

ADDIS ABABA UNIVERSITY
SCHOOL OF ALLIED HEALTH SCIENCE
DEPARTMENT OF MEDICAL LABORATORY SCIENCES



**Prevalence of Autoimmune Hemolytic Anemia in HIV Infected
Anemic Adults, a Cross Sectional study at Tikur Anbessa
Specialized Teaching Hospital from June 5, 2015 to September
10, 2015, Addis Ababa, Ethiopia**

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Abbreviations

Ab	Antibody
AIDS	Acquired immune deficiency syndrome
AIHA	Autoimmune hemolytic anemia
ANC	Antenatal care
ART	Anti-retroviral Therapy
AZT	Azidothymidine
CA-AIHA	Cold auto immune hemolytic anemia
CBC	Complete blood cell count
DAT	Direct anti human globulin test
D-Dabs	Drug-dependent antibodies
D-DHI	Drug-dependent hemolytic anemia
DIC	Disseminated intravascular coagulation
EDTA	Ethylene diamine tetra-acetate
EPO	Erythropoietin
G6PD	Glucose 6 phosphates dehydrognase
GA	Granulocyte agglutination
GIF	Granulocyte immunofluorescence
HAART	Highly active antiretroviral therapy
HCT	Hematocrit
HGB/Hb	Hemoglobin
HIV	Human immune deficiency virus
HSC	Hematopoietic stem cells
IRB	Institutional Review Board
MCH	Mean corpuscular hemoglobin

MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean cell volume
PBF	Peripheral blood film
PCH	Paroxysmal cold hemoglobinuria
RBC	Red blood cell
RNA	Ribonucleic acid
SC	Sickle cell
SLS	Sodium lauryl sulphate
WA-AIHA	Warm auto immune hemolytic anemia
WHO	World health organization
ZDV	Zidovudine

Operational Definitions:

HGB below 12.8 and 13.8 g/dl for females and male respectively is considered as anemia and classification of anemia was based on WHO criteria as follows by adjusting altitude above sea level.

Population	Anemia			
	Non –Anemia	Mild	Moderate	Severe
Non-pregnant women	12.8 or higher	11.8-12.7	8.8-11.7	lower than 8.7
Pregnant women	11.8 or higher	10.8-11.7	7.8-10.7	lower than 7.7
Men	13.8 or higher	11.8-13.7	8.8-11.7	lower than 8.7

- **DAT positive**:- from +1 to +4 agglutination of washed RBC with Anti-human globulin reagent.
- **Reticulocytosis** :- reticulocyte count greater than 1.8% .
- **Reticulocytopenia**:- reticulocyte count less than 0.4%.
- **Anemic**: - hemoglobin value less than 12.8 g/dl for female and less than 13.8g/dl for male.
- **AIHA**:- First, the anemia has to be Normocytic normochromic anemia (MCV= 90 ± 10 ; MCH= 27-32), second there has to be evidence for the presence of hemolysis in blood film such as:-spherocytosis or schistocytes (Helement cell, Karatocyte (horned cell) Triangular cell), nucleated RBC; third, DAT has to be positive and a Reticulocyte count must be greater than 1.8%. The presence of erythrophagocytosis by monocyte may be seen in severe cases and agglutinated RBC may be seen uncommonly in warm AIHA. These findings in blood film, with other parameters, used as evidence for the presence of AIHA.
- **Blood film finding interpretation**:- The presence of <1% fragments in the PBF is considered normal and 1-5, 6–14 and >15% finding of fragmented cell in blood film is reported as +1, +2 and +3 respectively. However, the finding of even a single spherocyte and nucleated RBC is considered as indicators since, they are usually absent in the blood films of healthy individuals. Reporting and interpretation was as follows, 1-5, 6–19, >20% finding of spherocyte in blood film was reported as +1, +2 and +3 respectively.

Summary

Background:- Hematologic manifestations in HIV/AIDS positive patients are many. A high prevalence of anemia and significantly reduced white blood cell and neutrophil counts were observed in HIV-infected individuals. Presence of auto antibody also contributes for the development of anemia in HIV positive individuals. Few studies are conducted to show the prevalence of anemia in HIV positive individuals, but there is no documented data that rule out the prevalence of AIHA in HIV positive individuals in Ethiopia.

Objective:- To determine the prevalence of autoimmune haemolytic anemia in HIV-infected adult patients at Tikur Anbessa Specialized teaching Hospital, Addis Ababa, Ethiopia.

Methods:- Hospital based cross sectional study was conducted on 384 HIV positive individuals using convenient sampling method at Tikur Anbessa Hospital from June 5, 2015 to September 10, 2015. Participants' socio-demographic data, CD4 result and some important information was extracted from participants' records; from patient card and request paper using Microsoft excel. And 5 ml of venous blood was collected for determination of HGB, RBC count, RBC indices, reticulocyte count and other CBC results. After all, DAT was performed and blood film examination was done to confirm hemolysis. Finally data was imported and analyzed using SPSS version 16 statistical software package, and Descriptive statistic and Chi-square tests were used.

Result:- Of 384 study participants, 9 (2.34 %) patients are found to be AIHA positive. Of these, 6 (66.7 %) were females and 3 (33.3 %) were males. Nineteen patients had positive DAT result; 10 (52.6 %) had mild anemia, 4 (21 %) moderate and 5 (26.3 %) severe anemia respectively. 37 (9.6 %) patients showed reticulocytopenia, while 31 (8.1 %) patients had shown reticulocytosis. From 26 patients who showed evidence for the presence of hemolysis in their blood film, two individuals showed +2 graded hemolysis whereas 24 patients showed +1 graded hemolysis.

Conclusion:- Prevalence of AIHA in HIV patients is not common. AIHA can be an etiological cause of anemia most of the time in patients with severe-moderate ranged anemia and in patients with low CD4 count.

Keyword:- Autoimmune haemolytic anemia, direct anti-globulin test, hemoglobin, reticulocytosis.

1. Introduction

1.1 Background

Hematological problem is one of the rapidly growing health issues including anemia and leukemia worldwide. Many study in different area around the world confirmed that this hematological problems are common in HIV positive individuals. Hematologic manifestations in HIV/AIDS positive patients are many. Major hematological abnormalities are; high prevalence of anemia and also significantly reduced white blood counts were observed in HIV-infected individuals (1).

Anemia is normally defined as a decrease of hemoglobin below the expected value in a given age and sex or incapability to fulfill the expected tissue oxygenation and sometimes it can be decrease in number of RBC or increase in their destruction. It is also associated with abnormal morphology of RBC. The cause for development of anemia can be varied including decrease production of RBC because of different reasons, such as stem cell failure in aplastic anemia, malignant transformation of stem cells in acute leukemia, acute blood loss after trauma, chronic blood loss in menstruating women, inadequate nutrition, chronic autoimmune conditions, chronic infections, and many other disorders (2).

Different mechanisms can be used for classification of anemia. One mechanism that is used in classification is based on the size of RBC; microcytic, normocromic and/or macrocytic. Even if anemia in HIV/AIDS positive individuals is expected to be classified under anemia of chronic disease, microcytic anemia, findings show that Normocytic normochromic anemia is most common type of anemia in these individuals. Second most common finding is microcytic hypochromic anemia and the third one is that of macrocytic anemia. The etiology of anemia in adult with HIV infection is multi factorial; it may be because of decreased RBC production, increased RBC destruction, and ineffective RBC production, as a result of this the management also should have to depend on these conditions (3, 4).

Hemolytic anemia is any situation in which there is a reduction in RBC life-span due to RBC destruction by different internal or external factors, and unlike the normal one the replacement production by bone marrow is unsatisfactory. Inability of compensatory marrow response for production of red cell results in anemia. Normal predominant site of RBC destruction, after completion of their life span, is red pulp of the spleen (5). Endothelial system of the liver and bone marrow are also sites for normal extra vascular RBC destruction. Loss of membrane plays a role in many types of pathologic hemolysis, like intravascular hemolysis, including autoimmune hemolytic anemia (6).

