THE PROGNOSTIC VALUE OF CD38 AND CD49d FLOW CYTOMETRY MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA: A RETROSPECTIVE 5-YEAR STUDY

By

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As the candidate's supervisor I have/have not approved this thesis for submission.

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Declaration

I,Siyabonga Voxeka, declare that

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

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Dedication

This work is dedicated to all patients suffering from Chronic Lymphocytic Leukemia

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Overview of the thesis:

Chronic Lymphocytic Leukemia (CLL) is a heterogeneous disease which has a highly variable clinical course. Most patients who show no clinical symptoms at the time of diagnosis survive several years without need for treatment, while others present with aggressive disease and have shorter life span. Understanding the prognostic factors associated with CLL plays an impactful role in the management of these patients. KwaZulu-Natal is a province in a resource limited South Africa and public health care faces many challenges. Patients with CLL present to health care facilities late for a number of reasons that include families seeking traditional options first, lack of insight into seriousness of the condition especially high risk patients who require treatment from the beginning of the disease and transport challenges to get to health care facilities.

There are different prognostic biomarkers in CLL, some of these include serum markers, immunophenotypic markers, immunoglobulin heavy chain variable (IGHV) gene mutation status, chromosome aberrations, gene mutations and microRNAs. Based on these biomarkers, classic prognostic models such as the Rai and Binet systems and the chronic lymphocytic leukemia – international prognostic index (CLL-IPI) were developed to improve risk stratification and tailor treatment intensity. The World Health Organisation generally recommends that all patients with CLL be tested for genetic abnormalities.

Many studies have proven that CD38 and CD49d high level of expression are independent poor prognostic markers with unfavourable outcomes in CLL patients.

This study drives the need to explore prognostic flow cytometry markers CD38 and CD49d that are used in prognostication of CLL patients as they are more easily available and cost-effective. This is the first study in South Africa that analyzed flow cytometry markers in CLL patients, and their association with other prognostic variables.

A 5-year retrospective analysis was performed on all newly diagnosed CLL patients. The expression of CD38 and CD49d were correlated with haemoglobin levels, platelet counts and Fluorescence in situ hybridisation (FISH) analysis. Patient charts were obtained from the haematology clinic for 2-year overall survival (OS) analysis, and described using Kaplan-Meier survival curves.

The recognition of these prognostic markers can be very helpful in predicting the clinical course of the disease thus facilitating appropriate and timely therapeutic interventions. In resource-constrained settings where molecular testing is not easily available, as demonstrated in this study, flow cytometry markers may have prognostic utility without significant additional cost or expertise required.

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ABBREVIATIONS:

11q-	11q deletion
13q-	13q deletion
17p-	17p deletion
BCL2	B Cell Lymphoma protein2
BMAT	Bone marrow aspirate and trephine
CD	Cluster of differentiation
CLL	Chronic Lymphocytic Leukemia
CLL-IPI	Chronic Lymphocytic Leukemia-International Prognostic Index
FISH	Fluorescence in situ hybridization
Hb	Haemoglobin
IALCH	Inkosi Albert Luthuli Central Hospital
IGHV	Immunoglobulin heavy chain variable
LIS	Laboratory information system
NHLS	National Health Laboratory Service
NK	Natural Killer cells
OS	Overall Survival
Plts	Platelets
TFS	Treatment Free Survival
TNF-a	Tumour necrosis factor-alpha
TP53	Tumour protein p53
VCAM-1	Vascular cell adhesion molecule 1
WCC	White cell count
β_2M	β2 micro-globulin

Part 1: The Review of Literature

History of CLL:

The first cases of chronic lymphocytic leukemia were discovered by Bennett and Virchow in the mid-19th century. In 1870 Ernst Neuman recognized the role of the bone marrow in leukemia and came up with the conception of the marrow as the source of blood cells. Paul Ehrlich in 1891 developed a tri-acid stain that allowed the clear definition of nucleus, cytoplasm and other cytoplasmic details and the stains allowed leukemias to be more clearly separated. In the twentieth century Minot and Isaacs produced a detailed description of the clinical features and natural history of 80 cases of CLL. In 1959 Richard Doll established that CLL was a disease of late middle age and is twice common in men. By the late 1960s haematologists knew all there was to know about the clinical features of CLL and its natural history.(1)(2)

Demographics of CLL:

Chronic lymphocytic leukemia is one of the most common adult leukemias affecting adults with a median age of approximately 70 years but can also present in younger adults.(3) Males are more affected than females with a ratio of 1.5-2:1.(4)

Pathophysiology of CLL:

Chronic lymphocytic leukemia is characterized by the clonal proliferation of CD5-positive B cells because of a failure in programmed cell death or apoptosis within the blood, bone marrow, lymph nodes and spleen.(5) CLL cells are resistant to apoptosis due to upregulation of the anti-apoptotic proteins such as B-Cell Lymphoma 2 protein (BCL-2)and downregulation of pro-apoptotic proteins such as BCLX.(4)(6) The micro-environment plays an important role in protecting CLL cells from death through a number of molecules such as b-cell receptor(BCR) signaling, activation via tumour necrosis factor (TNF) family members, tissue homing chemokine receptors and adhesion molecules.(7) Approximately 50% of cases the immunoglobulin heavy chain variable (IGHV) gene is unmutated. Unmutated cases are associated with auto reactivity and polyreactivity to certain molecules and have more proliferative pattern of a disease with a more aggressive clinical course.(4)

Clinical features of CLL:

Most of the patients are asymptomatic and are diagnosed during routine investigations such as bacterial infections. Patients present with painless, generalized lymphadenopathy and rarely during the diagnostic work-up of an autoimmune haemolytic anemia and less frequently an autoimmune thrombocytopenia. (4)

Diagnosis of CLL:

Patients with CLL have a persistent lymphocytosis of $\geq 5X\ 10^9$ /L of mature B lymphocytes in peripheral blood.(8)

Peripheral blood and bone marrow morphology of CLL:

Morphological examination is commonly the first step in the evaluation of a suspected CLL.(9) The cells in CLL are typically small mature lymphocytes with high nuclear-cytoplasmic ratio and clumped

nuclear chromatin with inconspicuous nucleoli in the peripheral blood, bone marrow, spleen and lymph nodes as shown in Figure 1. below.

