, Mann-Whitney test, p.value =NS

a compared to c, Mann-Whitney test, p.value =NS

NS= non significant

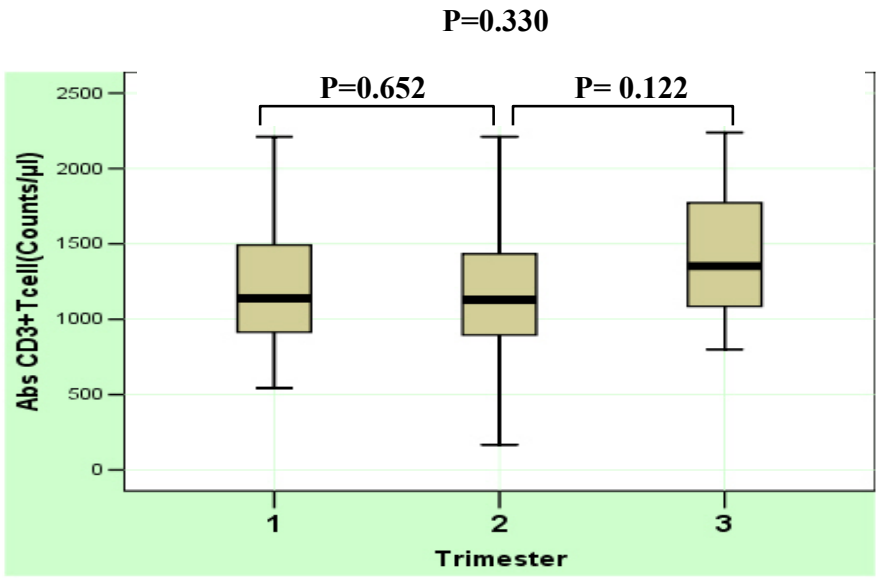


Figure 3.6 *Box plot showing absolute CD3+ T cell count by Trimester.*

3.2.4 $CD3^+$ T-cells Percentage

The mean and median of %CD3+ T-cells was 73.4 ± 10.3% and 76[IQR 71-80%), respectively. The 2.5^{th} and 97.5^{th} distribution was 39 and 85 %, respectively.

The median and IQR value of first, second and third trimester was 72% [IQR: 66-76%], 76% [IQR: 71-80%], 77.5% [IQR: 74-83%], respectively. The respective 95% ranges for the three trimesters were 48.5-81.5%, 39.1-85%, 69.0-83.7%, respectively (Table 3.5).

There was a positive correlation between % $CD3^+$ T cells and gestational age in which the CD3% show gradual increase as the gestational age increased. As shown in (Table 3.5) , the median percentage $CD3^+$ T cell vale in the first trimester is significantly lower compared to both the second (P=0.021) and the third trimester (P=0.023).

Table 3.5 *Trimester adjusted CD3+ T-cell percentage mean (SD), Median, 95%reference interval of subjects*

Trimester	***Number of subjects***	***Mean (SD)***	***Median***	***95% Reference range***
First[a]	24(20%)	70.3(9)	72.0	48.5-81.5
Second[b]	84(70%)	73.0(10)	76.0	39.3-85.0
Third[c]	12(10%)	76.0(4.3)	77.5	69.8-83.0
Total	120(100%)	73.4(10.3)	76.0	39.0-85.0