Auto immune hemolytic anemia is characterized by an increased breakdown of RBCs due to auto antibodies (auto-Ab's) with or without complement activation. Based on the optimal temperature, which favor for binding of autoantibody with RBC, AIHA is divided into a warm antibody AIHA (WA-AIHA), cold antibody AIHA (CA-AIHA), AIHA due to biphasic auto-Ab (mixed type) and drug immune hemolytic anemia (7,6).

1.2 Statement of the Problem

Anemia is the most common hematologic abnormality associated with HIV infection, affecting 60% to 80% of patients in late- stage disease. Anemia may manifest as a mere laboratory abnormality in some individuals, others may experience typical symptoms (4). Even patients with relatively severe hemolytic anemia may have only modest splenomegaly. However, in very severe cases, particularly those of acute onset, patients may present with fever, pallor, jaundice, hepatosplenomegaly, hyperpnea, tachycardia, angina, or heart failure (6). As a result of this, it is very important to follow the DAT result of HIV patients because, some findings say that the cases of autoimmune hemolytic anemia occurred only in patients who are severely immune suppressed. And also in the HAART era, when HIV-infected people are treated more and more early, autoimmune diseases can occur, mainly at the phase of immunological recovery (8).

Because of HIV virus or antiretroviral treatment, the pathogenesis of anemia becomes more severe in these individuals than the normal one. Symptom onset usually is slow, but occasionally a patient has sudden onset of symptoms of severe anemia and jaundice over a period of a few days. In primary, idiopathic, AIHA with only mild anemia that may results in physical examination seems to be normal. In secondary AIHA, the symptoms and signs of the underlying disease may overshadow the hemolytic anemia and associated features (6).

Major mechanisms of the HIV virus pathogenesis include depletion of the total body lymphocyte, primarily CD4+ cells which lead to immunodeficiency, and impaired production of RBC, because of its direct effect on HSCs (3). Immunological marker primarily CD4 count is useful in the understanding and evaluation of disease progression in HIV sero-positive patients (9). Studies show that, as the CD4 cell counts increase, the same thing will happen in hemoglobin levels and the total leukocyte counts (10).

Studies show that the prevalence of anemia in HIV seropositive individuals varies considerably, ranging from 1.3% to 95%, suggesting that this range depends on several factors, including the stage of HIV disease, socio-demographic status, presence of pregnancy, and injection-drug usage as well as the definition of anemia used for that particular case (11). From different kind of anemia the most prevalent kind of anemia in HIV positive individuals is not well studied. One

study which attempts to see iron deficiency anemia in HIV infected and non infected women showed that the prevalence of iron deficiency anemia in HIV positive women was 20.6% whereas in non HIV infected women was 14.7% (12). Even if the prevalence of AIHA is rare its presence can result in death or take many months to relief (13).

Autoimmune hemolytic anemia (AIHA) is one of the commonest autoimmune disorders which is seen in different individuals and mainly detected using human anti-globulin reagent *in vitro* in laboratories. It is very important to know the DAT result of HIV positive individuals even if they seem normal. Because this silent auto antibody may result in severe anemia after a while and studies indicate that the DAT-positive results may be specifically related to lower Hb levels in HIV+ patients (14). A negative DAT does not mean that there is no AIHA, as it may be of weak affinity antibody. Similarly, a positive DAT is also not in all cases associated with an overt hemolytic anemia (2). The immune related RBC clearance could be one of the multiple causes of anemia in HIV+ patients (14).

Since, anemia has great impact on economic and social well being of HIV positive individuals; because it facilitate death, weakness and disease progression to AIDS status (11, 15), it is very important to rule out and treat anemia in HIV patients appropriately. Finding suggested that iron deficiency were the cause of death in HIV patients and a 50% decline in CD4 cell count, and as they said on this study finding, anemia is an independent predictor of mortality, disease progression and it is responsible for low count of CD4; thus, it is necessary to specifically identify the type of anemia and treat it early (16).

Although the cause of autoimmunization remains obscure in 50% of patient and in more than half of cases it is associated with another disease (17, 18), finding proposed molecular mimicry is one mechanism in development of autoimmune disease in which exogenous infectious agent may have molecular similarity to self antigen which induces host's immune response (19). Another possible mechanism for autoimmune manifestations of HIV infection includes increased cytotoxic cell activity, increased expression of auto antigens and also alteration of RBC surface antigen by virus or drug, or possibly a cross reaction between antibody induced by infectious agent against RBC surface antigen (19, 20). Since, the primary function of Treg cell is

prevention of autoimmune disease by maintaining self-tolerance (21) change in balance between helper and suppressor T (Treg) cell also can result in autoimmune disease (20).

One study conducted in Ethiopia show that the prevalence of anemia in HIV/AIDS positive patients is 35% this ranges from mild to moderate anemia (22, 23). Of the anemic individuals 1.6%, 14.8%, 53.6% had severe, moderate and mild anemia respectively (23). Findings showed that one-third of HAART naive HIV positive patients were anemic and the increase in prevalence of anemia with decreased CD4 cell count was statistically significant (22).

Anemia, from the ancient Greek word and have a meaning of 'lack of blood', is defined as a decrease in the total amount of hemoglobin or the number of red blood cells in circulation. Iron deficiency anemia which is considered primarily as a nutritional deficiency is most prevalent cause of anemia worldwide (24). WHO has defined anemia in adults as a hemoglobin below 13 g/dL in males (a hematocrit [Hct] of about 39 and below) and <12 g/dL in females (Hct about 36 and less) at sea level below 1000m (25).

Therefore, early diagnosis to identify the type of anemia for appropriate treatment in these patients is essential. Clinicians need to routinely investigate and treat hematological abnormalities including anemia in those individuals particularly before and after treatment, and furthermore large scale based and identification focused studies are recommended to see the strength and then explore the problem in depth. Few studies are conducted to show the prevalence of anemia in HIV positive individuals, but there is no documented data that characterize the prevalence of AIHA in HIV positive individuals in Ethiopia. This study is planned to evaluate the prevalence of AIHA in HIV patients, as one type of anemia expected in HIV sero-positive individuals.

1.3 Significance of the study

This study would encourage ART clinicians and others to know the current burden of AIHA in HIV patients and help ART clinicians and laboratory personnel to suspect and investigate AIHA, as the possible etiology of anemia in HIV positive patients. This study also help researcher to use it as reference and encourage researcher to initiate and investigate more on related problem and to study further in depth. As a whole, it contributes to the reduction of the mortality and morbidity rate in HIV positive individuals because of AIHA that result from unidentified treatment of anemia without identifying the proper cause and type.

2. Literature review

Anemia is a problem at any age stage but it was also found to be a severe public health problem (>40% anemic) in African countries for children aged 7-14 (26). Hemolytic anemia is worldwide health problem and the cause for hemolysis can be different. One way is that hemolysis occurs extravascularly; this may be because of infections, medications, immunologic processes, erythrocyte membrane abnormalities, metabolic defects and hemoglobin structural abnormality and intravascular; presence of fibrin in DIC cases, infection such as malaria, toxin, severe burn and physical trauma (27). On the other hand hemolytic anemias can be classified into acquired and inherited types. The acquired forms are usually caused by the development of autoantibodies against red cells, whereas the latter forms are possibly due to red cell membrane defects, red cell enzyme defects, or abnormalities of the hemoglobin (Hb) molecule (2).

The global prevalence of HIV-1 has stabilized at 0.8%, with 33 million people living with HIV/AIDS, 2.7 million new infections, and 2.0 million AIDS deaths in 2007 (28). Global report on HIV epidemic reported that Worldwide an estimated 35.3 (32.2–38.8) million people were living with HIV in 2012. “There were 2.3 (1.9–2.7) million new HIV infections globally, showing a 33% decline in the number of new infections from 3.4 (3.1–3.7) million in 2001. At the same time the number of AIDS deaths is also declining with 1.6 (1.4–1.9) million AIDS deaths in 2012, down from 2.3 (2.1–2.6) million in 2005” (29). In sub-Saharan Africa which remains the most heavily affected region like other infections, with 67% of the global burden in HIV is in this area, and male-male sex, injection drug usage, and sex work were populated as the predominant risk factors (28).