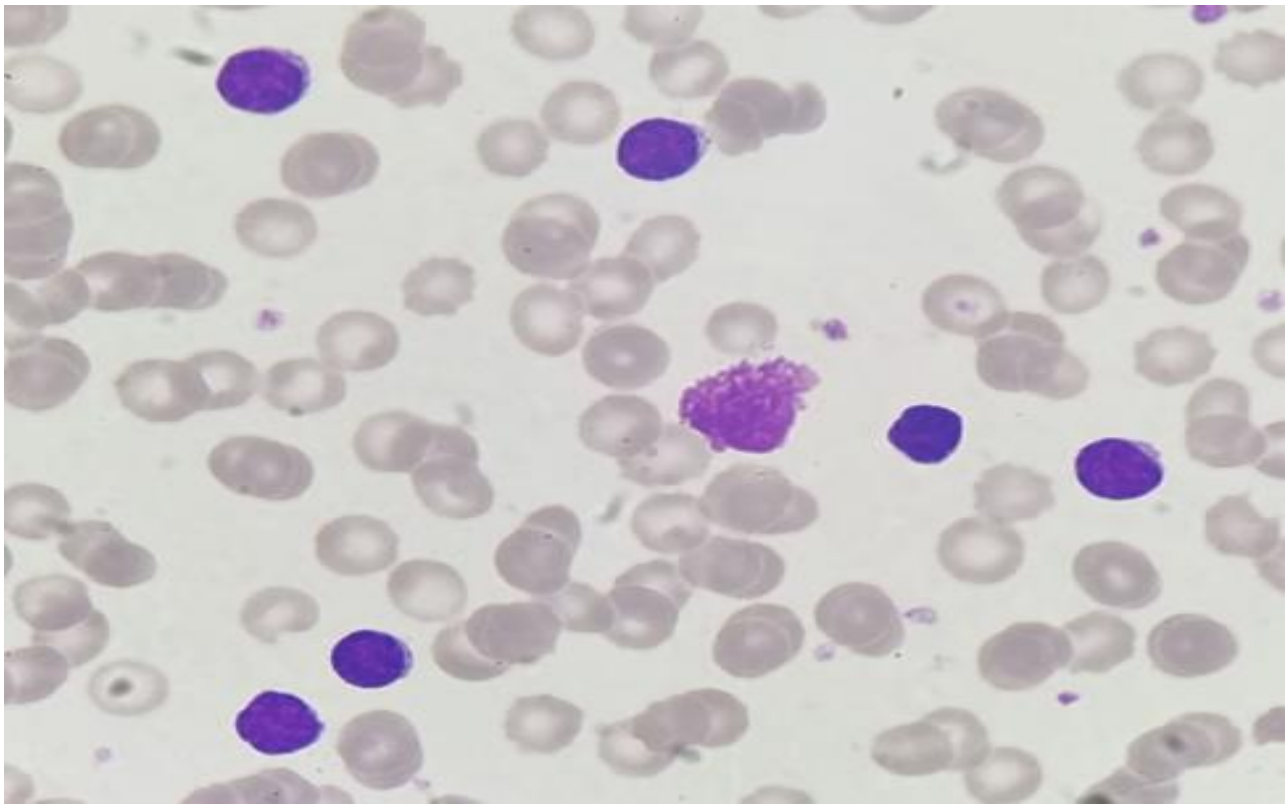

Figure 1: Peripheral smear of a typical case of CLL (magnification X 100)

In the tissue infiltrates, the CLL cells are admixed with prolymphocytes and para-immunoblasts forming proliferation centers.(3) The bone marrow biopsy may show interstitial, nodular, mixed (interstitial and nodular) or diffuse involvement. Diffuse involvement is usually associated with advanced disease.(3) Smudge cells are also present on the peripheral blood smear and bone marrow aspirate smear.(10) Morphologically suspected cases are then further evaluated by flow cytometry.

Immunophenotype of CLL:

The diagnosis of CLL is established on flowcytometry. When using the EuroFlow^{TM} Consortium B-Chronic Lymphoproliferative Disorder panel, tubes 1-5 (BCLPD1-5) is the diagnostic tube panel.(11) The leukemic cells in CLL express CD5 and CD19 with dim surface IgM/ IgD, CD20, CD22 and CD79b. They are also positive for CD43 and strongly positive for CD23 and CD200.(12) CD10 is negative and FMC7 is usually negative or weakly expressed. Each clone of leukemia cell expresses either kappa or lambda immunoglobulin light chains.(13) Some cases have atypical immunophenotype. These include negative expression of CD5/CD23, positive FMC7 and strong surface immunoglobulin or CD79b.(3)

Expression of CD38 in CLL:

There are various CD markers used for prognostication of CLL patients. CD38 and CD49d are both independent risk parameters in CLL. They influence CLL cell trafficking between blood and lymphoid organs as well as their survival within lymphoid organs and thereby imparting the pathophysiology of the disease.(14) Of the immunophenotypic markers, CD38 is a transmembrane type II glycoprotein

localized on the plasma membrane, cytoplasm and inner nuclear membrane of cells. It plays an important role in promoting proliferation and prolonging survival of leukemic cells. It is expressed on numerous haematopoietic cells such as lymphocytes, myeloid cells, natural killer cells, platelets and erythrocytes.(15) Patients with greater than 30% for CD38 have a poorer prognosis.

Expression of CD49d in CLL:

CD49d belongs to a family of integrin alpha subunits. It is an adhesion molecule expressed on the surface of B lymphocytes that can play a vital role in regulating cell-cell and cell-extracellular matrix interactions by binding to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) in the bone marrow. The extracellular matrix fibrinogen CD49d serves as a signaling receptor that influences B cell survival through upregulation of BCL-2 family members. Tumour necrosis factor-alpha can increase the binding of CD49d to VCAM-1 through increasing the expression of VCAM-1 on endothelial/ stroma cells. It also improves the survival of B cells of CLL patients by protecting them from apoptosis.(16) Patients are considered to be positive for CD49d when the expression is greater than 30% on flow cytometry. Recognition of these immunophenotypic markers can be very helpful in predicting the clinical course of the disease, thus facilitating appropriate and timely therapeutic interventions. (17)

Both CD38 and CD49d expression triggers signaling pathways preventing apoptosis and thus resulting in aggressive disease in chronic lymphocytic leukemia.(18)

Molecular analysis by FISH in CLL:

Fluorescence in situ hybridization (FISH), karyotype analysis and next-generation sequencing have been widely used in the diagnosis and risk stratification of CLL. The most common chromosomal aberrations in CLL include deletions of the long arm of chromosome 13, trisomy 12, deletions of long arm of chromosome 11 and deletions of the short arm of chromosome 17. Deletion 13, involving band 13q14 is the most frequently observed cytogenetic aberration, and is associated with good prognosis. Deletion 13q includes the miRNA miR-15a and miR-16-1 cluster which downregulates the anti-apoptotic gene BCL2 and thus promoting CLL survival. Trisomy 12 is the second most common chromosomal aberration and is associated with intermediate risk prognosis. Deletions 11q and 17p are associated with a poor prognosis.(19)(20)

Prognosis of patients with CLL:

Staging systems for CLL**:**

Two widely accepted staging methods, the modified Rai and Binet systems, are used in both patient care and clinical trials. Rai staging and Binet staging systems rely on physical examination and standard laboratory testing.

Rai Classification:

The Rai stage is used to determine the extent of CLL disease, and is classified from 0 to IV. This was based on lymphocyte count, presence of lymphadenopathy, hepatomegaly and cytopenias. The Modified Rai classification assessed patient's risk, and is classified as low, intermediate and high-risk. High risk disease includes patients with disease related anemia as defined by a haemoglobin <10 g/dL (formerly considered Rai stage III) or thrombocytopenia as defined by a platelet count of $<100 \times 10^9$/L

(formerly considered Rai stage IV) as shown in Table 1 Below. (21) The median survival declines sharply as the stage or risk category of CLL increases (shown in Table 1).

Rai Stage	Modified Rai Risk Status	Description	Median Survival (years)
0	Low	Lymphocytosis, with lymphoid cells >30% in the blood and/bone marrow	11.7
I	Intermediate	Stage 0 with enlarged node(s)	8.3
II	Intermediate	Stage 0-I with splenomegaly, hepatomegaly, or both	5.8
III	High	Stage 0-II with haemoglobin <110 g/L	2.0-4.0
IV	High	Stage 0-III with platelets <100 X 10^9/L	2.0-4.0

Binet staging system:

The Binet staging system is based on the number of involved lymphoid areas as defined by the presence of enlarged lymph nodes greater than or equal to 1cm in diameter, organomegaly and the presence of anemia or thrombocytopenia. In both Binet A and B stages, the haemoglobin and platelet levels are greater than 10g/dL and 100 $x10^9$/L, respectively, and these stages are associated with low and intermediate risk respectively. In Binet stage C, the haemoglobin is <10g/dL and the platelets count is <100 $x10^9$/L, and this stage is associated with high risk patients.(17)

CLL-International Prognostic Index:

A large number of biomarkers can provide prognostic information. The most relevant prognostic parameters are IGHV mutational status, serum β_2 -microglobulin, and the presence of del (17p) and or TP53 mutations. The identification of these new prognostic parameters led to the proposal of additional prognostic scores. One of these prognostic scores, the CLL- international prognostic index (CLL-IPI), studied approximately 28 prognostic variables among 3400 patients treated in clinical trials across the world, and was validated in two independent cohorts of patients, including the Mayo clinic and Scandinavian CLL cohort. Five factors including the clinical stage, age, IGHV mutational status, serum β_2M, the presence of del (17p) and or TP53 mutations were independently found to be associated with overall survival (OS), and four risk groups (low, intermediate, high and very high risk) were identified.(22)(23)(24)(25)

Survival of patients with CLL:

The median survival in years in the Rai classification for CLL patients varies according to the stage of a patient. In stage 0, the median survival is 11.7 years, stage I is 8.3 years, stage II is 5.8 years and stages III and IV the survival is 2 to 4 years respectively (Table 1). In the Binet system stage A, the

median survival is more than 10 years, Binet stage B is 5 years and Binet stage C OS > 2 to 4 years. Four risk groups (low, intermediate, high and very high risk) are identified in CLL-IPI) with 5 year OS of 93%, 79%, 63% and 23% respectively.(26)

There is no published data on prognosis associated with flow cytometry markers in CLL in KwaZulu-Natal, nor in South Africa. The purpose of this retrospective study was to assess the value of specific flow cytometry markers in CLL.

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Part 2: Manuscript submitted for publication.

Chapter 1 provided a background to the diagnosis and prognosis of CLL, and a rationale for this study. Chapter 2 contains the manuscript submitted for publication to the African Journal of Laboratory medicine, and is in the journal requested format.

TITLE

The Incidence and Significance of CD38 and CD49d expression in patients with Chronic Lymphocytic Leukemia.

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ABSTRACT

Background:

The prognosis of chronic lymphocytic leukemia (CLL) is determined by various prognostic markers. The importance of the immunophenotypic markers CD38 and CD49d on flow cytometry in CLL is well-established internationally. However, there is no data from South Africa on these markers.

Objective:

This study assessed the frequency of CD38 and CD49d expression in newly diagnosed CLL patients, and the correlation of these markers with other prognostic variables.

Methods:

A 5-year retrospective analysis was performed on all newly diagnosed CLL patients. The expression of CD38 and CD49d were correlated with haemoglobin concentration, platelet counts and markers by Fluorescence in situ hybridisation (FISH) analysis. Patient charts were obtained from the haematology clinic for 2-year overall survival (OS) analysis, and described using Kaplan-Meier survival curves.

Results:

Data from 86 newly diagnosed CLL patients were analyzed. Most of the patients, 70.9% (n=61), were between 60-79 years of age. The frequency of CD38 positivity was 29% (n=25), CD49d positivity was 15.1% (n=13), dual positivity for CD38 and CD49d was 15.1% (n=13) and dual negativity was 40.7% (n=35). Of the 37% (n=33) who had CLL FISH studies, seven had 13q deletion, ten had trisomy 12 and two had 11q deletion. CD49d expression correlated with trisomy12 with (p value 0.002).

Conclusion:
The incidence of CD49d expression in KwaZulu-Natal, was lower than that described in CLL internationally. Although there was some correlation with molecular abnormalities detected by FISH, further prospective studies are warranted to confirm if these immunophenotypic markers can be utilized as surrogate prognostic markers in CLL.

KEYWORDS
CD38, CD49d, Chronic Lymphocytic Leukemia, flow cytometry, survival analysis

INTRODUCTION
Chronic lymphocytic Leukemia (CLL) is a heterogeneous disease with a highly variable clinical course.(27) It is a clonal disease of mature, apoptosis-resistant B-cells and involves the blood, bone marrow and secondary lymphoid tissues such as spleen and lymph nodes.(22) CLL is one of the most common adult leukemias, affecting adults with a median age of approximately 70 years, but can also present in younger individuals.(3) Males are more affected than females with a ratio of 1.5-2:1.(4) Most patients show no clinical symptoms at the time of diagnosis, and they survive several years without the need for treatment, while others present with aggressive disease and have shorter life span.(28)(29)(30) The incidence and significance of the expression of flow cytometry markers in CLL has not been investigated in KwaZulu-Natal, nor in South Africa

CLL is suspected when a patient has a persistent lymphocytosis on the peripheral blood. Morphological examination is the first step in the evaluation, with confirmation of the diagnosis by flow cytometry. The patient must have a monoclonal B-cell count of $\geq 5 \times 10^9$/L, with the characteristic morphology and phenotype of CLL in the peripheral blood.(7) The clonal B-cell population in CLL typically express CD5 and CD19, with dim expression of surface Immunoglobulin (Ig) M or IgD, CD20, CD22 and CD79b. These cells also express CD43 and are strongly positive for CD23 and CD200. There is a lack of CD10 expression and FMC7 is usually negative or weakly positive.(3)

There are different prognostic biomarkers in CLL, some of which include serum markers, immunophenotypic markers, immunoglobulin heavy chain variable gene mutation status, chromosomal aberrations, gene mutations and microRNAs. Classic prognostic models such as the Rai and Binet staging systems and the CLL –International Prognostic Index (IPI), utilize these biomarkers.(8) The Binet staging system classifies the CLL patients by the number of affected lymphoid tissue groups and according to the haemoglobin concentration and platelet count.(21) In both Binet A and B stages, the haemoglobin concentration and platelet counts are greater than 10 g/dL and 100 $x10^9$/L, respectively. Binet stage A is associated with low risk group and Binet stage B is associated with intermediate risk group. In Binet stage C, the haemoglobin is <10 g/dL and platelets are <100 $x10^9$/L and this stage is associated with poor survival. These prognostic scoring systems were developed and adapted to improve risk stratification and tailor treatment intensity according to the dramatic advances in therapy for CLL.(23)(24)(31)(13)

Of the immunophenotypic markers, CD38 is a transmembrane type II glycoprotein localized on the plasma membrane, cytoplasm and inner nuclear membrane of cells. It plays an important role in promoting proliferation and prolonging survival of leukemic cells. It is expressed on numerous haematopoietic cells such as lymphocytes, myeloid cells, natural killer cells, platelets and erythrocytes.(15)

CD49d belongs to a family of integrin alpha subunits. It is an adhesion molecule expressed on the surface of B lymphocytes that can play a vital role in regulating cell-cell and cell-extracellular matrix interactions by binding to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) in the bone marrow. The extracellular matrix fibrinogen CD49d serves as a signalling receptor that influences B cell survival through upregulation of BCL-2 family members. Tumour necrosis factor-alpha can increase the binding of CD49d to VCAM-1 through increasing the expression of VCAM-1 on endothelial/ stroma cells. It also improves the survival of B cells of CLL patients by protecting them from apoptosis.(16)

Literature suggest that the dual expression of CD38 and CD49d in patients with CLL leads to an aggressive clinical course.(32) CD38 and CD49d are used for prognostication in CLL patients, although these markers are not formally included in the aforementioned prognostic scoring models.(33) Patients are considered to be positive for CD49d when the expression is greater than 30% on flow cytometry. Patients are considered to be positive for CD38 when the expression is greater than 20% and when the expression is greater than 30% patients have a poorer prognosis.(17)

Recognition of these immunophenotypic markers can be very helpful in predicting the clinical course of the disease, thus facilitating appropriate and timely therapeutic interventions.(34)(35) These markers, if correlated with prognosis, may be more cost-effective prognostic markers, especially in countries with economic constraints. The aim of this study was to assess the value of CD38 and CD49d flow cytometry markers in CLL, as there is no data on prognosis for flow cytometry markers in CLL in South Africa

METHODS

Ethical considerations

The study was approved by Biomedical Research Ethics Committee of the University of KwaZulu-Natal, South Africa (BREC/00004494/2022)

Study design and setting

This study retrospectively described the expression and significance of adhesion molecules CD38 and CD49d in all newly diagnosed CLL patients in the haematology laboratory at Inkosi Albert Luthuli Central Hospital, KwaZulu-Natal, South Africa from 1 September 2015 to 30 September 2020. Patient data was selected for inclusion for patients that were diagnosed with CLL in the study period and that were treatment naive.