a compared to b, Mann-Whitney test, p.value =0.021

b compared to c, Mann-Whitney test, p.value =NS

a compared to c, Mann-Whitney test, p.value =0.023

NS= non significant

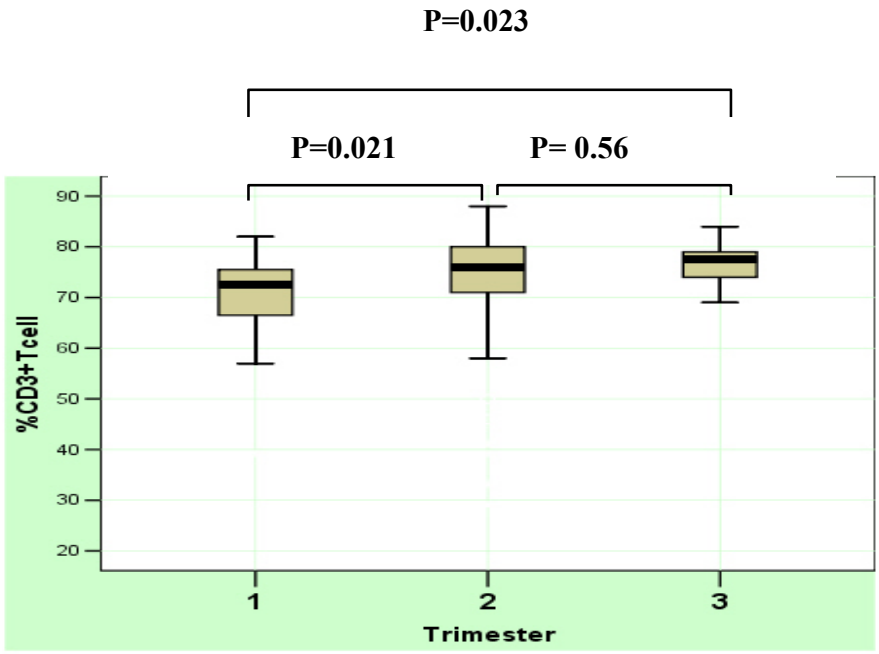


Figure 3.7 *Box plot showing percent CD3+ T cell by Trimester*

3.2.5 Absolute $CD45^+$ lymphocyte count

The mean and median $CD45^+$ lymphocyte absolute count was 1684cell/µl 1532.5 cells/µl respectively (IQR 1264.5-1999.0). The 2.5^{th} and 97.5^{th} percentile distribution 952.3-2984.9 cells/µl respectively.

After gestational age adjustment a decreased value of absolute count observed at the second trimester. In the third trimester an increased value was observed than the two trimesters, though it was not statistically significant (Table 3.6).

Table 3.6 *Trimester adjusted CD45+ lymphocyte count mean (SD), Median, 95% reference interval*

Trimester	***Number of subjects***	***Mean (SD)***	***Median***	***95% Reference range***
First[a]	24(20%)	1770(603)	1677	956-2874
Second[b]	86(70%)	1628(581)	1520	895-2981
Third[c]	12(10%)	1827(563)	1703	1086-2733
Total	120(100%)	1684(583)	1532	952-2985

a compared to b, Mann-Whitney test, p.value =NS

b compared to c, Mann-Whitney test, p.value =NS

a compared to c, Mann-Whitney test, p.value =NS

NS= non significant

4 Discussion

The current study was conducted on 120 HIV sero-negative pregnant women attending antenatal care at Yekatit 12 hospital. It is a general hospital and one of the first centers for ART and PMTCT programs. Since 2005 Ethiopia lunched free ART and PMTCT program for people living with HIV/AIDS, and prevention of mother to child transmission, all over the country. This study determines the values of $CD4^+$ T cells, $\%CD4^+$ T cells, $CD3^+$ T cells, $\%CD3^+$ T cells and $CD45^+$ lymphocytes of sero-negative pregnant women.

The study demonstrated 95% central reference interval of absolute $CD4^+$ T cell count to be 310-1185 cells/µl. The data therefore, showed that the lower cut-off is much below the clinically significant level of 500cells/µl set by WHO and adopted by Ethiopian MOH [46, 47]. It is also slightly lower than reference values established for Ethiopian non pregnant females (456-1368 cells/ µl) [14] , (386-1355 cells/µl) [65] , and also test kit manufacturer (410-1590 cells/µl).

In agreement with our findings other published studies reported low T cells reference range for pregnant than non pregnant women [66-71]. In contrary study from Germany [72], Brithish [82] reported increased $CD4^+$ T cell number during pregnancy. A study from Swiss [73] reported no significant differences in $CD4^+$ T cell counts between HIV-infected pregnant women and non pregnant controls.

The mean $CD4^+$ T lymphocyte count among pregnant women in our study was 671 cells /µl which is lower than that of Nigerian[66], Malawian[74], Tanzanian[75], German[72], Indian[54] pregnant women and higher than Botswanian [69] pregnant women. However, there is no published data in Ethiopian pregnant women though the value is lower than reported for non pregnant Ethiopian women [14]. This finding of variation in $CD4^+$ T cell levels with pregnancy has clinical implications. In the Ethiopian guideline as in other resource limited settings, the decision on when to initiate HAART is mainly based on the clinical stage and on the absolute $CD4^+$ T cell counts and the HAART starting criteria are the same in pregnant women as in other adults [47].