The national HIV prevalence in Ethiopia was 2.3% in 2009 (7.7% urban; 0.9% rural; 2.8% Female; and 1.8% Male), with 1.1 million people living with HIV. At an incidence rate of 0.28%, the number of new infections in 2009 was about 131,145 (30). HIV prevalence in Tigray was 1.8% in 2011; ANC data show that there had been a continuous decline in the prevalence of HIV in both urban and rural areas (urban: 14.9% in 2001 to 5.0% in 2009; rural: 5.2% in 2001 to

1.3% in 2009, ANC surveillance data) (31). In Addis Ababa from 243,791 tested individuals for HIV 15,588 were positive, hence it becomes 6.4% in 2010 (30).

Based on the study from Eastern India conducted by Pande A, *et al* in 2011, most common hematological abnormality in HIV positive patients was anemia, present in 74.7% and it was followed by leucopenia (38%), thrombocytopenia (23.33%) and pancytopenia (16%). Among the anemic 23.2% had severe anemia (<7g/dl). According to this study, from many etiologies that cause anemia in HIV positive patients, Anemia of chronic disease was the commonest etiology followed by HIV related myelodysplastic syndrome, iron deficiency anemia, bone marrow suppression due to direct involvement by some infective process (32).

Anemia in HIV/AIDS positive patients has multiple etiologies, these include: decreased production because of infiltration of bone marrow by other cell and as a result of impaired growth because of hormonal problem. Different explanations are given for this etiologies and some of them are: infiltration of the bone marrow by neoplasm or infection (listed later) including HIV infection itself, decreased production of endogenous erythropoietin or hemolytic anemia because of auto antibody and others (4, 33); Persistent infection with parvovirus (B19) (34), and also infection like *Mycobacterium avium* complex, *Pneumocystis carinii*, *bacterial pneumonia* (35) and others are mentioned as a risk factor for anemia may be because they can cause cytopenias by infiltrating the bone marrow, and impaired erythropoiesis resulting from increase relies of inflammatory cytokines and decrease production of hematopoietic growth factor are also other possible cause of anemia (36).

Thrombotic thrombocytopenic purpuras, which lead to intravascular hemolysis likely, and drug side-effect is also found to be the cause for cytopinea in these patients (37). On the other hand, immune and non-immune hemolysis, use of myelo suppressive medications such as zidovudine (ZDV) (4), ineffective production due to vitamin B12 or/ and folic acid deficiency or malabsorption are also considered as the possible causes for anemia on those individuals (38).

Individuals who are infected with HIV are known to have a high incidence of auto antibodies production. This may be because of the drug or obscured causes. Study conducted by Stroncek

DF *et al* in Minneapolis, to detect the presence of autoantibody in serum samples from 100 individuals with HIV infection show that there were positive for granulocyte antibodies (red cell antibodies, lymphocytotoxic antibodies, circulating immune complexes, and serum immunoglobulin G levels) by granulocyte agglutination (GA) method were 21%, and by granulocyte immunofluorescence (GIF) assays was 66% (39).

Auto antibodies, which contribute for the development of anemia, in HIV positive individuals can be directly against RBC itself or EPO. Study was conducted in Israel to see the association of circulating auto antibodies to endogenous erythropoietin with anemia. They found that circulating auto antibodies to EPO were present in 48/204 (23.5%) of the patients and they deduced that this circulating auto antibodies were an independent predictor of anemia (19). Tsiakalos A *et al* in 2010 also showed that Anti-EPO were detected in 46 individuals from a total of 113 which is 41% and based on their finding Anti-EPO has been associated with increased risk of anemia with (odds ratio [OR], 5.07; 95% [CI], 1.29-3.56) (40).

Normocytic anemia with decreased reticulocyte count was the most common type of anemia in most cases (4). The presences of warm and cold autoantibody contribute for severity anemia in HIV postive individual (13) and it is potentially lethal disorder requiring prompt diagnosis and treatment (41). These auto antibodies, which bind to the red blood cell membrane, results in a positive direct anti globulin test (DAT) in 34% to 85% of HIV-infected patients but it is rarely associated with significant hemolysis (42).

Individuals with HIV can develop drug-dependent antibody or antibody against the disease and different type of antibody can be involved IHA of HIV patients. Studies were conducted, by Gonzalez CA *et al* in 2003 in Argentina, to detect the presence of drug-dependent antibodies (D-DAbs) in 53 patients with AIDS who developed immune hemolytic anemia (IHA). According to their finding Drug dependent antibodies were detected in 43.4 percent (23/53) of the patients with IHA. Antibodies to more than one drug were detected in 60.8 percent (14/23) of patients with drug-induced IHA (D-IHA). The DAT was positive by RBC-bound IgG in eight patients, RBC-bound IgG/C3d in nine, IgG/IgA in three, IgG/IgA/C3d in two, and one patient had RBC-bound C3d only. No drug-independent antibodies were detected. Their study demonstrated that

patients with AIDS commonly develop D-DABs and they recommend that D-DIHA should be included in the differential diagnosis of a falling Hb in AIDS patients receiving drugs (43).

On the other hand antibodies present against the virus itself, anti-HIV antibody, can act as anti-RBC antibody and result in hemolysis. In Italy study was conducted by Biasinutto C *et al* to assess clinical significance of positive direct anti-globulin test in patients with HIV infection and they confirm the high prevalence of positive DAT, A positive DAT was found in 24 of 70 patients (34%, significantly higher compared to 0.1% in healthy controls), in patients with HIV antibodies. It suggests that a positive DAT might be a predictive factor in the clinical course of the disease (44). Study conducted in UAS by R.S. Root-Bernstein in 2004 also confirmed that antibody can be obtained from the virus itself directly; according to their deduction, antibodies elicited by HIV infection can interact with antibodies elicited by cofactor infections and can produce different problems (45).

Another study from Italy in 2006 by Lia M, indicated that the DAT-positive results may be specifically related to lower Hb levels in HIV+ patients. They found that patients with DAT-positive results showed lower Hb levels than DAT-negative patients and they rule out that the immunologic RBC clearance could be part of the anemic multi factorial condition in HIV+ patients (14).

Mild-to-profound anemia has been observed in patients with the acquired immune deficiency syndrome (AIDS). To investigate a possible immune mechanism, study was conducted by Macher AM *et al* and blood samples from 28 hospitalized AIDS patients were tested for the presence of atypical red cell antibodies. Eighteen AIDS patients (64%) had anti-i, nine (32%) had autoanti-U, and 12 (43%) had a positive direct antiglobulin test. The mean hemoglobin level of AIDS patients with anti-i or anti-U was significantly lower than the mean hemoglobin level of patients who did not have those antibodies (46).

Study conducted in South Western Nigeria to see DAT positivity in HIV and malaria infected individuals showed that from 20 only HIV infected individuals 3 (15%) of them had positive DAT (47). Study was done to demonstrate the frequency of AIHA in a cohort of adult Nigerian

HIV/AIDS patients and they found that the frequency of AIHA was 3.06%. 36.74% of study population was anemic and 11.22% had a positive DAT (48). These indicated that there may be an antibody which is not responsible for the development of autoimmune hemolytic anemia in these individuals, rather they simply attached to RBC surface and result in positive DAT result.

In order to advert anemia, if there is hemolysis, hematopoiesis should have to rise; this is indicated by high reticulocyte count. One study from Nigeria done to assess the prevalence of HIV related AIHA show that individuals with anemia had lower CD4 count (284.3 cell/l) and high mean reticulocyte percent (1.5%) than non anemic patient. According to their finding frequency of reticulocytosis was higher in female than males, and only 0.8% (2 of 250) of the study group screened positive to DAT (49).

3. Hypothesis

The prevalence of AIHA in HIV positive patient is rare.

4. Objective of the study.

4.1 General objective

To determine the prevalence of autoimmune hemolytic anemia in HIV-infected adult patients at Tikur Anbessa Specialized teaching Hospital from June 5 to September 10, 2015, Addis Ababa, Ethiopia.