Data collection

Data for all patients that met the inclusion criteria was obtained from the NHLS Laboratory Information System (LIS). The diagnosis of CLL was established using the EuroFlow^{TM} Consortium B-Chronic Lymphoproliferative Disorder panel, tubes 1-5 (BCLPD1-5).(11). Study parameters that were collected included demographic data (age, gender), clinical data (2-year survival analysis), laboratory data (haemoglobin concentration, platelet count) and FISH analysis for (13q,11q,17p and trisomy12).The tumour cells were assessed for the expression of CD38 and CD49d. The marker expression was recorded as positive if levels were >30%. The study cohort included 4 patient groups according to flow cytometry findings viz. solo CD38 positive:CD38 positive/ CD49d negative (CD38+/CD49-dualpositive:CD38positive/CD49dpositive (CD38+/CD49d+) and dual negative: CD38 negative/ CD49d negative (CD38-/CD49d-). Haemoglobin concentration <10 g/dL and platelet counts <100

x10^9/L were used as prognostic variables as described in the stage C Binet staging system. CD38 and CD49d expression was correlated with clinic-hematological parameters. Patient charts were obtained from King Edward Vlll Hospital Haematology Clinic for evaluation of the 2-year survival analysis.

Data analysis

Descriptive statistics were used to summarize the data. Frequencies and percentages were used for categorical data. Frequency distributions of numeric variables were examined for standard deviations (SD) or medians (IQR) were used. Demographic and laboratory characteristics associated with CD38 and CD49d were examined separately using Chi-square tests (Fisher's exact) for categorical variables and ANOVA or Mann-Whitney for numeric data. To determine the association of individual FISH abnormalities with CD38 and CD49 flow cytometry, the 5 patients with multiple FISH abnormalities were excluded from the analysis. Results were considered statistically significant if the p value and Fisher's exact were < 0.05. Stata v17 statistical software was used in the analysis. Overall survival (OS) was calculated from the time of diagnosis to death or last follow-up visit using a Kaplan-Meier survival curve.

RESULTS

There were 86 newly diagnosed CLL patients between 1 September 2015 and 30 September 2020.

Demographic data of the study cohort

The median age was of patients newly diagnosed with CLL was 68 years (range 36-88 years). Males comprised 52% (n=45) and females 48% (n=41). About 71% (n=61) of the patients presented between ages of 60-79 years of age as shown in **Figure 1**.

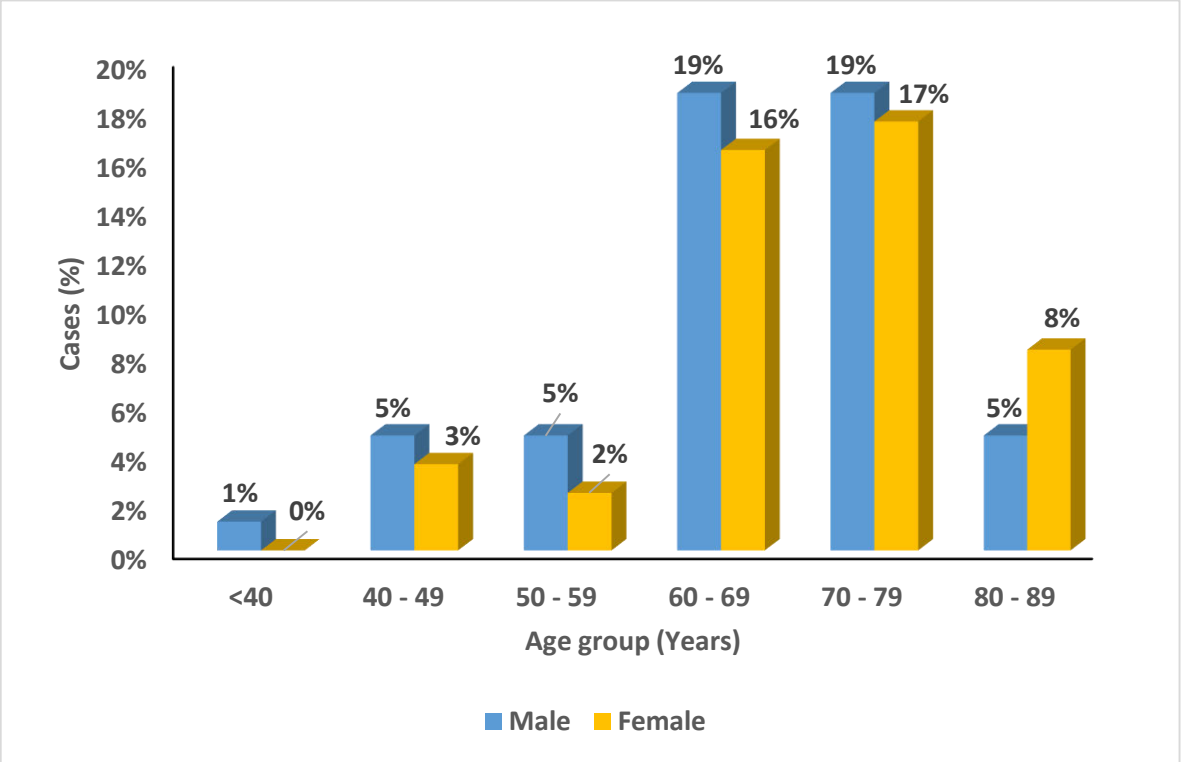

Figure 1: Distribution of chronic lymphocytic leukaemia cases by age group and gender.

Immunophenotypic expression of CD38 and CD49d by flow cytometry
The immunophenotypic expression of the 4 groups is shown in **Figure 2**

