

**COMPARISON OF β -2 MICROGLOBULIN WITH CD38 AND ZAP-70 AS
PROGNOSTIC MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA**

By

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A dissertation submitted to The University of Nairobi in part fulfillment of the requirement for the degree of Masters of Medicine in Human Pathology.

DECLARATION

I, Dr Deepa Patel, declare that this is my original work and as far as I am aware it has not been presented to any other institution.

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To my parents, my husband and my dear son Yash.

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TABLE OF CONTENTS

DECLARATION	i
SUPERVISORS DECLARATION	ii
DEDICATION	iii
TABLE OF CONTENTS	v
ABBREVIATIONS	ix
ABSTRACT	x
INTRODUCTION	1
LITERATURE REVIEW	3
Chronic lymphocytic leukemia	3
Epidemiology	3
Etiology of CLL	3
Clinical features	4
Laboratory features	5
Diagnosis	5
Prognosis	6
Clinical staging of CLL	6
Lymphocyte characteristics	7
Bone marrow infiltration	7
Beta 2 – Microglobulin	8
Other prognostic markers	9
CD38	10
Somatic IgVH gene mutation	10
ZAP –70	11
Treatment	11
Chemotherapy	12
Corticosteroids	13
Stem cell transplantation	13
Flow cytometry for ZAP-70 and CD38	14
Enzyme immunoassay for β 2 microglobulin	15
STUDY JUSTIFICATION AND RATIONALE	17
STUDY QUESTIONS	18
OBJECTIVES	18
Broad objective	18
Specific objectives	18
Secondary objectives	18
Study design	19
Setting	19
Study population	19
Inclusion criteria	19
Exclusion criteria	19
Sample size determination	20
Recruitment of study subjects	21
Data collection procedures	21

Quality assurance procedures	25
Preanalytical:	25
Analytical:	25
External quality assurances:	26
ETHICAL CONSIDERATION.....	27
DATA MANAGEMENT AND ANALYSIS.....	27
RESULTS	28
Baseline Characteristics	28
Clinical characteristics	29
Laboratory characteristics.....	30
Binet staging.....	33
DISCUSSION	45
CONCLUSIONS	49
LIMITATIONS.....	50
RECOMMENDATIONS.....	50
REFERENCES.....	51
APPENDICES.....	56
APPENDIX I: Laboratory values of normal adults	56
APPENDIX II: Staging systems in chronic lymphocytic leukaemia.....	57
APPENDIX III: Markers of poor prognosis in CLL	58
APPENDIX IV: Procedure for flow cytometry	59
APPENDIX V: Procedure for β 2 microglobulin test	73
APPENDIX VI: Calculation of GFR adjusted B2M	75
APPENDIX VII: Procedure for lactate Dehydrogenase (LDH) estimation.....	76
APPENDIX IX: Monoclonal antibodies panel.....	78
APPENDIX X: Data collection and recording sheet	79
APPENDIX XI: Laboratory tests data sheet	79
APPENDIX XII: Patient information and consent form	80
APPENDIX XI: Data collection instrument (study questionnaire)	85
APPENDIX XIV: Ethical approval	88
APPENDIX XV: Approval from department of Pathology - AKUHN	89

LIST OF FIGURES

Figure 1: Workflow.....	24
Figure 2: Absolute lymphocyte counts in the participants	32
Figure 3: Binet stage in participants at diagnosis	33
Figure 4: ZAP-70, CD 38, B2M and LDH results in participants.....	35
Figure 5: Correlation of B2M with CD38 in participants	36
Figure 6: Correlation of B2M with ZAP-70 in participants	37
Figure 7: Correlation of CD38 with ZAP-70 in participants.....	38
Figure 8: Correlation of LDH with B2M in participants.....	39
Figure 9: Correlation of LDH with CD38 in participants	40
Figure 10: Correlation of LDH with ZAP-70 in participants.....	41

LIST OF TABLES

Table 1: Patient recruitment sites.....	28
Table 2: Sex, Age and Race of patients with CLL	28
Table 3: Clinical findings in participants.....	29
Table 4: Haematology characteristics of participants.....	31
Table 5: Treatment received by participants.....	33
Table 6: B2M and LDH levels in participants.....	34
Table 7: ZAP-70 and CD38 expression in participants.....	35
Table 8: Sensitivity and specificity analyses of B2M against CD38.....	36
Table 9: Sensitivity and specificity analyses of B2M against ZAP 70.....	37
Table 10: Sensitivity and specificity analysis of CD 38 against ZAP 70.....	38
Table 11: Sensitivity and specificity analysis of LDH against B2M.....	39
Table 12: Sensitivity and specificity analysis of LDH against CD38.....	40
Table 13: Sensitivity and specificity analyses of LDH against ZAP-70.....	41
Table 14: Comparison of ZAP-70 with laboratory parameters.....	42
Table 15: Comparison of CD38 with laboratory parameters.....	42
Table 16: Comparison of Binet clinical staging with laboratory parameters.....	43
Table 17: Comparison of Binet clinical staging with median/mean of lab parameters.....	44

ABBREVIATIONS

AKUHN	Aga Khan University Hospital, Nairobi
AIHA	Autoimmune hemolytic anaemia.
B2M	β_2 microglobulin
BM	Bone marrow
CD	Cluster differentiation
CHOP	Cyclophosphamide, hydroxydaunorubicin, Oncovin & Prednisolone
CLL	Chronic lymphocytic leukemia
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELFA	Enzyme linked fluorescent assay
ELISA	Enzyme linked immunosorbent assay
FISH	Fluorescent in situ hybridisation
GFR	Glomerular filtration rate
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
IgVH	Immunoglobulin heavy-chain variable-region
IWCLL	International workshop on chronic Lymphocytic Leukemia
IQR	Interquartile range
KNH	Kenyatta National Hospital
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
MHC	Major Histocompatibility complex
MD	Monroe Dunaway
PBF	Peripheral blood film
RNA	Ribonucleic acid
SLL	Small Lymphocytic Leukemia
TFI	Treatment free interval
WHO	World Health Organization
ZAP	Zeta associated protein.

ABSTRACT

Background information: Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder of variable clinical course. Some patients do not need any treatment during the course of the disease, but some need intensive treatment. Clinical staging is used for prognosis and to initiate treatment, but it cannot identify patients who will run an indolent course or who will run progressive disease. There are prognostic markers, which can be used to identify patients who would benefit from early initiation of therapy irrespective of clinical stage. Elevated β -2 Microglobulin (B2M) and lactate dehydrogenase (LDH), expression of CD38 and ZAP-70 are markers associated with poor prognosis in CLL. B2M and LDH are inexpensive, affordable tests, while ZAP-70 and CD38 are expensive tests.

Objective: To compare β -2 Microglobulin with CD38 and ZAP-70 as prognostic markers for CLL and a secondary objective was to compare LDH with CD38 and ZAP 70 as prognostic markers.

Study design: A cross-sectional comparative descriptive study.

Setting: Thirty five (35) patients were recruited from Kenyatta National Hospital (KNH) hematology clinic and medical wards; ten (10) patients were sourced from hematologists clinic from the Aga Khan University hospital (AKUHN) and the MP Shah hospital.

Subjects: Forty-five participants diagnosed with CLL, on the basis of persistent lymphocytosis and peripheral blood morphology were enrolled in this study. The diagnosis was made as per International workshop of chronic lymphocytic leukemia (IWCLL) (2008) guidelines, which requires the presence of persistent lymphocytosis (in excess of 3 months) of at least 5×10^9 B lymphocytes/L (5000/ μ L) in the peripheral blood.

Methodology: Forty-five (45) eligible participants who were diagnosed with CLL were enrolled into the study. Clinical information as well as relevant laboratory test results at the time of diagnosis for Binet staging was extracted from the patient's records. Clinical features utilized in staging including the presence of lymph node enlargement, splenomegaly and hepatomegaly

were recorded into predesigned data extraction sheets. Four milliliter of venous blood was collected in ethylene diamine tetra acetic acid (EDTA) vacutainer and analysed for full blood counts, as well as CD38 and ZAP-70 expression at AKUHN laboratory. Another four milliliter of venous blood was collected in a plain tube. The serum was separated into a cryovial and frozen at -20°C at unit of clinical chemistry. B2M, LDH and creatinine were assayed in batches at university of Nairobi (UON) clinical chemistry laboratory at KNH using the above thawed serum sample.

Data was collected using the structured questionnaire, and a pre-designed data sheet. Data was entered into MS access database, cleaned and imported into STATA v.12 for analysis. Sensitivity, specificity and Kappa statistics were calculated.

Results: Forty-five eligible patients with the diagnosis of CLL were enrolled into the study. The median age was 62 years with a male to female ratio of 1:1. Twenty three (51%) patients were stage C of Binet staging. Twenty seven (60%) participants expressed ZAP-70, twenty two (49%) participants expressed CD38, thirty five (78%) participants had elevated B2M levels and twenty six (58%) participants had elevated LDH levels. When the comparison tests were done, there was poor concordance between B2M with ZAP-70 ($k=0.0$), CD38 ($k=0.07$) and LDH ($k=0.02$). There was marginal concordance between LDH with ZAP-70 ($k=0.12$) & poor concordance with CD38 ($k= -0.06$). The sensitivity of B2M when compared with ZAP 70 was 77.8% and CD38 was 81.8%. The specificity of B2M when compared with ZAP 70 was 22.2% and CD38 was 26.1%. The sensitivity of ZAP-70 was 65% when compared with LDH. Only mean CD 38 correlated with Binet staging ($p=0.021$), but there was no correlation when Binet stage A, B and C was compared with the positive and negative values of ZAP-70, B2M, LDH and ZAP-70/CD38 combinations.

Conclusion: There was poor correlation between B2M levels with CD38 as well as ZAP-70 expression. There was also poor correlation of LDH levels with CD38, ZAP-70 expression and B2M levels. Therefore B2M is not a suitable substitute for CD38 and ZAP-70 in prognostication of CLL. In addition LDH similarly is not a suitable substitute for CD38 and ZAP-70 as prognostic markers.

Recommendations:

According to this study the inexpensive B2M and LDH levels are inappropriate substitutes for ZAP-70 and CD38 expression in prognostication of CLL. A limitation in this study is the sample size due to budgetary and time limitations. A prospective study with a larger sample size is recommended.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder of small, mature B lymphocytes primarily involving peripheral blood, bone marrow, and lymph nodes (1). The World Health organization (WHO) classifies CLL as a neoplastic disease of B cells, with no involvement of T-cells (2).

Chronic lymphocytic leukemia is a disease of the elderly, which is relatively common in the western world with an annual incidence of 12.8 per 100,000 population. CLL is rare in Asia. There is paucity of data regarding the prevalence and incidence of CLL in Africa in general and Kenya in particular.

Chronic lymphocytic leukemia is a disease with a highly variable clinical course. Some patients never need treatment, while others require intensive treatment early after diagnosis (3). Clinical stage developed by Rai and improved on by Binet remains the strongest predictor of survival in patients with CLL (4). Patients with asymptomatic early stage disease and some patients of intermediate stage disease usually require only monitoring and follow up without therapy, until they develop evidence of disease progression. Patients with advance stage disease usually benefit from treatment.

Clinical stage however does not identify patients whose disease will remain stable or whose disease will progress rapidly, especially early in the disease. Majority of patients have early disease at diagnosis in Western countries. There exist other markers for identifying patients with poor prognosis, which may help take decisions on initiation of therapy as patients with poor prognostic markers derive benefit from early initiation of therapy irrespective of their clinical disease stage (3).

A number of biochemical, cytogenetic and molecular tests that are dependent on CLL tumour biology and tumour burden, as well the disease progression, have gained credence as valuable prognostic markers over the years. One of these factors is the mutation status of the CLL tumour cells, the Immunoglobulin heavy-chain variable-region (IgVH) mutation. Hypermutation is

associated with good prognosis (3). Other powerful prognostic factors include zeta associated protein 70 (ZAP 70) and CD38 expression of the tumour cells, which are both associated with poor prognosis. Elevation of beta-2 microglobulin (B2M) and of Lactate dehydrogenase (LDH) are also associated with poor prognosis in CLL (3,5,6)

Determination of IgVH mutation, ZAP-70 expression and CD38 expression are relatively expensive techniques requiring specialized equipment and skills (7, 8); thus these markers are unlikely to be affordable for routine use in public health facilities in Kenya. On the other hand, B2M and LDH are inexpensive tests that do not require sophisticated equipment or highly skilled personnel; hence can be undertaken in an average routine laboratory. No studies have previously been carried out on prognostic markers of CLL in Kenya, and indeed a literature search revealed paucity of similar information in the African continent.

LITERATURE REVIEW

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is one of the disorders included in the category of conditions grouped together as chronic lymphoproliferative disorders by the WHO. Chronic lymphocytic leukemia as a distinct clinical entity was first identified in 1903 by Turk who provided the criteria for its diagnosis and the features that differentiate it from the lymphomas (9). CLL is a lymphoproliferative disorder of the small, mature B lymphocytes, primarily involving peripheral blood, bone marrow, and lymph nodes (1). The WHO Classification of Tumours of Hematopoietic and lymphoid Tissues (2008) classifies CLL together with small lymphocytic lymphoma (SLL), being only distinguishable from SLL by its leukemic appearance. According to the WHO classification, CLL is a disease of neoplastic B cells, with no involvement of T-cells (2).

Epidemiology

In East Europe and USA the disease incidence of CLL is relatively high while in Asia CLL is rare. Chronic lymphocytic leukemia represents 30% of all leukemias in Caucasians (10). The overall incidence is 2-6 per 100,000 per year, increasing with age and reaching 12.8/ 100,000 at age 65. The mean age at diagnosis is 69.6 years and 80% of patients are aged over 60 years. The disease is rare in young people. The male to female ratio is 1.5-2:1 (2).

Sex and age distribution of CLL in tropical Africa differs from that in the western world. The overall male to female ratio of CLL in tropical Africa is 1:1(11). Below the age of 45 years, the male to female ratio is 1:2, while above 45 years; it is 2:1. CLL in younger adults has been associated with low socio-economic status and rural habitation. Clinical and hematological features are the same as in other continents (11). In KNH, leukemia is the second most common hematological neoplasia after lymphoma, with a relative frequency of 21.3% (KNH data 1979). However, there is no specific information on the prevalence of CLL.

Etiology of CLL

The etiology of CLL is not well understood. Unlike in other forms of leukemias, there is no clear evidence that exposure to chemicals or radiation, diet; cigarette smoking, viral infections or

autoimmune diseases are risk factors for the development of CLL. However, there is an increase in lymphoid malignancies, including CLL, and a subclinical monoclonal B-cell expansion in first and second degree relatives of patients with CLL (12). B-cell receptors of CLL cells demonstrate highly selected IgVH gene usage or even very similar entire antigen-binding sites coded by both heavy and light chain genes, and thus differ from much broader diversity found in normal B lymphocytes. This suggests a limited set of antigens promoting division of precursor cells and clonal evolution (2) .

Clinical features

Chronic Lymphocytic Leukemia is characterized by accumulation of nonproliferating mature-appearing lymphocytes in the blood, marrow, lymph nodes and spleen (13,14). Clinical presentation of CLL is variable, the most common being generalised lymph node swelling. Smaller numbers of patients report constitutional symptoms, (referred to as "B" symptoms), consisting of fever, weight loss, or night sweats. About 25% of CLL patients are asymptomatic at diagnosis, some of whom are picked up in tests of blood counts carried out during unrelated routine examination. The most common physical findings in patients with CLL include lymphadenopathy, splenomegaly, and hepatomegaly (9). Lymph nodes are usually discrete, freely movable and nontender. Painful enlarged nodes usually indicate superimposed infection (9). Bacterial infections (pneumonia) and viral infections are frequent, due to hypogammaglobulinaemia & dysfunction of lymphocytes (15,16). Patients can present with features of anaemia related to bone marrow replacement or, more rarely, to associated autoimmune hemolysis or bone marrow failure. Patients may have bruising or bleeding, secondary to thrombocytopenia, acquired vonwillebrands disease or an acquired inhibitor to factor VIII (9).

Richter transformation occurs when CLL transforms into another lymphoid malignancy such as prolymphocytic leukemia, diffuse large cell lymphoma, hodgkin disease, acute leukemia, multiple lymphoma. Richter syndrome refers to the development of a diffuse large B cell lymphoma or its immunoblastic variant in a patient with CLL (9)

Laboratory features

The diagnosis of CLL requires the presence of persistent lymphocytosis of at least 5×10^9 B lymphocytes/L ($5000/\mu\text{L}$) in the peripheral blood (9,14). Typically at the time of diagnosis, lymphocyte count ranges from 50 to 200×10^9 / L but the count may occasionally be higher (15). The leukemia cells in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. These cells may be found admixed with larger or atypical cells, cleaved cells, or prolymphocytes. Gumprecht nuclear shadows, or smudge cells, found as cell debris, are other characteristic morphologic features found in CLL on the peripheral blood film (17). About 10% of patients may present with a Coombs-positive autoimmune hemolytic anemia. Ten percent of patients may have hypogammaglobulinemia, and another 15% may have hypergammaglobulinemia or even a monoclonal gammopathy. Most asymptomatic patients are identified on the basis of an absolute lymphocytosis on routine complete blood count. Bone marrow findings in CLL include normal to high cellularity with a B lymphocyte population that is monoclonal for kappa or lambda light chain expression (18).

Chronic Lymphocytic Leukemia cells co express the T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23. The levels of surface immunoglobulin, CD20, and CD79b on CLL cells are characteristically low compared with those found on normal B cells (17). These B cells which have relatively low surface immunoglobulin form rosettes with mouse but not sheep erythrocytes (15). Each clone of leukemia cells is restricted to expression of either kappa or lambda immunoglobulin light chains (17). Patients with IgVH unmutated genes (unmutated-CLL) have a more malignant condition, including evidence of advanced, progressive disease, atypical peripheral blood cell morphology, adverse cytogenetic features, clonal evolution, and resistance to therapy than those with mutated IgVH genes (mutated-CLL) (7).

Diagnosis

The diagnosis of CLL as per International workshop of chronic lymphocytic leukemia (IWCLL) (2008) guidelines requires the presence of persistent lymphocytosis (in excess of 3 months) of at least 5×10^9 B lymphocytes/L ($5000/\mu\text{L}$) in the peripheral blood. An additional requirement is that the clonality of the circulating B lymphocytes should be confirmed by flow cytometry and

the lymphocytes should show characteristic lymphocyte morphology and immunophenotype (12,17). Where facilities allow, immunophenotyping should be carried out routinely. As immunophenotyping is expensive and facilities are not yet available widely, the clinical presentation, coupled by lymphocytosis and morphology of lymphocytes on peripheral blood remains the mainstay of diagnosis in our setting.