4.2 Specific objective

- To determine retics count in HIV positive anemic individuals.
- To determine DAT positivity in HIV positive anemic individuals.
- To assess the severity of anemia in HIV patient.
- To determine the CD4 count in HIV positive anemic individuals.

5. Materials and Methods

5.1 Study Area

The study was conducted in Tikur Anbessa specialized teaching Hospital, Addis Ababa, Ethiopia. Addis Ababa contains 10 sub-cities; Tikur Anbessa specialized Hospital is found in Lideta sub-city. It is one of the largest last referral and teaching hospital of the country. The hospital is established in November 3, 1973 E. C (50). The laboratory has 72 staffs (1 doctor, 8 MSc holders 40 technologists, 10 technicians and 13 supporting staffs) and this specialized teaching hospital laboratory gives its service using different units. On the average Hematology and immuno hematology Unit of the hospital laboratory perform 25 CBC testes per day for HIV sero positive individuals only and 5 to 7 number of people will be allocated in hematology and immuno hematology unit at a time. The responsibility of hematology and immuno hematology is performing CBC, blood film, morphology examination, CD4, ESR, cross match, deliver blood component and blood grouping.

DAT test was conducted in national blood bank of Ethiopia. National blood bank of Ethiopia is established in 1996 E.C by Ethiopian red cross society since 2004 E. C it has been transferred to Federal Minister of health Ethiopia. Blood donor service management, Human resource and general service, Quality control which perform quality control test for blood bank product are few of the institution's departments. The laboratory has ABO, infectious and screening and component preparation case teams and it contributes about 50 % safe blood demand of the country. The responsibility of this laboratory is screening the collected blood for infection (HIV, HBV and syphilis), carried out blood typing, preparing blood component (concentrated red cell, fresh frozen plasma, platelets and cryoprecipitate), distribute save blood for health institutions and control local blood bank in the country (51, 52). Generally, this institution has 149 staff of them 38 are laboratory personnel including one hematologist.

5.2 Study period

The study was carried out from June 5 to September 10, 2015.

5.3 Study Design

Hospital based Cross Sectional study was conducted in HIV positive patients at Tikur Anbessa Specialized Teaching Hospital, Addis Ababa, Ethiopia.

5.4 Population

5.4.1 Source population

HIV positive individuals who were sent to laboratory of Tikur Anbessa Specialized Teaching Hospital during the study period were the source population.

5.4.2 Study population

HIV positive individuals who came to hematology unit, who developed anemia, met the inclusion criteria and volunteered patients were the study population.

5.5 Sample size determination and sampling technique

5.5.1 Sample size

The required sample size for this study was calculated using single population proportion formula and by considering the following assumptions.

- Since, there is no study conducted in Ethiopia to show the prevalence of AIHA we use Proportion 50%.
- With 95% confidence interval and 5% marginal error, sample size (n) was be determined using the following statistical formula

$$n = \frac{Z^2 P (1 - P)}{d^2}$$
$$n = \frac{1.96^2 (0.5)(1-0.5)}{0.05^2} = 384$$

Where n = sample size,

Z = Z statistic for a level of confidence,

P = expected prevalence or proportion,

d = precision, margin of error between the sample and the population.

5.5.2 Sampling technique

- Convenient sampling technique was used.

5.6 Inclusion and Exclusion Criteria

5.6.1 Inclusion Criteria

- HIV positive patients who were not transfused in previous three months
- Those who were volunteers and
- Who develop anemia.

5.6.2 Exclusion Criteria

- Participant whose age is below 14 were excluded from the study
- Individuals who came to the laboratory more than once during the study period
- Hemolysed and inappropriate proportion with EDTA samples

5.7 Data collection procedure

Participants' socio-demographic data, CD4 result and some important information was extracted from Participants' records, from patient card and request paper, using Microsoft excel. About 5 ml of venous blood was drawn from each participant into sodium ethylene diamine tetra-acetate (EDTA) test tube. This sample was used to measure full blood count using automatic hematology analyzer, Sysmex XT2000i Analyzer, made by Sysmex Cooperation Kobe, Japan; for hemoglobin measurement, RBC count, RBC indices and others. For reticulocyte count manual method was used; finally, blood film examination was performed using collected blood and DAT was analyzed.

Three to four ml of venous blood is collected in to EDTA test tube and within 3-4 hour of collection CBC was done then blood film preparation and examination was done. In blood film we were looking for the presence of schistocytes, spherosyt, nucleated RBC and in the mean time we cross check for morphology with analyzer result. Finally, reticulocyt count was performed using Brilliant Crestal Blue by incubating 3 drop of blood with 3 drop of reagent for 25-30 minute, after all smear prepared and retics count was performed.

5.8 Data analysis and result interpretation

Data entry and analysis were done by using SPSS statistical software version 16. Descriptive statistics; for CD4, retics, severe to moderate ranged anemia, and χ^2 (for CD4 and retics, evidence for presence of hemolysis and CD4) test were used. The results were presented in words, percentages, graphs and tables. Based on the study result, conclusions and recommendations were made.

5.9 Data quality control

Pre-analytical

To assure the quality of the data, training was given to the data collectors to minimize technical and observer bias. Standard operating procedures were strictly followed during specimen collection and laboratory procedures.

Analytical

Quality control for working equipments and reagents was ensured using standard controls as well as standard operational procedures were implemented and for Sysmex normal back ground reading was also included daily. The result of each and every test was properly recorded. The same procedure, for DAT, was repeated with Coomb's positive and negative cells as control for each batch.

Microscopic Smear review was performed to check RBC morphology and to see distribution if there is doubt in result or for extremely outranged result. As a quality control for retics count, 500 cells was counted on two slides each and result should agree within range of $\pm 15\%$ each other. If they do not, reticulocyte count was repeated on the third smear.

Post analytical

Every day, the collected data was checked for completeness and accuracy by the principal investigator. During the entry of data it was cross checked to assure the right data was entered correctly.

5.10 Ethical Consideration

The study was approved after it is ethically reviewed by the Department of Laboratory Sciences Research Ethical Review Committee of Addis Ababa University. Permission was also obtained for this study from Tikur Anbessa Specialized Hospital medical director, ART department head and laboratory head, document and record officer and also from national blood bank administrator and laboratory head. All the information obtained from the study participants were coded to maintain confidentiality and data were collected after written informed consent was obtained. Full explanation about the purpose of the study was made to persons who full fill the inclusion criteria and expected to participate in the study. These candidates were informed of their right to refuse or agree to be part of the study, or discontinue their participation whenever they feel the need.

5.11 Dissemination of results

The study report would be submitted to College of Health Science, School of Allied Health Science, Department of Medical Laboratory Science of Addis Ababa University and also the results would be submitted to the study site. The study abstract would be submitted to local associations like EMLA to present the results of the survey during continuous medical education events organized through this association. Extra paper would be submitted to international or national peer reviewed journal for publication.

