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Immunophenotype of Chronic Myeloid Leukemia patients on Tyrosine Kinase Inhibitor treatment at Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by AzebTarekegn, entitled: **Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia,** and submitted in partial fulfillment of the requirements for a Master of Science degree in Clinical Laboratory Sciences (Hematology andImmunohematology) complies with the regulations of the university and meets the accepted standards concerning originality and quality.

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Abbreviations

AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
ALL	Acute Lymphocytic Leukemia
AML	Acute Myeloid leukemia
BM	Bone marrow
BP	Blast Phase
CBC	Complete blood count
CD	Cluster of differentiation
CHR	Complete hematologic response
CML	Chronic Myeloid Leukemia
CP	Chronic phase
FACS	Fluorescence-activated cell sorting
FCA	Flowcytometry Analysis
OS	Overall Survival
PB	Peripheral Blood
PBS	Phosphate buffer saline solution
RT-PCR	Reverse transcription-polymerase chain reaction
SOP	Standard operating procedures
SPSS	Statistical Package for Social Science
TASH	TikurAnbessa Specialized Hospital
TKI	Tyrosine kinase inhibitor
WHO	World Health Organization

Abstract

Background: **Background**: Chronic myeloid leukemia (CML) is a cancer affecting blood-forming cells in the bone marrow and blood. It is linked with an abnormal chromosome referred to as Philadelphia (Ph) chromosome, which occurs due to chromosomal translocation t (9; 22).

Objective: The study aimed to assess the immunophenotype of CML patients in chronic or accelerated phase during treatment with Tyrosine Kinase Inhibitor (TKI) drugs at TikurAnbessa Specialized Hospital.

Methods: A total of 38 chronic myeloid leukemia patients on TKI treatment and 15 healthy control (HC) subjects were enrolled. Peripheral blood samples were collected, and whole blood was stained with monoclonal antibodies. A number of cell surface markers were evaluated on both CD45dimCD34+ leukemic stem cells and normal lymphocytes. Differences in the frequency of cell subsets between leukemia patients and controls were analyzed using the non-parametric Kruskal-Wallis test. Data were summarized as median (interquartile range), and a P-value of less than 0.05 was taken as a statistically significant difference.

Result: The percentage of CD45dim cells was significantly higher in CML patients than HCs ($p=0.0001$). Among CD45dim cells the percentage of CD34+ CD38+ and CD34+ CD38- cell among CML patients was substantially elevated as compared to the healthy controls ($p=0.0006$ and $p=0.0008$ respectively). Normal CD19+ B cells were significantly reduced ($p = 0.0014$), and CD56+ NK and NKT cells were significantly elevated ($p = 0.02$ and $p = 0.0001$, respectively) in CML patients relative to controls. CD38 but not CD25 or CD27 was significantly associated with ABL-BCR genotype b2a2; however, none of three markers was associated with levels of the ABL-BCR transcript levels.

Conclusion: According to the study, we observed significant associations with leukemic stem cell markers, and progression of CML from the chronic to imatinib resistant. Moreover, CML patients had an altered distribution of normal B, NK and NKT cells relative to healthy controls. These results suggest that flow cytometric characterization of CML patients may have prognostic value in predicting disease progression, and potentially alerting clinicians that blast crisis may be imminent.

Keywords: Immunophenotype, CML, Flowcytometry, TKI

1. Background

1.1 Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the translocation of the Abelson (ABL1) gene from chromosome 9 to the long arm of chromosome 22, the Breakpoint Cluster Region (BCR) [t(9;22)(q34;q11)], generating the so-called Philadelphia chromosome (1). CML is also defined as stem cell-derived leukemia in which neoplastic cells exhibit the Philadelphia chromosome and the related oncoprotein BCR-ABL1 (2). The BCR-ABL1 fusion gene is the molecular hallmark and causative event of CML. More than 95% of CML patients express the transcript e14a2 (b2a2) or e13a2 (b3a2), namely major BCR-ABL1 coding for p210 protein (3).

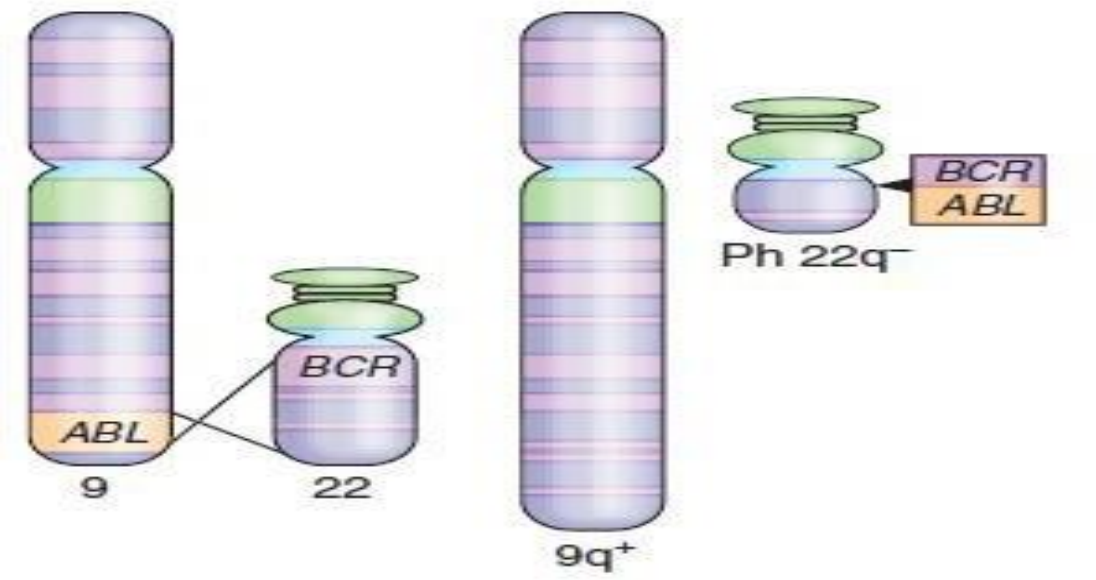

Figure 1: Formation of Philadelphia chromosome (4)

About 85% to 90% of CML cases are represented by chronic phases (CP). However, the remaining progressed to an accelerated phase and then to either myeloid or lymphoid blast crisis over a 5-year time frame (5). However, the mechanism of disease progression is complex, and disease behavior is highly variable for individual patients, with some progressing within a few months and others remaining in stable CP for up to 20 years (6). The progression of CML has three stages: chronic phase, accelerated phase, and blast crisis. The chronic phase is very early,

and approximately 85% of patients are diagnosed in this phase. The second phase is an accelerated phase in which the levels of immature white blood cells are higher than in the chronic phase at about 5-30 %. The blast crisis is CML's final and most severe phase (7).

CML is usually diagnosed in the chronic phase, but patients with CML can rarely present directly in a blast crisis (BC). The blast crisis represents an advanced phase of CML. Progression of CML is frequently accompanied by cytogenetic evolution, with an extra copy of the Philadelphia chromosome, trisomy 8 and 19, and isochromosome (17p) commonly detected (8). Extramedullary blast crisis, as the initial presentation of CML with bone marrow remaining in the chronic phase, is an unusual event (1). In this disease, the peripheral blood and bone marrow (BM) are filled with myeloid precursor cells.

Clinically, 90%-95% of cases of CML have the characteristic t(9;22) (q34.1; q11.2) translocation that leads to the Ph chromosome. Besides, 20% and 2% of adult patients with acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML) have the same cytogenetic marker, respectively (9). The distribution of patients according to immunophenotype was: myeloid (67%), lymphoid (28%), mixed lineage (4%), megakaryocytic (0.5%), and undifferentiated (0.5%) immunophenotype occurred less frequently. The immunophenotype was unknown in 19 male patients (10).

Currently, immunotherapy strategies aim at improving the anti-tumor immune responses of the body and have achieved remarkable efficacy in treating malignant diseases. Tyrosine kinase inhibitors (TKIs) imatinib, nilotinib, and dasatinib are used as first-line treatment in CML. These small molecule inhibitors block the adenosine triphosphate-binding site of the Bcr-Abl tyrosine kinase and prevent phosphorylation of downstream effector proteins (11). Imatinib has shown to be highly efficacious in the treatment of chronic myeloid leukemia (CML) but continuous dosing and patient adherence is essential treatment success (12).

Clinical response to treatment is assessed initially by monitoring the reduction of the peripheral white blood cell count, and subsequently by measurement of BCR-ABL1 transcript levels against a control gene (11). This immune status of patients with CML is essential for choosing an effective drug regimen, evaluating the therapeutic response, and predicting prognosis in patients with CML (13).

1.2 Statement of the problem

Leukemia accounted for approximately 3.4% of all new cancer cases. An estimated 437 thousand new cases and 309 thousand cancer deaths from leukemia worldwide occurred in 2018. CML accounts for approximately 15% of adult leukemia.Reliable data on the exact prevalence of CML are still scarce (14). Globally, the incident cases of CML were increased dramatically by 54.1% in the past 30 years from 42.7×10^3 in 1990 to 65.8×10^3 in 2019 (15).The incident cases, death cases, and Disability-adjusted life years (DALYs) of CML showed an upward trend in themiddle Socio-demographic Index (SDI), low-middle SDI, and low SDI quintiles due to population growth (16).

As per the report in the United States, CML is a myeloproliferative neoplasm with 1-2 cases per 100,000 adults, accounting for approximately 15% of newly diagnosed cases of leukemia in adults (17). Another study also identified CML as a rare stem cell disease with 1 to 2 cases per 100,000 per annum, peaking in the sixth and seventh decades of life (5). An epidemiological study conducted in Germany stated that the prevalence of CML is not well known but has been estimated to be 10-12 cases per 100,000 inhabitants (18). A report also stated that the disease affects all population groups with a median age of 53 years (range, 16 to 84); of this, 64% were male (10).

A study conducted at TikurAnbesa Hospital shows that 40 acute leukemia cases were phenotyped by (FC) flowcytometry; 21 were classified as AML and 19 as ALL. Of the ALL cases, there were 10 (52.6 %) identified as B lineage leukemia cells (B-ALL), and 9 (47.5%) defined as T-lineage cells (T-ALL). Concerning gender frequency, females represented 47.5% and males 52.5% of the cases (19).