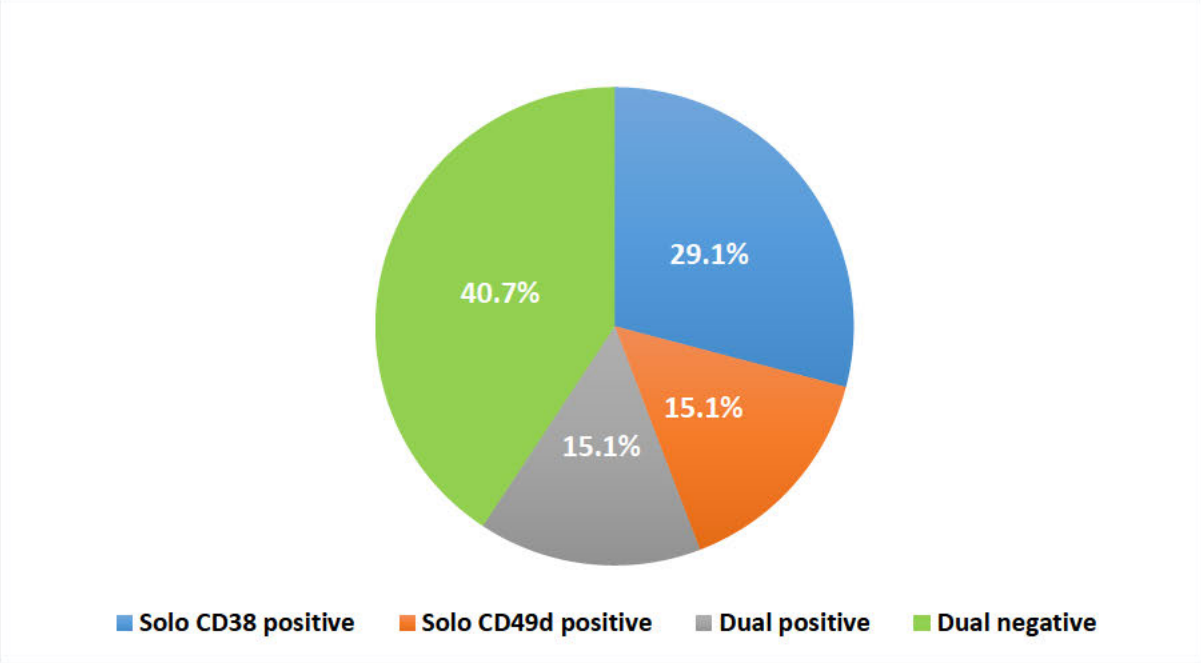

Figure 2. Expression of CD38 and CD49d for all CLL patients from 2015 to 2020

Abbreviations: CLL-Chronic lymphocytic leukemia

Haemoglobin concentrations and Platelet counts of the study cohort
The haemoglobin concentrations and platelet counts of patients with CLL and their comparison with the 4 groups according to CD38 and CD49d expression are shown in **Table 1**.

Table 1. haemoglobin concentration and platelet counts according to flow cytometry markers

	Haemoglobin					**Platelet count**				
Flow cytometry	**<10 g/dL**		**>10 g/dL**			**<100 x 10^9/L**		**>100x10^9/L**		
markers	n	%	n	%	Total	n	%	n	%	Total
SoloCD38 positive	11	44.0%	14	56.0%	25	6	24.0%	19	76.0%	25
SoloCD49d positive	8	61.5%	5	38.5%	13	5	38.5%	8	61.5%	13
Dual positive	8	61.5%	5	38.5%	13	6	46.2%	7	53.8%	13
Dual negative	20	57.1%	15	42.9%	35	11	31.4%	24	68.6%	35
Total	47	54.7%	39	45.3%	86	28	32.6%	58	67.4%	86
Fisher's exact	**0.663**					**0.526**				

Molecular markers analysis by FISH in CLL

Of the 86 patients, 37 (n=33) had had FISH analysis performed. Seven patients had isolated 13q deletion, ten patients had only trisomy 12, two patients had 11q deletion as the sole molecular abnormality, nine were negative for all markers and five patients had multiple FISH abnormalities. Of the five patients who had multiple FISH abnormalities, one patient had solo CD49d expression and was positive for all the CLL FISH panel markers. The remaining four patients comprised one patient 13q and 11q with solo CD38 positivity,2 had 13q and 11q with dual negative and one had 13q, trisomy 12 and 11q with dual negative flow cytometry markers. The comparison of the 4 flow cytometry groups with patients with single FISH abnormalities is shown **in Figure 3.**

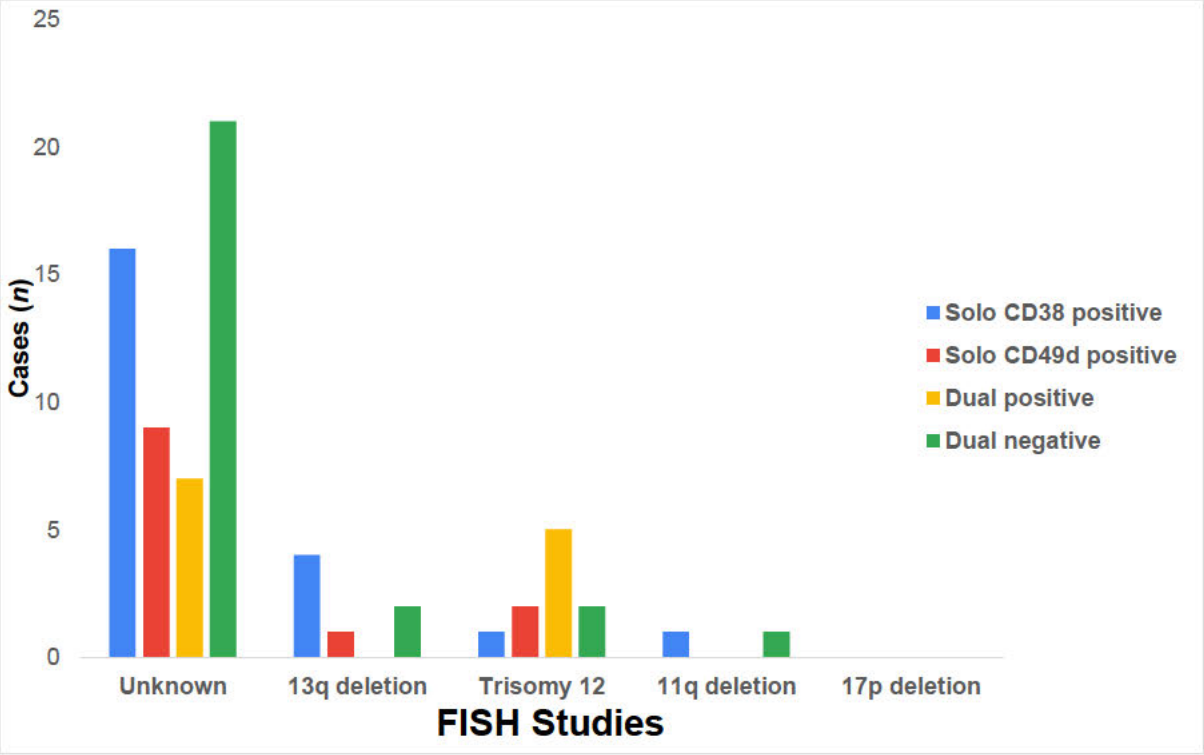

Figure 3: Comparison of flow markers with molecular studies for CLL

The expression of CD49d significantly correlated with trisomy 12 (p value 0.002) and CD38 expression was not significantly associated with CLL molecular abnormalities (p value of 0.8) excluding five patients who had multiple FISH abnormalities as shown on **Table 2.**

Table 2. Correlation of FISH studies with CD38 and CD49d flow cytometry markers

		CD 38				CD 49			
		> 30%		< 30%		> 30%		< 30%	
		n	%	n	%	n	%	n	%
FISH analysis	13q deletion	4	28.6%	3	21.4%	1	11.1%	6	31.6%
	Trisomy 12	6	42.9%	4	28.6%	7	77.8%	3	15.8%
	11q deletion	1	7.1%	1	7.1%	0	0.0%	2	10.5%
	All negative	3	21.4%	6	42.9%	1	11.1%	8	42.1%
	total	14	100.0%	14	100.0%	9	100.0%	19	100.0%
p value					**0.8**				**0.02**

Abbreviations: FISH-Fluorescence in situ hybridization

*5 Patients with multiple FISH abnormalities were excluded from the statistical analysis in Table 2.

Overall survival analysis

Of the total cohort, about half of patient charts were available for analysis of 2-year survival. Seven patients demised within 2 years of diagnosis. The CD49d positive/CD38 negative patient with all positive molecular markers demised within 2 months after the diagnosis of CLL. The Kaplan-Meier 2-year survival curve according to immunophenotypic expression of CD38 and CD49d is depicted in Figure 4.