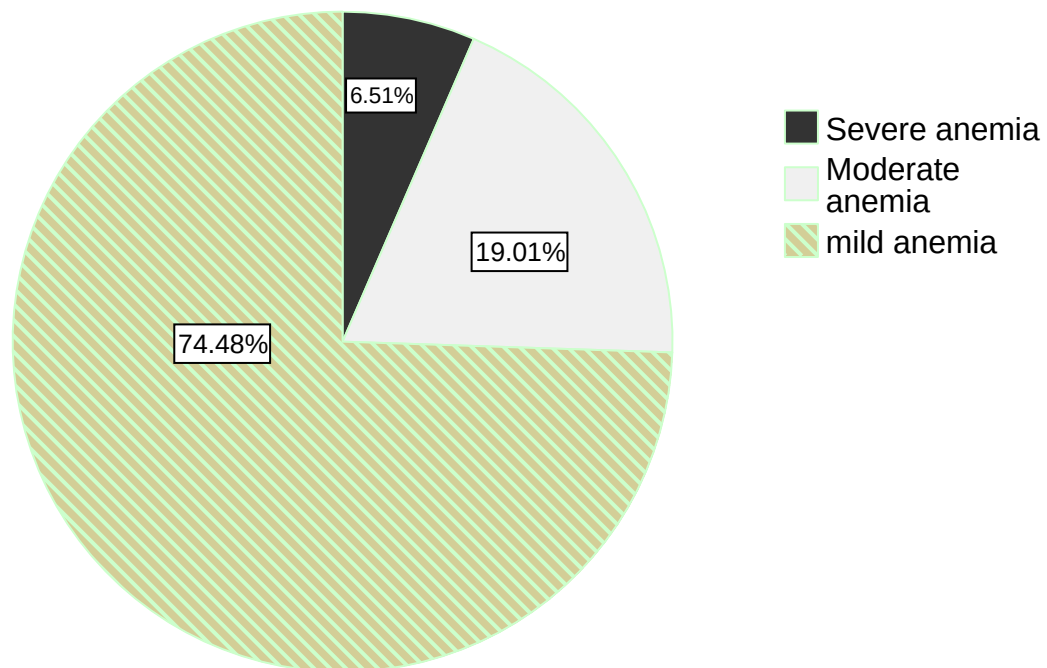
6. Result

The study is done in total of 384 patients, which was made up of 100 (26.1%) males and 284 (73.9%) females. All female participants were non- pregnant during the study time. The mean age of the study participant were 36 +/- with SD of 12 years, 116 (30.2 %) being in the age group 36 - 42 years; age and sex distribution of the study population is shown in table 1. From all participants of the study, 9 (2.34 %) were found to be AIHA positive, 6 (66.7 %) females and 3 (33.3 %) males respectively, and only 10 other patients had positive DAT without being AIHA positive.

Table 1:- The demographic characteristic of the study population at Tikur Anbessa Specialized Teaching Hospital; Addis Ababa, Ethiopia, from June 5, 2015 to September 10, 2015.

Age(year)	Male (%)	Female(%)	TOTAL (%)
15-21	23 (6.0)	31 (8.1)	54 (14.1)
22-28	11 (2.9)	34 (8.9)	45 (11.7)
29-35	10 (2.6)	68 (17.7)	78 (20.3)
36-42	22 (5.7)	94 (24.5)	116 (30.2)
43-49	12 (3.1)	17 (4.4)	29 (7.5)
50-56	14 (3.6)	31 (8.1)	45 (11.7)
57-63	4 (1.0)	4 (1.0)	8 (2.0)
64-70	2 (0.5)	5 (1.3)	7 (1.8)
71-77	2 (0.5)	0 (0)	2 (0.5)
Total	100 (26.1)	284 (73.9)	384 (100)

Highly frequent AIHA positive patients, 3 (33.3 %) were seen in age group 43-49 followed by 50-56 and 29-35 age group with 2 (22.2 %) AIHA positive patient each and lastly in age group



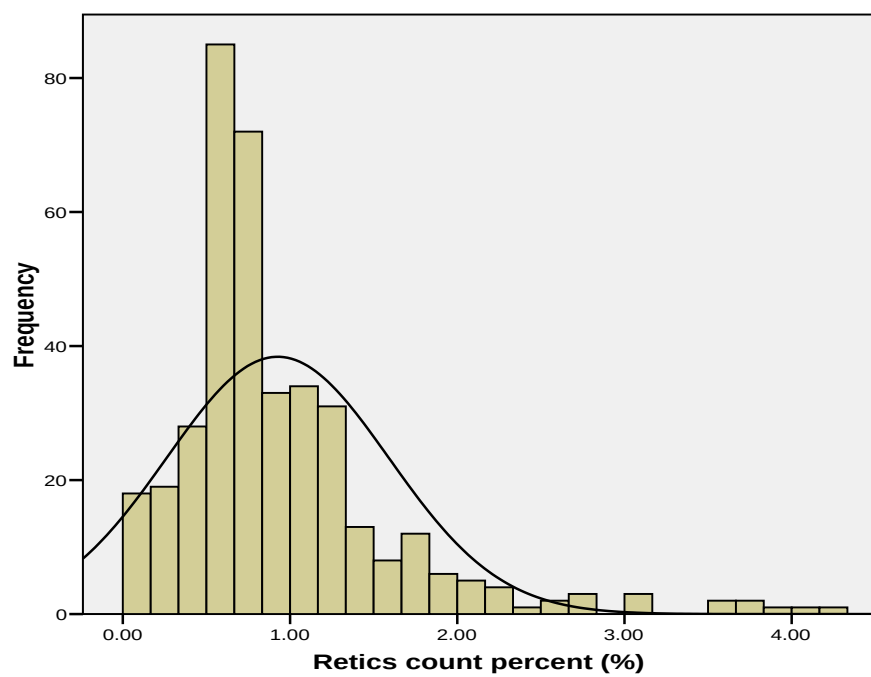
Using this study definition, the most prevalent type of anemia was normocytic normochromic anemia which accounted for 216 (56.25 %) and followed by macrocytic normochromic anemia, 107 (27.9 %), and microcytic hypochromic, 22 (5.73 %), respectively.

The CD4 distribution of the study participant with DAT result and HAART usage in different age and sex group is shown in table 2. Nineteen (4.9 %) patients had positive DAT result; 10 (52.6 %) had mild anemia, 4 (21 %) moderate and 5 (26.3%) severe anemia respectively. And from those whose DAT was positive, 14 (73.6 %) patients had a finding for presence of hemolysis in blood film and only 5 (26.31 %) patients had positive DAT result without the presence of evidence for hemolysis in their blood film. Twelve patients were negative for DAT result but showed evidence for hemolysis in their blood film finding.

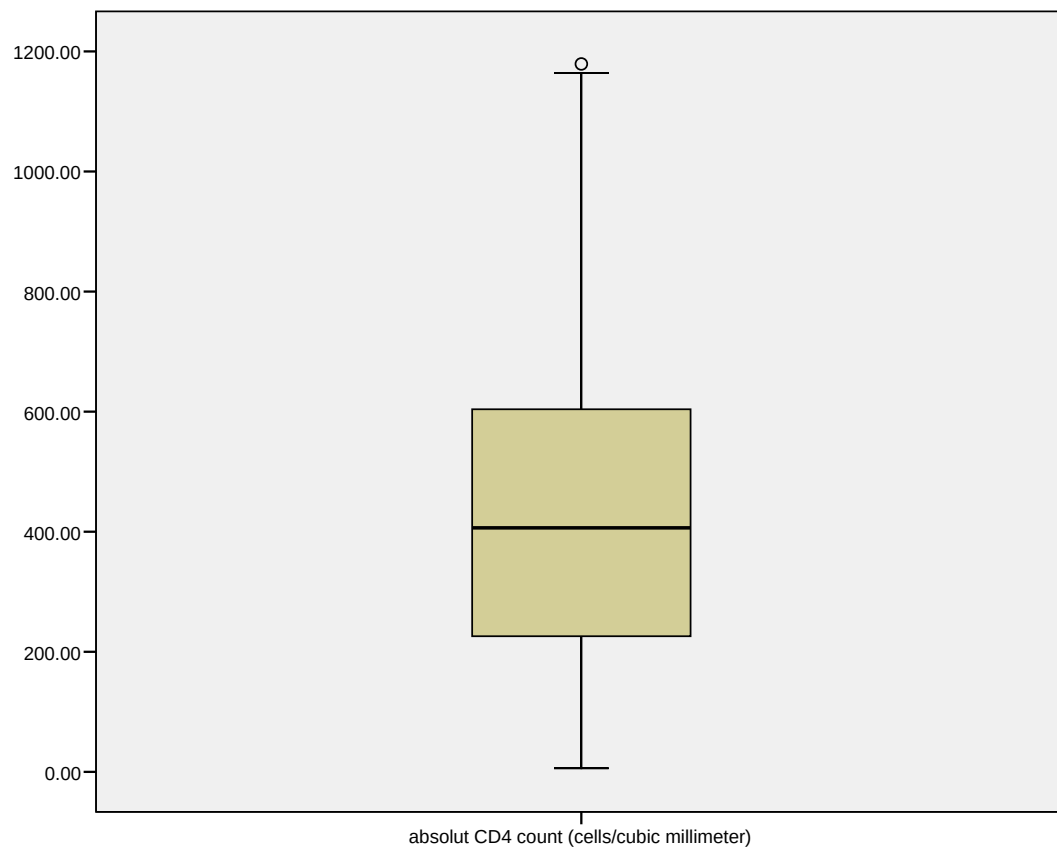
Table 2:- CD4 count, DAT result and HAART usage of the study participants in different age and sex distribution of the study population at Tikur Anbessa Specialized Teaching Hospital; Addis Ababa, Ethiopia, from June 5, 2015 to September 10, 2015.