CML is an orphan disease in Africa because of the inaccessibility to specific treatmentand the high cost of diagnosis and monitoring patients (20). In Ethiopia, national data on the prevalence and incidence of CML are lacking. However, patient attendance and medical admission rates are rising. Gleevec International Patient Assistance Program (GIPAP) supported the CML patient registry at TikurAnbessa Specialized Hospital, the only center in the country treating CML; accordingly, more than 1,800 patients with CML were diagnosed so far. There is lack of study that describes imunophenotyping of CML patients by flowcytometry in Ethiopia, however only

one study on ALL however Tegengen etal have showed the diagnostic utiity of immunophenotyping for diagnosis and classification of Acute Leukemia.

Since Imatinib was the first TKI approved and is currently considered a very effective frontline option, TKIs has radically changed the management of patients with CML and have markedly improved their outcome. Therefore, the immunophenotype of the CML patients with TKIs to optimize outcomes for treatment failure and suboptimal response cases with the recommended laboratory method is crucial. It is vital to choose an effective drug regimen, evaluate the therapeutic response, and predict prognosis in patients with CML.Limited data are available on hematologic, cytogenetic, andmolecular responses of TKI-treated patients in resource-limited settings (21).

Immunophenotyping remains necessary for assigning specific lineage to blast in patients of CML_BC and thus providing clinical information regarding the treatment protocols and prognosis of the patients. However the immune changes induced by TKIs in patients with CML have not been investigated in Ethiopia. Hence, the current study aimed to profile the peripheral immunophenotype of CML patients treated with TKIs in TASH.

1.3. Significance of the study

The optimal use of current therapeutic opportunities for chronic myeloid leukemia patients requires integrating clinical and laboratory monitoring. Assessment of immunophenotype by flowcytometry is one means of monitoring the efficacy of tyrosine-kinase inhibitor treatment which has increasing support in the literature although is not yet accepted as mainstream clinical management. Immunophenotyping enables comprehensive characterization of the cell surface phenotype of leukemic stem cells (LSCs), and potentially predict imminent blast crisis, a stage requiring significant changes in disease management. The present study aimed to define CML stem cells in the Ethiopian setting in order to assess its potential of disease monitoring.

2. Literature review

As per a report from the United States using FC among 110 participants with chronic phase (CP-CML), 33 exhibited aberrant populations. Two of these 33 patients expressed lymphoid markers, and 31 expressed aberrant myeloid markers. From Seven patients with CML and abnormal B lymphoid blasts (ABLB) identified by FC studies, ABLB ranged from 0.006% to 3.4%, typically demonstrated an immunophenotype with increased CD10 and CD19 and decreased CD38 without myeloid antigens (22). In contrast to aberrant myeloid markers, the detection of lymphoid markers by FC at the time of the diagnosis of CP-CML appears to be associated with early progression to lymphoid BP (23).

In a cohort study by Donatella et al., 243 patients with leukocytosis suspected of CML were tested for circulating PB CD26+ LSCs. After FC evaluation, 211/243 samples scored positive for the presence of the CD26 antigen. The expression of CD26 on the CD34+/CD38− population was detectable in 211/211 PB and 84/84 BM samples of subsequently confirmed BCR-ABL+ CP-CML patients. None of the 32 samples suspicious for CML but scored negative for circulating CD26+ LSCs were diagnosed as CML after conventional cytogenetic and molecular testing. To validate their results, they checked for PB CD26+ LSCs in patients affected by other hematological disorders, and they all scored negative for CD26 expression. Finally, they conclude FC evaluation of CD26 expression on PB CD34+/CD38− population as a new rapid, reproducible, and powerful diagnostic tool for diagnosing CML (24).

Likewise, a retrospective study conducted among 51 patients with CML-BP diagnosed and treated at healthcare institutions between 1988 and 2013 described that the Immunophenotype analysis of blast cells in BM and PB specimens was 36 (70.6%) of myeloid BP, 12 (23.5%) lymphoid BP, and 3 (5.9%) mixed phenotype BP. In addition, patients with lymphoid BP presented with a higher BM blast cell count than those with myeloid BP and a lower platelet (25).

A study at TikurAnbessa Hospital identified aberrant expression of myeloid antigens in some ALL cases. CD33, CD13, and cytoplasmic myeloperoxidase (cMPO) were observed within the B-ALL group in 10%, 40%, and 60% of the cases, respectively. In none of the cases was more than one myeloid marker detected. Two of the 9 cases of T-ALL displayed cMPO; 1 case was positive for CD19, and one positive for CD117. Forty-four percent of T-ALL cases revealed

CD10 expression; these were all negative for CD19. Of the 9 cases of T-ALL, two expressed CD33 and CD13. One case was positive for the B-lineage marker CD19, and 4 of 21 (19%) were positive for the B-lineage marker cCD79a (19).

A recent review protocol by Sadovnik showed that the data suggest CML leukemic stem cells (LSCs) aberrantly express the interleukin-2 receptor alpha chain CD25. Whereas normal CD34+/CD38L BM stem cells display only low amounts of CD25 or lack CD25 altogether, CD34+/CD38L LSCs express CD25 strongly in more than 90% of all patients with untreated CML. Consequently, CD25 can be used to identify and quantify CML LSCs. Besides, the growth of CML LSCs is negatively regulated by CD25 (2).

A quantitative assessment by Culen investigated CD26 markers in a cohort of 31 unselected CML patients. A*ccordingly, BCR/ABL1* positivity was analyzed in highly enriched stem cell fractions using fluorescence *in situ* hybridization (FISH) and reverse transcription PCR (RT-PCR). The proportion of CD26+ LSCs and CD26– HSCs varied considerably among the patients analyzed, and the percentage of CD26+ cells correlated with leukocyte count. In addition, the CD26 expression robustly discriminated LSCs from hematopoietic stem cells (HSCs) (26).

The overall antigen expression among myeloid BP patients was reported as follows: CD13, 85%; CD15, 41%; CD33, 71%; CD117, 40% and myeloperoxidase, 72%. Seven patients (19.4%) expressed megakaryocyte-associated antigens. In 6 patients (16.7%), myeloblasts and monoblasts were demonstrated in the BM aspirate and expressed CD11b and CD64. One case (2.8%) was classified as erythroleukemia based on glycophorin and CD71 (transferrin receptor) expression. The overall expression of B-cell-associated antigens was as follows: CD10, 75%; CD19, 83%; CD20, 41%; and CD22, 67%. Two cases were classified as B-cell lymphoid/myeloid lineage. In both cases, blast cells expressed CD13, CD117, myeloperoxidase, CD19, and CD79a. One case was classified as an uncommon case of mixed lymphoid B/T phenotype based on the expression of B-cell markers CD10, CD19, and CD22 and T-cell markers CD5 and CD7 (25).

A retrospective study involving 71 male participants (between April 2017 and Mar. 2018) aimed to highlight CML in blood samples. Accordingly, the immunophenotypic result based on CD34 & CD7 expression showed that the rate of CD7+ expression was 100% (30/30), 92.7% (38/41), and 100% (30/30) among the newly diagnosed patients, treatment group, and control group,

respectively. Alternatively, all samples (100%) from newly diagnosed patients and the control group had a negative expression of the CD34 marker compared to the treated group with 92.7% (38/41) negative or 7.3% (3/41) positive expression [20]. Implying that TKI treatment is effective in reversing the observed deviation in values of blood parameters to normal or very close to values of healthy individuals (27) .

Tyrosine kinase inhibitors have revolutionized the management of patients with CML and have markedly improved their overall survival (OS). Imatinib was the first TKI approved and is currently considered a very effective frontline option for most patients. Newer TKIs have revolutionized in response to many patients discontinuing Imatinib, either due to disease progression/ resistance or secondary to intolerance (28). Most CML patients are diagnosed in the chronic phase, and approximately 10% to 30% of these patients will, at some time in their course, meet the definition criteria of resistance to Imatinib (29) .

The study conducted to determine the blast lineage using both morphologic and immunophenotypic features identified that 44 (30.1%) patients with non–TKI-based therapy developed blast phase and were treated with traditional modalities such as hydroxyurea or bone marrow transplantation. Furthermore, 23 (9.8%) patients with CML who were treated with TKI evolved into the blast phase. The reduced cumulative incidence of blast phase in TKI-treated patients was significantly lower than in those treated in the pre-TKI era (30). In the era of TKI, the blasts were the most frequent type other than the usual myeloid or lymphoid types compared to the pre-TKI era. The blast phase in TKI-treated patients was associated with a higher peripheral WBC count and a lower blast percentage in the bone marrow (30).

A study conducted in China investigated that, after dasatinib treatment, six of nine patients achieved a better response level, while three did not show improved response levels. Among the nine patients, there were no significant differences in Th1, Th2, and Treg cell levels, whereas CD8þT cell levels significantly increased after dasatinib treatment compared with before treatment. When we analyzed the six patients who obtained a better response level, Th1 and CD8þT cell levels were significantly increased after dasatinib treatment, but Th2 and Treg cell levels did not change. The other three patients who did not have improved response levels showed a decreased Th1 and an increased Treg cell level after treatment (13). There is also a report of a series of 8 patients who developed chronic peripheral lymphocytosis, identified as

natural killer cells or natural killer/T-cells based on their large granular lymphocyte morphologies and CD16+, CD56+, CD3– or CD3+ immunophenotypic profiles, out of 18 patients receiving dasatinib therapy. All cases that developed large granular lymphocyte lymphocytosis achieved optimal molecular response (8/8 in large granular lymphocyte+ patients vs. 3/10 in large granular lymphocyte– patients (31).

As El Rassi et al. reported, of the 33 patients who received Imatinib (85%), dasatinib (12%), or nilotinib (3%) as their first-line treatment; 2/2 patients with lymphoid markers and 3/31 patients with aberrant myeloid markers experienced a transformation to lymphoid BP at a median of 11 months after the initiation of TKI therapy. Although both cases with detectable lymphoid markers rapidly progressed to lymphoid BP, the positive predictive value of BP transformation by detecting aberrant myeloid cells with FC was only 10% (22).

A cohort study involving 42 confirmed cases of CML patients identified two types of transcripts, e13a2 (b2a2) and e14a2 (b3a2) using a multiplex RT-PCR. Accordingly, e13a2 or e14a2 fusion transcripts were demonstrated in all patients with RT-PCR, demonstrating 100% concordance with their baseline Ph cytogenetic status (positive). Moreover, the baseline TLC (201-600x103/µl) and platelet counts (201-900x103/µl) were more associated with the e14a2 (b3a2) than the e13a2 (b2a2) transcript type (32).