Prognosis

Clinical staging of CLL

Clinical staging systems by Rai and Binet [appendix II] (19,20) are the mainstay for assessing prognosis based on physical examination and the results of a routine blood count in patients with CLL. Patients with low-risk disease [Rai stage 0, Binet stage A] have a median survival that is close to 15 years, those with intermediate-risk disease [Rai stage I or II; Binet stage B] have a median survival of 5–7 years, and most patients with high-risk disease [Rai stage III or IV; Binet stage C] have a life expectancy less than 3–4 years (7). Of note, assigning a clinical stage to a given patient only requires a physical examination and a blood count. They however, have some limitations; for example, patients who will have progressive disease and those in whom the leukemia will run an indolent course are not identifiable by the stage or staging criteria (7).

Although clinical stage (i.e. Rai and Binet stages) is the strongest predictor of survival, additional prognostic parameters, including patterns of bone marrow (BM) infiltration, lymphocyte doubling time, lymphocyte morphology, immunophenotype and cytogenetics, have now been identified (4, 7). The prognosis of a given patient ultimately depends on complex relationships between the characteristics of the patient (age, gender, comorbidity, performance status), the disease (burden, kinetics and biology of the tumor), as well as sensitivity of the disease to treatment (7).

Age and sex

Older age has consistently been shown to confer a poor prognosis in CLL. Most studies suggest that females with CLL survive longer than males. The reasons for this difference are not clear (4).

Lymphocyte characteristics

Approximately 20% of patients have atypical CLL. These patients have a more advanced stage, higher proliferative index and poorer prognosis. Survival decreases with increased lymphocyte count. Lymphocyte doubling time (LDT) is a useful measure of disease aggressiveness (9). Rapid lymphocyte doubling time is usually defined as the doubling of peripheral blood absolute lymphocyte count in less than 12 months. Montserrat et al. reported a median survival time of over 118 months for patients with slow lymphocyte doubling times, while patients with rapid doubling times had a median survival time of only 61 months (18).

Bone marrow infiltration

The pattern of bone marrow infiltration separates CLL patients into two different prognostic groups. Patients with diffuse infiltration have a median survival ranging between 2 and 4 years, while this value is between 8 and 10 years for those with a non-diffuse pattern. The pattern of bone marrow [BM] infiltration correlates with clinical stage and therefore predominates in advanced cases. Furthermore, patients in early clinical stages may be subclassified into two different prognostic subgroups on the basis of BM histology - patients in early clinical stages with diffuse BM histology tend to progress rapidly towards a more advanced clinical stage. The BM histologic pattern is useful not only in the diagnosis and assessment of prognosis of CLL patients, but it may provide important information for evaluating response to therapy. Nodular or focal lymphoid infiltration is compatible with a complete response (4). It is notable however that some recent studies suggest that the prognostic value of BM biopsy may now be superseded by new prognostic markers (17).

Considering that disease stages and other clinical prognostic factors (e.g., degree of bone marrow infiltration, blood lymphocyte levels, lymphocyte doubling time, lymphocyte morphology) merely reflect the variable clinical course of the disease, the focus of recent research in prognostic factors of CLL has changed from clinical to biological factors, the so-called biomarkers or classifiers (7).

Several biologic markers have been associated with a more aggressive form of CLL. These include rapid lymphocyte doubling time, cytogenetic findings. For example deletions of 6q chromosome are associated with a poor treatment outcome and isolated deletion of 13q14.3 is associated with favourable outcome. Serum beta-2 microglobulin (B2M), CD38 expression, unmutated (germline) immunoglobulin heavy chain gene, and ZAP-70 expression are all associated with a bad prognosis (18).

Beta 2 – Microglobulin

Beta 2-microglobulin (B2M) is a low molecular (11.6 kDa) polypeptide which binds to α chain of the major histocompatibility complex class I (MHC-I) molecules. It is a structural subunit that stabilizes the tertiary structure of the MHC-I molecule on the cell surface of nucleated cells. Metabolism and degradation of HLA leads to B2M dissociation from the heavy chain and its release into the extracellular fluids in free form, which can be measured using immunoassay techniques (21–23) . Half the plasma B2M originates from lymphocytes. Circulating B2M is filtered at the glomerulus and most (99%) is reabsorbed and catabolized by proximal tubular cells. Raised plasma B2M indicates either decreased glomerular filtration or increased synthesis due to membrane turnover. In malignancies this turnover is frequently associated with tumour mass and growth rate. Elevated plasma B2M levels have been reported in several haematologic malignancies including multiple myeloma, lymphomas, myelodysplastic syndromes, chronic myeloid leukemia and chronic lymphocytic leukemia (21–25). Some reports indicate that raised B2M is a predictor of poor survival in several of these malignancies.

B2M is constantly shed by lymphocytes and it is expected that its levels steadily increase together with the progressive expansion of the leukemic clone suggesting a close correlation between clinical stage and B2M levels in CLL (26). In CLL B2M levels correlates with tumor mass and elevated levels predict resistance to chemotherapy (5,27). A significant B2M level reduction is seen after treatment, whereas its increase could detect a relapse. Serum B2M determination is therefore useful in follow-up either during treatment or remission (28,29).

Although a correlation between B2M and the disease stage in CLL likely exists, a study conducted by Gentile et.al found a substantial proportion (33%) of patients with high B2M levels

already at Binet stage A (26). Also a retrospective study conducted on a series of untreated patients from the MD Anderson Cancer Center found B2M was the strongest predictor of 5-year survival on multivariate analysis which controlled for age, stage, and performance status [5-year survival for Rai stages I-II with elevated B2M was 54 months versus 116 months for patients without elevated B2M] (30). Both studies recommended that the role of B2M as a prognostic tool should be re-evaluated possibly in prospective studies involving larger patient cohorts (26).

Given the relative ease with which GFR-corrected B2M can be measured, its recommend to be determined in all CLL patients at diagnosis in order guide the frequency of follow-up visits and identify those patients at greatest risk for early progression (31).

Other prognostic markers

Other serum markers include lactate dehydrogenase (LDH), serum thymidine kinase and soluble CD23. Serum LDH and CD23 levels presumably also reflect tumour burden (5,27). Their value is currently limited either by the lack of a standard assay method, variable cut-off points between series or the lack of validation in a prospective study (12).

Lactate dehydrogenase is an enzyme in the glycolytic pathway, which is released following cell damage. LDH elevation has been reported in several hematological and non-haematological malignancies. Although LDH elevation is usually non specific, it has been shown to correlate with the tumour mass thus providing prognostic indication of disease progression (32).

Some studies have compared LDH and B2M in patients with CLL. A study by Shen et al on 141 patients with CLL in China showed that serum LDH and B2M levels of patients in Binet C were significantly higher than those in Binet A. Binet C and high LDH level were associated with significantly shorter overall survival. The overall survival time in group of elevation of both LDH and B2M levels was shorter than that in group of normal levels of both LDH and B2M. The study concluded that serum LDH level and Binet stage are important prognostic factors for CLL (33).

CD38

CD38 is a cell surface molecule frequently expressed on leukemic cells in patients who experienced relatively early CLL progression (18). CD38 interacts with CD31 and inhibits apoptosis. When CD38 is upregulated, and reaches signal threshold, it can deliver proliferation signal, thus expression of CD38 in CLL is associated with poor prognosis (14,34). The optimum cut-off level with greatest prognostic significance is uncertain. Different studies have chosen values of 7%, 20% or 30% of cells by flow cytometry (12,18). CD38 positivity is associated with significantly shorter overall survival and progression-free survival times, and also a poor response to fludarabine (18). Ghia et al. reported that patients with CD38+ CLL had a 75% probability of progression at a median follow-up of 90 months, compared with 13% probability in patients without CD38 expression (35). CD38 expression may vary during the course of the disease. A correlation exists between high CD38 expression and unmutated IgVH genes, however CD38 is not considered a surrogate marker for IgVH gene status (12).

Somatic IgVH gene mutation

In CLL two broad prognostic categories are recognised based on the presence or absence of somatic mutations in IgVH genes. The presence of unmutated genes in CLL cells is associated with poor prognosis (8). About 50 to 70 percent of patients with CLL have evidence of somatic hypermutation in the IgVH genes of the leukemic cells. These patients probably constitute a subgroup in whom the leukemic cells have passed through the germinal center, the site of IgVH hypermutation (36). In one study, patients with mutated IgVH genes had a median survival of 25 years compared with 8 years for patients with unmutated IgVH genes (37). However, there is still controversy as to the percentage of mutations which best correlates with clinical outcome [between 98% and 95% homology to the germline gene] (12). Most general laboratories, however, are unable to determine IgVH sequences because this requires highly skilled personnel and sophisticated equipment, rendering it into more of a research tool. Moreover, even when the technique is available, it is too costly and time consuming to be included in the standard workup of CLL patients. These considerations have necessitated a search for a surrogate of IgVH mutation in CLL (36). More easily detectable cellular markers have been investigated as surrogates, in particular, the expression of ZAP-70 and CD38 by flow cytometry (8).

ZAP –70

ZAP-70 (zeta associated protein) is a member of the Syk-ZAP-70 protein tyrosine kinase family. It is expressed by normal T cells and natural killer cells and it plays a critical role in the development and differentiation of these cell types. There is no ZAP-70 expression on normal B-lymphocytes, but it was detected on B-CLL cells. It was suggested that the expression of ZAP-70 could not only predict IgVH mutational status, but it could also serve as a prognostic factor in B-CLL and its expression would be stable during the course of the disease. Recently the role of ZAP-70 as a surrogate marker for IgVH mutation to help identify patients with a more aggressive clinical course was reported (3).

There is a 70 to 90% correlation between ZAP-70 expression and absence of IgVH mutation. Measured by flow cytometry (>20% of cells positive), western blot analysis or immunohistochemistry, positivity correlates moderately with CD38 positivity and the presence of poor risk cytogenetics i.e. deletion 11q22-q23, del 17p13 and trisomy (17,38). Thus, patients who express both markers would have the poorest prognosis, those in whom none of these markers is present would have good outcome, and discordant cases would fall in an intermediate-risk category. These results should be validated in prospective studies (7).

Treatment

In general, newly diagnosed patients with asymptomatic early-stage disease (Rai 0, Binet A) should just be monitored and followed up without therapy. Treatment is only initiated if these patients show evidence of disease progression. In general use of alkylating agents in patients with early-stage disease does not prolong survival. This finding was established through several meta-analysis studies. In one study, treated patients with early-stage disease had an increased frequency of fatal epithelial cancers compared with untreated patients (17).

Whereas patients at intermediate (Rai stages I and II) and high risk (Rai stages III and IV) or the equivalent Binet stage B and C usually benefit from the initiation of treatment. However, even some of these patients with more advanced disease (in particular Rai intermediate risk or Binet stage B) can also be monitored without therapy until they have evidence of progressive or symptomatic disease (17).

Criteria for institution of therapy in CLL include (17);

1. Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia.
2. Massive splenomegaly (at least 6 cm below the left costal margin) or progressive or symptomatic splenomegaly
3. Massive nodes (at least 10 cm in longest diameter) or progressive or symptomatic lymphadenopathy.
4. Progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) of less than 6 months. LDT can be obtained by linear regression extrapolation of absolute lymphocyte counts obtained at intervals of 2 weeks over an observation period of 2 to 3 months. In patients with initial blood lymphocyte counts of less than $30 \times 10^9/L$ ($30,000/\mu L$), LDT should not be used as a single parameter to define a treatment indication. In addition, factors contributing to lymphocytosis or lymphadenopathy other than CLL (e.g., infections) should be excluded.
5. Autoimmune anemia and/or thrombocytopenia that is poorly responsive to corticosteroids or other standard therapy.
6. Constitutional symptoms, defined as any one or more of the following disease-related symptoms or signs:
 - a. Unexplained weight loss of 10% or more within the previous 6 months;
 - b. Significant fatigue or inability to work or perform usual chores.
 - c. Fever higher than $100.5^\circ F$ or $38.0^\circ C$ for 2 or more weeks without evidence of infection.
 - d. Night sweats for more than 1 month without evidence of infection.

Chemotherapy

Alkylating agents are widely used, the most commonly used being chlorambucil. Alkylating agent-based combination (39) are also effective in reducing disease bulk in majority of cases although resistance may develop (16).

Fludarabine is the most extensively studied Purine analogue for the treatment of patients with CLL (39). It is the first line drug of choice in younger patients (16) . Fludarabine is also an effective alternative in patients with disease resistant to chlorambucil. The combination of fludarabine with cyclophosphamide (FC) is more effective than fludarabine alone. Fludarabine, cyclophosphamide and rituximab (FCR) improves the response rate and duration of response (16,39).

Monoclonal antibodies

Rituximab, is a monoclonal Antibody targeting CD20. Relatively low levels of CD20 are expressed on CLL B cells, compared to normal B or neoplastic B cells of other lymphomas (39). In addition, soluble CD20 has been demonstrated in plasma of patients with CLL; this may inhibit the capacity of rituximab to bind to CLL B cells, thereby resulting in rapid clearance and negatively affecting pharmacokinetics (39). Standard-dose rituximab has very limited activity for patients with CLL. Dose-intense and dose-dense single-agent rituximab has been shown to increase efficacy (9,39). Rituximab enhances the activity of purine analogue–based therapies and has been incorporated into combination regimens of chemoimmunotherapy (9,39).

Patients with 17p deletion lack p53 function and are resistant to treatment with standard antileukemia drugs such as chlorambucil, purine analogs, and rituximab (39). Alemtuzumab is the humanized monoclonal antibody targeting CD52, a highly expressed antigen on CLL B cells and normal T and B lymphocytes. It is the only FDA-approved drug that reportedly has activity in treating patients with leukemia cells that lack p53 function (39).

Corticosteroids:

Steroids kill CLL cells with p53 mutation; they can be useful for palliation in patients who are resistant to alkylating agents and nucleoside analogues (9).

Stem cell transplantation:

Haematopoietic stem cell transplantation is normally considered in few patients. Stem cell transplantation is worthy of exploration as CLL is not curable, particularly in younger patients. Identification of biologic risk factors indicative of aggressive disease permits the recognition of

patients having poor prognosis with standard therapy and may benefit from early transplantation (9).

Other forms of treatment are radiotherapy, combination chemotherapy (CHOP), Cyclosporin, Splenectomy, Immunoglobulin replacement and Stem cell transplantation [an experimental approach in young patients. May be curative but has a high mortality rate] (16).

Although the indications for treatment are presently based on clinical guidelines, the new prognostic factors markers e.g., mutation status of the immunoglobulin heavy chain variable gene (IgVH); expression of ZAP-70; expression of CD38; plasma level of β 2-microglobulin (β 2M) and presence of chromosome abnormalities such as 17p deletion and 11q deletion can indicate which patients are likely to progress, thereby providing tools to identify patients for whom early treatment may be indicated (39).

Flow cytometry for ZAP-70 and CD38

ZAP-70 and CD38 expression on CD19⁺/CD5⁺ leukemic cells is analyzed using flow cytometry. Multi-parametric immunophenotyping allows the detection of aberrant antigen coexpression and the analysis of heterogeneity and clonality of malignant cells in leukemias (9).

Flow cytometers are instruments that are used primarily to measure the physical and biochemical characteristics of biological particles. Flow cytometry 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 and granularity (represented by right-angle scatter) can resolve certain cell populations. They make measurements on thousands of individual cells/particles in a matter of seconds (40).

Flow cytometers are very complex instruments that are composed of four closely related systems. The *fluidic system* transports particles from a suspension through the cytometer for interrogation by an *illumination system*. The resulting light scattering and fluorescence is collected, filtered, and converted into electrical signals by the *optical and electronics system*. The

data storage and computer control system saves acquired data and is also the user interface for controlling most instrument functions (41).

Basic principle: (42)

- Prepared single cell or particle suspensions are necessary for flow cytometric analysis. Various immunofluorescent dyes or antibodies can be attached to the antigen or protein of interest. The suspension of cells or particles is aspirated into a flow cell where, surrounded by a narrow fluid stream, they pass one at a time through a focused laser beam.
- The light is either scattered or absorbed when it strikes a cell. At the interrogation point, a beam of monochromatic light, usually from argon laser that generates light at 488nm, intersects the cells.
- Light scatter is dependent on the internal structure of the cell and its size and shape. Fluorescent substances absorb light of an appropriate wavelength and reemit light of a different wavelength. Fluorescein isothiocyanate (FITC), Texas red, and phycoerythrin (PE) are the most common fluorescent dyes used in the biomedical sciences.
- Light and/or fluorescence scatter signals are detected by a series of photodiodes (FL1, FL2, FL3 and FL4) and amplified. Optical filters are essential to block unwanted light and permit light of the desired wavelength to reach the photodetector.
- The resulting electrical pulses are digitized, and the data is stored, analyzed, and displayed through a computer system. The end result is quantitative information about every cell analyzed. Since large numbers of cells are analyzed in a short period of time (>1,000/sec), statistically valid information about cell populations is quickly obtained.

Enzyme immunoassay for β 2 microglobulin

Antigen-coated solid phases are incubated with test serum to trap specific antibodies and, after washing, with anti-immunoglobulin antibody-enzyme conjugates to measure the specific antibodies. The amount of specific antibodies is correlated to the enzyme activity bound to the antigen-coated solid phase (43).

In the second most widely used conventional enzyme immunoassay method (immunoglobulin capture method), antiimmunoglobulin antibody-coated solid phases are incubated with test serum to trap both specific and nonspecific immunoglobulins and, after washing, with antigen-enzyme conjugates to measure the specific antibodies. The enzyme activity bound to the anti-immunoglobulin antibody-coated solid phase increases with increasing amounts of specific antibodies (43).

Enzyme immunoassays are solid-phase assay systems that are similar in design to radioimmunoassay but utilize an enzyme rather than a radioactive isotope as the immunoglobulin marker (44).

STUDY JUSTIFICATION AND RATIONALE

It has recently become increasingly clear that younger patients who have early stage CLL (Rai stage 0 or Binet stage A), and who have adverse prognostic features derive significant survival benefit from early initiation of treatment. In the past, it has been the practice to just follow up all patients with early stage CLL, without treatment. Younger patients, those with higher stage disease or with adverse prognostic factors may benefit from more aggressive combination regimens and consideration for stem cell transplant early in the disease course. It is therefore important to identify patients with poor prognostic markers, so that they can benefit from early and appropriate treatment.

In recent years, B2M, ZAP-70 and CD38 have been identified as reliable prognostic markers in CLL patients, which identify patients with poor prognosis, who would derive survival benefit from early treatment. The new markers ZAP-70 and CD38 are increasingly being used in the developed world to assess the prognosis of CLL. They have provided more refined prognostic information allowing CLL patients to be separated into three subgroups: good, intermediate and poor prognosis, with respect to the treatment-free interval (TFI) from diagnosis to first treatment. The three groups with long, intermediate and short median TFI also show long, intermediate and short median survival from diagnosis: 13+ years, 8 years 8 months and 5 years 5 months, respectively (8). These markers also help in early initiation of therapy where necessary.

Performance of ZAP-70 and CD38 is expensive. It requires specialized equipment and reagents as well as highly skilled personnel. These techniques may not be affordable in public health facilities in resource challenged developing world. However both B2M and LDH are affordable easy to set up tests, that can be carried out in routine laboratories.