Variable	CD4		DAT		HAART	
	<200	>201	Positive	Negative	Naive	User
Age						
15-21	6	48	0	54	0	54
22-28	11	34	4	41	1	44
29-35	25	53	4	74	2	76
36-42	22	94	3	113	1	115
43-49	8	21	3	26	3	26
50-56	6	39	4	41	2	43
57-63	1	7	0	8	0	8
64-70	0	7	1	6	0	7
71-77	1	1	0	2	0	2
SEX						
Male	22	78	4	96	3	97
Female	58	226	15	269	6	278

The mean of reticulocyte count was 0.93 with 0.67 SD. The distribution frequency for reticulocyte count is shown in fig 2. From 31 patients whose reticulocyte count was greater than 1.82, 4 (12.9 %) patients had severe while 7 (22.6 %) and 21(67.7 %) patients had moderate and mild anemia respectively. On the other hand, from this 31 previously mentioned patients 19 (61.3 %) of them were negative for DAT while only 12 (38.7 %) patients were positive for DAT test. Reticulocytosis was more common in females, 20 (64.5 %), than in males. Chi-square test was performed to see the association for severity of anemia with reticulocyte count of the patients, and no statistically significant association was found between severe and moderate type of anemia with reticulocyte count , χ^2 (1, N= 384)= 1.76 , $p= 0.184$.



Mean =0.93
Std. Dev. =0.665
N =384



7. Discussion

The three most basic mechanisms for the pathology of anemia in HIV patient according to Meidan M *et al* in 2012 are decreased RBC production, increased RBC destruction, and ineffective RBC production, and they are the risk factor for Normocytic Normochromic anemia (4). Based on our finding Normocytic Normochromic anemia was present in 56.25 % of the patients, this agree with the study conducted in Mexico by Mata-Marin JA *et al* in 2010 to study risk factor and correlates for anemia in treatment naïve HIV infected patients. According to their finding normocytic normochromic anemia was found in most (85 %) of their participants (33). This also agrees with the study conducted in Ethiopia By Enawgaw B *et al* in 2014 on determination of immunological and hematological parameters among HIV positive patients taking HAART and treatment naïve in ART clinic of Gonder university and they found that the prevalence of normocytic normochromic was 43.3 % and they concluded that it is the dominate type of anemia in their patients (53).

The prevalence of AIHA on this study was 2.34 % it is consistent with Volberding PA *et al* in 2003 finding in which they confirmed that AIHA is not common in HIV patients (54). This value also agree with previous studies conducted in Nigeria by Olayemi E *et al* in 2008 to study the prevalence of AIHA in HIV infected patient and they found that AIHA was rare with the prevalence of 3.06 % (48), and also it is comparable with the study conducted by Adewumi AA *et al* in 2014 to assess the prevalence of HIV associated AIHA and they found that from the total of 350 HIV infected participant none of them were found to be AIHA positive patients (49). According to studies autoimmune disorders are said to be rare in indigenous African population and they assumed that this may be because of the widespread infectious disease which impaired host T-cell immunity (29). Other study conducted by Telen MJ *et al* suggested that the frequent lack of reticulocytosis may lead to un diagnoses of the disease in this population (55).

Most of AIHA developed patients were female, 6 (66.7 %), which agrees with the Eaton WW *et al* finding in 2007 to assess the Epidemiology of Autoimmune diseases in Denmark in which they deduced that most Auto immune disease are more common in female (56). From all AIHA developed patients 5 (55.5 %) of them were above 40 years, and this is similar to Gunawardena D *et al* finding in 2013 in which they state that AIHA can occur at any age stage but mostly

AIHA is found in individuals whose age is above 40 (57). From all 9 AIHA developed patients 2 (22.2 %) of them had severe and 3 (33.3 %) of them had moderate anemia respectively, this is comparable with Salawn L *et al* study in 2002 in which they found that all AIHA positive patients had severe-moderate anemia (29). And it is also in agreement with Kamesaki T *et al* finding in which they say that hemoglobin level was significantly low in patients with AIHA (58).

From those 9 AIHA patients most of them, 6 (66.7 %), had CD4 count less than 200 cell/cubic mm. This finding seems to agree, not perfectly, with Iordache L *et al* finding in 2014 where in their cases all of autoimmune hemolytic anemia occurred only in patients severely immunodepressed (8). But our finding completely disagree with Shoenfeld Y *et al* finding in which they proposed that Autoimmune diseases occur when there is no immunosuppression such as during acute infection (Stage I), when the CD4 count > 200 (Stage II), or following highly active antiretroviral therapy (Stage IV). Autoimmune disease is not reported in patients with profound immunosuppression (Stage III) (19). This significant variation may be because they include many Auto-immune diseases in addition to AIHA. Further longitudinal and large scale study should have to be initiated to resolve this controversy.

Based on our findings only 2 (22.2 %) patients had severe anemia with AIHA and this result is lower than Koduri PR *et al* finding in 2002 where, in which they evaluated all AIHA patient infected with HIV-1 had severe anemia and even they required blood transfusion (13). This significant variation may be because of some of their patient were bled due to DIC and others may also have obscured cause of anemia.

Based on our finding severity of anemia was related with CD4 count and non-perfectly with DAT positivity of patients. From a total of 25 severe anemia developed patients, 16 (64%) of them had CD4 count below 200 cell/ cubic mm and from a total of 19 DAT positive individuals 9 (47.4 %) of them had severe-moderate ranged anemia. This finding is consistent with Ferede G *et al* finding in which they state that increase in prevalence of anemia with decreased CD4 cell count was statistically significant (22) and agree, not perfectly, with Lai M *et al* in 2006 finding which states that Anemia was related with the DAT results (14). The reason for this non perfect

agreement may be the HAART drug and life style effect on hemoglobin count of economically varied countries.

Based on this study finding from 19 patients whose DAT was positive 12 (63.2 %) of them had CD4 count below 350; this shows that high prevalence of DAT positivity as the clinical stage of the disease increased. This find is similar with Volberding PA *et al* declaration in which they states that the prevalence of DAT positivity increased with more advanced stage of HIV infection (54) and also consistent with Angelis VD *et al* finding in 1994 in which they found that DAT positive worsen the clinical condition of HIV patients (44).

Nineteen (4.9 %) patients in this study had positive DAT. This is highly lower than Angelis VD *et al* finding in which they found that DAT positivity is shown in 34 % of their participant (44). It was slightly lower than Nigerian studies in which they found that 11.22 % of their participant was positive for DAT (48) but it is higher than Adewumi A *et al* study in which they found that only 2 out of 350 (0.6 %) patients were positive for DAT (49).

Rheingold SR *et al* in 2004 stated that positivity of direct anti globulin test (DAT) in HIV infected patient ranges from 34% to 85% (42) and it contradict with our finding, which was only 4.9 %. Based on our study none of the participant met other criterion of AIHA except DAT positivity or in other word no patient developed DAT negative AIHA, Kamesaki T *et al* in 2013 finding show that from a total of 216 AIHA diagnosed patients only 62 of them were DAT+ AIHA patients where as 154 of them were DAT- AIHA patients (58). Thedsawad A *et al* in 2011 also found that about 2-10% of patient with WAIHA exhibit a negative DAT (false negative) and they say that more sensitive test is required for diagnosis (59).