There are now indications that, in a portion of patients achieving deep molecular responses, TKI treatment can be stopped without signs of relapse, indicating that these drugs may indeed induce a cure. It is of particular importance since adverse events related to long-term TKI therapies, compromising the quality of life, are now being increasingly recognized (33). The median overall survival was 12 months, and the median failure-free survival was five months (10); similar to this, the median overall survival (OS) in patients whose disease responded to treatment was seven months, with a median disease-free survival of 5 months (25). All seven patients responded to TKI therapy, and no patients progressed to the clinical lymphoid blast phase (23).

In multivariate analysis, myeloid immunophenotype, prior TKI treatment, age >=58 years, lactate dehydrogenase level >=1227 IU/L, platelet count < 102 K/muL, no history of stem cell transplantation, transition to BP from chronic phase/accelerated phase, and the presence of chromosome 15 aberrations, predicted for a significantly increased risk of death (10). Similar multivariate analysis also revealed that lymphoid BP and TKI therapy had a statistically

significant positive impact as prognostic factors for complete hematologic response (CHR). Conversely, in the multivariate analysis, age > 60 years, hemoglobin< 10g/dL, and complex karyotype were statistically significant negative prognostic factors for OS (25).

Overall, achieving a significant hematologic response and/or complete cytogenetic response to first-line treatment was predictive of better survival. In addition, combining a TKI with intensive chemotherapy followed by stem cell transplantation appeared to confer the best outcome (10).

3. Objectives

3.1 General Objective

- To assess the Immunophenotype of CML patients during treatment with TKI drugs at TikurAnbessa Specialized Hospital.

3.2 Specific objectives

1. To determine the phenotype and prognostic value of leukemic stem cells (CD45, CD38, CD34, CD25, CD27) among CML patients attending the TASH Haematology clinic.
2. To enumerate the percentages of peripheral blood cell subsets population such as B- T-, NK- & PMN-cells using their marker (CD3, CD19, CD56, CD15, CD45) among CML patients attending TASH Hematology clinic.
3. To assess the association of phenotype with PCR–determined BCR-ABL genotype, and levels with CML progression.

4. Hypothesis

We Hypothesize that there exists unique CML stem cell and associated host immune system phenotype which is related to CML progression and prognosis.

5. Materials and Methods

5.1 Study area

The study was conducted at Tikur Anbessa Specialized Hospital (TASH) hematology unit in Addis Ababa, Ethiopia. Tikur Anbessa Specialized Hospital was established in 1972. It is the largest teaching hospital affiliated with the College of Health Sciences, Addis Ababa University, and serves as a training center for undergraduate and postgraduate students. TASH delivers diagnosis services and treatment for approximately 500,000 patients per year in its 20 outpatient specialty clinics, inpatient, and emergency unit. From those, the hematology clinic is one of the largest units which deliver comprehensive specialty service for leukemia patients mostly CML patients. Three to five newly diagnosed patients per week and a total of more than 60 CML patients visit the clinic. Outpatient services to CML patients were given in the clinic four times per week. All CML patients took Imatinib free of charge from the oncology pharmacy.

5.2 Study Design and Period

A hospital-based comparative cross-sectional study design was conducted from January 2020 to January 2023 at TASH.

5.3 Population

5.3.1 Source population

All CML patients attended the TASH in the hematology unit during the study period.

5.3.2 Study population

All consented patients aged 18 years and above who were confirmed with CML and patients who were in the follow-up clinic of hematology-oncology for treatment of CML at TASH during the study period.

5.4 Inclusion and Exclusion criteria

5.4.1 Inclusion criteria

For CML patients

- Adult patients with confirmed chronic myeloid leukemia who were in the follow-up clinic of haematology-oncology.

- Patients were willing to participate in the study and gave informed consent.

For Healthy controls

- Participants who were willing to participate in the study and gave informed consent
- Patients who had normal CBC result.

5.4.2 Exclusion criteria

- ✓ Patients who had recently been drawn blood (to avoid repeated vein puncture).
- ✓ Seriously ill patients were excluded from the study. E.g., Severe anemia.
- ✓ Patients who came to the study site for the second time during the study period.

5.5 Study Variables

5.5.1 Dependent Variable

The level of CD markers expression was the dependent variable of the study.

5.5.2 Independent Variable

- Socio-demographic variables
- Clinical variables, such as treatment status, disease phase
- Multiplex RT-PCR result
- BCR-ABL transcript (Qt-PCR) IN % International scale(IS)

5.6 Measurement and Data collection procedure

5.6.1 Sample size determination and sampling method

A convenient sampling technique was used for this study, where fifty-three study participants who fulfilled the eligibility criteria during the study period were recruited (CML, n=38 and healthy control, n=15).

5.6.2 Data collection tools and procedure

All study participants were adequately informed about the study and asked for consent. Then, data on socio-demographic variables and other necessary information, including laboratory investigations, were collected from patients' medical records (charts) using structured formats.

The data collection was conducted by the principal investigator and trained data collector. All the data collection formats were filled in the Hematology-Oncology center of TASH.

5.6.3 Specimen collection laboratory analysis

5.6.3.1. Whole blood collection

Two ml of peripheral blood was collected from both CML patients and apparently healthy individuals, respectively, and transported to Armauer Hansen Research Institute (AHRI) for laboratory investigations on the same date.

5.6.3.2. Flowcytometry

Peripheral blood was stained with surface antibodies (CD19-FITC, CD56-PE, CD3-APC, CD15-FITC, CD34-APC, CD38-PE, CD25-PE, CD27-FITC, and CD45-Percp). All antibodies were purchased from (BD Bioscience, San Jose, CA, USA) and analyzed by BD FACS Canto II with Diva software and FlowJo (Version 10.7.1).

5.6.3.2.1. Gating strategy

Flow cytometry data analysis is fundamentally based upon the principle of gating. Gates and regions are placed around populations of cells with common characteristics, usually forward scatter, side scatters, and marker expression, to investigate and quantify these populations of interest.

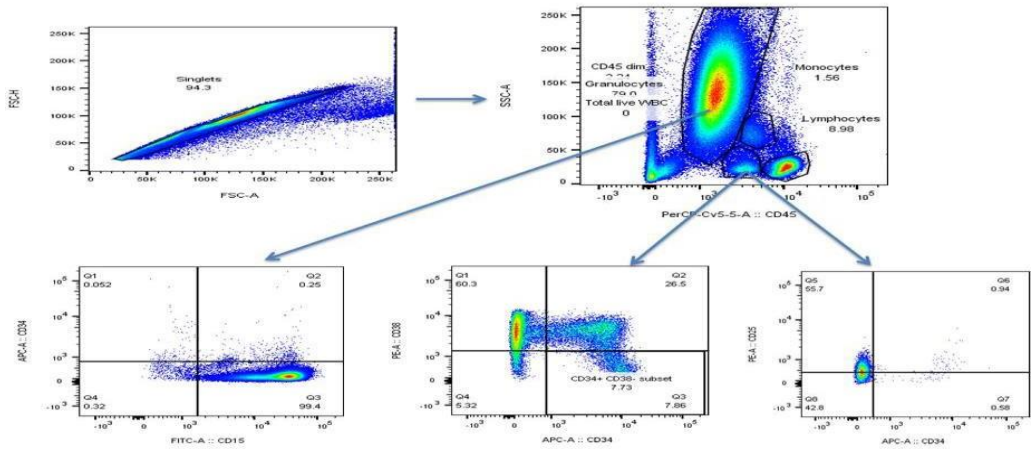

Figure 2. Typical example of gating Strategy Granulocyte and Blast cells**:** First singlet gate using forward scatter (FSC-A) and Forward Scatter FSC-H, followed by a gate on CD45.

Subsequently, granulocytes were identified using CD15 and CD34. Finally, blast cells were identified using CD34 and CD38 and the alternative subset of blast cell gate on CD34 and CD25.

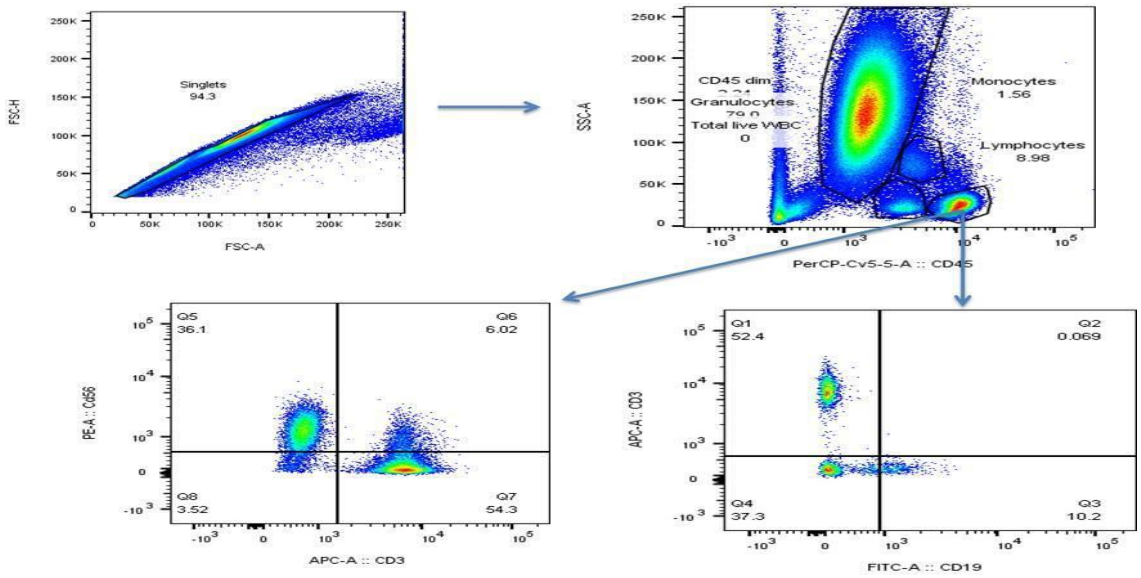

Figure 3: A typical example of gating Strategy is Lymphocyte and NK cells: First singlet gate using forward scatter (FSC-A) and Forward Scatter FSC-H, followed by a gate on CD45. Subsequently, lymphocytes were identified using CD3 and CD19. Finally, NKcells were identified using CD3 and CD56.