Our study found a marginally significant association between age and absolute $CD4^+$ T cell count, this is consistent with a study [13] However; the study in Ethiopia [14],in Zimbabwe [71] reported no correlation between age and $CD4^+$ T cells.

The documented relationship between gestational age and $CD4^+$ T cell levels in pregnant women varies in the literature. Our study did not found significant association between gestational age and $CD4^+$ T cell count. In agreement with our study from Nigerian [66] reported no association between $CD4^+$ T cells count and gestational age; a study from Kenia [67] reported no relationship between gestational age and $CD4^+$ T cell counts in both HIV-positive and HIV-negative women; but another study [78] reported an increase in $CD4^+$T cell counts of 2.76 cells/µl per week of pregnancy.

Our study observed decreased mean of $CD4^+$ T cell counts in the second trimester than the first and third (715, 643,785 cells/µl first, second, third respectively). This pattern of observation is similar to reports from Italy [79], from Malawi [74], and from Germany [72]. The increased $CD4^+$ T cells value in the third trimester may be due to immune activation in the late pregnancy period as suggested [80].

In this study the mean $CD4^+$ T-lymphocyte percentage (CD4%) was 40.8% and the central 95% reference range was 17.9–54.0%. This is different from the values given by test kit manufacturers (31-60%) and from that reported for Ethiopian Adult females (29-55%) [65]. In agreement with our study [81] reported decreased value of CD4 % in pregnant women compared to their non-pregnant counterparts. On the other hand, contrary to our study higher values of CD4% during pregnancy was also reported from Kenya [67], from British [82].
The study in India [54] reported no difference in the value of CD4% between pregnant and non pregnant women.

In our study CD4% remains stable in all three trimesters (mean 41%,40%,43%) this is in agreement with [57,72,74] , and others have reported decrease percentage of $CD4^+$ T cells during early pregnancy and increased in later pregnancy [82]. Our findings of stable %CD4 across the different trimesters and that the absolute $CD4^+$ T cell count could experience

variations during pregnancy has implications in the management of HIV infected pregnant women in Ethiopia. The finding indicates that the CD4 percentage could be more reliable than the absolute count in pregnant women in accurately targeting the most appropriate time to start HAART.

Targeting of the time to start HAART in pregnant women is important because of the sensitive ratio between the risks and the benefits of HAART during pregnancy. Starting HAART earlier could lead to benefits in preventing MTCT. On the other hand, starting HAART too early may expose non infected children to drug side effects that still need to be fully assessed [60, 61]. Different side effects including rash and hepatic toxicity with Neverapine (NVP) containing HAART, the most commonly used first-line regimen in resource-limited settings, has been suggested to be significantly higher in female patients with higher CD4+ T cell counts including during pregnancy [64].
In our study, $CD4^+$ T cell % did not show significant level of association with age as well as gestational age. This agrees with studies reported elsewhere [57, 74, 77]

In this study mean absolute $CD3^+$ T- cell count was 1213 cells/µl, and the central 95% reference interval was 663-2061 cells/µl. The upper limit is different from the values given by test kit manufacturers (690-2,540 cells/µl), but both the lower and the upper limit in our study is lower than that reported for non pregnant Ethiopian females (871-2,413 cells /µl) [14], (741-2,329 cells /µl)[65]. Our absolute $CD3^+$ T cell count showed a slight negative correlation with age and this is in agreement with other study [52]. Although it was not statistically significant, absolute $CD3^+$ T cell count decreased at the second trimester than the first and third (mean 1222, 1183, 1413 cells/µl respectively for first, second and third trimesters). This is similar to the study in Germany [72]. In contract, elevated value in $CD3^+$ T cell count in pregnant women than non pregnant with significant increase in the first trimester were reported from studies elsewhere [74, 83].
The mean % $CD3^+$ T-cells was 73.4%.The central 95% reference interval was 39-85%. The lower limit of the present study was lower than that of test kit manufacturer (55-84%). In our study the $CD3^+$ T cells percentage tends to positively associate with the gestational age, though the association was not statistically significant. A statistically significant difference was observed

between trimesters; The %$CD3^+$ T cells value of the first trimester was significantly lower than the values in the second (P=0.02), and third trimesters (P=0.03). The difference between the second and the third trimesters was not statistically significant. Similar result was reported [83] and his group. This may be because of the immune activation during late pregnancy [80].