Segel GB *et al* in 2013 had explained the reason for all this previously mentioned significant variations, concerning DAT, and some of them are:-IgG sensitization below the threshold of detection by the commercial antiglobulin reagent, low affinity IgG removed by preparatory washes and rarely monomeric IgM which not accompanied by complement fixation, thus it cannot be detected by a commercial antiglobulin reagent that contains anti-IgG and anti-C3 are the possible reason for false DAT negative result (60). The number of molecule bounded around

RBC play a significant role in DAT result of the patients thus; Barcellini W in 2015 said that in order to be DAT positive at least 400 immunoglobulin molecules should have to sensitize each RBC molecule (61).

From 19 DAT positive patients only 14 (73.7 %) of them showed evidence of hemolysis in their blood film where as 5 (26.3 %) patients have positive DAT without hemolysis this may be because some compliments (like C3) don't seem to have any hemolysis even if DAT is positive (62) and positive DAT not in all cases associated with an overt hemolytic anemia (12). Likely Kamesaki T *et al* in 2013 found that generally DAT+AIHA developed patients suffer slightly by hemolysis than patients with DAT-AIHA developed (58). Statistically significant association had been found between reticulocyte count and CD4 count of the study participant, as CD4 increase or decrease the same thing will be happen on retics count, the strong suspicion for this significant association is that because of cytopenia is more common in HIV positive patients (63).

Based on this study, 37 (9.6 %) patients had shown reticulocytopenia and this finding is not consistent with finding in 2003 by Volberding PA *et al* in which they stated that reticulocytopenia is sufficiently frequent in those with HIV infection (54). According to Thedsawad A *et al* finding in 2011 this variation may be because of the course of infection influences on reticulocyte count that reticulocyte count may not be elevated early in the course of the infection of the disease (59). Unlike both our and Volberding PA *et al*; Koduri PR *et al* study in 2002 showed that all their patients who develop AIHA had reticulocyte count above 11% which show that reticulocytopenia may not be seen frequently in this patients (13). Even though reticulocyte count is used as a hall mark for detection of AIHA this finding show that it doesn't have to be used as indicator and large scale study have to be conducted to resolve these nonconformities. The primary cause of reticulocytopenia in HIV patient was suspected because of suppression of erythropoiesis by the HIV virus, ART drug or other infection (49, 54).

From a total of 98 patients who had severe to moderate ranged anemia only 11 (11.2 %) of patients showed reticulocytosis which was normally expected in case when anemia is present even though no statistically significant association is showed between severity of anemia and reticulocyte count. From 25 patients who had severe anemia few of them, only 7(28 %) patients, had reticulocytosis this show that insufficient RBC production contribute for the development of

anemia this finding is consistent with Meidani M *et al* in 2012 declaration in which they stated that pathophysiology of HIV-associated anemia may involve decreased RBC production (4). Based on our finding reticulocytosis is more common in female than in male, 20 (64.5 %), this finding is in agreement with AdewumiAA *et al* result according to their finding frequency of reticulocytosis was higher in female than males (49).

Based on this study finding from a total of 26 patients showed evidence for presence of hemolysis in their blood film only 8 (30.8%) patients had CD4 count below 200 cell per cubic millimeter and no statistically significant association was found between the presence of evidence for hemolysis in blood film and CD4 count. This finding contrast with Koduri PR *et al* finding in 2002 in which all their participant had CD4 count below 200 and all patients were presented with the acute onset of severe hemolytic anemia (13). This variation is because of although many warm autoantibody fix compliment, intravascular hemolysis is unusual in WA-AIHA (64) and extra vascular hemolysis predominate intravascular hemolysis.

8. Strength and limitation

8.1 Strength of the study

- ✓ Although there are studies which tries to rule out the prevalence of AIHA elsewhere, to the best of our knowledge this is the first study in Ethiopia that specifically study the prevalence of AIHA in HIV patient.

8.2 Limitation of the study

- ✓ Only intracellular hemolysis, blood film, is ruled out to confirm the presence of hemolysis.
- ✓ In appropriate storage of patient record severely limited our study to generalize some findings.
- ✓ It is one hospital based study thus; it is difficult to conclude findings.
- ✓ Although hemolysis by warm antibody is the most prevalent type in immune related hemolytic anemia, our study exclude cold antibody initiated hemolytic anemia.

9. Conclusion and recommendation

9.1 Conclusions

Based on the findings of this prospective study AIHA in HIV patient is rare with only the prevalence of 2.34% %. However, missing AIHA in diagnosis will be critical and life treating. Our finding showed that some patients have antibody without developing AIHA, DAT positivity was 4.9, and based on our finding reticulocytosis is not common even if anemia is severe. In addition, even though AIHA occur mostly in advanced age stage and more in females, this study also showed that AIHA can be developed at any condition.

9.2 Recommendations

Some patients may develop AIHA without reticulocytosis we strongly recommend that physician and ART clinic staff or any concerned body to be not dependent on reticulocytosis to rule out the presence of AIHA. In addition to this we recommend that first to use more sensitive test like flowcytometry and advanced molecular testes which also help to quantitate amount of RBC bounded antibodies to know the probability for hemolysis. AIHA test has to be done specifically before blood cell transfusion in HIV patients. For future, we recommend that large scale study be done using highly sensitive test method by incorporating both intracellular and extracellular hemolysis.

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11. Annexes

Annex I: Laboratory procedure

I. Laboratory reagents, supplies and equipment

1. 3 or 4 ml Purple vacutainer tube (EDTA)
2. Needle
3. Vacutainer holder
5. Alcohol swab
6. Cotton balls
7. Normal saline
8. Sysmex CBC Analyzer with reagents.
9. Mixer
10. Brilliant Cresyl Blue
12. Antihumangloblin reagent.
13. Wright stain
14. Heparinized/EDTA capillary tube
15. Disposable glove
17. Tourniquet
18. Microscope with 100X objective
19. Microscope slides
20. Glass test tubes
21. Centrifuge
22. Plastic pasture pipette
23. Distilled water
24. Microscopic oil immersion

N. B commercially prepared liquid Reticulocytes Stain (Brilliant Cresyl Blue) solution was used. It was stored in a brown bottle. If precipitate had been a problem on the smear, the stain was filtered prior to use.

II. Principle of different tests

Principle the instrument: - Sysmex perform hematology analysis according to Hydro dynamic focusing, flow cytometry and SLS hemoglobin method. DC method, which is for RBC measurement for this paper, is work based on the following condition. The blood sample, which is suspended in diluted sample, will pass through the apparatus causing DC resistance. As this occur change in blood cell size is detected as the electrical pulls and blood cell count is calculated by counting pulls.

In Sysmex hematology analyzers RBC are first hemolyzed, and hemoglobin released is converted to a single stable form such as oxyhemoglobin, cynmethemoglobin or SLS hemoglobin. Thos stabilized hemoglobin are measured by spectrophotometrical methods. From RBC indices MCV is directly measured by instrument whereas, others are calculated as follows.

- $MCH (pg) = [Hgb (g/dl) / RBC (x10^{12}/L)] \times 10$
- $MCHC (g/dl \text{ or } \%) = [Hgb (g/dl) / Hct(\%)] \times 100$

Principle for Reticulocyte count: - reticulocyte, non-nucleated immature erythrocytes, contain nuclear remnants of RNA. To detect the presence of this RNA, the red cells must be stained while they are still living. This process is called supravital staining. With supravital staining, the RNA appears as a reticulum within the red cell.

Principle for Wright stain: - This stain is methyl alcohol solution of acid, eosin red in color, dye and basic, methylene blue which is blue in color. Eosin component stain cytoplasm and methylene blue component stains nuclear material, granule and inclusions.

DAT is performed based on the following principle. Sometimes Red cell coated by compliment or IgG antibody, this cell are said to be sensitized with IgG or compliment, does not agglutinate when centrifuged. In order for agglutination to occur, with FC portion of IgGAb or c3b or c3d component of compliment, anti human antibody must be added to the system. This will form a bridge between antibody or compliment, coating the red cell, and antibody on the other cell causing agglutination to be visualized. In the direct form of antihuman globulin test, which is applicable in this study, sensitization occurs *in vivo*.