5.7 Data Quality Assurance

The necessary quality control measures were undertaken before starting the procedure. Standard operating procedures (SOP) were used to maintain the quality of tests. Reagents and whole blood were optimized for flowcytometry. Also,the quality of generated data was assured by double entry. The data is confidential and also maintained in the database of AHRI.

5.8 Data analysis and interpretation

Data were analyzed by JMP software. A difference in the frequency of PB cell subsets between the two groups was analyzed using the non-parametric Kruskal-Wallis tests. Data aresummarised as median (Interquartile range), and a P value of less than 0.05% was taken as a statistically significant difference.

5.9 Ethical considerations

Ethical approval was obtained from the AAU department of SMLT and / AHRI/ ALERT Ethics Review Committee. All participants gave informed consent prior to data and specimen collection. For all study participants, ethical issues, privacy, potential risk, safety, confidentiality, beneficence, and justice were strictly observed to ensure the study is under the legal framework of the AAU department of MLT and AHRI Ethics Review Board.

5.10. Dissemination of results

The result of this study will be submitted to the AAU Department of Medical Laboratory Sciences, College of Health Sciences, and Hematology-Oncology Clinic, and AHRI. The findings will also be presented in seminars and conferences, and published in peer reviewed Journals.

5.11. Operational definition

Immunophenotyping is a technique used to analyze heterogeneous populations of cells to identify the presence and proportions of the various protein expressed by cells.

Flow cytometry measures cellular properties as they move in a fluid stream (flow) past a stationary set of detectors.

An antigen is any substance capable, under appropriate conditions, of inducing a specific immune response and reacting with the products of that response with a specific antibody.

CD (cluster of differentiation) is a marker used for identifying and investigating cell surface molecules, providing a target for the immunophenotyping of cells.

5.12 Conceptual framework

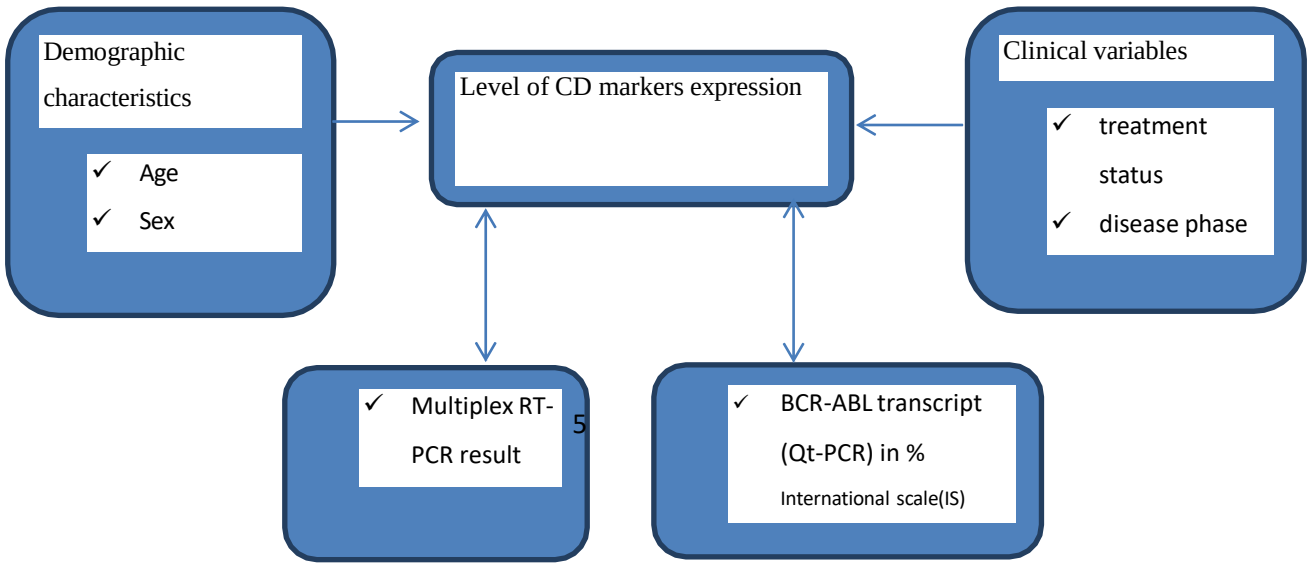

Fig.4. Conceptual frame work

6. Result

6.1 Demographic Characteristics

In this study, 53 participants were enrolled in two groups: CML patients (n=38) and healthy controls (n=15). Nearly half (49.1%) of the study participants were within the age group of 30 - 45 years. The median age of the participants was 34 years, with an interquartile range of 10. More than half of the participants were male (56.6%) Table 1.

Table 1. Socio-demographic characteristics of the study participants

Variables		Type of study participants			
		Cases N (%)	**Controls N (%)**	**TotalN (%)**	***P-value***
Sex	Male	22.0(57.9)	8.0 (53.3)	30.0 (56.6)	0.763
	Female	16.0(42.1)	7.0 (46.7)	23.0 (43.4)	
	Total	38.0(100.0)	15.0 (100.0)	53.0 (100.0)	
Age in years	< 30	10.0(26.3)	7.0 (46.7)	17.0 (32.1)	0.216
	30 -45	19.0(50.0)	7.0 (46.7)	26.0 (49.1)	
	>45	9.0(23.7)	1.0 (6.7)	10.0 (18.9)	
	Total	38.0 (100.0)	15.0 (100.0)	53.0 (100.0)	

Our findings revealed that most (92.11%) of the study participants were in the chronic phase during the first diagnosis and had a median initiate blast count of 5 with an interquartile range (IQR) of 4. All of the 38 patients were on TKIs (Imatinib) treatment, but 4 patients were Imatinib resistant at sample collection time. The result of the study shows a high frequency of b3a2 cells

(57.9 %), followed by b2a2 cells (42.1 %). The rate of treatment interruption and treatment regimen change was 18.42% and 21.05%, respectively (Table 2).

Table: 2 Clinical characteristics of the CML patients visiting Tikur Anbessa specialized hospital, 2021.

Variables		Freq.	Percent
Stage of disease at diagnosis	Chronic Phase	35	92.11
	Accelerated Phase	1	2.63
	Blast crisis	2	5.26
Treatment regimen change	Yes	8	21.05
	No	20	52.63
	Unknown	10	26.31
Treatment interruption	Yes	7	18.42
	No	21	55.26
	Unknown	10	26.31
Status of disease at sample collection	Chronic Phase	32	84.21
	Imatinib Resistant	4	10.52
	Blast crisis	1	2.63
	Unknown	1	2.63
BCRABL type	b2a2	15	42.1
	b3a2	23	57.9

The mean value of WBC count was considerably higher among CML patients than in healthy control (230.39 10^3cells/ mm^3 vs. 5.7910^{3}cells/ mm^3) at the time of diagnosis. Likewise, among CML patients, the WBC counts at the time of diagnosis were higher than the WBC count during the follow-up period (36.7410^3 cells/ mm^3), which may indicate the improvement of the Patient. Furthermore, in patients with CML, a lower (10.03gm/dl) mean value of the hemoglobin level was observed at the time of diagnosis compared to the mean value during the follow-up period

(11.74gm/dl) and in healthy controls (13.88gm/dl), respectively. This may again shows positive progress for the CML patients. The finding also shows a considerable difference in platelet count between patients and healthy controls, with 383.87 10^3cells/ mm^3 and 260.5310^{3}cells/ mm^3, respectively. However, there was no significant difference in platelet count at the time of diagnosis compared to the follow-up time (Fig. 3).

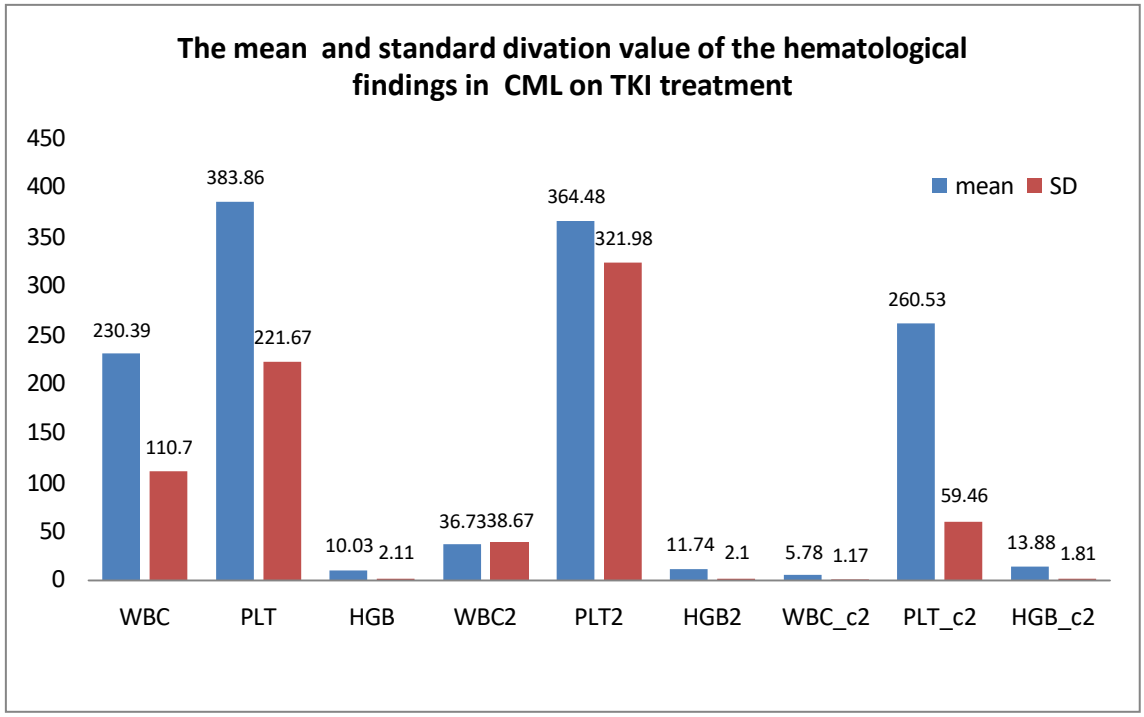

Figure 5: The mean and standard deviation value of the hematological finding in CML on TKI treatment and healthy control.