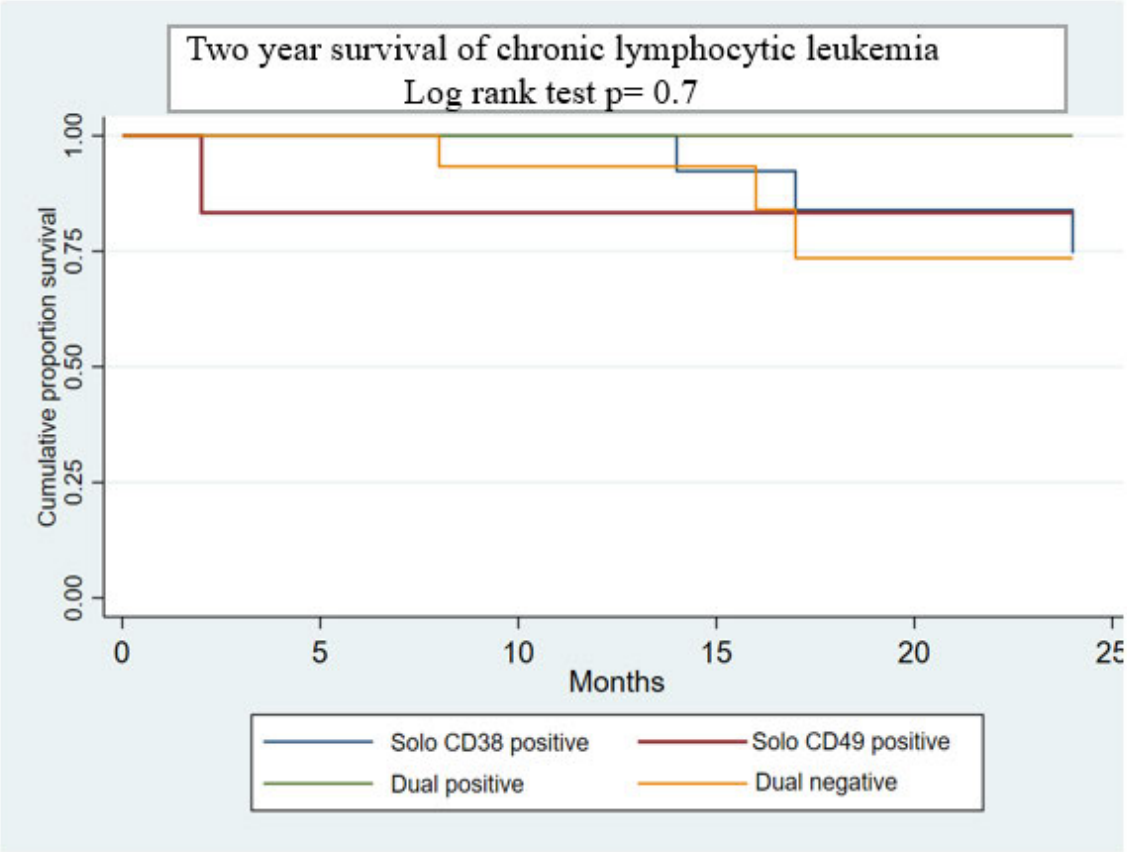

Figure 4: Kaplan-Meier 2-year survival curve for Chronic Lymphocytic Leukemia according to CD38 and CD49d expression

DISCUSSION

Although CLL has a stronger male predisposition, our study demonstrated an almost equal gender incidence over the 5-year period. This finding is similar to a study done on 80 newly diagnosed CLL patients in Cape Town, South Africa.(9) Other countries in Africa, show a variable gender distribution; with Cote d'Ivoire and Nigeria, having a higher incidence in females, whereas Senegal and Sudan had a higher incidence in males.(36)(37)(38)(39) The strong female representation in our cohort is most likely reflective of the population demographics in Kwa-Zulu Natal and South Africa (40)(41)

The incidence of CD38 expression in our study is similar to that described in Egypt and Pakistan; reporting CD38 expression of 48.9% and 49% respectively.(10)(42). However, our values are higher than those reported in USA and Italy with 29% in both countries.(34)(43). CD49d was positive in our study in just under a third, whereas other studies conducted in Italy, India and Iran had higher CD49d expression of 38.6%,44% and 52% respectively.(44)(45)(46)The sample size of our study was similar to these whose cohorts ranged from 25 to 140. Further prospective studies are therefore warranted to determine whether the incidence of CD49d in our population is truly lower than other countries.

The comparison of haemoglobin concentration and platelet counts with the flow cytometry markers demonstrated that the majority of the patients in the solo CD38 positive group had haemoglobin concentration of more than 10 g/dL and platelets > 100 $x10^9$/L. Whilst this was not statistically significant, it was an unusual finding for this group, since multiple studies have shown that CLL patients expressing CD38 have a poorer prognosis.(47)(48)(49) In the dual positive cohort (both CD38 and CD49d positive), the majority of these patients had a haemoglobin concentration of less than 10g/dL (61.5%), and fewer had a platelet count of < 100 x 10^9/L (46.2%). This latter finding regarding the platelet count is also not in keeping with other studies, which have demonstrated a significant decrease in both haemoglobin concentration and platelet counts in patients with dual expression of immunophenotypic markers.(18)(14) A possible reason for these discrepant findings could be the small sample size of our study.

As many patients in our study were diagnosed coincidentally, this led to some of the patients not having haematology follow up, or the complete CLL work-up, including FISH studies. This could be attributed to some patients being asymptomatic with early stages of the disease, non-compliance with follow-up or death. FISH studies at diagnosis of CLL provides prognostic indications that are important in the management of the disease. The World Health Organization generally recommends that all patients with CLL be tested for genetic abnormalities.(3) However, in low and middle–income countries this is often challenging, with a lack of skilled capacity and resource limitations. This drives the need to explore other more easily available and cost-effective prognostic markers.

In our study CD49d had statistically significant correlation with trisomy 12 positivity. This is similar with other studies demonstrating trisomy 12 in association with CD49d positivity.(50)(51) A single patient who expressed CD49d, was positive for all the FISH panel studies and demised within 2 months of diagnosis. This patient was also found to have low levels of haemoglobin and platelets. Patients with CD49d expression are characterized by an aggressive course and short survival.(20) Candidates with 17p deletion have an inferior prognosis and require novel therapies, which are not accessible to public or state patients in South Africa.(23)(24)(25)

Of the 44 haematology charts that were available for survival data, there was an 84% 2-year OS in CLL, which is compatible with current literature.(52) Of the immunophenotypic prognostic markers, our

study showed no correlation with CD38 and CD49d and OS. This contrasts with the poor outcome associated with both CD38 and CD49d in other studies.(53)(20) Our study finding is most likely secondary to a combination of a limited sample size as well as only half of the patients having survival data.

LIMITATIONS

The limitations of our study need to be acknowledged. This study had a small patient cohort which most likely affected the statistical outcome. The study was done in a single centre which, whilst performing the flow cytometry for all patients attending the public health sector in KwaZulu-Natal, does not represent patients in the private sector in the province. The diagnosis of CLL for many patients was made at the base hospital, and some of these patients were not referred for further work-up or follow-up. This impacted on determining the association of the immunophenotypic markers with molecular findings, as well as the OS for these patients. Less than 40% of CLL patients had FISH analysis performed.

CONCLUSION

This is the first study in South Africa that analyzed flow cytometry markers in CLL patients, and their association with other prognostic variables. This study showed a lower incidence of CD49d expression in patents with CLL compared to international data. Many studies have proven that CD49d is an independent poor prognostic marker with unfavorable outcomes and can be considered in addition to the international prognostic index. The recognition of these prognostic markers can be very helpful in predicting the clinical course of the disease thus facilitating appropriate and timely therapeutic interventions. In resource-constrained settings where molecular testing is not easily available, this immunophenotypic marker may have prognostic utility without significant additional cost or expertise required.

Further prospective studies should be done on larger groups of patients to evaluate the frequency and prognostic significance of these prognostic markers in the local population.

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CONFLICT OF INTEREST

There was no conflict of interest among the authors

AUTHOR'S CONTRIBUTION

S.V. conceived the idea of the study and presented to S.M. S.V. and S.M. developed the theory, methodology and protocol of this study. N.R. reviewed the work and advised on the study design and the implementation of the research. S.M. supervised and N.R. co-supervised the research. All authors contributed to the analysis of the results and the writing of the final manuscript.