This study aimed at comparing B2M with ZAP-70 and CD38 as prognostic markers of CLL in Kenyan patients, so the study aims at determine whether the inexpensive B2M marker could be a useful substitute of ZAP-70 and CD38, among Kenyan patients with a diagnosis of CLL.

STUDY QUESTIONS

- a. How do serum B2M levels compare with ZAP-70 and CD38 expression in CLL patients as prognostic markers?
- b. How do serum LDH levels compare with ZAP-70 and CD38 expression in CLL patients as prognostic markers?

OBJECTIVES

Broad objective

To compare serum B2M levels with ZAP-70 and CD38 expression as prognostic markers in patients with CLL

Specific objectives

1. To determine serum B2M levels in patients with CLL.
2. To determine ZAP-70 and CD38 expression in CLL.
3. To compare serum B2M levels with ZAP-70 AND CD38 expression as prognostic markers.
4. To compare B2M, ZAP-70 and CD38 expression with clinical stage of CLL (Binet staging).

Secondary objectives

1. To determine serum LDH levels in patients with CLL.
2. To compare serum LDH level with ZAP-70 and CD38 expression as prognostic markers.
3. To compare serum LDH level with serum B2M level as prognostic markers.

METHODOLOGY

Study design

This was a cross-sectional descriptive study.

Setting

Participants were recruited from the hematology clinic and medical wards at the Kenyatta National Hospital (KNH), Kenya's major teaching and tertiary referral Centre. In order to get a sufficient sample size within the available time additional participants were also recruited from the hematology clinic and wards of the Aga Khan University Hospital (AKUH) and patients from other two private hospitals, MP Shah hospital and Avenue hospital of Nairobi. Laboratory tests were carried out at the following areas:

- Complete blood counts– Unit of Hematology and Blood Transfusion, Department of Human Pathology and Hematology Laboratory, AKUHN.
- Flow cytometry – Hematology Laboratory, AKUHN. Flow cytometry procedure for leukemias is already well established at AKUHN.
- B2M, LDH, etc - Unit of Clinical Chemistry, Department of Human Pathology and General Chemistry Laboratory, KNH.

Study population

The study population comprised all the patients already diagnosed with CLL at KNH, AKUHN and the other two private hospitals, using the diagnostic criteria of absolute persistent lymphocytosis of $5 \times 10^9/l$ in association with appropriate morphological features on PBF.

Inclusion criteria

- Patients with diagnosis of CLL.
- Patients not currently (in the last 2 weeks) on treatment for CLL
- Patients aged 18yr and above who gave consent to participate.

Exclusion criteria

- Patient who declined to give consent to participate.
- Patients with increased prolymphocytes or increased blast count suggesting disease transformation.

- Patients with severe leukopenia ($< 0.5 \times 10^9$).
- Patients who had been treated with monoclonal antibodies.
- Patients with documented evidence of renal and hepatic failure.

Sample size determination

Since there was no study or evidence as to the percentage agreement between B2M and CLL staging, we assumed a percentage agreement of 50%.

Preliminary enquiries at KNH & AKUH indicated that there were approximately 50 new patients with CLL. This is a finite population. A finite population correction was undertaken so as to adjust the sample size to suite the context.

The following formula (45) was used to estimate the sample size;

$$\begin{aligned}
 n' &= \frac{NZ^2P(1-P)}{d^2(N-1) + Z^2P(1-P)} \\
 &= \frac{50 \times (1.96)^2 \times 50 (1-50)}{5^2 (50-1) + (1.96)^2 \times 50 (1-50)} \\
 &= 44.24
 \end{aligned}$$

Where,

n' = the desired sample size after the finite population correction.

N = the estimate of the population size (number of patients who would be attended to with CLL diagnosis at the Haematology clinic numbering 50)

Z = is the standard normal deviation at the 95% confidence level (1.96).

p = is the estimated proportion of agreement between B2M and CLL staging. Since the agreement is not known, p was estimated at 50% so as to yield a larger sample size

d = margin of error (degree of precision) ($\pm 5\%$).

$n' = 45$

A minimum of 45 patients was sufficient to describe the percentage accuracy of the use of B2M, ZAP-70, CD38 and LDH in the prediction of CLL staging with 95 % confidence interval ($\pm 5\%$).

Recruitment of study subjects

Participants were recruited into the study consecutively until the sample size was achieved. Patients were mainly recruited from among those attending the hematology clinic (clinic 23, KNH). Notices of the study requesting recruitment of patients were also put up in medical wards and medical residents were also requested to assist in the recruitment of patients.

Data collection procedures

The principal investigator identified patients who met the inclusion criteria and the participants gave an informed consent as an initial step into the study. The information of each participant was then obtained by administration of a questionnaire. The questionnaire included the patient's demographic characteristics and other relevant clinical information about CLL from the patient's records [Appendix XIII]. Thereafter the principal investigator did a thorough physical examination on the patient.

Demographic and Clinical details

- a. Patient's name, identification number and address.
- b. Age, sex and race of the patient.
- c. Brief clinical information from the patients file – specifically looking for fever, night sweats, weight loss, lymphadenopathy, splenomegaly, hepatomegaly and any other relevant symptoms and signs.
- d. Basic relevant laboratory test results at the time of diagnosis for staging of the patient – haemogram (haemoglobin, WBC count, platelet count).
- e. Rai or Binet staging of the disease [Appendix II] was determined by review of the patients file or by performing general physical examination of the patient where the patients were newly diagnosed. Splenomegaly and hepatomegaly were determined by palpation of abdomen and ultrasound of the abdomen when available.

Eight ml of venous blood was collected aseptically by venipuncture after explaining the collection procedure to the patient. Four milliliter of blood was dispensed in ethylene diamide

tetra acetic acid (EDTA) vacutainer tube and the other 4ml into a plain vacutainer tube. Venous blood in the EDTA vacutainer was mixed adequately to ensure that there was no clotting of blood. EDTA samples were transported at room temperature in a cool box to haematology lab of AKUH.

The samples in plain vacutainers were used to determine levels of B2M, LDH and creatinine. The samples in plain vacutainer were transported in a leak proof container without exposing to extremes of temperatures or sunlight to the clinical chemistry unit. The sample was allowed to clot and serum was separated by centrifuging the sample within two hours of collection. Serum was aliquoted into a cryovial labeled with patient identification details. The sample was then stored at -20°C at unit of clinical chemistry for later assay of B2M, LDH and creatinine levels. These tests were run in batches at regular intervals using the frozen sample. For the samples collected at other hospitals, the serum sample for biochemistry tests were separated and transported to clinical chemistry unit, UON to be frozen at -20°C for further processing.

Laboratory procedures

The laboratory tests were performed by the Principal Investigator, with the aid of qualified laboratory personnel at the Haematology and Biochemistry laboratories.

1. Haematology

Full Haemogram was performed at AKUH using Sysmex XT4000i haematology analyzer, within 24 hours. Blood films were made and stained according to standard protocol using Leishman stain. The principal investigator and the supervisors examined the films independently for the morphological diagnosis. The results of the haemogram were interpreted using reference ranges for men and women [appendix I].

2. Immunophenotyping [appendix IV, appendix VII]

The immunophenotyping using multi-color Beckman-coulter FC 500 flow cytometer was carried out on peripheral blood sample within 24hr for CD19 and CD5. Further immunophenotyping for ZAP-70 and CD38 was done on CD 19⁺/CD5⁺ cells. Gating was done by the principal investigator and further reviewed by the supervisor. The flow

cytometry data was displayed in the form of dot plots or histograms and interpreted according to kit manufacturer's instructions. ZAP-70 and CD38 was interpreted as positive when they were expressed on more than 20% and 30% of CD19+/CD5+ cells respectively (2, 9) [appendix I].

3. B2M [appendix V]

Serum B2M was determined in batches by enzyme immunoassay using Mini Vidas (BioMerieux, France) at General Chemistry Laboratory, KNH and GFR adjusted B2M level was calculated. B2M was interpreted as positive when the values were >2.1mg/L [appendix I].

4. Serum LDH [appendix VII]

Serum LDH was determined in batches using automated chemistry analyzer Olympus AU 640 at General Chemistry Laboratory, KNH. The normal ranges for LDH was 200 – 480U/l. [appendix I].

5. Creatinine [appendix VIII]

Serum Creatinine measurement was used as an indicator for GFR. Serum creatinine was determined in batches using automated chemistry analyzer Olympus AU 640 at General Chemistry Laboratory, KNH. The normal ranges for creatinine was 45 – 115µmol/l. [appendix I].

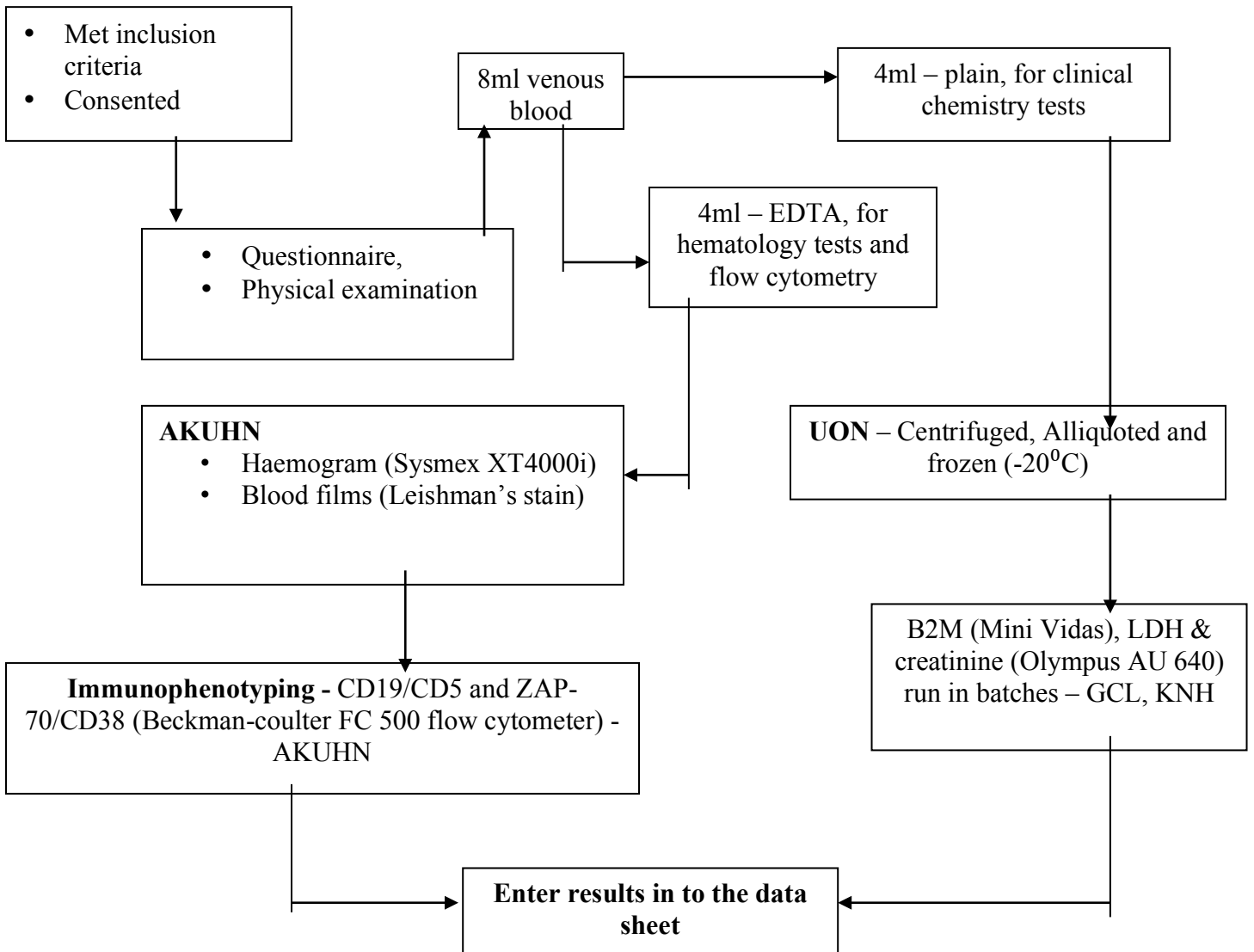


Figure 1: Workflow of collection of data and samples and processing.

Quality assurance procedures

Internal quality control:

Preanalytical:

Venous blood was collected aseptically using the correct technique. Proper volume (4ml) was dispensed into EDTA vacutainers and mixed adequately to ensure that there was no clotting of blood.

Analytical:

Haemograms were run after ensuring that the internal quality controls were within ± 2 standard deviation index (SDI). The WBC counts were compared with the peripheral blood film picture. Blood films were made on new clean glass slides within two hours of collection of blood. A quality control slide was included for all the staining of peripheral blood with Leishman staining. The supervisors and the Principal Investigator reviewed all the slides.

Quality control analysis for flow cytometry was done according to the manufacturer's instructions.

1. Flow check beads were run to verify instrument alignment.
2. PMT voltages were set using flow set and PC7 beads
3. Compensation was set with the compensation tubes prepared.
4. Verify tube (normal blood) was run, compensation setting & sample preparation was confirmed

For serum B2M assay, calibration was performed using the calibrator provided in the kit. Serum B2M were run in two batches and each batch included positive and negative controls.

For serum LDH and serum creatinine, calibration of the machine was performed. They were run in two batches after ensuring that the internal quality controls were within ± 2 standard deviation index (SDI).

All the laboratory results were entered into a pre-designed data sheet.

External quality assurances:

- KNH laboratory participates in external QA quarterly – human Quality Assessment Service (HUQAS)
- AKUHN is ISO 15189 accredited by – South African National Accreditation System (SANAS)

ETHICAL CONSIDERATION

Approval for the study protocol was obtained from the Kenyatta National Hospital/University of Nairobi - Ethical and Research Committee (KNH/UON – ERC) prior to commencement of the study (**P155/05/2010**, Appendix XIV). In addition approval was obtained from AKUHN to conduct the study at AKUH laboratory (Appendix XV).

Informed consent was obtained from each study participant. Samples were collected according to standard procedure. Patient details and results were kept confidential. Patients did not incur any expenses due to participation in this study.

All the results were communicated to the referring doctors to assist in the patient management.

DATA MANAGEMENT AND ANALYSIS

Data was collected with the structured questionnaire, and pre-designed data sheet [Appendix – X, Appendix - XIII] and entered into MS access database after which the data was cleaned and verified. Finally it was imported into STATA v.12 (Stata Corp, College Station, Texas USA) for analysis. Descriptive statistics; Means (Standard Deviations) for normally distributed continuous variables while Medians (Interquartile Range) for non-normally distributed continuous variables. Count and proportions were used for the tabulation of categorical variables.

Bivariate associations were assessed using parametric tests (chi-square, fishers test) and Non-parametric tests (Mann Whitney, Kruskall Wallis and Ranksum) as appropriate.

For diagnostic comparisons, sensitivity, specificity and kappa statistics were calculated. With the results of CD38 and ZAP-70 from flow cytometry as the standards, sensitivity of B2M and LDH against each of them was calculated as true positives (true positive + false negatives), and specificity as true negatives (true negatives + false positives). In the comparison between B2M and LDH, B2M was used as the standard.

RESULTS

During the period between September 2011 and July 2012, 68 patients files were scrutinized for participation in the study. A total of 45 participants met the inclusion criteria and were enrolled into the study. Among the thirteen (13) patients who were not recruited, three (3) patients had severe leucopenia; one (1) patient had increased blast count and the other nine (9) patients were not available. Of these participants, thirty-two (32) were recruited from hematology clinic (clinic no: 23) at KNH, three (3) from various wards at KNH, six (6) from AKUHN & four (4) from other hospitals. Majority 32 (71.1%) were from the KNH hematology clinic (Table 1).

Table 1: Patient recruitment sites

Hospital (n=45)	n (%)
KNH Haematology clinic	32 (71.1%)
KNH wards	3 (6.7%)
AKUHN	6 (13.3%)
MP Shah and Avenue hospital	4 (8.9%)

Baseline Characteristics

As shown in table 2, the median age in years was 62 years. Patient's age ranged from 42yrs to 95yrs. About half of the patients were males 23(51.1%); with male to female ratio was 1:1. Majority 44 (97.8%) were black Africans with only 1 (2.2%) being of Asian origin.

Table 2: Sex, Age and Race of patients with CLL

Characteristic (n=45)	
Age	
Median age	62yrs
Age range	42 – 95yrs
Sex	
n (%)	
Male	23 (51.1%)
Female	22 (48.9%)
Race	
n (%)	
African	44 (97.8%)
Asian	1 (2.2%)

Clinical characteristics

As shown in table 3, the commonest clinical features were lymph node enlargement, splenomegaly and weight loss. Most commonest site of lymph node enlargement was of cervical region followed by axillary region.

Table 3: Clinical findings in participants

Clinical symptoms and findings (n=45)	
Characteristics	n (%)
Weight loss	18 (40%)
Night sweats	13 (28.9%)
Fever	13 (28.9%)
Lymphadenopathy	25 (55.6%)
Cervical Lymphadenopathy	15 (33.3%)
Axillary Lymphadenopathy	7 (15.6%)
Inguinal Lymphadenopathy	6 (13.3%)
Generalised Lymphadenopathy	5 (11.1%)
Splenomegaly	23 (51.1%)
Hepatomegaly	14 (31.1%)
Hepatosplenomegaly	11 (24.4%)

Laboratory characteristics

The mean haemoglobin level was 10.5g/dL (SD \pm 2.6; Range 3.7 – 14.6 g/dL) (table 4). Majority 40 (88.9%) of the patients were anaemic, with only 5 (11.1%) having a normal haemoglobin level.

The mean WBC count was $70.5 \times 10^9/l$ (SD \pm 74.9; Range 7.19 – 320×10^9), (table 4). Majority of the patients had high WBC count with only six patients (13.3%) having normal levels.

As shown in table 4, all the patients (100%) had absolute lymphocytosis (Range 5.6 - $297 \times 10^9/l$) and increased differential lymphocyte count (Range 45.3% – 97%). Majority 28 (62.3%) of the patients had absolute lymphocyte counts below $50 \times 10^9/l$. Eleven patients (24.4%) had absolute lymphocyte counts in between $50 - 100 \times 10^9/l$ and six (13.3%) patients had absolute lymphocyte counts more than $100 \times 10^9/l$ (figure 2).

The mean platelet count was $156.4 \times 10^9/l$ (SD \pm 79.2; Range 39 – 409×10^9), (table 4). Twenty-five (55.5%) of the patients had thrombocytopenia and twenty patients (44.4%) having a normal platelet count.