6.2 Percent of CD 45 dim Cells

Percentage of CD 45 dim cells were significantly higher in CML patients than HC control (*p=0.0001*).The result show significantly high percentage of the CD45 dim/ CD34+ CD38+ and CD45 dim/CD34+ CD38- and cell among CML patients as compared to the Healthy Controls (HCs) (*p=0.0006 and p=0.0008* respectively). CD45 dim/ CD34+ CD25+ and CD45 dim/ CD34+ CD25- cells were not statistically significant (*p=0.6999* and *P=0.3534* respectively) (Table 3).

6.3 Proportion of CD45 dim/ CD27+ CD34+, and CD45 dim/ CD27- CD34+ in CML and HC

There were no differences in CD45 dim/ CD27+ CD34+ cells, and CD45 dim/ CD27- CD34+ percentages between CML and HCs (*p=0.3213 and p=0.8901* respectively) (Table 3). In the contrast, the frequency of CD45 dim/ CD27+ CD34- cells were significantly lower in the CML group compared to the HCs (*P=0.0429*).

6.4 Percentage of T-cells, B-cells, and NK in CML and HC

In this study, within a lymphocyte population, T-cells, B-cells, and NK cells were defined as CD19-CD3+ cells, CD19+ CD3- cells, and CD3- CD56+ cells, respectively. The median percentage of T cells, B-cells, and NK cells were assessed in whole blood in CML and HCs. Accordingly, there were no differences in T-cell percentages between CML and HCs (*p=0.7446*). Significantly low proportions of B cells were observed in the CML group compared to the HC group (*P=0.0014*) (Table 3). However, CML groups showed a significant increase in NK cells (CD3-Cd56+) compared to the HC groups (*p=0.0220*) (Table 3).The NKT-like (CD3+Cd56+) cell had a relatively high median value (4.79) proportion among the CML patients than (1.43) of HCs.

6.5 Frequency of CD15+ CD34- on granulocyte

The percentage of the CD15+ CD34- (*P= 0.1053*) exhibited no differences between CML patients and HCs (Table 3).

Table 3: Test results (p-value) of the Wilcoxon rank-sum test among the study participants in Addis Ababa, Ethiopia, 2022

Leukocyte cells subsets	CML	HCs	P=value
	Median (IQR)	Median (IQR)	
CD45 dim	3.88(2.24-7.9)	1.02(0.55-1.37)	0.0001
CD45 dim/ CD34- CD38+	79.5(58.1-89.475)	64.2(43.8-87)	0.1386
CD45 dim/CD34+ CD38-	0.995(0.255-3.02)	0.26(0.083-0.43)	0.0008

CD45 dim/ CD34+ CD38+	3.73(0.808-11.625)	0.65(0.25-1.01)	0.0006
CD45 dim/ CD34+ CD25+	0.185(0.045-0.85)	0.21(0.043-0.41)	0.6999
CD45 dim/ CD34+ CD25-	3.64(0.607-7.86)	1.6(0.95-3.7)	0.3534
CD45 dim/ CD27+ CD34+	0.033(0.005-0.153)	0.12(0-0.25)	0.3213
CD45 dim/ CD27- CD34+	2.535(0.33-10.148)	2.31(0.7-16.3)	0.8901
Granulocytes (SSC H CD45 med)	59.15(44.68-74.53)	43.7(40.3-54.7)	0.0070
Granulocytes/ CD15+ CD34-	95.95(89.4-99.03)	98(97.3-99)	0.1053
Lymphocytes (SSC L CD45 bright)	9.24(5.348-17.4)	32(24.1-43)	<.0001
Lymphocytes/ CD19- CD3+	81.95(66-86.33)	77.5(73-81.1)	0.7446
Lymphocytes/ CD19+ CD3-	2.72(1.505-4.025)	6.14(4.41-8.34)	0.0014
Lymphocytes/ CD3- CD56+	9.695(6.228-18.525)	7.52(4.06-9.38)	0.0220
Lymphocytes/ CD3+ CD56+	4.79(3.665-7.603)	1.43(0.98-4.01)	0.0001

CML, chronic myeloid leukemia; IQR, Interquartile range; HC, healthy control

6.6 BCR-ABL type and level of cell expression in CML

The finding of this study shows significant differences on the CD38 cell marker expressions between BCRABL type 1(b2a2) and type 2 (b3a2) with p-value = 0.0159 < 0.05. BCRABL type 1 (b2a2) has a higher median value of CD38 cell marker expressions as compared to type 2 (b3a2) (figure 1(a)). We also analyzed the median value of the BCRABL type 1 (b2a2) and type 2 (b3a2) in CD27 and CD25 cell marker expressions. However, the finding of the current study shows that the BCRABL type 1 (b2a2) and type 2 (b3a2) had no observed difference in the median value of CD27 and CD25 cell marker expressions (Figure 6(B&C).

1=BCR-ABL type 1(b2a2)

2=BCR-ABL type 2(b3a2)

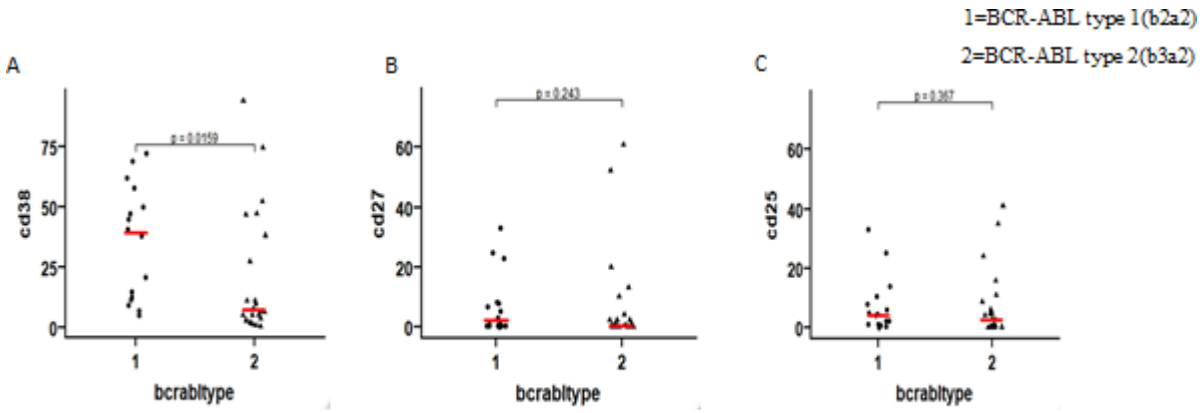

Figure 6. The median value of BCR-ABL expression in chronic myelogenous leukemia (CML) cells: (A) CD38, (B) CD27, (C) CD25

6.7. Normalized Bcr-Abl1 (IS) vs. Cell expression level

CD38 and CD25 cell marker expressions have indirect relationship with PCR result (normalized Bcr-Abl1 (IS)). Figure 7(a) shows that the CD38 cell marker expressions decrease when the Bcr-Abl1 (IS) percentage level increased. CD25 cell marker expression has also a slightly decrement when PCR result increase (Figure 7(c)). However, CD27 cell marker has direct relationship with

Bcr-Abl1 (IS) percentage level. Figure 7(b) shows the direct relationship in which the CD27 cell marker expression increases when the Bcr-Abl1 (IS) percentage level increases.

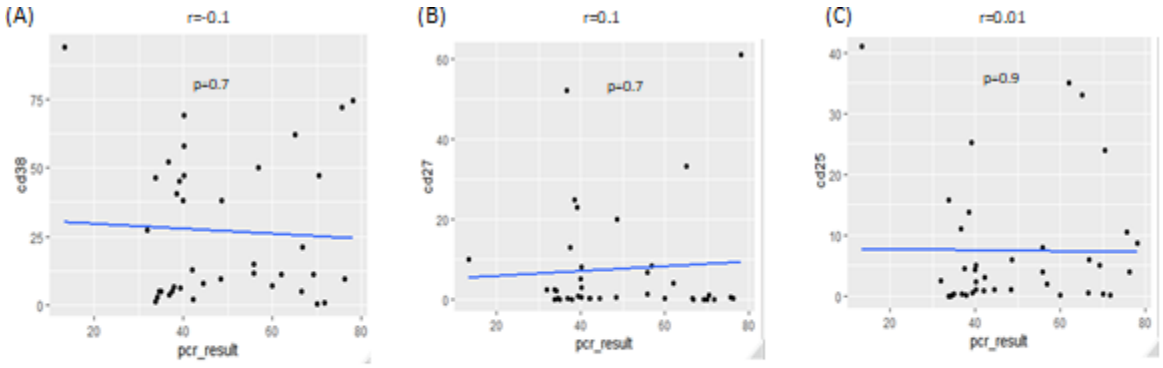

Figure 7. The relationship between cell marker and amount of BCR-ABL1(IS) of ,(A) CD38 ,(B) CD27, (C) CD 25
Lack of relationship between RNA levels and (A) CD38, (B) CD27 and (C) CD25. The correlation coefficients and p=values were A) r = -0.05, **p = 0.73**, B) r = 0.06, **p = 0.69**, and C) r = -0.01, **p = 0.94**

6.8. Disease phase and cell marker expression relationships

The finding of current study shows that number 1 (chronic phase) has a higher median value of CD38 cell marker as compared to number 2 (Imatinib resistant) with a p-value = 0.0128 (Figure 6(a)). Similarly graph 3(b) shows that the median value of the CD27 cell marker have significant differences between the chronic and Imatinib resistant with high median value among chronic phases (p-value= 0.00437). However, the result of the current study has no significant difference in the median value of the CD25 cell markers in different disease phases.