In our study the mean $CD45^+$ T-lymphocyte count was 1684 cells/µl and the central 95% reference range was 952-2985 cells/µl. This was lower than that reported for Ethiopian Adult females [14] (mean 1857 cells/µl), and 95% reference range (1,032-3,432 cells/µl); Zimbabweans' [91] (mean 1800 cells /µl). In agreement with our study others [79] reported decreased absolute count in the second trimester than the first and third trimesters.

5 Limitations of the study

The limitation in our study was disproportionate number of participants between the three trimesters which made difficulty in comparison between such groups. Different statistical approaches, method and instrumentation used by the different studies also made comparison of results with previous studies difficult.

6 Conclusion

Our results for T lymphocyte populations in pregnant women were different from those of test kit manufacturer and the values obtained for non-pregnant Ethiopian women.

The reference values obtained were 310-1185 cells/µl for absolute $CD4^+$ T cells; 17.9-54.0% for $CD4^+$ T cells percentage; 690-2540 cells/µl for absolute $CD3^+$ T; 55-84% for $CD3^+$ T cells percentage; 952-2985 cells/ µl for absolute $CD45^+$ lymphocyte.

7 Recommendations

The clinicians should be aware of decreased value of $CD4^+$ T cells during pregnancy is a physiological phenomenon. Before deciding to initiate ART in pregnant women that have a lot of side effects in the fetus life, the low back ground $CD4^+$ T cell levels in pregnant women should be taken into considerations.

For the assessment of immune suppression in HIV infected pregnant women $CD4^+$ T cell percentage (CD4%) is more reliable than absolute count in pregnant women.

The findings of the present study though important, are based on small sample size at specific geography and cannot be generalized. Studies on larger sample size need to be done to confirm these findings.

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Ethical Considerations

The study protocol was evaluated and Ethical clearance obtained from Addis Ababa University Medical Faculty Institutional Review Board, Addis Ababa university Black lion specialized hospital gynecology and obstetrics department and Addis Ababa Health Bureau ethical clearance committees. Before enrollment, written consent was obtained from subjects for questionnaire and blood sample collection. Subjects positive for syphilis and/or HIV were linked to the ANC clinic.

Annex I Consent form

Participant's Code number_____

Addis Ababa University, faculty of medicine, department of Biochemistry has designed a study on lymphocyte subset (CD4+ lymphocyte)

I have been informed about the study, which plans to establish reference interval of CD4+ lymphocytes in pregnant women. The objective and the application of the study were explained to me. I am also informed that all information contained within the questionnaire is to be kept confidential. Moreover, I have also been well informed of my right to refuse information, decline to cooperate and drop out of the study if I want and that none of my actions will have any bearing at all on my overall health care and hospital access.

It is therefore with full understanding of the situations that I agreed to give the informed consent voluntarily to the researcher to use the specimen (8 ml blood) for the mentioned study.

I also agreed that the specimen might be stored for further investigation to be done. Moreover I have had the opportunity to ask questions about the project and I have received clarifications in a language I understand.

I was also told that results for the hematological tests and other laboratory investigations would be reported timely to the nurse in charge of counseling and that I may ask my result if I want.

I----------hereby give my consent for giving of the requested information and specimen for the purpose of the study.

	Signature:	Date
Participant:	_____	_____
Nurse (counselor)	_____	_____

Annex II Consent form (Amharic Version)

በጥናት ለመሳተፍ ስምምነት ቅጽ

የተሳታፊ ቁጥር_____

የአዲስ አበባ ዩንቨርስቲ ህክምና ፋኩልቲ የባዮ ኬሚስትሪ ትምህርት ክፍል በነጭ ደም ህዋሳት (ሲ ዲ 4 እና ሲ ዲ 8) ላይ ጥናት ለማካሄድ አቅዷል፡፡