Table 4: Haematology characteristics of participants

Haematology results (n=45)	
Characteristics	
Haemoglobin (g/dL), mean (SD)	10.5 (2.6)
Haemoglobin categories	n (%)
Normal	5 (11.1%)
Anaemia	40 (88.9%)
White blood cell count ($\times 10^9/l$) mean (SD)	70.5 (74.9)
WBC count categories	n (%)
Normal WBC count	6 (13.3%)
High WBC count	39 (86.75%)
Lymphocyte count, ($\times 10^9/l$) mean (SD)	75.2 (14.7)
Absolute lymphocytosis n (%)	45 (100%)
Platelet count ($\times 10^9/L$), mean (SD)	156.4 (79.2)
Platelet count categories	n (%)
Normal n (%)	20 (44.4%)
Thrombocytopenia n (%)	25 (55.6%)
Peripheral blood film n (%)	
Normocytic normochromic anaemia	26 (57.8%)
Microcytic hypochromic anaemia	19 (42.2%)
Lymphocytosis	45 (100%)
Platelets	
Adequate	26 (57.8%)
Mild thrombocytopenia	6 (13.3%)
Moderate thrombocytopenia	8 (17.8%)
Severe thrombocytopenia	5 (11.1%)

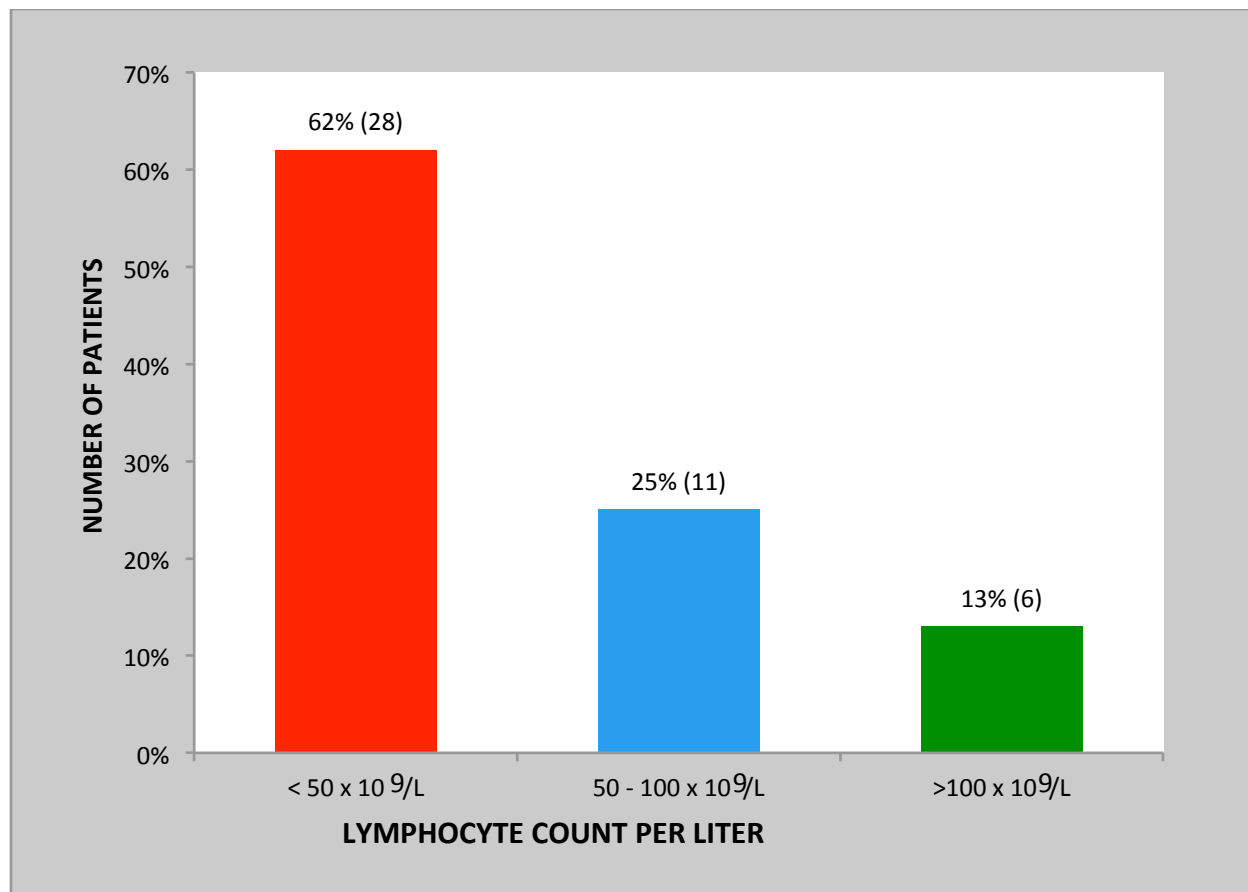


Figure 2: Absolute lymphocyte counts in the participants (n = 45)

Binet staging

More than half 23 (51.10%) were in Binet stage C, 17 (37.78%) in Binet stage A and 5 (11.11%) were in Binet stage B (Figure 3).

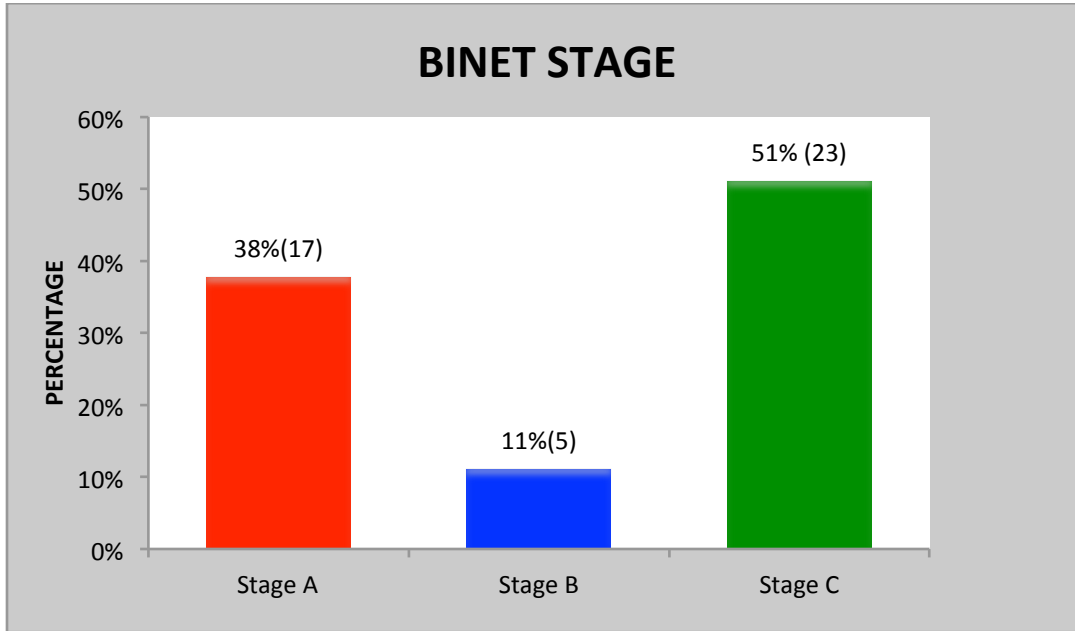


Figure 3: Binet stage in participants at diagnosis (n=45)

Over three quarters 32 (71.1%) patients had been on treatment in the prior two weeks. Among them majority 27 (60%) were treated with Chlorambucil (table 5).

Table 5: Treatment received by participants who had been treated before the study

Treatment received by participants	
Treated patients	32 (71.1%)
Type of treatment (n = 32)	
Chlorambucil	27 (60%)
CHOP	4 (8.9%)
Cychlophosphamide & Fludarbine	1 (2.2%)

CHOP - Cyclophosphamide, hydroxyoduanorubicin, Oncovin & Prednisolone

The mean B2M was 4.1mg/L (SD±3.2; Range 1.3 – 22.3mg/L) and the median B2M was 3.55. Thirty-five (77.8%) had high B2M. The mean LDH IU/L was 593.9IU/L (SD±352; Range 68.4 - 1610/L). Twenty-six (57.8%) had high LDH (table 6, Figure 4).

Table 6: B2M and LDH levels in participants

Results of B2M and LDH (n=45)		
Characteristics		95% confidence Limits
β 2 microglobulin (mg/L), median	3.55	
β 2 microglobulin (mg/L), mean (SD)	4.1 (3.2)	3.13 – 5.08
B2M Positive n (%)	35 (77.8%)	
LDH (IU/L), mean (SD)	593.9 (352.4)	487.9 – 699.7
LDH Positive n (%)	26 (57.8%)	

Patients were considered positive for ZAP-70 when the expression was found in 20% or more CD19+, CD5+ leukaemic cells. The mean ZAP-70 expression was 43.5 (SD±32.7; Range 2 – 100%). ZAP-70 was expressed in 27 patients (60%) (Table 7, figure 4).

Patients were considered positive for CD-38 when the expression was found in 30% or more leukaemic cells (CD19+, CD5+). The mean CD 38 expression was 32.5 (SD±20.6; Range 1.3 – 73%). CD-38 was expressed in 22 (48.9%) (Table 7, figure 4).

Patients were divided into four groups. In 30 patients (66.7%) there was a complete concordance of ZAP-70 and CD38 expression: seventeen patients (37.8%) were ZAP-70+/CD38+ and thirteen patients (28.9%) showed a ZAP-70-/CD38- phenotype. Ten patients (22.2%) were characterized by ZAP-70+/CD38- phenotype and five patients (11.1%) showed a ZAP-70-/CD38+ phenotype.

Table 7: ZAP-70 and CD38 expression in participants

Results of ZAP-70 and CD38 (n=45)		
Characteristics		95% confidence Limits
ZAP 70 (%), mean (SD)	43.5 (32.7)	33.7 – 53.3
ZAP 70 Category Positive (>20%) n (%)	27 (60%)	
CD38 (%), mean (SD)	32.5 (20.6%)	26.2 – 38
CD 38 Category Positive (>30%) n (%)	22 (48.9%)	
Combinations of ZAP-70 and CD38		
	n (%)	
ZAP70-, CD38-	13 (28.9%)	
ZAP70+, CD38+	17 (37.8%)	
ZAP70-, CD38+	5 (11.1%)	
ZAP70+, CD38-	10 (22.2%)	

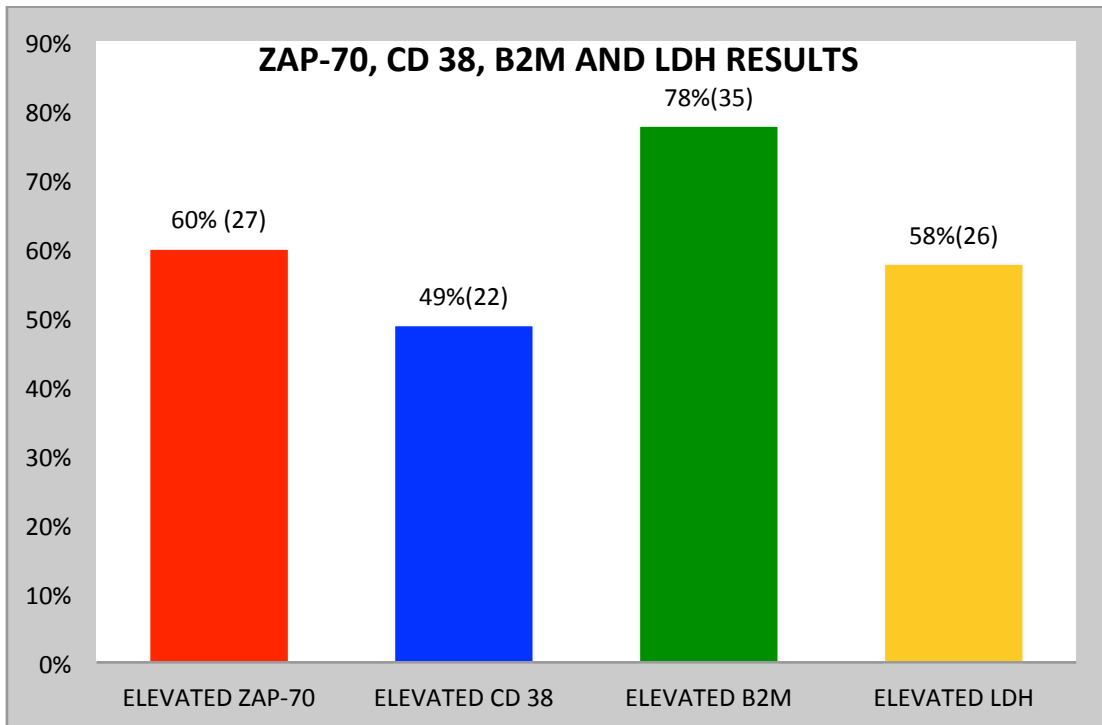


Figure 4: ZAP-70, CD 38, B2M and LDH results in participants (n=45)

Using CD38 with B2M as reference standard, the sensitivity of B2M was 81.8% and a Specificity of 26.1% (Table 8). The average agreement between two tests was 53%. (37.8 – 68.3%) but kappa showed poor Concordance between B2M and CD38 ($k=0.07$)

Table 8: Sensitivity and specificity analyses of B2M against CD38

CD38			
B2M	Positive	Negative	Total
Positive	18	17	35
Negative	4	6	10
Total	22	23	45
Statistic			[95% Confidence limit]
Sensitivity	Pr (+A)	81.8%	59.72% - 94.81%
Specificity	Pr (-N)	26.1%	10.23% - 48.41%

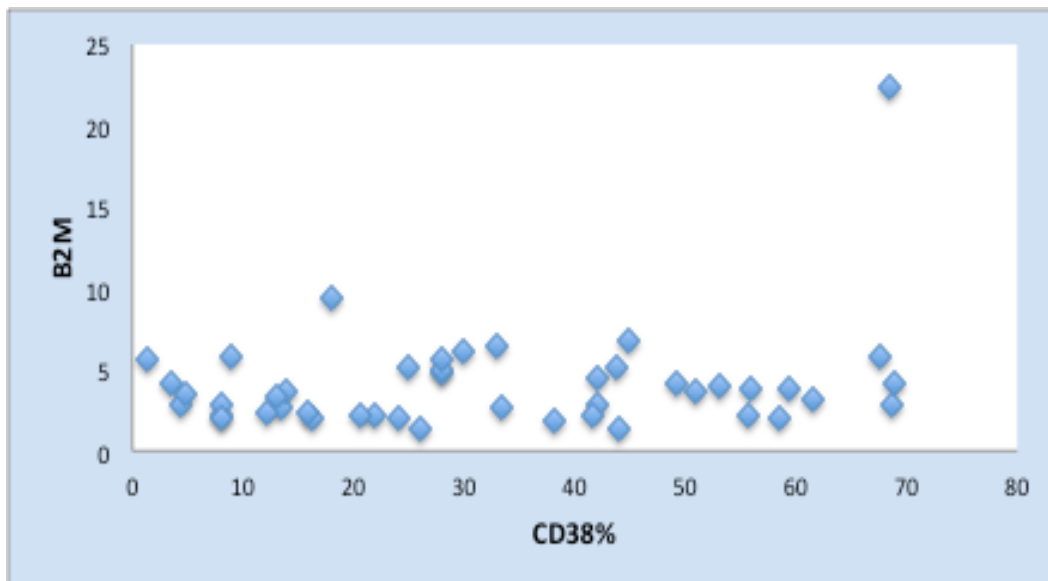


Figure 5: Correlation of B2M with CD38 in participants (n=45)

Using ZAP-70 with B2M as reference standard, the sensitivity of B2M was 77.8% and a Specificity of 22.2% (Table 9). The average agreement between two tests was 55.6%. (40% – 70.35%). There was poor Concordance between B2M and ZAP-70 ($k=0.00$)

Table 9: Sensitivity and specificity analyses of B2M against ZAP 70

ZAP 70			
B2M	Positive	Negative	Total
Positive	21	14	35
Negative	6	4	10
Total	27	18	45
Statistic			[95% Confidence limit]
Sensitivity	Pr (+A)	77.8%	57.4 % – 91.3%
Specificity	Pr (-N)	22.2%	6.41% - 47.64%

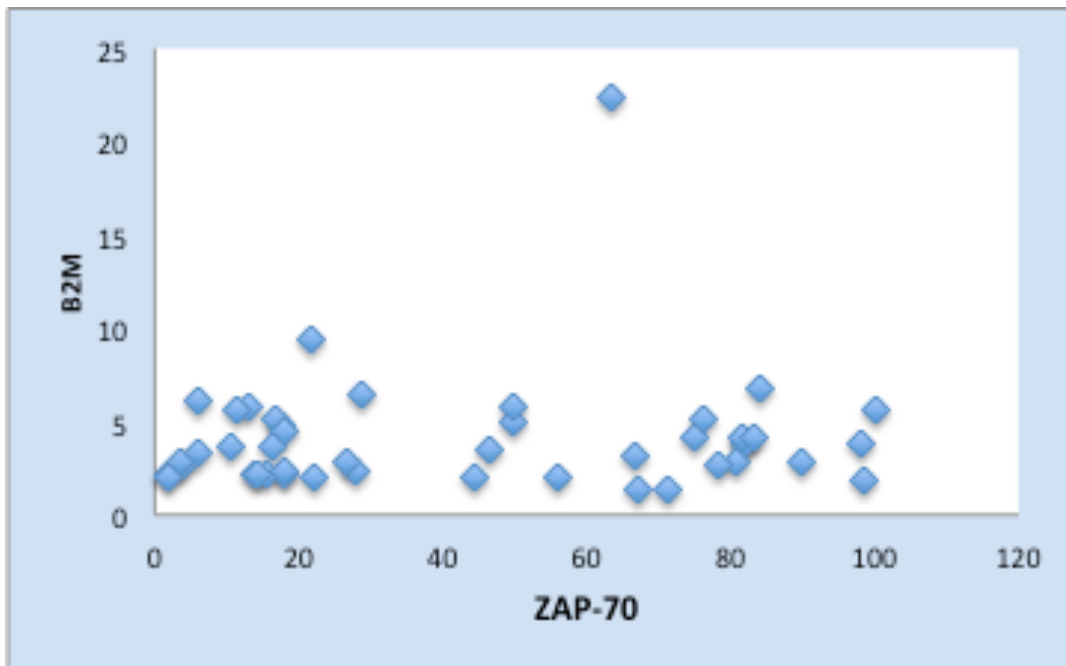


Figure 6: Correlation of B2M with ZAP-70 in participants (n=45)

For ZAP-70 as the reference, the sensitivity of CD38 was high at 62.9%, while the specificity was 72.2% (Table 10). Concordance between CD38 vs. ZAP-70 was marginal ($k=0.33$).