Even though intracellular hemolysis is there in IAHA, most of the time hemolysis is found Extracellularly. You can find intracelural hemolysis, this is because of the auto antibodies alteration in membrane will be occurred; causing loss of membrane which results in spherical shape erythrocytes (spherocytes). These spherocytes are susceptible for hemolysis and as a result of this one can find fragmented cells. Also you can find macrophage engulfed RBC and Schistocytes if hemolysis occurs intracellularly.

III. Procedure:

For CBC

1. Specimen will be collected into EDTA (purple) vacutainer (3 or 4 ml volume). This procedure will be performed by strictly adhering to SOP and all expected safety rule will be applicable.
2. Well mix blood with EDTA and perform CBC within at most before 8 hour of collection.
3. Register CBC result on provided sheet.

For reticulocyt manual count

4. Add 3-4 drops of Brilliant Cresyl Blue solution to 3-4 drops of thoroughly mixed EDTA anticoagulated blood to a glass test tube.
5. Mix the contents by gently shaking and allow incubating at room temperature for a minimum of 25 minutes.
6. At the end of 25 minutes, gently mix the blood/stain solution.
7. Using a capillary tube, place a drop of the mixture on each of three slides near the frosted edge as it would be done when making a peripheral smear.
8. Using the wedge smear technique, make acceptable smears not too thick or thin.
9. Label the slides with code number
10. Allow to air dry.
11. Count the Reticulocytes Cells within 1000 (500 on each slide) red cells in oil immersion fields. Record the number reticulocytes seen.

$$\% \text{ reticulocytes} = \frac{\text{Number of reticulocytes counted}}{10}$$

Result interpretation will be: **Reticulocytosis** if reticulocyte count greater than 1.8% and **Reticulocytopenia** if reticulocyte count less than 0.4% (65).

For Blood film preparation:-

1. Prepare thin blood film
2. Allow to air dry.
3. Add wright stain and cover the all film by stain.
4. Add buffer solution equal proportion to stain after 2 minute.
5. After 10 minute wash and air dry
6. Examine blood film in 100x objective.

Finally, Perform DAT.

DAT will be carried out as follows: 3 drop of RBC will be done on test tube then the test cells will be washed three times with a minimum of 10 ml of saline per wash, as much of the supernatant as possible will be removed after each wash to achieve maximum dilution of residual serum and then 3% suspension will be done using normal saline. Two volumes of the anti globulin reagent will be added to two volumes of the 3% cell suspension; the test tube would be immediately centrifuged after thorough mixing and finally reading for presence of agglutination will be done macroscopically.

AIHA result interpretation

The anemia has to be Normocytic normochromic anemia ($MCV = 90 \pm 10$ $MCH = 27-32$) (66,67,68), evidence for the presence of hemolysis in blood film such as schistocytes (Helement cell, Karatocyte (horned cell) Triangular cell) or spherosytosis or nucleated RBC (64,69,70), DAT positive and a Reticulocyt count greater than 1.8%. The presence of erythrophagocytosis by monocyte may be seen in severe cases and agglutinated RBC may be seen uncommonly in warm AIHA (64) as the result of such findings in blood film, with other parameters, used as evidence for the presence of AIHA.

Annex II: English Version Informed Consent

Informed Consent Form

Informed consent form before data collection on assessment of prevalence of AIHA in HIV
positive patient Tikur Anbessa Specialized Hospital

Identification: _____

Name of faculty _____

Institution code _____

Hello, how are you? My name is _____ I am currently a student of Addis Ababa University, Department of Medical Laboratory Sciences. And I am going to conduct a survey on prevalence of AIHA in HIV positive patients. The aim of conducting this study is to advance the diagnosis of anemia in these individuals and know the current status of the problem in Ethiopians. I would like to use your CD4 result and blood sample to perform CBC, DAT and retics count that help me in doing this investigation. Your cooperation and willingness to be participant of the study will be very helpful in understanding current prevalence of problem. Your name will not be written in the form and I assure you that all the information will be kept strictly confidential. Your participation is voluntary based and you are not obligated to participate. If you are not comfortable please feel free to refuse.

Do I have your permission to continue? Yes ☐ No ☐

If yes, continue to the next page for the interview

Name of investigator _____ Signature _____

Date of investigation _____

Supervisors name _____ Signature _____

Thank you for your cooperation.

Annex III: Amharic Version Informed Consent

በ ኤች.አይ.ቪ ታማሚዎች ላይ በሚደረገው የAIIHA ግኝታዊ ጥናት ላይ የሚወል መጠይቅ ፎርም

ጥቁር አንበሳ እስፔሻላይዝድ ሆስፒታል

መለያ _____

የጤና ድርጅቱ ስም _____

የ ጤና ድርጅቱ ኮድ _____

የተከበሩ ተሳታፊዬ እንደምን አሉ? የኔስም _____ ይባላል። በአሁኑ ሰአት በአዲስ አበባ ዩኒቨርሲቲ የህክምና የትምህርት ክፍል የህክምና ላብራቶሪ ተማሪ ስምን AIIHA የሚባል በሽታ በኢትዮጵያ ያለበትን ደረጃ ለማወቅ በጥናት ላይ እገኛለሁ። የጥናቱ አላማ HIV ታካሚዎች ላይ የሚደረገውን ምርመራ ለማሻሻል ሲሆን ለዚሁ ይረዳኝ ዘንድ እኔ የእርስዎን CD4 ቁጥር እና የደም ሴል ቁጥር የምጠቀም ሲሆን በተጨማሪም የእርስዎን DAT ውጤት እጠቀማለሁ። የእርስዎ ፈቃደኝነትና ትብብር በጣም ያስፈልጋል። ስሞት የትም ቦታ የማይጠቀስና ሁሉም የሚሰጡት መረጃ በሚስጢር የተጠበቀ ይሆናል። ተሳትፎዎት ሙሉ በሙሉ በእርስዎ ፈቃደኝነት ላይ የተመሰረተ ሲሆን ካልተመቸዎት በነጻነት አለመሳተፍ ይችላሉ።

ፈቃደኛ ኖት አዎ ☐

አይደለሁም ☐

አዎ ካሉ ይቀጥሉ

የጠያቂው ስም _____ ፊርማ _____

ቀን _____

የተቆጣጣሪው ስም _____ ፊርማ _____

ስለተሳትፎዎ አመሰግናለሁ።

Annex IV: Patient Data Extraction form

Patient Data Extraction form on the investigation of prevalence of AIHA in HIV positive Adult patient at Tikur Anbessa Specialized teaching hospital.

This data was extracted from patient history card, request and laboratory result at Tikur Anbessa specialized Hospital, Addis Ababa Ethiopia.

Data extraction form		
1.	Patient card number:_____	Patient code:_____
2.	Sex of the patient	1. Male 2. Female
3.	Pregnancy	1. Pregnant 2. Non pregnant
4.	Age of the patient	
5.	HIV status	
6.	Have you ever transfused blood?	
	If yes in No 6, how long it is?	
7.	Are you taking HAART drug? If yes then, how long :-	Not started----- 3month of usage----- 6month----- 9 month----- A year----- Other----- If other please specify-----
CD4 result		
8.	Cell/mm ³	
	%	
Laboratory result		
9.	CBC result	
	RBC (cell/ l)	
	HGB (g/dl)	

	HCT (%)	
	MCV	
	MCHC	
	WBC	
	LYM total	
	LYM %	
	PLT	
10.	Retics count	
	Number (%)	
	Absolute count (thousand/mm ³)	
11.	DAT result	Positive Negative
12.	Blood film result	

Annex V: Declaration

I the undersigned, declare that this is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been acknowledged.

Name: MignotTaye (BSc) Signature _____

Place: Department of Medical Laboratory Sciences, College of Health Sciences, Addis Ababa University, Ethiopia.

Date of submission: _____

This thesis has been submitted with my approval as University advisor.

Name: Mintewab Hussein (MSc)

Signature _____

Jemal Alemu(MSc)

Signature _____