የዚህ ጥናት ዓላማ የነፍሰ ጡር እናቶችን የሲድ 4 እና የሲድ 8 ነጭ የደም ሴል ህዋሳትን ልኬት መጠን ለመወሰን እንደሆነና ጥናቱ ሲጠናቀቅ የሚሰጠው ጥቅምና አገልገሎት ተገልፆልኛል፡፡ በተጨማሪ የሰጠሁት ማንኛውም መረጃ በሚስጥር እንደሚጠበቅልኝ የተገለፀልኝ ሲሆን በጥናቱ ያለመሳተፍ መብቴ እንደሆነ፣ ከዚህ በተጨማሪም የማልፈልገውን መረጃ ያለመስጠት እንደምችልና ከጥናቱ በማንኛውም ጊዜ ማቋረጥ እንደምችል ይህንንም በማድረጌ ከጤና ድርጅቱ በማገኘው አገልግሎት ላይ ምንም ዓይነት ተፅዕኖ እንደማያስከትልብኝ ተብራርቶልኛል፡፡ በተጨማሪም ጥያቄ እንድጠይቅ ዕድሉ ተሰጥቶኝ በማውቀው ቋንቋ ማብራሪያ ተሰጥቶኛል፡፡

በዚሁ መሰረት የጥናቱን ዓላማ በመረዳት ለጥናቱም የሚሆን 8 ሚ.ሊ የደም ናሙና ልሰጥና የሰጠሁት ናሙና ለቀጣይ ምርመራ ቢያስፈልግ በማንኛውም መልኩ እንዲቀመጥ ተስማምቻለሁ፡፡

የሰጠሁት የደም ናሙና ውጤት በአማካሪ ነርስ በኩል ማግኘት እንደምችልና አስፈላጊ ሆኖ ከተገኘ ውጤቱ ለጤና ክትትል ወደሚመለከተው ህክምና ክፍል ሪፖርት እንደሚደረግልኝ ተገልፆልኛል፡፡

እኔ_____በዚህ ጥናት ለመሳተፍ መስማማቴን በፊርማዬ አተጋግጣለሁ፡፡

	ፊርማ	**ቀን**
ተሳታፊ	_____	_____
ነርስ	_____	_____

Annex III Questionnaire

Information for the participant:

Dear participants of this study, we appreciate in advance for being part of this study and providing genuine information. This study is initiated by AAU, Faculty of Medicine, department of Biochemistry.

SECTION 1-questionner identification

(1) Respondent's Identification code ----------

(2) Where is your Birth place? Region_____

(3)Urban/Rural

(4)For how long have/had you been in your birth place?_____ years

(5)Age_____ (yrs) (6) Sex-----

SECTION 2- Socio economic and demographic characteristics

(7) What is your marital status? Single, Married, Divorced, widowed

(8) What is your Religion? Orthodox/Muslim/Catholic

Protestant/Others ----------

(9) What is your ethnic group? Amhara /Oromo/Tigrię

South/Others --------

SECTION 3- Anthropometrical and Clinical information

(10) Height _____,

(11) Weight _____,

(12) Hemoglobin %----------

(13) Blood pressure; Systolic/Diastolic _____/_____

(14) Heart Beat _____/minute

(15) Body Temperature _____

(16) Week of gestation----------

(17) Have you ever been sick for the last one month? Yes/ No

If yes, what was your illness? Febrile illness, Respiratory. Others specify ----------

SECTION –4 Alcohol drinking habit.

If you don't drink alcohol at all skip this section.

(18) How often do you drink alcohol? Daily, Every weekend occasionally

SECTION –5 Smoking habit

If you do not have smoking habit skips this section.

(19) How often do you smoke cigarettes? Chain smoker, Social smoker

Thank you!

End

Annex IV. Information sheet

For participants of the study entitled reference value determination of CD4+ lymphocyte and %CD4 Lymphocytes on sero-negative Ethiopian pregnant women.

Information sheet (English version)

Addis Ababa University, Faculty of medicine department of Biochemistry Addis Ababa, Ethiopia

Principal Investigator: - Taye zewdie

Advisors: - Dr Yesehak Worku, Dr R. chawla, Dr. Wondwossen Amogne, Dr. Aster Tsegaye, Dr. Wakgari Deressa, Dr. Dawit Desalegn

Name of the sponsor. Addis Ababa University, faculty of Medicine and department of Biochemistry.