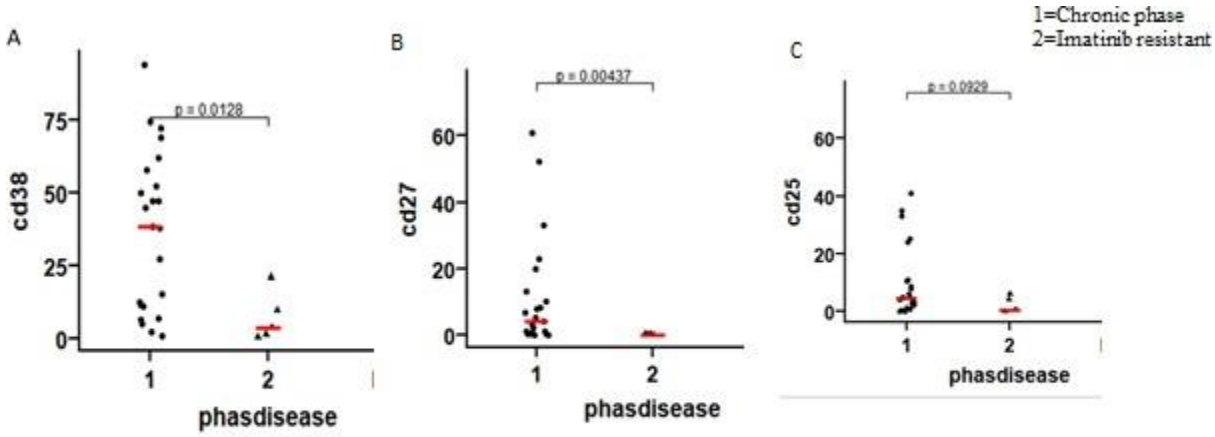

Figure 8:The median value of cell marker expression and disease phase:(A) CD38 ,(B) CD27,(C) CD25

7. Discussion

Chronic myeloid leukemia accounts for 15-20% of newly diagnosed cases of leukemia in adults. The majority of CML cases are characterized by cells carrying the Ph chromosome, which contains the oncogenic BCR-ABL fusion protein. CML and TKI therapy induce changes in the phenotype and function of immune cells. Hence, we conducted this study to assess the Immunophenotypes of CML patients during treatment with TKIs by enumerating nonleukemic cell subset and leukemic stem cell subsets and looking at the association of those cells' phenotypes and genotype and BCR-ABL level.

CD38 is an important cell marker in human hematopoietic stem cells, the finding of current study shows that the phase 1 (chronic phase) has a higher median value of CD38 cell marker as compared to the phase 2 (accelerated phase) with a p-value = 0.0128 (Figure 6(a)). Similarly graph 3(b) shows that the median value of the CD27 cell marker have significant differences between the chronic and accelerated phase with high median value among chronic phases (p-value= 0.00437). However, the result of the current study has no significant difference in the median value of the CD25 cell markers in different disease phases. The study conducted in USA examined the expression of CD38 during CML progression and resulted in CD38 level in chronic phase, but reduce in blast crisis.

The finding of this study shows significant differences on the CD38 cell marker expressions between BCRABL gene type, type 1(b2a2) and type 2 (b3a2) with p-value = 0.0159 < 0.05. BCRABL type 1 (b2a2) has a higher median value of CD38 cell marker expressions as compared to type 2 (b3a2) (figure 1(a)). We also analyzed the median value of the BCRABL type 1 (b2a2) and type 2 (b3a2) in CD27 and CD25 cell marker expressions. However, the finding of the current study shows that the BCRABL type 1 (b2a2) and type 2 (b3a2) had no observed difference in the median value of CD27 and CD25 cell marker expressions.

The result of the current study identified that high 57.9% of b3a2 cells followed by 42.1% of b2a2 cells. In parallel, a cohort study involving 42 confirmed cases of CML patients identified two types of transcripts, e13a2 (b2a2) and e14a2 (b3a2), using a multiplex Reverse Transcriptase-Polymerase Chain Reaction technique (RT-PCR)(32). Sazawal et al. recently

report that more than 95% of CML patients express the transcript e14a2 (b2a2) or e13a2 (b3a2) (3).

We also found no significant difference in the percentage of T cells between CML patients and healthy controls (81.95% vs. 77.50%, *p=0.7446).* However, a significantly lower proportion of B cells was demonstrated among CML patients than in the healthy controls (2.72% vs. 6.14%, *p=0.0014).* However, the proportion of NK cells (CD3- Cd56+) among CML patients was estimated to be high (9.7%) than the proportion of (7.52%) of healthy controls with *P=0.0220*. A related study reported the median proportion of NK cells among lymphocytes was increased in patients compared with controls (16 vs 11%, P = 0.003), Patients whose relative proportion of NK cells was higher than median had increased molecular relapse-free survival compared with the patients with lower NK-cell proportion (73% vs 51%). In contrast, both B-cell and T-cell numbers were decreased, so similar association was not observed (34).

The number of NK cells positively correlated with the successful achievement of TFR in CML. Therefore, NK cells can attack against leukemic cells in CML, and NK cell immunity can have a pivotal role in CML (35).

NKT-like cells (CD3+CD56+) are large granular lymphocytes, and CD1dun restricted, effectively killing cancer cells in a non-MHC-restricted fashion and capable of cytokine production. We analyzed the proportion of NKT-like cells in TKIs treated CML patients and healthy Controls by multiparametric flow cytometry and found a relatively high median value of NKT-like (CD3+ Cd56+) cell proportion among CML patients than in HCs (4.79% vs. 1.43%). However, our findings strongly disagree with the report that revealed that NKT-like cells are decreased in Imatinib-treated CML patients compared to healthy controls (36).

8. Strength and Limitations of the study

8.1. Strength

- All lab works for this study was done with follow up and close supervision of seniors immunologist.
- The study also facilitates the learning of advanced immunological techniques and flow data analysis software (Flow Jo)

8.2. Limitation

- This study primary limitation is the uneven distribution of sample sizes across the three disease phases, which might have an impact on our predictions. However, the results suggest that flow cytometric characterization of CML patients may have prognostic value in predicting disease progression,

9. Conclusion

The percentage of CD45dim cells was significantly higher in CML patients than HCs. Among CD45dim cells the percentage of CD34+ CD38+ and CD34+ CD38- cell among CML patients was substantially elevated as compared to the healthy controls. According to the study, we observed significant associations with leukemic stem cell markers, In general CD38 cell marker predominantly expressed on leukemic steam cells, which is a promising prognostic marker for CML patient. Moreover, CML patients had an altered distribution of normal B, NK and NKT cells relative to healthy controls.

10. Recommendations

These results suggest that flow cytometric characterization of CML patients may have prognostic value in predicting disease progression, and potentially alerting clinicians that blast crisis may be imminent. Other studies that investigate the immunpheotype of CML patients before treatment and after treatment is recommended to further know the effect of the drug. A large scale study is required to confirm and translate this finding into clinically applicable tests.

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11. Annexes

Annex I: English version of study participant information sheet for CML patients.

Addis Ababa University, College of health science, School of Allied Health Science,

Department of medical laboratory science

E-mail: azititar@gmail.com

Tel. +251 912-07-57-12

Participant Information sheet for adults (≥18 years)

1. Study title:

Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

2. Invitation paragraph:

You have been invited to take part in this research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask questions if there is anything that is not clear or if you would like more information.

3. Introduction of the disease

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by increased proliferation and accumulation of immature myeloid cells in the peripheral blood (PB) and bone marrow (BM) of CML patients, without the loss of their capacity to differentiate. It can occur in all age groups but is predominantly a disease of adults; accounting for 20% of adult leukemias. Crowding due to such cells makes the bone marrow unable to produce healthy blood cells and causes serious illness. The immunophenotypic categories of CML are particularly important because they may use for monitoring of the drug outcome and to identify distinctive treatment.

4. The purpose of the study

I am investigating to assess the immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia. I hope that this will help to understand more about the disease progression and prognosis.

5. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time, without giving a reason. A decision not to take part or to withdraw at any time, will not affect the standard of care you receive.

6. What is the study procedure?

If you take part in the research the demographic data assembled by your physician, morphologic result of your bone marrow aspirate and 2 ml blood will be used for the study.

7. What are the possible benefits of taking part?

If you participate in this research, you may not get any direct benefit but anything found in the study based on your laboratory results will be communicated to you and your physician. In addition, your participation is likely to help us in utilization of flowcytometry for monitoring the drug out come and treatment selection.

8. What are the possible disadvantages and risks of taking this part?

There is no major risk in participating in this research, but the minor pain and bleeding that may occur during blood collection will be avoided, as the procedure is carried out by trained and experienced health professionals on the standard good clinical practice.

9. Will my taking part in the study is kept confidential?

The information that we collect from this research project will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. Which number belongs to which name will be kept separately in a password protected data management file and it will not be revealed to anyone except the principal investigator and your treating physician. Your personal information will not be disclosed even during the reporting of the findings. Reports will be written and disclosed anonymously.

10. What will happen to any samples I give?

As already described, during the laboratory analysis we will use your given code not your name for your sample. The samples are immediately processed and analyzed. If there is an abnormal

result, it will immediately be communicated to you and your Doctor, so as your Doctor will take the appropriate action. The data collected will be written and published in peer-reviewed scientific journals.

11. Who is organizing and funding the project

The cost of this research project is covered by AHRI.

12. How to give my consent if you have interest to take part in this research?

The PI or the delegated person will be available at the hematology clinic and will provide you the consent form which you can sign if you agree to participate.

You will be given a copy of the information sheet and a signed consent form to keep

Thank you in advance for considering taking part in this study

Study coordinator and Principal investigator

AzebTarekegn, MSc fellow

Mobile: 091207571

Annex II: Amharic version of study participant information sheet for CML patients.

የመረጃቅጽ

1. የጥናቱመጠሪያ

Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

2. በጥናቱእንድሳተፉስለመጋበዝ

በዚህጥናትሊይእንዲሳተፉእንጋብዘዎታለንነገርግንበጥናቱከመሳተፍዎበፊትየጥናቱንአላማናአስፈላጊነትበቅ ድሚያመረዳትያስፈልገዎታል።እባክዎጊዜወስደውየሚከተለውንመረጃያንብቡ።ማንኛዉምጥያቄወይምግ ሌፅ ያልሆነ ነገር ካለመጠየቅ ይችላሉ::

3. የበሽታው ምንነት

ሥር የሰደደየማይሎይድሉኪሚያ (ሲኤምኤል)በሽታመቅኔበደንብያልደረሱ(ያላደጉ) የደምሕዋሣትንያለአግባብበመጨመርሲያመርትናበደምዝውውርውስጥሲያጠራቅምይከሰታል።በሁሉምየ ዕድሜክልሎችውስጥሊከሰትይችላልነገርግንበብዛትየአዋቂዎችበሽታነው።

20%የበሽታውተጠቂዎችአዋቂዎችናቸው::በእንደዚህዓይነትሕዋሳትመጨናነቅምክንያትመቅኔጤናማየደም ሴሎችንማፍራትእንዳይችልእናከባድህመምያስከትላል።ትክክለኛውንመድሃኒትለመምረጥናየመዳኒቱንምለ ውጥለመከታተልስለሚጠቀሙበጣምአስፈላጊናቸው::

4. የጥናቱዓላማ

እኔአሁንየማጠናዉሥርየሰደደየማይሎይድሉኪሚያ

(ሲኤምኤል)በሽታንፍሎሳይቶሜትሪዘዴንበመጠቀምሲሆንበሽታውያለበትንደረጃበመለየትትክክለኛውን መድሃኒትለመምረጥናየመዳኒቱንምለውጥለመከታተልይጠቅማል።የዚህምጥናትዉጤትስለበሽታውየተሻ ለግንዛቤእንዲገኝይረዳል።

5. በዚህጥናትላይለመሳተፍየግድያስፈልጋል?