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DISCLAIMER

The views expressed in this article are those of the authors and not of the institution (University of KwaZulu-Natal).

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CHAPTER 3: CONCLUSION

Chapter 2 contained the manuscript submitted for publication, and Chapter 3 highlights the pertinent study findings and recommendations.

3.2.Recommendations:

3.2.1. **Limitations.**

3.2.1.1. This study had a small patient cohort which most likely affected the statistical outcome.

3.2.1.2. The study was done in a single centre. Although the haematology laboratory at IALCH performs all the immunophenotyping for state or public patients, the number of patients in this study may not represent all public patients with CLL during the study time. Moreover, patients in the province from the private sector were not included in the study.

3.2.1.3 The diagnosis of CLL for many patients was made at the base hospital, and some of these patients were not referred for further work-up or follow-up and this impacted on determining the association of the immunophenotypic markers with molecular findings, as well as the OS for these patients.

In view of the findings of this study, we recommend:

1. That a prospective, long-term study be conducted to verify the findings.
2. The laboratory reporting CD38 and CD49d should indicate the prognostic relevance of these markers once these findings have been verified in a large study.

3. More studies in other provinces of South Africa to get to allow for comparison of these flow cytometry markers with other countries; hence collaboration with other centres is recommended.
4. Impact of HIV on the immunophenotype in CLL needs to be analysed in a prospective study
5. Comparison and correlation of CD38 and CD49d with CLL FISH molecular results in a prospective study.

3.3 Conclusion:

This is the first study in South Africa that analyzed flow cytometry markers in CLL patients, and their association with other prognostic variables. Although this was a small study sample, the study showed a lower incidence of CD49d expression in patents with CLL compared to international data. As CD49d is known to be associated with a poor prognosis, it may add to the currently utilized prognostic tools, if it can be included or used as a surrogate prognostic marker. These immunophenotypic markers may add value without significant additional resources required, and may impact on treatment decisions.

Further prospective studies should be done on larger groups of patients to evaluate the frequency and prognostic significance of these prognostic markers in the local and South African population.

APPENDICES

Appendices should be used for illustrative material which is not included in the main body of the thesis, or for material which interrupts the flow of the text, such as list of abbreviations, pertinent documents and extensive raw data. If several separate items are relegated to appendices, each one should form a separate appendix. If the appendices contain further references, a separate list should be included at the end of each appendix.

Appendix 1, 2 and 3 below are standard for all theses.

Appendix 1: The final Study Protocol (Include the final protocol which was given full approval by Brec and/or the postgrad office)

III

PROTOCOL NUMBER:

(BREC/00004494/2022)...

BIOMEDICAL RESEARCH ETHICS COMMITTEE EXPEDITED APPLICATION

Application to the UKZN Ethics Committee for of new research projects

(For research on human

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

FORM[1]

Research ethics review

participants)

RESEARCH OFFICE CONTACT DETAILS: Biomedical Research Ethics Administration, Westville Campus, Govan Mbeki Building, Private Bag X 54001, Durban, 4000, KwaZulu-Natal, South Africa; Tel: +27 31 2602486; Email: BREC@ukzn ac za ; Website: http://research ukzn ac za/Research-Ethics/Biomedical-Research-Ethics aspx

SECTION A:

APPLICANT/PRINCIPAL INVESTIGATOR: ** For UKZN statistical reporting purposes*

[1] Note: This application must be self-sufficient. Sections marked "see protocol" are unacceptable and will be returned to the applicant.

Title: Mr		Ms		Mrs		Dr	√	Prof		*(Select option)*

Name : Siyabonga Voxeka			
*Gender: Male			
*Race: African			
UKZN College: Health Sciences			
UKZN School/Discipline:	UKZN School of Medicine	NA	
Hospital/Institution where employed:	Inkosi Albert Luthuli Central Hospital	NA	
Professional status:	Medical Doctor		
Postal address:	47 Montgomery Drive, Winston Park, Gillitts		
Contact phone Numbers: Office:	031 240 2657		
Mobile number:	082 829 1127		
Fax number:			
Email address:	siyavox@gmail.com		
Full/Part time Employment: Registrar – Department of Haematology			

Current HPCSA Number (or equivalent): MP0554693

*if registration is pending, submit proof of application

Purpose of research: If postgraduate degree *(Please tick)*	Hons	MMedSc	MMed	MSc	MFamMed	MBChB	PhD	N/A
			√					

Other degree not listed above:

Student Number and year of study: (*if applicable)*

If for postgraduate degree, please confirm whether the application has been reviewed and approved by your school's Academic Leader (Research):	Yes		No	

If yes, provide approval date and attach approval letter:

Title of research project: ***The Incidence and Significance of CD38 and CD49d expression in patients with Chronic Lymphocytic Leukemia***

Name and qualifications of Supervisor: Dr S Murugan
Consultant/ Lecturer : MBChB, FCHaem PATH(SA), MMED

e-mail address of Supervisor: Stephanie.murugan@nhls.ac.za

Name and qualifications of Co-supervisor: Dr Nadine Rapiti
MBChB, FCHaem PATH(SA),PHD
e-mail address of co-supervisor:nadine.rapiti@nhls.ac.za

If not for degree purposes, state other (example, self-initiated research):

Has this study been, or is it likely to be, submitted to any other Research Ethics Committee?	Yes		No	√	N/A	

If yes, please name the Committee/s and or institution and give outcome - i.e. approved/rejected/pending/not applicable?
(If approved, attach approval letter)

FUNDING OF THE RESEARCH:

Has funding been secured?	Yes		No	√

Amount: R N/A

Name of funder: *(full details) N/A*

Is this project funded from a US DHHS funding source?	Yes		No	√

If yes, name the federal fundingagency:

Can this project proceed without funding? *(give a brief explanation)*	Yes	√	No	

Has an application for funds been made to other sources to support this project?	Yes		No	√
If yes, state name/s of funding agency and amount requested: N/A				

Note:
For all US Federally funded studies (e.g. NIH, CDC, NIAID, DAIDS, NIMH, etc), one complete copy of the original funding application and approval must accompany the BREC ethics application.

All University contracts need to be uploaded on the Contracts Management online submission form with either the signed **Approval letter** (non-research) **or Form 1**(research related). The website link to the system is http://legalservices.ukzn.ac.za/ContractsManagement.aspx

If you require assistance with the completion of the online submission form, or with any aspect of the new system, please contact Mr Rendra Phalad on Ext 7455 for all contracts (non-research contracts), and Mr Deon Moodley on Ext 8199 (for research contracts).

FAILURE TO MAKE FULL FINANCIAL DISCLOSURES WILL DELAY ETHICS APPROVAL

Please indicate whether a BREC review fee is applicable for this study? (See Fee Schedule on BREC Website)	Yes		No	√
If Yes, is the study covered by your Centre/Unit's annual levy fee to BREC? N/A	Yes		No	

Note:
* Expedited review only applies to minimal risk studies – e.g. retrospective chart reviews, studies on stored samples etc., for details see BREC ToR and SoP at

http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

SECTION B:

NATURE OF STUDY

Quantitative

Type of Study: *(please tick)*	Epidemiological	Observational clinical study	Experimental	Observational	
	Retrospective Chart Review √	Prospective Chart Review	Laboratory study on stored samples	Audit	Other:(Specify)

Qualitative

1. THE PROTOCOL FOR STUDY

1.1 Full title of research project:

- *The Incidence and Significance of CD38 and CD49d expression in patients with Chronic Lymphocytic Leukemia*

1.2 Where will the Research be carried out?

- Inkosi Albert Luthuli Central Hospital

1.3 Aim

- To investigate the expression and prognostic value of adhesion molecules CD38 and CD49d in Chronic Lymphocytic Leukemia(CLL) patients

- To study the association between immunophenotypic markers (CD38 and CD49d) with other laboratory prognostic parameters (Haemoglobin(Hb), Platelet count(Plts), White cell count(WCC), lymphocyte count, β2 micro-globulin(B2M), Bone marrow aspirate and trephine(BMAT), Fluorescence in situ hybridization(FISH) molecular studies [13q deletion(13q-),11q deletion(11q-),trisomy 12 and 17 p deletion(17p-)/Tumour protein p53(TP53)]

2

2.1 Hypothesis to be tested, or Research Question to be answered:

- This is a retrospective chart review. It is therefore a descriptive study and cannot have a hypothesis that is being tested.