Table 10: Sensitivity and specificity analysis of CD 38 against ZAP 70

ZAP 70			
CD38	Positive	Negative	Total
Positive	17	5	22
Negative	10	13	23
Total	27	18	45
Statistic			[95% Confidence limit]
Sensitivity	Pr (+A)	62.9%	
Specificity	Pr (-N)	72.2%	

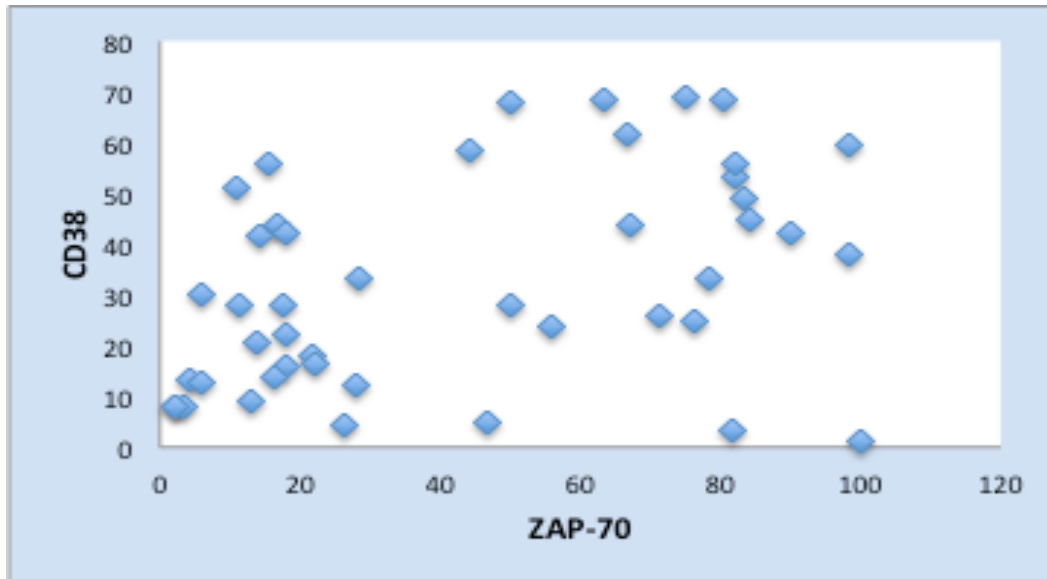


Figure 7: Correlation of CD38 with ZAP-70 in participants (n=45)

For B2M as the reference, the sensitivity of LDH was 57.1% and Specificity was 40% (Table 11). The average agreement between two tests was 53.3%. (37.87% – 63.34%). There was poor Concordance between B2M vs. ZAP-70 ($k=0.02$)

Table 11: Sensitivity and specificity analysis of LDH against B2M

		B2M		
LDH		Positive	Negative	Total
Positive		20	6	26
Negative		15	4	19
Total		35	10	45
Statistic				[95% Confidence limit]
Sensitivity	Pr (+A)		57.1%	39.40% - 73.7%
Specificity	Pr (-N)		40.00%	12.20% - 73.8%

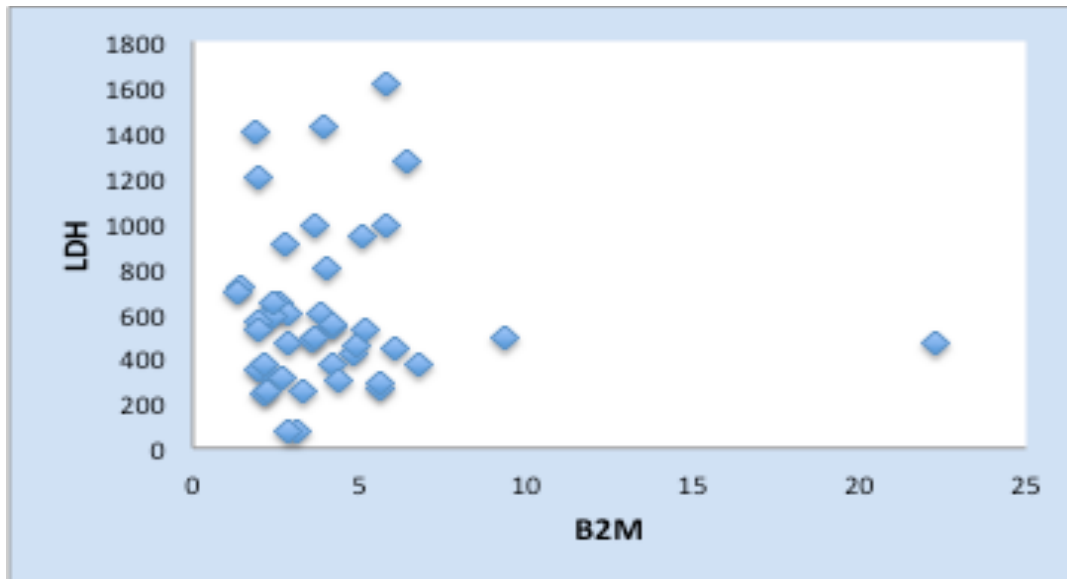


Figure 8: Correlation of LDH with B2M in participants (n=45)

Using CD 38 as the reference, the sensitivity of LDH was 54.5% and specificity of 39.3% (Table 12). The average agreement between two tests was 46.67%. (31.6 % – 62.13%). There was poor Concordance between LDH vs. CD38 ($k = -0.06$).

Table 12: Sensitivity and specificity analysis of LDH against CD38

CD38			
LDH	Positive	Negative	Total
Positive	12	14	26
Negative	10	9	19
Total	22	23	45
Statistic			[95% Confidence limit]
Sensitivity	Pr (+A)	54.5%	32.21% - 75.61%
Specificity	Pr (-N)	39.3%	19.71% - 61.46%

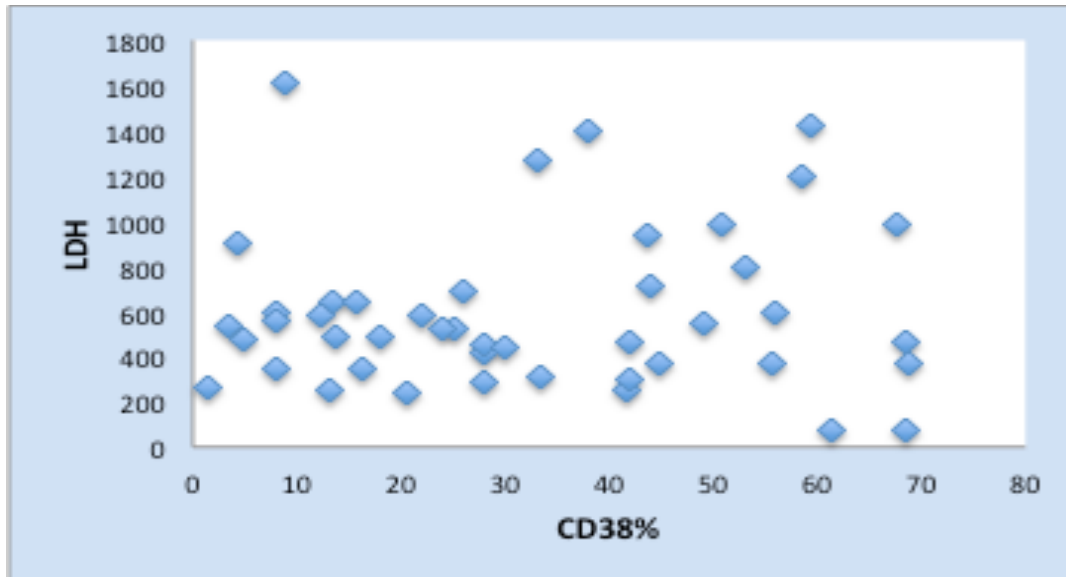


Figure 9: Correlation of LDH with CD38 in participants (n=45)

For ZAP-70 as the reference, the sensitivity of LDH was 62.9% and a specificity of 50% (Table 13). The average agreement between two tests was 57.8%. (42.15% – 72.34%). Concordance between LDH vs. ZAP-70 was marginal ($k=0.12$).

Table 13: Sensitivity and specificity analyses of LDH against ZAP-70

ZAP 70			
LDH	Positive	Negative	Total
Positive	17	9	26
Negative	10	9	19
Total	27	18	45
Statistic			[95% Confidence limit]
Sensitivity	Pr (+A)	62.9%	42.37% - 80.6%
Specificity	Pr (-N)	50%	26.02% - 73.98%

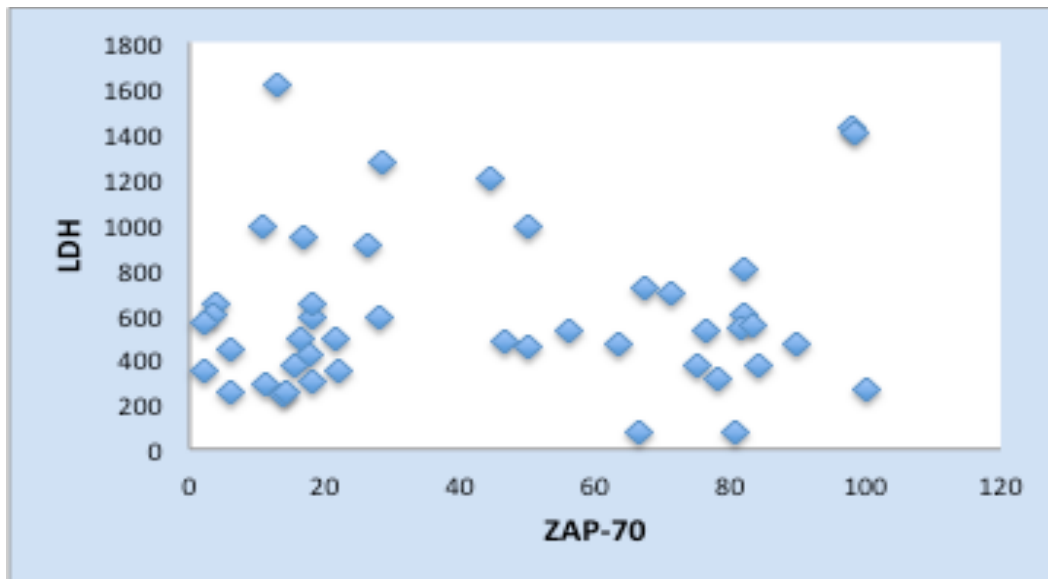


Figure 10: Correlation of LDH with ZAP-70 in participants (n=45)

With reference to CD38, the ZAP-70 positive, patients had a significantly higher median, P=0.049. There was no significant difference in the median values of B2M and LDH (Table 14).

Table 14: Comparison of ZAP-70 with laboratory parameters

ZAP-70				
Lab parameters	Negative	Positive	Test stat.	P value
	n= 18	n= 27		
B2M			Mann-Whitney U-test	0.618
Median (IQR)	3.1 (2.2,4.7)	3.8 (2.6,5)		
CD 38 %			t-test (43 df) = 2.03	0.049*
Mean (SD)	25.1 (15	37.4 (22.2)		
LDH			Ranksum test	0.372
Median (IQR)	461.7 (314.4,624.8)	523.8 (407.7)		

Significant P-values are marked by asterisk ()*

The mean ZAP-70 was significantly higher among those with CD38 positive, P <0.001 (Table 15). However there was no significant difference in median B2M and LDH with respect to CD38 categories.

Table 15: Comparison of CD38 with laboratory parameters

CD38 Category				
Lab parameters	Negative	Positive	Test stat.	P value
	n= 23	n= 22		
B2M			Mann-Whitney	0.394
Median (IQR)	2.9 (2.1,4.9)	3.9 (2.8,4.9)		
ZAP-70			t-test (43 df) =	< 0.001*
Mean (SD)	27.6 (27.2)	60.2 (29.9)		
LDH			Ranksum test	0.489
Median (IQR)	485.5 (382.6,590.2)	532 (365.4, 970.4)		

Significant P-values are marked by asterisk ()*

In Binet clinical staging there was no significant difference in ZAP-70, CD38, LDH, B2M and combinations of ZAP-70 and CD38 (table 16).

Table 16: Comparison of Binet clinical staging with laboratory parameters

Binet Clinical Staging					
	A	B	C	Test stat.	P value
Total	n= 17	n= 5	n= 23		
ZAP 70 % >20				Fisher's exact test	0.059
Negative	8 (47.1)	4 (80)	6 (26.1)		
Positive	9 (52.9)	1 (20)	17 (73.9)		
CD 38 % >30%				Fisher's exact test	0.099
Negative	12	3 (60)	8 (34.8)		
Positive	5 (29.4)	2 (40)	15 (65.2)		
LDH Category				Fisher's exact test	0.386
Negative	5 (29.4)	3 (60)	11 (47.8)		
Positive	12	2 (40)	12 (52.2)		
B2M Category				Fisher's exact test	0.534
Negative	4 (23.5)	2 (40)	4 (17.4)		
Positive	13	3 (60)	19 (82.6)		
ZAP-70 and CD38				Fisher's exact test	0.075
ZAP-70-/CD38-	6 (35.3)	3 (60)	4 (17.4)		
ZAP-70-/CD38+	2 (11.8)	1 (20)	2 (8.7)		
ZAP-70+/CD38-	6 (35.3)	0 (0)	4 (17.4)		
ZAP-70+/CD38+	3 (17.6)	1 (20)	13 (56.5)		

There was a significant difference in the mean (SD) of CD38, with Stage A having the lowest 22.2 (15.7) followed by stage B 32.2 (27.8) while stage C 40.1(19.7) had the highest mean, P=0.021 (Table 17). There was no significant difference in B2M and ZAP-70 with respect to Binet staging.

Table 17: Comparison of Binet clinical staging with median/mean of lab parameters

Binet Clinical Staging					
	A	B	C	Test stat.	P value
Total	n= 17	n= 5	n= 23		
B2M				Kruskal-Wallis	0.123
Median	2.9 (2.2,4.4)	2.4 (2.1,2.9)	4 (2.9,5.3)		
ZAP-70				Kruskal-Wallis	0.085
Median	21.6 (12.9,71.4)	15.7 (6,18)	56 (22.1,80.1)		
CD 38 %				ANOVA F-test	0.021*
Mean (SD)	22.2 (15.7)	32.2 (27.8)	40.1 (19.7)		

Significant P-values are marked by asterisk ()*

DISCUSSION:

Chronic lymphocytic leukemia is predominantly a disease of the elderly, usually diagnosed in the fifth to sixth decade (18). In this study, the median age of the patients was 62years, which is comparable to reports from previous studies. The peak age in the study conducted by Mukiibi et al. in Central Africans was between 60 – 64yrs (46). The male to female ratio was 1:1; which is in agreement with the review conducted by Alan Fleming in CLL patients in Africa (11) and also with this study.

Among the 45 patients recruited, only six (13.3%) patients were diagnosed incidentally during regular health checkup or when routine haemogram was done for other causes. The rest presented with suggestive clinical features.

The most common presenting symptom in CLL is lymph node enlargement, while smaller numbers of patients report “B” symptoms (fever, weight loss, or night sweats), and 25% of patients are asymptomatic (18). The general physical examination details from patient’s files revealed that 56% of the patients had lymphadenopathy at the time of diagnosis, mostly cervical lymphadenopathy in 32% of the patients. Half (51%) of the patients had splenomegaly and one third (31%) had hepatomegaly. In the study conducted by Mukiibi et al (46) in central Africans, the major clinical findings included lymphadenopathy (33.3%), splenomegaly (68%) and hepatomegaly (37.3%) (46). Since lymphadenopathy is sign of disease progression, the fact that a larger proportion of patients in this study had lymphadenopathy suggests that they had more advanced disease than patients in previous studies.

Forty (89%) patients at the time of diagnosis were anaemic, possibly due to bone marrow involvement, autoimmune haemolytic Anaemia (AIHA) and splenomegaly or even previous treatment, but the exact cause could not be determined. Thirty-nine (87%) patients had leukocytosis and all the patients (100%) had absolute lymphocytosis as expected in CLL. Majority (62%) had lymphocyte count below 50×10^3 and only six (13%) patients had more than 100×10^3 . Twenty-five (56%) patients had thrombocytopenia again probably due to bone marrow failure, autoantibodies, and possibly hypersplenism or previous treatment.

The clinical staging in this study was done using Binet staging, which is based on number of involved areas, the level of haemoglobin, and the platelet count (9). In this study, Half (51%) of the patients were in Binet stage C, one third (38%) in stage A and remaining 5 (11%) patients were in stage B. In the study by Mozaheb et al. 37% of participants were in Binet stage C, 37% in Binet stage A and 26% in Binet stage B (47). Most of the overt clinical features of CLL (lymphadenopathy, organomegaly) are associated with late stage of the disease (9,48). Late presentation of cancer patients is not uncommon in Kenya which may reflect the health seeking behavior of patients (49).

Three quarters (71%) of the patients in this study had previous treatment. The treatment was based on staging and clinical picture. It is estimated that one third of CLL patients never require treatment (9). Mozaheb et al study had 55% of their study participants on treatment (47). This difference is reflecting high proportion of subjects in stage C in this study compared to 37% in Mozaheb et al study. Most commonly used treatment in CLL is the alkylating agent chlorambucil (9). Among the treated patients, twenty-seven (60%) patients were on chlorambucil, four (9%) on CHOP and only one (2.2%) on cyclophosphamide and fludarabine (2%).

Beta-2 Microglobulin and LDH levels

In this study the mean B2M was 4.1mg/dL (SD±3.2; Range 1.3 – 22.3mg/L, Reference range < 2.1mg/L). B2M values greater than 2.4mg/L were considered elevated. Gentile et al reported that the cut-off value of B2M that could discriminate between CLL patients whose disease progressed from those with stable disease was 2.4 mg/dL ((26)). Majority (77.8%) of the patients in this study had elevated B2M levels which is comparable to the study done by Z Mozaheb et al., where B2M was elevated in 70% of patients diagnosed with CLL (47).

The mean LDH value was 593.9IU/L (SD±352; Range 68.4 - 1610/L). Twenty-six patients (58%) had high LDH levels (Reference range: 200 – 480 U/L). Other studies have reported increased LDH levels in 20 -25% of CLL patients at the time of enrollment into the study

(50,51). In the study by Z Mozaheb et al., LDH was elevated in only 20% of the patients (47). A possible explanation for the high proportion of patients with elevated LDH in this study may be the contribution of non-specific LDH increase. Serum LDH is elevated in many disorders such as myocardial infarction (MI), heart failure, pulmonary infarction, hemolytic jaundice and in some muscular disorders. It's main use in diagnostic enzymology is as a general indicator of the existence and severity of acute or chronic tissue damage (50). In this study anaemia was found in 89% of patients. The attendant reduction in the tissue oxygenation in the affected subjects may have contributed to this finding.

ZAP-70 and CD38 levels

CD38 expressed in 22 patients (49%) with a mean CD38 value of 32.5. ZAP 70 was expressed in 27 patients (60%) with a mean ZAP 70 value of 43.5. In the study done by Hus et al. (3), CD38 was expressed in 33.3% patients and ZAP-70 was expressed in 36.5% patients. The difference in expression of ZAP – 70 and CD 38 between this study and that of Hus and colleagues maybe explained by the fact, that, in the study conducted by Hus et al. (3) majority of the patients were of lower clinical stage compared to patients in this study, where majority of the patients were in more advanced stage. Moreover CD38 expression may vary during the course of the disease. When followed over time, many CD38- patients become CD38+, which is associated with 17p13 deletion (9,12). Even though molecular studies for 17p13 were not carried out, its probable that the higher ZAP-70 and CD38 expression is reflection of more advanced disease.