This information sheet is prepared by a researcher for a project with the aim of developing reference values of CD4+T lymphocytes and %CD4 Lymphocyte in sero-negative Ethiopian pregnant women

Aim of the study

Reference values are used for comparism of laboratory results for medical diagnosis or therapeutic management.CD4+ lymphocytes are predictor of HIV disease progression, and essential for decision making when to initiate anti retroviral treatments, and to evaluate the out come of the treatments. This study plan to establish reference intervals for CD4+ lymphocytes in sero-negative pregnant women. Establishing those intervals in this group of population will help in the management of HIV infected pregnant women

Study design and procedure

If you agree to take part in the study, the investigator or a health worker will give you verbal and\or written information about the study and you will be given the consent form to sing. The nurse or health professional will ask you some questions about your general health and perform a complete medical examination and assess whether you qualify to participate in the study. If you are fit for the study blood samples of 8 ml will be collected and test for HIV and syphilis. If negative CD4+ cell enumeration conducted.

Risk and discomfort

Participating in this project will not cause more discomfort than required you could go through for routine examination and ANC service and no need of extra sample other than samples taken

for ANC management but there will be minor pain during blood drawing which disappear with in few minutes. The amount of blood taken from each volunteers is 8 ml which will not affect your health. There is no major risk in participating this research, as the whole procedure is carried out by health professionals following the standard procedure.

Risk and benefit

The result of the laboratory finding will be communicated to your nurse or health professional for use in the management. You will have the chance to know your general health status from the medical examination. The study could benefit HIV positive pregnant women and the concerned bodies that design treatment strategies of the disease. You will not be provided any direct incentives for your participation in the research.

Confidentiality

All information's abut the patient will be kept confidential. Lab log books will not have names but cods.

Right to refuse or withdrawal

You have full right to refuse or with draw from participating in this study at any time before and after consent with out explaining the reason .your decision will not affect your right to get health service you have to get.

Whom to contact

This study protocol is reviewed by Addis Ababa University ethical clearance committee to make sure that research participants are protected from harm. For more information contact the chair person of the committee ----------.

To know more information abut the study you can contact any of the following individuals.

1. Taye Zewdie (+251-91-1176729)
2. Dr. Yesehak Worku (+251-11-5539049)
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6. Dr. Wakgari Deressa (+251-91-1483714)
7. Dr. Dawit Desalgn (+251-91-1234896)
8. Addis Ababa University Medical Faculty Institutional Review Board (IRB)

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Annex V. Information sheet (Amharic version)

የጥናቱ ዓላማ

የሲዲ 4 እና ሲዲ 8 ነጭ የደም ሴሎች የኤች አይቪ በሽታ ደረጃ ለማወቅና የፀረ ኤች አይ ቪ መድሀኒትን ውጤት ለመከታተል የሚረዱ የሰውነታችን ሴሎች ናቸው። የዚህ ጥናት ዓላማ በጤነኛ እናቶች ውስጥ የሚገኘውን የየሲዲ 4 እና የሲዲ 8 ትክክለኛ መጠን በመወሰን በኤች አይ ቪ ለተያዙ እናቶች የበሽታውን ደረጃ ለማነፃፀሪያነት ለመጠቀም ነው።

በጥናቱ ውስጥ የተሳትፎ ሁኔታ

ለመሳተፍ ከተስማሙ ነርሱ ወይም የጤና ባለሙያው ጤነኛ መሆንዎን ለማረጋገጥ የጤና ምርመራ ያደርግልዎታል። ከዚያም መሣተፍ እንደሚችሉና እንደማይችሉ ይነግርዎታል። በጥናቱ መሣተፍ ከቻሉ፣ጥናቱ መሰረት የሚያደርግበት 8 ሚሊ ሊትር ደም በጤና ባለሙያው ይወሰድልዎታል። የናሙና አወሳሰዱ ማንኛውንም ደም ለወላድ ክትትል ለመመርመር ከሚከተሉት ሂደት የተለየ አይደለም። በጥናቱ በመሳተፍዎ ለምርመራ ከሚሰጡት የተለየ ተጨማሪ ናሙና መስጠት አይጠበቅብዎትም። ከሰጡት የደም ናሙና ላይ የኤች.አይ.ቪ፤ ቂጢኝ ምርመራ ይደረጋል። የደምናሙና የሚሠጡት የምክር አገልግሎት ካገኙ በኋላ ሲሆን፣ የምርመራ ውጤቱን እንዲያውቁ አይገደዱም። ውጤትን ማወቅ ከፈለጉ ከምርመራ በኋላ በሠለጠኑ ባለሙያ የምክር አገልግሎት እንዲያገኙ ይደረጋል።