በጥናቱላይለመሳተፍየግድአያስፈልግም፤በፍላጎትላይብቻየተመሠረተነው።በጥናቱላይለመሳተፍከወሰኑይ ህመረጃናመስማማትዎንየሚገልጽቅጽይሰጠዎታል።መረጃውንካነበቡናየሚጠይቁትጥያቄካለምበመጠየቅ በሚገባከተረዱበኃላመስማማትዎንይገልጻሉ።ከጥናቱበፈለጉትጊዜናሰዓትያለምንምቅድመሁኔታማቋረጥ

ይችላሉ።እራስዎንከጥናቱበማግለሎምክንያትለህመምዎየህክምናእርዳታከማግኘትአያግድም።እንደማንኛ ውምታካሚአስፈሊጊውንየህክምናእርዳታያገኛሉ።

6. ከእርስዎምንይጠበቃል?

በጥናቱለመሳተፍከተስማሙዶክተርዎእርስዎንበመጠየቅየወሰደውማስታወሻለጥናቱይውላልእንዲሁምከ ላይየተገለፀውንየመቅኔምርመራውጤቱንእናአንድየሻይማንኪያያልሞላየደምናሙናእንዲሰጡይጠበቃል። እነዚውጤቶችእናናሙናዎችለጥናቱምርምርይውላለ።

7. በጥናቱላይቢሳተፉጥቅማጥቅምአገኛለሁኝን?

በዚህጥናትላይበመሳተፍዎየተለየጥቅምበግልአያገኙም።ነገርግንበጥናቱወቅትየተገኘውንየላቦራቶሪውጤት ለእርስዎናለዶክተርዎይገለጻል።እንዲሁምየእርስዎበጥናቱመሳተፍስለበሽታውአይነትያለውንጥሩግንዛቤእን ዲኖረን፤የመድሃኒቱዉጤትምንእንደሚመስልናተገቢውንመድሃኒትበመምረጥበሽታውንለማከምጠቃሚ መረጃለመስጠትይጠቅመናል።

8. በጥናቱላይበመሳተፌየሚደርስብኝጉዳትአለ?

ጥናቱላይበመሳተፍዎየሚደርስብዎትልቅጉዳትየለም፤ነገርግንየደምናሙናበሚወሰድበትወቅትሊፈጠርየሚ ችለውንአነስተኛህመምናየደምመፍሰስለማስወገድልምድባላቸውእናስልጠናበተሰጣቸውባለሙያዎችይከና ወናል።

9. በዚህጥናትመሳተፍበሚስጥርይያዛልን?

አዎንለዚህጥናትየሚሰበሰበውናሙናእናየናሙናውጤትበሚስጥርይያዛል።ስለእርስዎየሚገልጽማንኛውም ነገርበናሙናምሆነበውጤቱላይአይጻፍም።ውጤትሲገለጽስምአልባይሆናል።ለእያንዳንዱናሙናልዩመለያቁ ጥርወይምምልክትይሰጠዋል።የትኛውቁጥርየማንእንደሆነዋናተመራማሪውብቻያውቃል።ስለናሙናዎው ጤትከዋናተመራማሪውበተጨማሪእርሶንየሚከታተለውሀኪምምሊያውቀውይችላል።

10. እኔየምለግሰውናሙናምንይሆናል?

የርስዎውጤትናናሙናየተለየቁጥርይሰጠዋል።በናሙናውላይየርስዎስምአይጻፍም።አብዛኛውናሙናወዲያ ውኑስራላይይውላል።በጥናትየሚገኘውመረጃበህትመትመልክለጤናባለሙያናለሳይንትስቶችይደርሳል።ው ጤትበጅምላበሚገለጽበትጊዜየማንንምውጤትአይወክልም።

11. የጥናቱንወጪየሚደግፈው?

የጥናቱወጪየሚደገፈውበአርማውርሀንሰንየምርምርማእከልነው

12. ፈቃደኝነቴንለመግለፅ?

ጥናቱላይየመሳተፍፍላጎትካለዎትከሚታዩበትክፍልከጥናቱዋናአስተባባሪወይምከተወከለውሰውየስምምነ ትቅፅበመውሰድፊርማዎትንበማሰፈርስምምነትዎንንማረጋገጥይችላለ።

Annex III: English version of study participant information sheet for healthy control.

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Department of medical laboratory science

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Participant Information sheet for adults (≥18 years)

1. Study title:

Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

2. Invitation paragraph:

You have been invited to take part in this research study. Before you decide whether to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Ask questions if there is anything that is not clear or if you would like more information.

3. The purpose of the study

I am investigating to assess the immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia. I hope that this will help to understand more about the disease progression and prognosis.

4. Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are free to withdraw at any time, without giving a reason.

5. What is the study procedure?

If you agree to participate in the study, you are requested to provide 2ml of venous blood. Then, socio demographic information will be taken.

6. What are the possible benefits of taking part?

If you participate in this research, you may not get any direct benefit but anything found in the study your participation is likely to help us in utilization of flowcytometry for monitoring the drug out come and treatment selection.

7. What are the possible disadvantages and risks of taking this part?

There is no major risk in participating in this research, but the minor pain and bleeding that may occur during blood collection will be avoided, as the procedure is carried out by trained and experienced health professionals on the standard good clinical practice.

8. Will my taking part in the study is kept confidential?

All information that we collected for this research project will be kept confidential. Your personal information will not be disclosed even during the reporting of the findings.

9. What will happen to any samples I give?

As already described, during the laboratory analysis we will use your given code not your name for your sample. The samples are immediately processed and analyzed. The data collected will be written and published in peer-reviewed scientific journals.

10. Who is organizing and funding the project

The cost of this research project is covered by AHRI.

11. How to give my consent if you have interest to take part in this research?

The PI or the delegated person will be available and provide you the consent form which you can sign if you agree to participate.

You will be given a copy of the information sheet and a signed consent form to keep

Thank you in advance for considering taking part in this study

Study coordinator and Principal investigator

AzebTarekegn, MSc fellow

Mobile: 091207571

Annex IV: Amharic version of study participant information sheet for healthy control.

የመረጃቅጽ

1. የጥናቱመጠሪያ

Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

2. በጥናቱእንድሳተፉስለመጋበዝ

በዚህጥናትሊይእንዲሳተፉእንጋብዘዎታለንነገርግንበጥናቱከመሳተፍዎበፊትየጥናቱንአላማናአስፈላጊነትበቅ ድሚያመረዳትያስፈልገዎታል።እባክዎጊዜወስደውየሚከተለውንመረጃያንብቡ።ማንኛዉምጥያቄወይምግ ሌፅያልሆነነገርካለመጠየቅይችላሉ::

3. የጥናቱዓላማ

እኔአሁንየማጠናዉሥርየሰደደየማይሎይድሉኪሚያ

(ሲኤምኤል)በሽታንፍሎሳይቶሜትሪዘዴንበመጠቀምሲሆንበሽታውያለበትንደረጃበመለየትትክክለኛውን መድሃኒትለመምረጥናየመዳኒቱንምለውጥለመከታተልይጠቅማል።የዚህምጥናትዉጤትስለበሽታውየተሻ ለግንዛቤእንዲገኝይረዳል።

4. በዚህጥናትላይለመሳተፍየግድያስፈልጋል?

በጥናቱላይለመሳተፍየግድአያስፈልግም፤በፍላጎትላይብቻየተመሠረተነው።በጥናቱላይለመሳተፍከወሰኑይ ህመረጃናመስማማትዎንየሚገልጽቅጽይሰጠዎታል።መረጃውንካነበቡናየሚጠይቁትጥያቄካለምበመጠየቅ በሚገባከተረዱበኃላመስማማትዎንይገልጻሉ።ከጥናቱበፈለጉትጊዜናሰዓትያለምንምቅድመሁኔታማቋረጥ ይችላሉ።

5. ከእርስዎምንይጠበቃል?

በጥናቱለመሳተፍከተስማሙአንድየሻይማንኪያያልሞላየደምናሙናእንዲሰጡይጠበቃል።እነዚውጤቶችእ ናናሙናዎችለጥናቱምርምርይውላለ።

6. በጥናቱላይቢሳተፉጥቅማጥቅምአገኛለሁኝን?

በዚህጥናትላይበመሳተፍዎየተለየጥቅምበግልአያገኙም።እንዲሁምየእርስዎበጥናቱመሳተፍስለበሽታውአይ ነትያለውንጥሩግንዛቤእንዲኖረን፤የመድሃኒቱዉጤትምንእንደሚመስልናተገቢውንመድሃኒትበመምረጥበ ሽታውንለማከምጠቃሚመረጃለመስጠትይጠቅመናል።

7. በጥናቱላይበመሳተፌየሚደርስብኝጉዳትአለ?

ጥናቱላይበመሳተፍዎየሚደርስብዎትልቅጉዳትየለም፤ነገርግንየደምናሙናበሚወሰድበትወቅትሊፈጠርየሚ ችለውንአነስተኛህመምናየደምመፍሰስለማስወገድልምድባላቸውእናስልጠናበተሰጣቸውባለሙያዎችይከና ወናል።

8. በዚህጥናትመሳተፍበሚስጥርይያዛልን?

አዎንለዚህጥናትየሚሰበሰበውናሙናእናየናሙናውጤትበሚስጥርይያዛል።ስለእርስዎየሚገልጽማንኛውም ነገርበናሙናምሆነበውጤቱላይአይጻፍም።ውጤትሲገለጽስምአልባይሆናል።ለእያንዳንዱናሙናልዩመለያቁ ጥርወይምምልክትይሰጠዋል።የትኛውቁጥርየማንእንደሆነዋናተመራማሪውብቻያውቃል።

9. እኔየምለግሰውናሙናምንይሆናል?