Both ZAP-70 and CD38 were positive in three (3) patients of Binet stage A, one (1) in Binet stage B and thirteen (13) in Binet stage C. The patients were divided as having concordant or discordant results based on CD38 and ZAP-70 positivity and negativity. Thirteen (29%) patients were negative for both CD38 and ZAP-70, 17 (38%) were positive for both and 22 (48%) had discordant results. In the study done by Hus, 53.2% showed a ZAP-70⁻/CD38⁻ phenotype and 22.4% were positive for ZAP-70 and CD38. Twenty two patients (24.4%) were characterized by either ZAP-70⁺/CD38⁻ phenotype or ZAP-70⁻/CD38⁺ phenotype. This slight variation in CD38 and ZAP-70 expression rates may be due to methodological differences in analysis and reporting of flow cytometry (52).

When B2M was compared against ZAP 70 and CD38, the sensitivities were found to be 82% and 78% respectively but it had very low specificities of 22% and 26% respectively for ZAP 70 and CD38. Kappa correlation statistics showed poor concordance between B2M with ZAP-70 ($k=0.0$) and CD38 ($k=0.07$). The Expression of CD 38 varies during the course of the disease, which may explain the low specificity of B2M against CD38 in this study. Other studies reported similar lack of correlation between B2M and CD 38 or ZAP 70 (3,53) . Morabitto et al in a study on 194 Binet A stage patients who were not on treatment, serum levels of B2M did not correlate with CD38 or ZAP-70 expression. But patients with increased serum levels of B2M ($p=0.001$), higher CD38 ($p<0.0001$) or ZAP-70-expression ($p<0.0001$) showed a significantly shorter 4-year time to first treatment (53). Also B2M did not show any correlation with CD38 ($p= 0.083$) and ZAP 70 ($p=0.071$) in the study conducted by Hus et al (3) . These findings indicate that ZAP-70, CD38 cannot be a substituted for by B2M. The need to combine other laboratory/clinical prognostic indicators with ZAP-70 and CD38 expression has been reported from other studies (3,48).

LDH when compared against B2M had a sensitivity of 57% and specificity of 40%. When LDH was compared against ZAP 70, the sensitivity was 39% and specificity was 47%. LDH when compared against CD38, the sensitivity was 50% and specificity was 57.8%. The lack of correlation was supported by low kappa values - marginal concordance between LDH and ZAP 70 ($k=0.12$), poor concordance between LDH and CD38 ($k= -0.06$), LDH and B2M (0.02).

CD38 and ZAP-70 correlated well with each other. There was correlation, comparing mean value of ZAP-70 positive to CD38 positive patients ($P=0.001$). There was however no correlation when median values of CD38 and ZAP-70 were compared to B2M and LDH. Correlation between CD38 and ZAP-70 has also been reported in other studies (3,54). Prognostic information given by ZAP-70 and CD38 expression is complementary, and can be used to guide treatment decisions especially in early clinical stages of the disease (54)

Staging and prognostic markers

When B2M, CD38, ZAP-70 and LDH were correlated with the Binet staging, only CD 38

showed a correlation with the staging, the mean CD38 values being lowest in Binet Stage A 22.2 % followed by stage B 32.2 % while stage C had the highest mean of 40 % (P=0.021). To conclude this needs further exploration in view of relatively small size of the sample. There was no correlation when median of B2M and ZAP-70 were compared with Binet staging. There was no correlation when Binet stage A, B and C was compared with the positive and negative values of ZAP-70, CD38, B2M, LDH and ZAP-70/CD38 combinations. These findings suggest that, Binet staging does not compare with the prognostic markers employed in this study and hence it is important to analyze prognostic markers in whichever stage the patient presents.

When B2M, CD38, ZAP-70 and LDH were correlated with the Binet staging, only CD 38 showed a correlation with the staging, the mean CD38 values being lowest in Binet Stage A 22.2 (15.7) followed by stage B 32.2 (27.8) while stage C had the highest mean of 40.1(19.7) (P=0.021). This study was powered for comparison between B2M and ZAP-70/CD 38. Could be a larger sample size is needed to demonstrate differences in the markers in various Binet Classes.

CONCLUSIONS:

1. This study found poor correlation between B2M levels and ZAP-70 expression ($k=0$), B2M levels and CD38 expression ($k=0.07$), the findings of this study do not support substitution of CD38, ZAP-70 by B2M as prognostic markers.
2. This study found poor correlation of LDH levels with ZAP-70 ($k=0.12$) expression, LDH levels with CD38 expression (0.06) and LDH levels with B2M levels ($k=0.02$). Similarly ZAP-70 and CD38 cannot be substituted by LDH as prognostic markers.
3. This study found positive correlation between mean CD38 and Binet stage ($p=0.021$). This study however did not reveal any relationship between the Binet stage of CLL and these prognostic markers (ZAP-70, B2M and LDH).

LIMITATIONS:

1. The proportion of subjects in advanced Binet stage was relatively high which was not anticipated and may have influenced the findings. A stratified sampling approach to ensure adequate representation of all disease stages may give more of the entire disease spectrum. However this was not possible because of limited sample size due to budget constraints and limited time period to do the dissertation.
2. Budget constraints also limited the use of wider panel of Immunophenotypic markers. Additional use of CD23, clonality markers (kappa and lambda) would have given more information.
3. Ideally the patients should have been followed up, this was not possible due to time limitation

RECOMMENDATIONS:

1. According to this study B2M and LDH levels are inappropriate substitutes for ZAP-70 and CD38 expression in prognostication of CLL. It is therefore recommended for purposes of prognostication, all the four prognostic tests should be performed in a patient with CLL when possible.
2. A longitudinal study using a larger cohort of newly diagnosed untreated patients is recommended to further assess prognostic value of ZAP-70, CD38 and B2M in our setup.

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APPENDICES

APPENDIX I: Laboratory values of normal adults

Haemoglobin	
Men	14 – 18g/dl
Women	13 – 17g/dl
White blood cell count	3 – 10 x 10 ⁹ /l
Absolute lymphocyte count	1 – 3 x 10 ⁹ /l
Platelet count	150 – 450 x 10 ⁹ /l
B2M	2.1mg/L
LDH	200 – 480U/L
SERUM CREATININE	45 - 115µmol/L
ZAP-70	0 - 20%
CD 38	0 - 30%

APPENDIX II: Staging systems in chronic lymphocytic leukaemia

	Characteristics
Binet stage	
A	<3 lymphoid areas* involved
B	>3 lymphoid areas involved
C	Haemoglobin < 10g/dl or Platelets < 100
Rai stage	
0	Lymphocytosis only
I†	Lymphadenopathy
II†	Hepatomegaly or splenomegaly ± Lymphadenopathy
III‡	Hemoglobin < 11 g/dl
IV‡	Platelet < $100 \cdot 10^9/l$

* The five lymphoid areas comprise unilateral or bilateral cervical, axillary and inguinal lymphadenopathy, hepatomegaly and splenomegaly.

†Risk group at low level.

‡Risk group at intermediate level.

‡ Risk group at high level

APPENDIX III: Markers of poor prognosis in CLL

IN USE
Advanced Rai or Binet staging
Male
Atypical morphology of CLL/PLL
Peripheral lymphocyte doubling time of < 12months
CD38 ⁺
High β_2 - microglobulin level
Diffuse marrow histology
High lactate dehydrogenase
Fludarbine resistance
INVESTIGATIONAL
Lack of IgVH gene mutation
Expression of ZAP-70 protein
Del 11q22-q23 (loss of ATM gene), del 17p13 (loss of P53), or trisomy 12
Increased serum levels of CD 23, tumour necrosis factor, and thymidine kinase

APPENDIX IV: Procedure for flow cytometry

Samples:

Venous blood was taken using sterile tubes containing EDTA as the anticoagulant. The samples were kept at room temperature (18-25°C) and not shaken. The samples were homogenized by gentle agitation prior to taking the test sample. The samples were analyzed within 24 hours of venipuncture.

Procedure of ZAP70 test:

Material:

1. Fluorochrome –conjugated antibody
 - Surface markers (vial 1) - CD5-FITC/CD19-PC5/CD3+CD56-PC7 - 10 ul/test
 - ZAP70 (vial 2) - ZAP70-PE - 10 ul/test
2. Protease free bovine serum albumin (BSA)
3. 10% triton X
4. Fixative – 10% formaldehyde
5. Flow reagents
 - Flow set
 - PC 7 set up kit
 - Flow-check fluorospheres
 - QuickCOMP 2 kit
 - CD-45 PC7
 - CD-45 PC5
6. Polypropylene tubes
7. Automatic pipettes with tips (20,100,500ul)
8. Centrifuge and vortex
9. Flow cytometer

Working solution prepared prior to sample processing

1. PBS WORKING SOLUTION

Dissolve 1 sachet of PBS buffer in 500ml of distilled water

2. FIXATIVE (FA) – 1: 10
E.g. – 50ul of Iotest solution in 450ul PBS
3. LYSE – PERMEABILIZATION BUFFER
 - Prepare fresh daily
 - Add 1ml Triton X to 9ml PBS
 - Pipette 233ul of the above solution and make up to 10ml of PBS (aspirate 233ul of PBS & add 233ul of 10% Triton X)
4. WASHING BUFFER – 2% (refrigerate at 4⁰)
 - Prepare 2% of BSA
 - 3ml 30% BSA solution + 42ml PBS
 - Powder form – 0.5g BSA in 25ml of PBS
5. VIAL 2 WORKING SOLUTION
 - For one sample - mix 10ul of vial 2 solution, add 90ul of wash buffer

Procedure:

1. All working solutions were prepared
2. Four 12x75 tubes for compensation reagents (CD45 – FITC, PE, PC5 & PC7), one flow set tube, one verify tube (normal blood) & one tube for each sample were labelled.
3. Compensation reagents were pipetted to the appropriately labeled compensation tubes using the volumes recommended on the reagent vial.
4. 10ul of vial 1 of ZAP70 reagent (CD5/CD19/CD3/CD56) was added to each sample tube and the verify tube.
5. 100ul of normal blood was added to the compensation and verifying tubes and 100ul of sample blood was added to appropriately labeled tubes. Then mixed gently, vortexed & incubated for 20mins in the dark.
6. 110ul of fixative was pipetted to each tube and mixed gently, vortexed & incubate for 10mins in the dark.
7. 2ml of lyse (prewarmed buffer) was pipetted & vortexed. Incubated for 15mins in the dark.

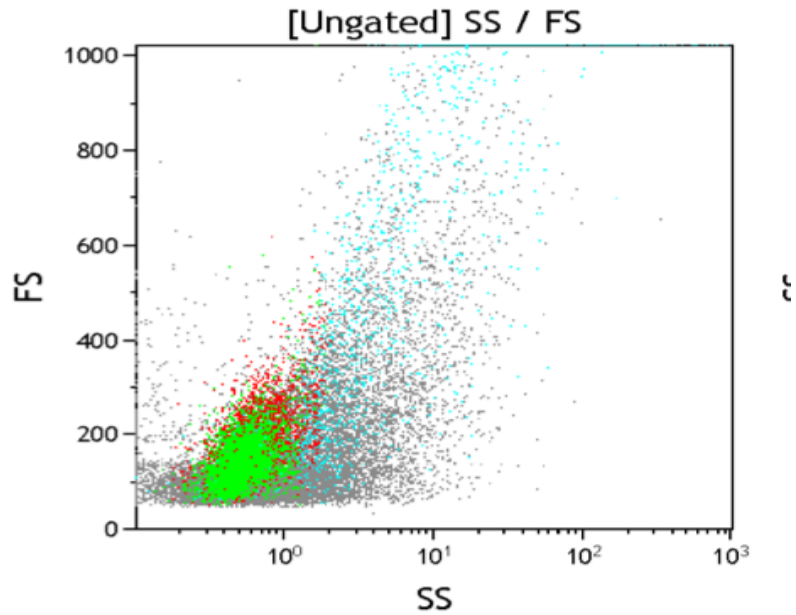
8. 1ml of wash buffer was pipetted & vortexed. Then Centrifuged at 2500g for 5mins. Supernatant was discarded and blotted (There may be some residual red cells remaining in the pellet, these typically dissipate during the subsequent washes).
9. 3ml of wash buffer was again pipetted & the same procedure was repeated.
10. 100ul of vial 2 working solution was pipetted to each sample tube & verify tube. Then mixed gently & vortexed. Incubated for 30mins in the dark at room temperature.
11. 3ml of wash buffer was pipetted to verify and sample tube. Then vortexed & centrifuged at 2500g for 5mins. Supernatant was discarded & blotted.
12. 1ml of PBS was added.
13. The sample was run in the FC 500 flow cytometer and analyzed within 2 hours.

Sample analysis recommended procedure:

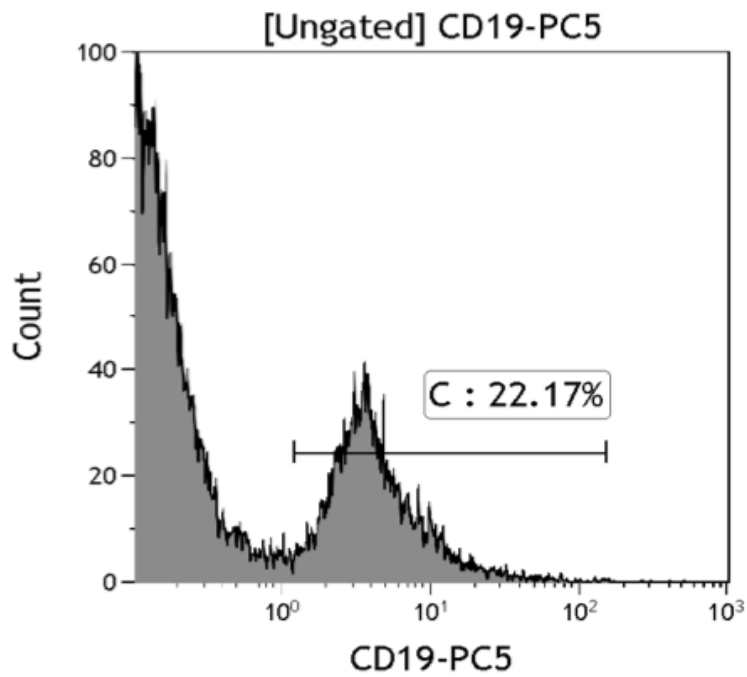
1. Flow check beads were run to verify instrument alignment.
2. PMT voltages was set using flow set and PC7 beads
3. Compensation was set with the compensation tubes prepared.
4. Verify tube was run to confirm compensation setting & sample preparation.

ZAP-70 analysis protocol:

1. **Histogram 1** – FS vs. SS histogram. Stop count was set at 100,000 lymphocytes.

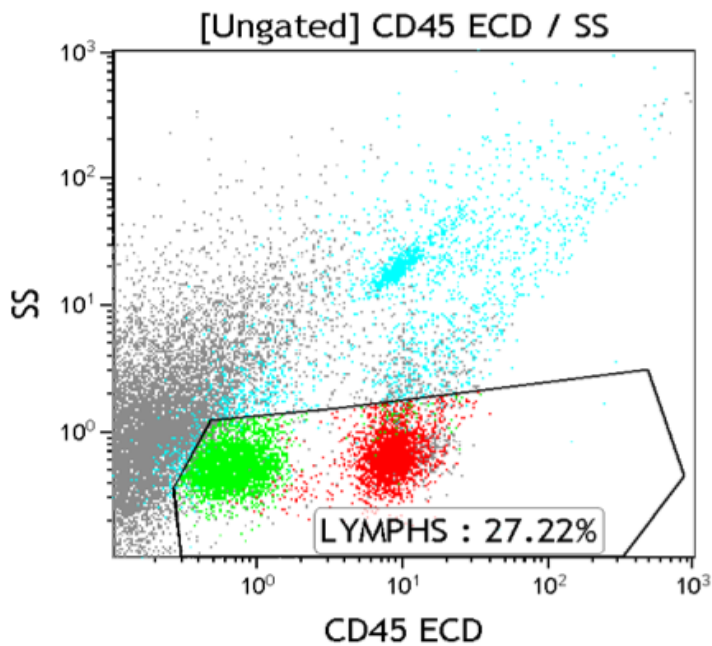


2. **Histogram 2** - single parameter histogram for gating CD19+ cells (C)

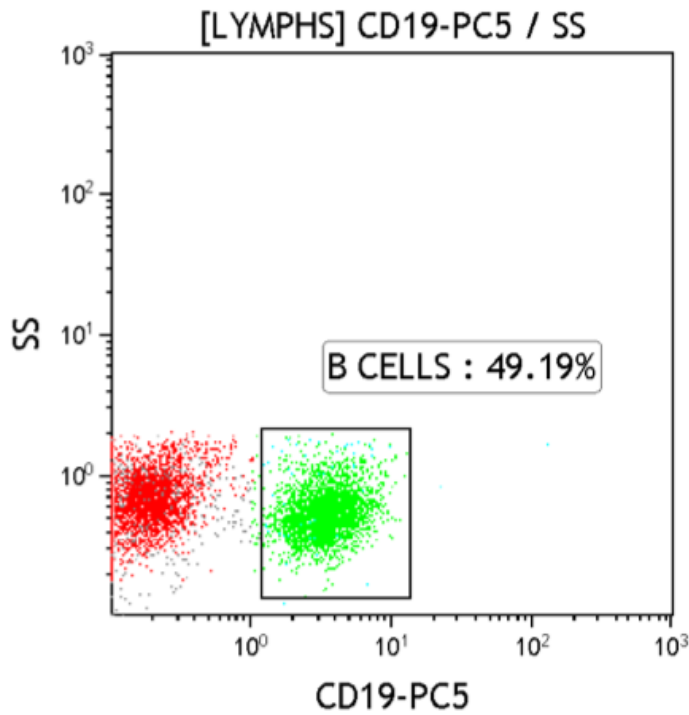


3. **Histogram 3** - SS vs. CD45.

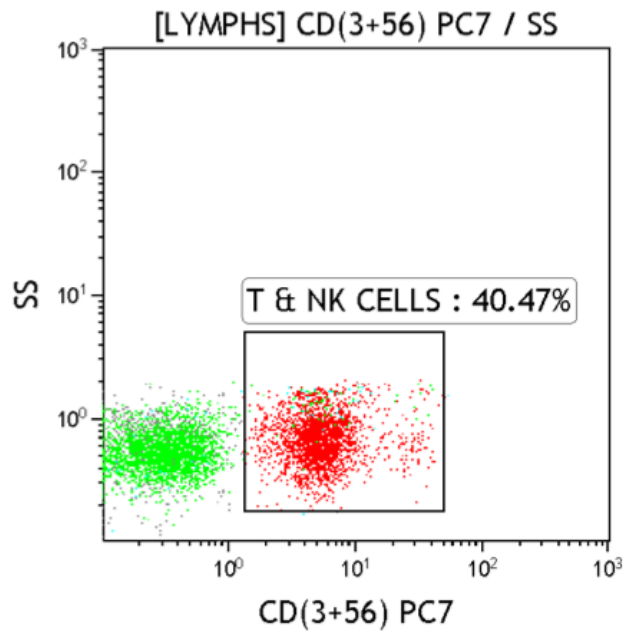
- a. A gate was placed on lymphs to include the dim CD45 population. During ZAP-70 treatment, the malignant cells shed CD45 epitope, which are expressed as dim CD45.
- b. To know that the dim CD45+ populations are CD19+, A gate was placed on CD45 ECD vs. SS plot on lymphs. Lymphs gate was removed after the population was identified.