ሊከሰቱ ስለሚችሉ ስጋቶች እና የምቾት መጓደል

በዚህ ፕሮጀክት ተሳታፊ መሆንዎ እንደተለመደው ለእናቶች ጤና ክትትል ከሚያደርጉት የተለየ የምቾት መጓደል አያስከትልብዎትም። ነገር ግን ናሙና በሚወሰድበት ጊዜ በጥቂት ደቂቃ ሊጠፉ የሚችል መጠነኛ የሆነ ህመም ስሜት ሊያስከትል ይችላል። እንዲሁም የናሙና አወሳሰዱና ሌሎች ቅደም ተከተሎች የሚከናወኑት በነርሱ ወይንም በሰለጠኑ የህክምና ባለሙያ የህክምና ደንብ በሚፈቅደው የንጽህና አጠባበቅ ደረጃ በመሆኑ ይህ ነው የሚባል ስጋት በእርስዎም ሆነ በልጅዎ ላይ የለም።

ጥቅሞችና ማካካሻ

ከላብራቶሪ ምርመራ የተገኘው ውጤት ከጤና ባለሙያው ጋር በመነጋገር በሽታውን ለመቆጣጠር ጥቅም ላይ ሊውል ሊችላል። አጠቃላይ ጤና ምርመራ ይደረግልዎታል። በዚህ ምርመራ የተለየ ወይን ያልተጠበቀ ውጤት ቢታይ አስፈላጊ ህክምና የሚያገኙበት ሁኔታ ይመቻችልዎታል። በዚህ ጥናት ተሣታፊ በመሆንዎ የተለየ ጥቅም አያገኙም። ነገር ግን ከጥናቱ በተያያዘ ለሚደረግልዎ አጠቃላይ የጤና ምርመራ ወጪ በፕሮጀክቱ ይሸፈናል።

ሚስጥር ስለመጠበቅ

ሁሉም የሰጡን መልሶች በሚስጥር ይጠበቃሉ።በቤተ ሙከራ የሚቀመጠው መዝገብ ምንም አይነት የህመምተኛ ሥም አይኖረውም። የህመምተኛውን ስምና ኮድ የያዘው የመረጃ ቅፅ ተቆልፎ ይቀመጣል። ይህ መረጃ በምንም አይነት ከዋናው ተመራማሪና ከጤና ባለሙያው በስተቀር ለማንም አይገለፅም። የተሰበሰበው ናሙና ከጥናቱ ዓላማ ውጪ ለሌላ ዓላማ አይውልም። የጥናቱ ሪፖርት ይፋ በሚሆንበት ጊዜ የእርስዎ ስም አይገለፅም ።

በጥናቱ ያለመሣተፍና እራስን የማግለል መብት

በጥናቱ ያለመሣተፍ ሙሉ መብት አለዎት። ጥናቱ ከተጀመረ በኋላ በማንኛውም ሰዓት ራስዎን ከጥናቱ ማግለል ይችላሉ። ይህን በማድረግዎ ምንም ዓይነት የእንክብካቤ መጓደል አያስከትልብዎትም። ለሚወስኑት ውሣኔ ምንም ሰው ምክንይቱን እንዲገልፁ አይስገድዶትም።

መረጃ ስለ ማግኘት

ይህ ጥናት፣ ከአዲስ አበባ ዩኒቨርሲቲ የህክምና ፋክልቲ ኢቲካል ኮሚቴ ክሊራንስ አግኝቷል። የዚህ ኮሚቴ ዋና አላማ የጥናቱ ተሳታፊዎች ከጉዳት መጠበቃቸውን ለማረጋገጥ ነው። በማንኛውም ግዜ ጥያቄ መጠየቅ ከፈለጉ ከዙህ በታች ከተጠቀሱት አንዱን ማናገር ይችላሉ።

1. ታዬ ዘውዴ (251-911-176729)
2. ዶ/ር ይስሀቅ ወርቁ (251-911-539049)
3. ዶ/ር ሪቻውላ (251-911-505248)
4. ዶ/ር ወንድወሰን አሞኘ (251-911-406179)
5. ዶ/ር አስቴር ፀጋዬ (251-911-696085)
6. ዶ/ር ዋቅጋሪ ደሬሳ (251-911-483714)
7. ዶ/ር ዳዊት ደሳለኝ (251-911-234896)