የርስዎውጤትናናሙናየተለየቁጥርይሰጠዋል።በናሙናውላይየርስዎስምአይጻፍም።አብዛኛውናሙናወዲያ ውኑስራላይይውላል።በጥናትየሚገኘውመረጃበህትመትመልክለጤናባለሙያናለሳይንትስቶችይደርሳል።

10. የጥናቱንወጪየሚደግፈው?

የጥናቱወጪየሚደገፈውበአርማውርሀንሰንየምርምርማእከልነው

11. ፈቃደኝነቴንለመግለፅ?

ጥናቱላይየመሳተፍፍላጎትካለዎትከሚታዩበትክፍልከጥናቱዋናአስተባባሪወይምከተወከለውሰውየስምምነ ትቅፅበመውሰድፊርማዎትንበማሰፈርስምምነትዎንንማረጋገጥይችላለ።

Annex V: English version of Informed Consent form for CML patient.

Title: Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

I have been given enough information on the proposed study and understood that all information collected will be kept confidential. I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result and also I authorize the investigator to disclose the results of my participation in the study, but not my name. I understand that I have autonomy to withdraw from the project at any time. None of this would affect the care you receive from Hematology clinic of TASH.I have been informed that the investigators will be collect 2 ml of a blood sample which is equivalent to less than one teaspoon. Therefore, I consent to participate voluntarily in the research project.

Name:_____ Signature:_____ Date: _____

The participant is unable to sign. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

I confirm that I have fully explained the purpose of the study and what is involved t

_____ _____ _____

Name of Impartial Witness (if required)Signature Date

Thank you in advance for your cooperation!

Name and signature of data collector _____Date_____

Name and signature of Investigator _____Date_____

I have given the above named copy of this form together with the information sheet.

Contact Address: AzebTarekegn -0912075712

A copy of the signed informed consent form will be given to the study participant

Annex VI: Amharic version of Informed Consent form for CML patients

የፈቃደኝነትማረጋገጫቅጽ (አማርኛቅጅ)

Title: Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

ስለጥናቱበቂየሆነመርጃአግኝቻለው፤ከእኔየሚወሰደውማንኛዉምአይነትመርጃበሚስጥርእንደሚጠበቅተረ ድቻለው።ጥያቄየመጠየቅመብትየመወያየትእድሌከአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱሊ ይበቂምክርናውይይትአድርጌያለሁእናምተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ።ነገርግ ንስምመጠቀስየለበትም።።።በዚህጥናትለመሳተፍምሆነላለመሳተፍበመወሰኔበሆስፒታሉዉስጥበማገኙዉማ ንኛዉምአገልግሎትላይምንምአይነትተጽዕኖየማይኖረዉሲሆንከጥናቱበመማንኛውምሰዓትመውጣትእን ድምችልምተነግሮኛል።አጥኚዎቹአንድየሻይማንኪያያልሞላየደምናሙናእንደሚወስዱተነግሮኛል።ስለዚህበ ፈቃደኝነትበጥናቱለመሳተፍተስማምቻለው።

ስም_____ፊርማ_____

ቀን_____

ይህበጥናቱየሚሳተፈውሰውመፈረምስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊ ውምለመሳተፍመስማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ።

ስለተሳተፉእናመሰግናለን!

የመርጃሰብሳቢውስም _____ፊርማ _____ቀን_____

የተመራማሪውስም _____ፊርማ _____ቀን _____

የተፈረመበትየፈቃደኝነትየማረጋገጫቅጽለተሳታፊውይሰጣል!

Annex VII: English version of Informed Consent form for healthy control.

Title: Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

I have been given enough information on the proposed study and understood that all information collected will be kept confidential. I have given the opportunity to ask questions and discuss the study with the investigator or their deputies on all aspects of the study and I have understood the advice and information given as a result and also I authorize the investigator to disclose the results of my participation in the study, but not my name. I understand that I have autonomy to withdraw from the project at any time. I have been informed that the investigators will be collect 2 ml of a blood sample which is equivalent to less than one teaspoon. Therefore, I consent to participate voluntarily in the research project.

Name:_____ Signature:_____ Date:

_____

The participant is unable to sign. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

I confirm that I have fully explained the purpose of the study and what is involved t

_____ _____ _____

Name of Impartial Witness (if required)Signature Date

Thank you in advance for your cooperation!

Name and signature of data collector _____Date_____

Name and signature of Investigator _____Date_____

I have given the above named copy of this form together with the information sheet.

Contact Address: AzebTarekegn -0912075712

A copy of the signed informed consent form will be given to the study participant

Annex VIII: Amharic version of Informed Consent form for healthy control.

የፈቃደኝነትማረጋገጫቅጽ (አማርኛቅጅ)

Title: Immunophenotype of CML patients on TKI treatment at TikurAnbessa Specialized Hospital, Addis Ababa, Ethiopia.

ስለጥናቱበቂየሆነመርጃአግኝቻለው፤ከእኔየሚወሰደውማንኛዉምአይነትመርጃበሚስጥርእንደሚጠበቅተረ ድቻለው።ጥያቄየመጠየቅመብትየመወያየትእድሌከአጥኚዎችወይምከነሱተወካይጋርተሰጥቶኝበጥናቱሊ ይበቂምክርናውይይትአድርጌያለሁእናምተመራማሪዎቹየጥናቱንውጤትይፋእንዲያደርጉእፈቅዳለሁ።ነገርግ ንስምመጠቀስየለበትም።።በዚህጥናትለመሳተፍምሆነላለመሳተፍበመወሰኔምንምአይነትተጽዕኖየማይኖረ ዉሲሆንከጥናቱበመማንኛውምሰዓትመውጣትእንድምችልምተነግሮኛል።አጥኚዎቹአንድየሻይማንኪያያል ሞላየደምናሙናእንደሚወስዱተነግሮኛል።ስለዚህበፈቃደኝነትበጥናቱለመሳተፍተስማምቻለው።

ስም_____ፊርማ_____

ቀን_____

ይህበጥናቱየሚሳተፈውሰውመፈረምስለማይችልከላይየተዘረዘሩትመረጃዎችለተሳታፊውየተሰጡናተሳታፊ ውምለመሳተፍመስማማቱንገለልተኛታዛቢበመሆንአረጋግጣለሁ።

ስለተሳተፉእናመሰግናለን!

የመርጃሰብሳቢውስም _____ፊርማ _____ቀን_____

የተመራማሪውስም _____ፊርማ _____ቀን _____

የተፈረመበትየፈቃደኝነትየማረጋገጫቅጽለተሳታፊውይሰጣል!

Annex IX: Clinical data collection sheets

I: Data Collection form from Patient Interview

Patient ID (card No.) _____

I care card no. _____

Patient Name _____

Age _____

Sex _____

Place of Residence _____

Occupation _____

II: Data collection Form from patient card

First enrolled date dd_____mm_____yyyy_____

WBC count ×10^3 cells/ mm^3

Neutrophils, %_____Lymphocyte, %_____Monocyte, %_____

Eosinophil, % _____Basophils, % _____

Platelet count × 10^3 cells/ mm^3 _____

RBC count × 10^6 cells/ mm^3 _____

Hgb g/dl _____

Morphology result _____

Duration of treatment _____

Stages of Disease at diagnosis

1. Chronic Phase 2.Accelerated Phase 3.Blast Crisis Phase (Make circle the number)

About Regimen change History

I. Was the regimen changed? Yes_____ No _____

II. Was treatment interrupted? Yes_____ No_____

Annex X: Standard operating procedures for laboratory test

Immunofluorescence Staining of Whole Blood

Scope

Use this method to detect cells bearing specific membrane antigens. Begin by adding whole blood to fluorochrome-conjugated monoclonal antibodies that bind specifically to cell surface antigens. Next, treat the stained sample with FACS Lysing Solution to lyse erythrocytes under gentle hypotonic conditions while preserving the leucocytes; then wash the sample to remove excess antibody and debris. Finally, analyze the cells by flow cytometry.

Principle of Flowcytometry

Flowcytometry measures optical and fluorescence characteristics of single cells or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation avelengths and different emission wavelengths (or "colors"), allows several cell properties to be measured simultaneously.

Materials and reagents

Antibodies will be used for following surface markers.

- CD19, CD56, CD3, CD45, CD38, CD15, CD34, CD27, CD25, IgG 1(Isotype control)
- Facs buffer or PBS
- Facs lysing solution
- 2% Formaldehyde
- Facs tubes
- Appropriate volumes of pipette man
- Pipette tips

Procedures for surface staining

1. Add CD19 FITC 10µl, CD56 PE 10µl, CD45 Percp5µl andCD3 APC 5µl(Panel 1)

2. Add CD15 FITC5µl, CD38 PE 10µl, CD45 Percp, 5µl and CD34 APC10µl (Panel 2)

3. Add CD27 FITC 10µl, CD25 PE 10µl, CD45 Percp, 5µl andCD34 APC10µl (Panel 3)

4.Add CD26 FITC5µl, CD38 PE 10µl, CD45 Percp, 5µl and CD34 APC10µl (Panel 4)

5. Add IgG1 FITC10µl, IgG1 PE10µl, CD45 Percp, 5µl and IgG1 APC 2.5µl (Panel 5)

6. Add 100 µl of whole blood in P1, P2, P3, P4 and P5 tubes, vortex well and incubate for 20 minutes in dark area,

7. Add 1 ml FACS lysing solution in all tubes and incubate for 10 minutes.

8. Add 3 ml FACS buffer or PBS.

9. Centrifuge at 1700rpm for 5 minutes at room temperature.

10. Decant supernatant by inverting tube and allowing liquid to drain in to waste container, and repeat procedure 8, 9 and 10. Then,

11. Add 400 µl FACS buffer solution and mix thoroughly store at 2° to 8°C until analyzed.

12. Acquire on a FACS BD flowcytometery. Mix samples thoroughly before acquisition.

(Source: BD Technical Support Protocol, 2002)

Declaration

I, the undersigned agree to accept responsibility for the scientific ethical and technical conduct of the research project and for the provision of required progress reports as per terms and conditions of the research publications office.

M.Sc. candidate: **AzebTarekegn (B.Sc.)**
Signature: _____

Date of submission: _____

This proposal has been submitted with our approval as advisors.

Advisor: **Samuel Kinde (MSc, PhD candidate)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Aster Tsegaye (MSc, PhD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: **Rawleigh Howe (MD,PHD)**

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.