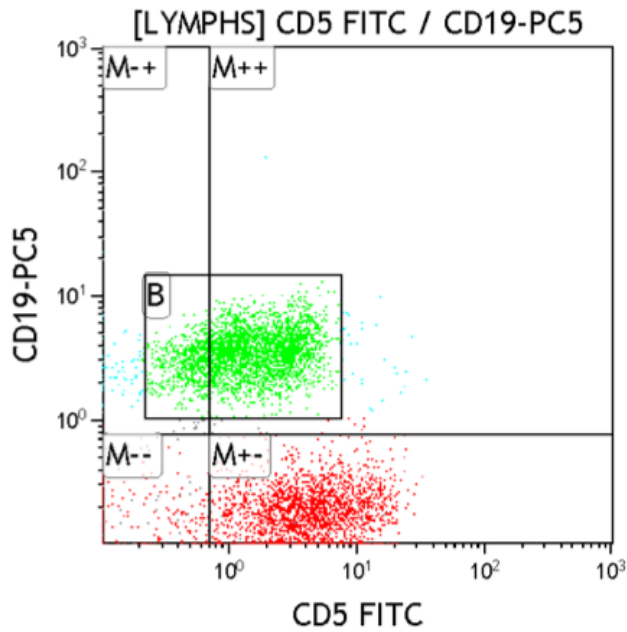
4. Histogram 4 – SS vs. CD19. A gate was placed on CD19+ population (B cells)



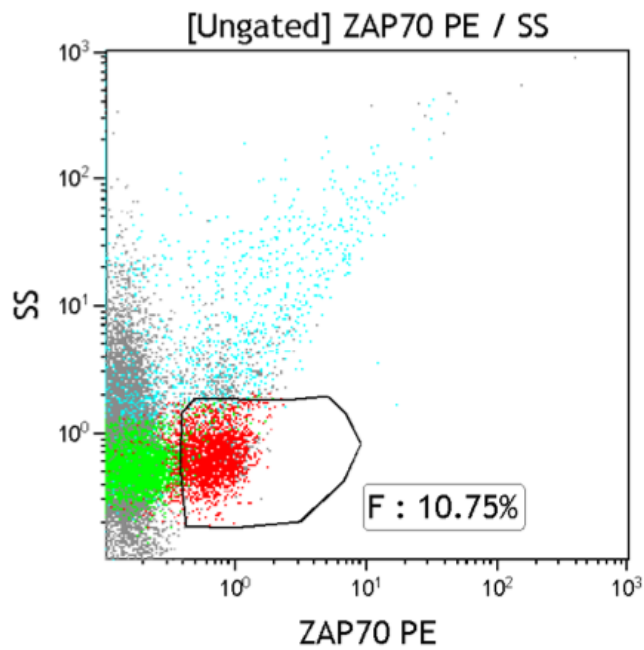
5. Histogram 5 – SS vs. CD (3+56). A gate was placed on CD (3+56)+ population (T & NK cells)



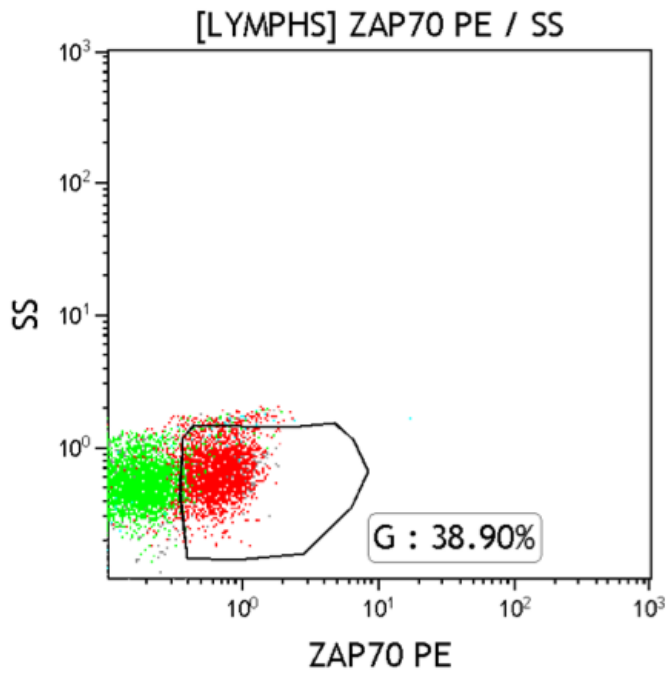
6. Histogram 6 – CD19 vs. CD5. A gate was placed on CD5+CD19+ population (B)



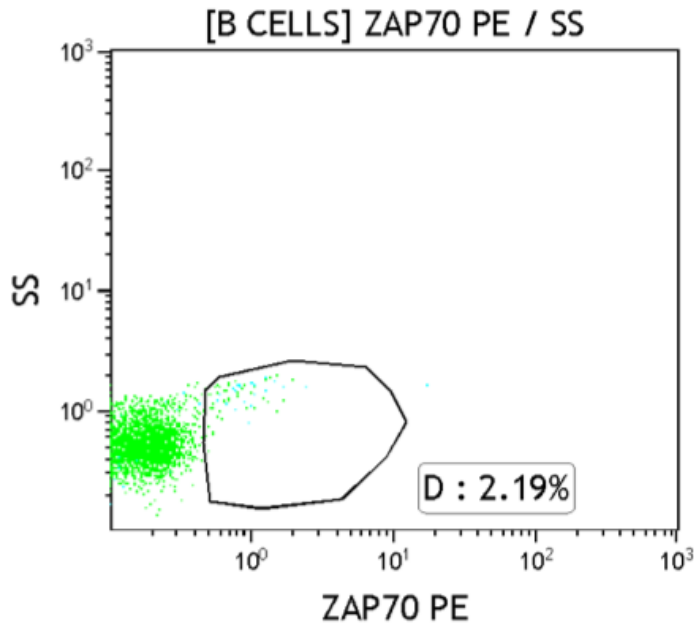
7. Histogram 7 – SS vs. ZAP-70. A gate was placed on ZAP-70 + population (F)



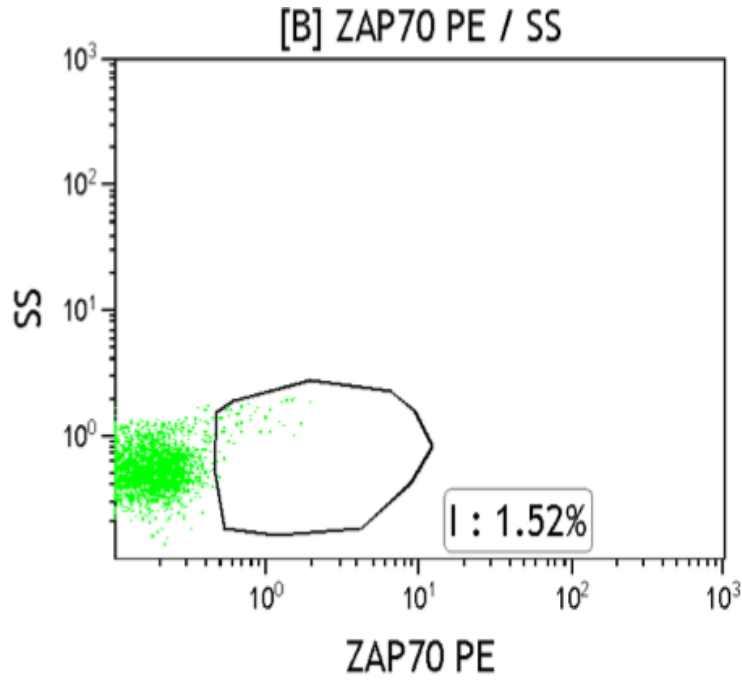
8. Histogram 8 - SS vs. ZAP-70. A gate was placed on ZAP-70 + lymphocytes [lymphs](G)



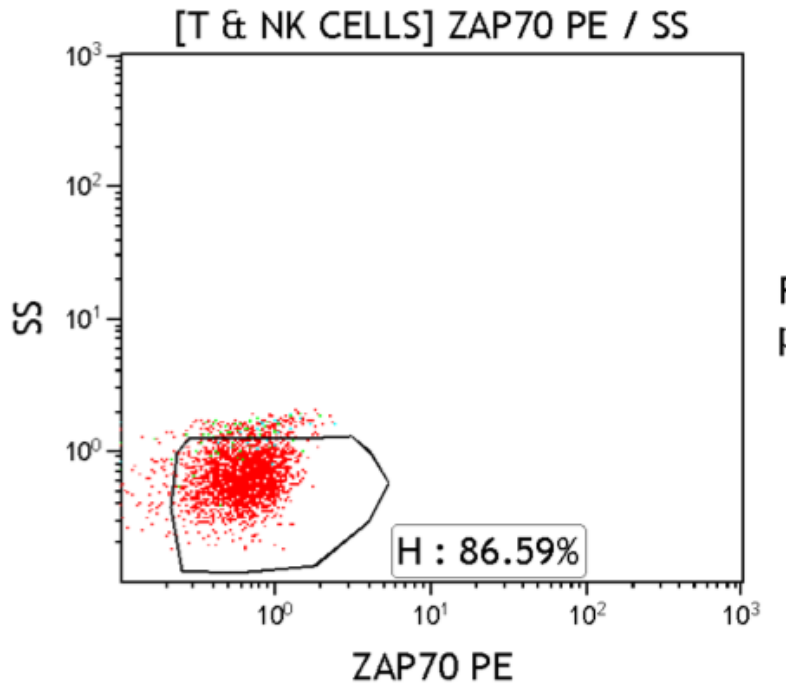
9. Histogram 9 – SS vs. ZAP70. A gate was placed on ZAP-70+ B cell population [region B cells] (D)



10. Histogram 10 – SS vs. ZAP70. A gate was placed on ZAP-70+ CD19+/CD5+ population.
[Region B]. (I)



11. Histogram 11 – SS vs. ZAP70. A gate was placed on ZAP-70+ CD (56+3)+ population.
[Region T & NK cells]. (H)



Procedure of CD38 test:

Material:

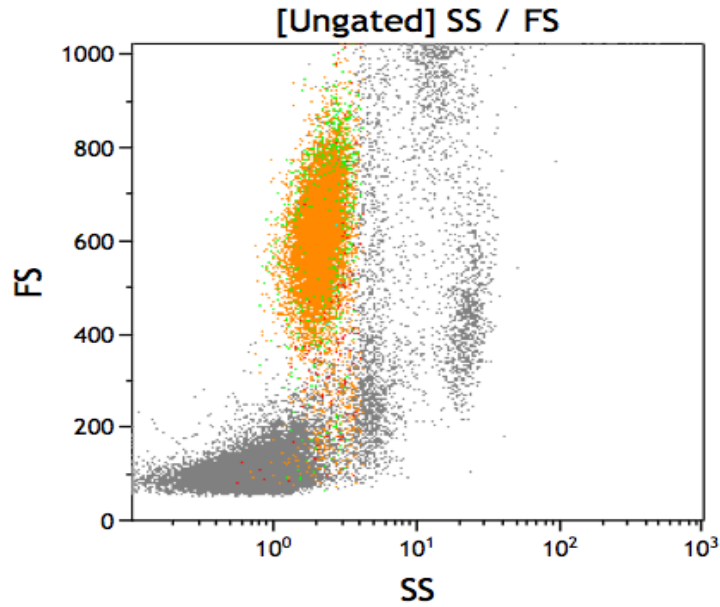
1. Fluorochrome –conjugated antibody
 - IOTest 3 conjugated antibodies, CD-38FITC/CD56-PE/CD45-ECD (20µL/test)
 - CD5-PC5 (10µL/test)
 - CD19-ECD (20µL/test)
2. Versalyse
3. Flowset
4. Leucocyte fixation reagent – IOTest3
5. PBS buffer
6. Polypropylene tubes
7. Automatic pipettes with tips (10, 100, 500ul)
8. Centrifuge and vortex
9. Flow cytometer

Procedure:

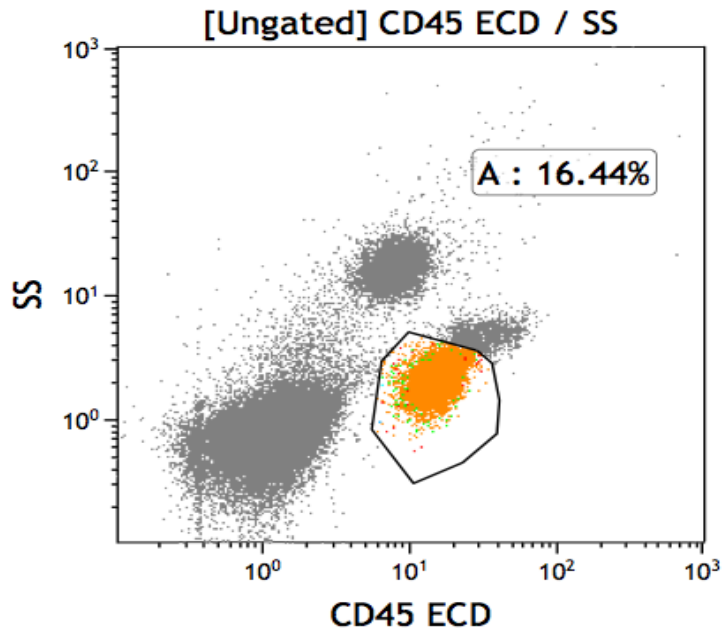
1. 10ul of CD5-PC5 was pipetted to each sample tube and the control tube.
2. 20ul of IOTest 3 conjugated antibody (CD-38FITC/CD56-PE/CD45-ECD) was pipetted to each sample tube and the control tube.
3. 20ul of CD19-ECD was pipetted to each sample tube and the control tube.
4. 100ul of sample blood was pipetted to appropriately labeled tubes. Then mixed gently, vortexed and incubated for 15mins in the dark.
5. One ml versalyse was added for lysis of red cells. Incubated for 10mins in the dark.
6. Centrifuged for 5mins at 150 x g at room temperature. Supernatant removed by aspiration.
7. Cell pellet was suspended using 0.5ml of PBS.
8. Preparations were analysed within 2hrs.

CD38 analysis protocol:

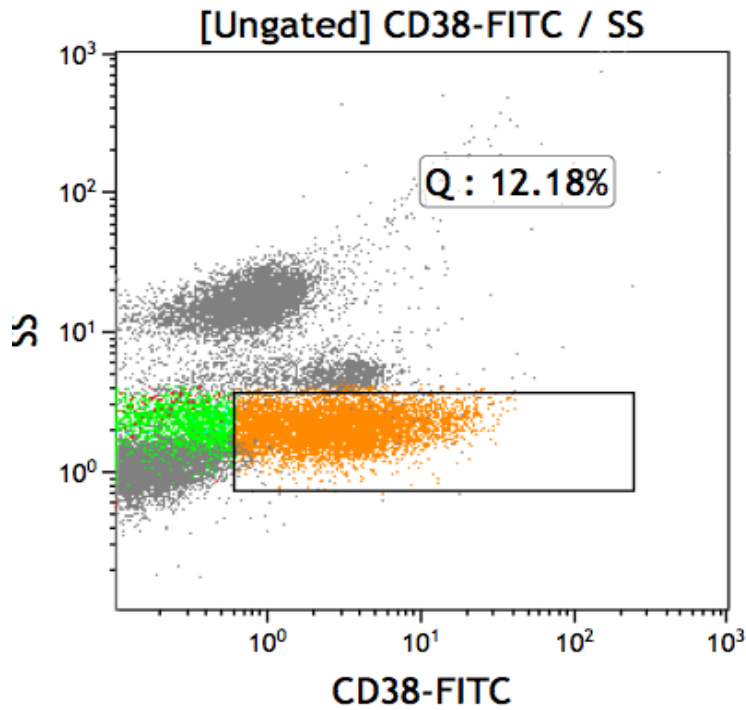
1. **Histogram 1** – FS vs. SS histogram. Stop count was set at 100,000 lymphocytes.



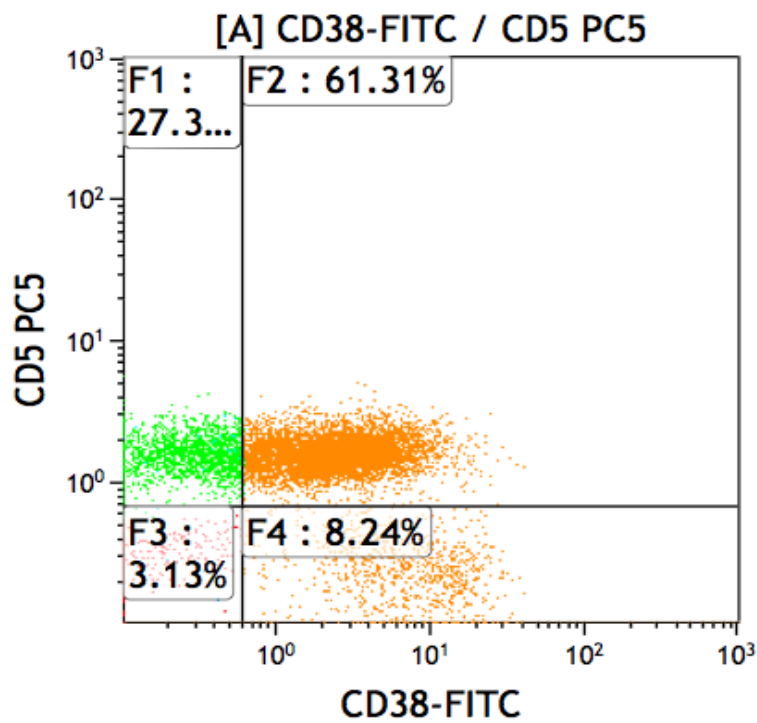
2. **Histogram 2** – FS vs. CD45 histogram. A gate was placed on CD45+ lymphocytes



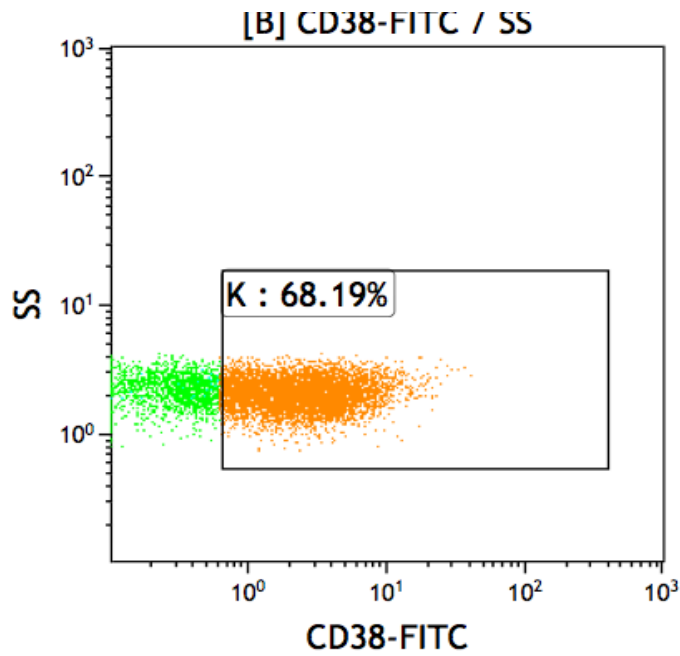
3 Histogram 3 – CD56 vs. CD38 histogram.



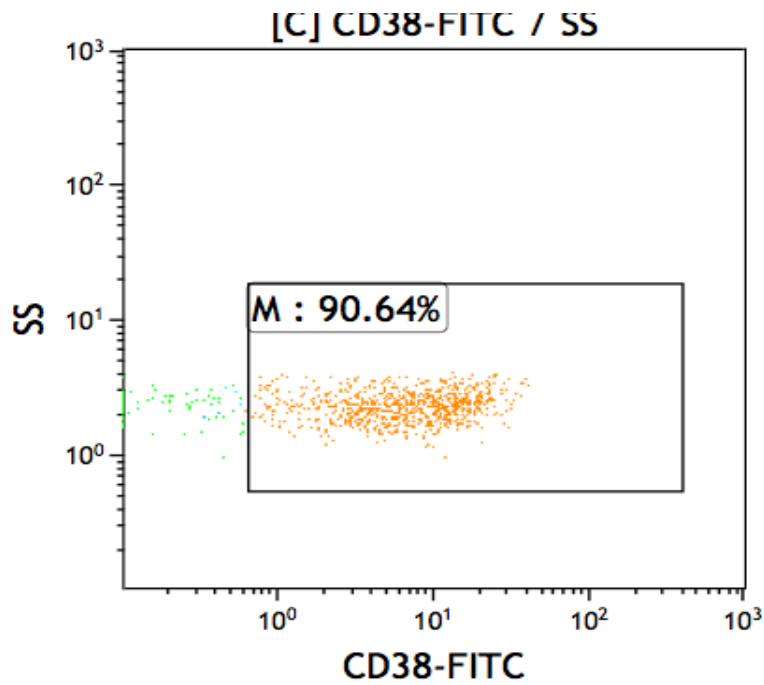
4. Histogram 4 – CD5 vs. CD38 histogram.



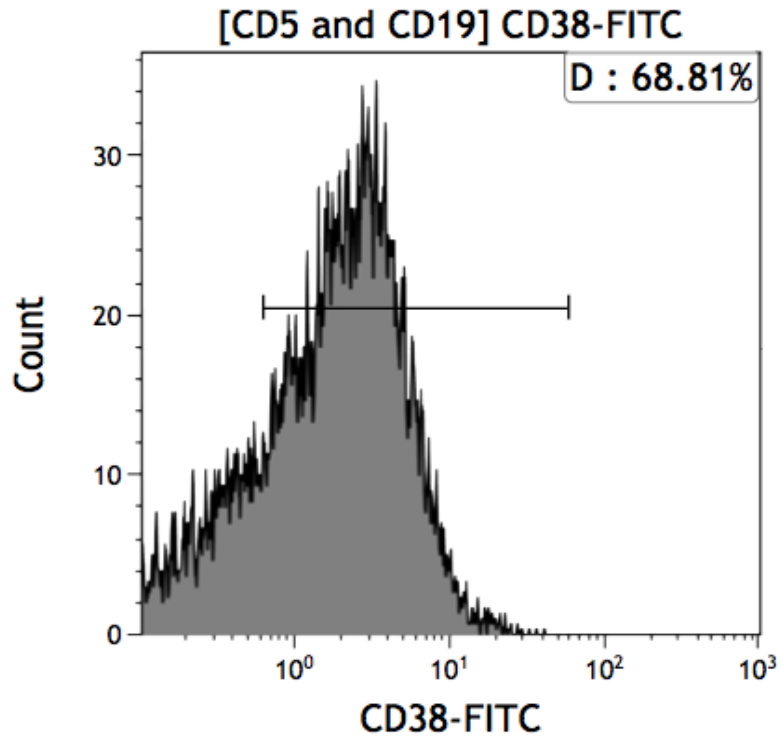
5. **Histogram 5** – SS vs. CD38 histogram. A gate was placed on CD5+ lymphocytes (region K)



6. **Histogram 6** – SS vs. CD38 histogram. A gate was placed on CD56+ lymphocytes



7. **Histogram 7** – a single parametric histogram for gating CD38+ cells on CD19/CD5+ cells



APPENDIX V: Procedure for β 2 microglobulin test

The serum B2M levels of the selected patients will be assessed using Mini Vidas immunoassay instrument

Principle:

The assay principle combines a two-step enzyme immunoassay sandwich method with a final fluorescent detection (ELFA). The Solid Phase Receptacle (SPR), serves as the solid phase as well as the pipetting device for the assay. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All the assay steps are performed automatically by the Mini Vidas instrument. The reaction medium is cycled in and out of the SPR several times.

After diluting the sample, the β 2 microglobulin in the sample binds with the specific monoclonal antibody coating the interior of the SPR. Unbound components are eliminated during the washing steps. The β 2 microglobulin retained is revealed by an alkaline phosphatase-labeled polyclonal anti-human β 2 microglobulin antibody (sheep). Unbound conjugate is eliminated during the washing phase. During the final detection step, the substrate (4-Methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone), the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of β 2 microglobulin present in the sample.

At the end of the assay, results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

Specimen type and collection:

Use serum or plasma (validated anticoagulants: lithium heparinate, EDTA).

- Do not use contaminated or inactivated sera (after 30mins at 56°C, the concentration of β 2 microglobulin in the sample is reduced by 18 to 50%).
- Samples containing impurities must be centrifuged before analysis.

Specimen stability - Serum and plasma samples can be stored at

- 2-8°C for up to 5 days;
- If longer storage is required, freeze at $-25 \pm 6^{\circ}\text{C}$ (avoid successive freezing and thawing)

Material:

1. B2M reagent strips with SPR.
2. Pipette with disposable tip calibrated to dispense 100 μl .
3. B2M high positive control.
4. B2M low positive control.
5. B2M calibrator.
6. Powder less, disposable gloves.
7. Centrifuge machine.

Procedure:

1. Required reagents were removed from the refrigerator and were allowed to come to room temperature for at least 30 minutes.
2. One "B2M" strip and one "B2M" SPR was used for each sample, control or calibrator to be tested the storage pouch was resealed after the required SPRs were removed).
3. B2M test code was selected on the machine. The calibrator was identified as "S1", and tested in duplicate. For low positive control was identified by "C1" and the high positive control was identified by "C2".
4. Calibrator, controls and samples were mixed using a Vortex-type mixer.
5. 100 μl of calibrator, sample or control was pipetted into the sample well.
6. The SPRs and strips were inserted into the instrument. Then checked to make sure the color labels with the assay code on the SPRs and the Reagent Strips were matching.
7. The assay was initiated as directed in the Operator's Manual. All the assay steps were performed automatically by the instrument. The assays were completed within approximately 40 minutes.
8. After the assay was completed, the SPRs and strips were removed from the instrument and disposed appropriately.

APPENDIX VI: Calculation of GFR adjusted B2M

1) Serum B2M and creatinine levels were measured on the same peripheral blood sample.

2) GFR was estimated using the modification of diet in renal disease equation:

$$\text{GFR (ml/min/1.73 m}^2\text{)} = 186.3 \times \text{serum creatinine } (\mu\text{mol/l divided by } 88.4)^{-1.154} \times \text{age (years)}^{-0.203} \times 1.212 \text{ if black, } \times 0.742 \text{ if female.}$$

3) GFR-adjusted B2M (GFR-B2M) was calculated using the following equation (2)

$$\text{GFR-B2M} = \text{B2M (mg/l)} \times \text{GFR (ml/min)/100}$$

APPENDIX VII: Procedure for lactate Dehydrogenase (LDH) estimation

The serum LDH of patients was estimated using Olympus AU 640 analyzer.

Principle: lactate dehydrogenase catalyses the reduction of pyruvate by NADH, to form lactate and NAD⁺. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340nm.

Specimen type and collection:

Use serum or plasma. Serum or plasma must be separated from the clot as soon as possible. Do not use hemolysed samples. For plasma samples, heparin is used as anticoagulant.

Specimen stability – LDH in serum or plasma is stable for 2 days at room temperature, for 24 hours at 2-8⁰ C and for longer storage at -20⁰ C.

Material:

1. Olympus AU 640 analyser
2. Pipette with disposable tip.
3. LDH high positive control.
4. LDH low positive control.
5. Powder less, disposable gloves.
6. Centrifuge machine.

Procedure:

1. The blood samples collected in the plain bottle were centrifuged to separate the serum.
2. Serum was aliquoted in to a pre labeled cryovial and stored at -20⁰ C.
3. The serum was thawed at room temperature at the time of estimation.
4. The sample was loaded in the Olympus AU 640 analyzer, which automatically calculates the activity of each sample with a specified valid calibration factor from calibration process.
5. The results were interpreted as U/L.

APPENDIX VIII: Procedure for creatinine estimation

The serum creatinine of patients was estimated using Olympus AU 640 analyzer.

Principle: creatinine in the sample reacts with picrate in alkaline medium forming a coloured complex. The complex formation rate is measured in a short period to avoid interferences.

Specimen type and collection:

Use serum or plasma. Serum or plasma must be separated from the clot as soon as possible. Do not use hemolysed samples. For plasma samples, heparin, EDTA and fluoride may be used as anticoagulant.

Specimen stability – creatinine in serum or plasma is stable for 24 hours at 2-8⁰ C and for longer storage, stable at -20⁰ C.

Material:

- 1 Olympus AU 640 analyser
- 2 Pipette with disposable tip.
- 3 Creatinine high positive control.
- 4 Creatinine low positive control.
- 5 Powder less, disposable gloves.
- 6 Centrifuge machine.

Procedure:

1. The blood samples collected in the plain bottle were centrifuged to separate the serum.
2. Serum was aliquoted in to a pre labeled cryovial and stored at -20⁰ C.
3. The serum was thawed at room temperature at the time of estimation.
4. The sample was loaded in the Olympus AU 640 analyzer, which automatically calculates the activity of each sample with a specified valid calibration factor from calibration process.
5. The results were interpreted as µmol/L.

APPENDIX IX: Monoclonal antibodies panel

Tube 1	CD5-FITC/CD19-PC5/CD3+CD56-PC7, ZAP70-PE
Tube 2	CD19, CD 5, ZAP70, CD56+3

APPENDIX X: Data collection and recording sheet

Sl no	Name	Age/ Sex	Race	Relevant history – fever, weight loss, night sweats	Lymphadenop athy	Splenomegaly	Hepatomegaly	Hb g/dl	WBC count x 10 ⁹ /L	Platelet count x 10 ⁹ /L	Binet staging	Treatment
01												
02												
03												

APPENDIX XI: Laboratory tests data sheet

Sl no	Peripheral smear	CD 45	CD 19	CD 5	ZAP 70	CD 38	B2M mg/L	GFR adjusted B2M	LDH U/L	Hb g/dl	WBC count x 10 ⁹ /L	Absolute lymphocyte count X10 ³	Differential lymphocyte count %	Platelet Count x 10 ⁹ /L	Binet staging
01															
02															
03															
04															

APPENDIX XII: Patient information and consent form

This Informed Consent Form has two parts:

1. Information Sheet (to share information about the study with you)
2. Certificate of Consent (for signatures if you agree to take part)

Consenting patients will be given a copy of the signed informed consent form.

PART I: Information Sheet

Introduction

My name is **Dr. Deepa Patel**. I am a postgraduate student in the department of human pathology at the University of Nairobi. I am conducting this study so as to understand if a particular substance in the blood of someone with the condition that you have can be used to make important decisions regarding treatment of the condition, to improve the treatment outcome. The condition that you are being treated for is called Chronic Lymphocytic Leukemia. Chronic lymphocytic leukemia (CLL) is a type of cancer of the blood and bone marrow — the spongy tissue inside bones where blood cells are made.

We invite you to participate in this study. You do not have to decide today whether or not you will participate. Before you decide, you can talk to anyone you feel comfortable with about the study.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask me, at any time.

Type of Research Intervention

This study involves the analysis of blood samples from 45 patients with this condition called CLL. In the course of your treatment, we will take a little of your blood. The blood will be about a table spoonful. The blood will then be sent to the laboratory to measure the amount of this substance under study (B2M, CD38, ZAP-70). The blood sample that you will give us will be disposed off after the tests.. When we take the small amount of blood, you will feel a little pain, like a prick. This pain is temporary and will be over in about five minutes. We are using fresh and clean needles which will be appropriately disposed off after the test.

Participant selection

We shall recruit adult patients who come to the Haematology clinic, or are admitted at KNH and AKUHN who have been diagnosed with CLL.

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the medical care you are receiving will continue with no change whatsoever. You are free to withdraw from the study at any time.

Procedures and Protocol

Eligible participants will be interviewed. The interview will be followed by physical examination. The patients will be requested to sign the consent form, and thereafter you will be asked some questions which will be recorded in a questionnaire regarding patient's characteristics. Thereafter venous blood (5 - 10ml) from the forearm will be collected in two specimen bottles. The blood collection will be done aseptically.

Side Effects

There are no side effects expected in this study. All we will do is to ask you a few questions and get measurements from the blood sample.

Risks

There will be no risks expected in this study. The only discomfort will be from the needle prick as we take a small blood sample. The pain should go away in a few minutes. Rarely there may be a small hematoma formation, which usually subsides within days. We do not anticipate infection because we use new needles and sterile equipment.

Benefits

There may not be any benefit for you as an individual, but your participation is likely to help improve the care of patients with CLL in general.

Reimbursements

You will not be given any money or gifts to take part in this research.

Confidentiality

We will not share the identity of those participating in the research. The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name.

Sharing the Results

The knowledge that we get from doing this research will be shared with you through your doctor. We will publish the results in order that other interested people may learn from our research. However your identity will not be revealed.

Right to Refuse or Withdraw

If you decline to participate in this study, this will not affect your treatment in any way. You will still have all the benefits that you would have otherwise. You may stop participating in the research at any time that you wish, without losing any of your rights as a patient here.

Who to Contact

If you have any questions regarding the participation in this study at any time, you may contact any of the following people:

1. Dr. DEEPA PATEL (Principal investigator) on telephone number 0724214350 or my supervisors Dr. KITONYI G. (telephone number 0722385336) and Dr AMAYO A (telephone number 0733617678), Dr. PAMNANI RITESH (telephone number 0733743792).

If you have further questions about the study you can contact Prof. BHATT K, Chairperson of Ethics Committee on telephone number 02726300 (extension 44102).

You can ask me any more questions about any part of the research study, if you wish to. Do you have any questions?

PART II: Certificate of Consent

I have read the foregoing information, or it has been read to me. I have had the opportunity to raise questions about my participation in the study, and any questions that I have asked have been answered to my satisfaction. My rights have been explained to me I consent voluntarily to participate as a participant in this study.

Print Name of Participant _____

Signature of Participant _____

Date _____

Day/month/year

If illiterate,

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness _____

AND

Thumb print of participant

Signature of witness _____

Date _____



Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands what the research is all about.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability.

I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent _____

Signature of Researcher /person taking the consent _____

Date _____

Day/month/year

APPENDIX XI: Data collection instrument (study questionnaire)

Study title: correlation of β -2 Microglobulin, ZAP-70, CD38 AND LDH with clinical staging of CLL

1. Study number

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2. Code

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Information from patient's and patient's files:

3. Patient's name _____ .

4. IP/NO.

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5. Age

--	--

 (years).

6. Gender _____

7. Race _____

8. Address and phone number _____

9. Ward/ unit:

- Medical ward.
- Hematology clinic
- Other _____.

10. Diagnosis / presumptive diagnosis _____

11. Disease stage _____

History:

1. Fever _____
2. Weight loss _____
3. Night sweats _____
4. Others _____

Physical examination:

1. General physical examination
 - a. Pallor _____
 - b. Icterus _____
 - c. Cyanosis _____
 - d. Lymphadenopathy _____
 - e. Oedema _____
2. Splenomegaly _____
3. Hepatomegaly _____
4. Any abdominal masses _____
5. Any other findings _____

Relevant laboratory tests from the patient file:

1. Hematological tests
 - a. Hb _____ g/dL
 - b. WBC count _____ $\times 10^3/L$

- c. Absolute lymphocyte count _____ x 10³/L
- d. Differential lymphocyte count _____ %
- e. Platelet count _____ x 10⁹/L
2. Serum B2M level _____ mg/L
3. Serum LDH level _____ U/L
4. Any other tests _____

Treatment:

1. Is patient on treatment? _____
2. If so, when was it started? _____
3. Details of treatment _____

APPENDIX XIV: Ethical approval



Ref: KNH-ERC/ A/620

Dr. Deepa Y. Patel
Department of Human Pathology
School of Medicine
University of Nairobi

Dear Dr. Patel

**Research proposal: "Comparison of B-2 microglobulin with CD38 and ZAP-7 as prognostic markers
in chronic lymphocytic leukemia"**
(P155/05/2010)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal for the period 3rd November 2010 – 2nd November 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

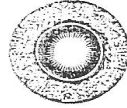
PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

c.c. The Deputy Director CS, KNH
The Dean, School of Medicine, UON
The Chairman, Dept. of Human Pathology, UON
The HOD, Records, KNH
Supervisors: Dr.A. Amayo, Dept.of Human Pathology, UON
Dr. Pamnani Ritesh, Dept.of Human Pathology, UON

KENYATTA NATIONAL HOSPITAL
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Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP", Nairobi.
Email: KNHplan@Ken.Healthnet.org
3rd November 2010

APPENDIX XV: Approval from department of Pathology - AKUHN

Noted
Copies to Dr. Kabera
4/27/10



THE AGA KHAN UNIVERSITY

Faculty of Health Sciences

Post Graduate Medical Education



30th April, 2010

Dr. J. Githanga
Chairperson
Department of Human Pathology
Kenyatta National Hospital
P.O. Box 19676-00202 KNH
Kenya

Dear Dr. Githanga,

REF: DR. DEEPA Y. FATEL

Reference is made to your letter of 21st April, 2010 requesting us to allow Dr. Patel access to the Flow Cytometer. We will be glad to assist and please let her liaise with one of our residents, Dr. Beatrice Kabera who will guide her through the relevant techniques on the equipment.

I am copying this letter to Dr. Francis McOdimba, (Administrative Director – Laboratory) and Mr. Josephat Wambua (Laboratory Manager) for their information and facilitation.

Sincerely,

Peter J. Ojwang
Chair – Department of Pathology - AKUHN