2.2 Summary of the proposed research methodology (restrict to 100 words)

- The data will be collected using laboratory information system (LIS) on National Health Laboratory Services (NHLS) database identifying all newly diagnosed patients with Chronic Lymphocytic Leukemia (CLL) from 1 September 2015 to 30 September 2020. Data with specific prognostic markers of CLL will be extracted. Other documented parameters (haemoglobin levels, platelet counts, white cell counts, lymphocyte count, β2 micro-globulin levels, bone marrow aspirate and trephine and FISH molecular studies will also be obtained to investigate correlation with CD38 and CD49d markers which are used for prognostication of CLL patients.

2.3 Keywords

- Chronic Lymphocytic Leukemia (CLL), flow cytometry, white cell count, lymphocyte count, bone marrow aspirate and trephine

2.4 Background and Literature Review (maximum 1 page):

Chronic Lymphocytic Leukemia (CLL) is a heterogeneous disease which has a highly variable course. It is one of the most common adult leukemias(8).It is a clonal disease of mature B cells characterised by lymphocytosis with a monoclonal B cell count of $\geq 5 \times 10^9$/L.(16)) It involves the blood, bone marrow and secondary lymphoid tissues such as spleen and lymph nodes.

Most patients who show no clinical symptoms at the time of diagnosis survive several years without need for treatment, while others present with aggressive disease and have shorter life span.(16)CLL affects adults with a median age of approximately 70 years but can also present in younger adults. Males are more affected than females with a ratio of 1.5-2:1 (3).

There are different prognostic biomarkers in CLL, some of these include serum markers, immunophenotypic markers, immunoglobulin heavy chain variable (IGHV) gene mutation status, chromosome aberrations, gene mutations and microRNAs. Based on these biomarkers, classic prognostic models such as the Rai and Binet systems and the chronic lymphocytic leukemia – international prognostic index (CLL-IPI) were developed to improve risk stratification and tailor treatment intensity (1).

The diagnosis of CLL is made using flow cytometry. The leukemic cells in CLL express CD5 and CD19 with dim surface IgM/ IgD, CD20, CD22 and CD79b. They are also positive for CD43 and strongly positive for CD23 and CD200. CD10 is negative and FMC7 is usually negative or weakly expressed. (3)

CD38 is a transmembrane type II glycoprotein localized on the plasma membrane, cytoplasm and inner nuclear membrane of cells. It plays an important role in promoting proliferation and prolonging survival of leukemic

cells. It is expressed in numerous cell types of haematopoietic system such as lymphocytes, myeloid cells, natural killer (NK) cells, platelets and erythrocytes as well as in solid tissues. CD38 plays a critical role in diverse immune functions such as in T-cell activation, neutrophil chemotaxis, dendritic cell migration and monocyte chemokine production (15).

CD49d belongs to a family of integrin alpha subunits. Integrins are heterodimers of non-covalently linked alpha and beta subunits. It is an adhesion molecule expressed on the surface of B lymphocytes which can play a vital role in regulating cell-cell and cell-extracellular matrix interactions by binding to fibronectin and vascular cell adhesion molecule 1 (VCAM-1) in the bone marrow. The extracellular matrix fibrinogen CD49d serves as a signalling receptor that influences B cell survival through upregulation of BCL-2 family members. Tumour necrosis factor-alpha (TNF-a) can increase the binding of CD49d to VCAM-1 through increasing the expression of VCAM-1 on endothelial/ stroma cells. It also improves the survival of B cells of CLL patients by protecting them from apoptosis(2).

The dual expression of CD38 and CD49d leads to aggressive clinical course. CD38 and CD49d are used for prognostication in CLL patients. Patients are considered to be positive for CD49d when its level is >30%(42,51).Recognition of these prognostic markers can be very helpful in predicting the clinical course of the disease thus facilitating appropriate and timely therapeutic interventions.

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2. PLAN OF INVESTIGATION FOR STUDY

* In the case of Higher Degrees, please state name and School of person consulted regarding the design:

2.1	Is this a retrospective chart review with no human contact?	Yes	✓	No	
2.2	Is this a study of stored tissue?	Yes		No	✓
2.3	Are host genetic factors being studied?	Yes		No	✓

2.4 How many hours per week will the PI devote to this project?

- 6 hours

2.5 Describe in detail your data collection methods for the research project

- A data collection tool will be used by the investigator to extract CLL prognostic biomarker data of all patients that meet the inclusion criteria from the electronic records of the LIS. The data collection tool will elicit information that includes patient demographic and CLL prognostic biomarker information.

3. STATISTICAL PLANNING AND DATA ANALYSIS

3.1 Has this project been approved by a professional statistician? If No, please justify.	Yes	✓	No	

3.2 If answered "yes" to (3.1), provide the name of the statistician:

- Cathy

3.3 Please provide a brief overview of statistical and data analytic considerations, including:

- Descriptive statistics will be used to summarize the data. Frequencies and percentages will be used for categorical data. Frequency distributions of numeric variables will be examined for normality and means (SD) or medians (IQR) used as appropriate. Demographic and clinical characteristic associated with CD38 and CD49d will be examined separately using Chi square tests (Fisher's exact) for categorical variables and ANOVA or Mann-Whitney for numeric data. Logistic regression will be used to identify independent factors associated with each expression. Patients will then be categorized further into 4 subgroups depending on the absence/presence of each expression. Clinical and demographic factors will be compared as above. Stata v17 statistical software will be used in the analysis

3.4 For *qualitative* studies: What is the framework/approach to be used for analysis of the data?

4. PARTICIPANTS IN THE STUDY

4.1 Is this a multi-national study?	Yes			No	✓	

4.2 List all sites in South Africa in which the project will be carried - Kwazulu- Natal (IALCH)

4.3 Source: *(Please indicate number per group)*	Inpatients	Outpatients ✓	Volunteers

4.4 Age (human studies) *(Please indicate number per group)*	Neonates	Infants	Children	Adolescent	Adults ✓

4.5 Is there a control group(s)?	Yes		No	✓	

4.6 Demographic profile of participants *(please tick ALL appropriate boxes below.)*

4.6.1 Gender: Female √ Male √

4.6.2 Population Group: Black √ Coloured √ Indi √ Wh √

4.6.3 Language Group/s: Specify…All languages ….

4.7 Describe the recruitment process in detail for all groups. • All adult patients with diagnosis of Chronic Lymphocytic Leukemia						
4.8 Will incentives be offered to facilitate recruitment?	Yes		No	✓	N/A	
4.9 Will participants be reimbursed in some way for participation?	Yes		No	✓	N/A	
4.10 Will reimbursement for participants and investigators be in accordance with: Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa: Department of Health (2006) and; • Ethics in Health Research: Principles, Structures and Processes: (2015) • Current SA DoH Guidance on reimbursement (*See BREC website*)	Yes		No		N/A ✓	
4.11 Will participants be insured against research related injury?	Yes		No		N/A ✓	

4.12 List in detail the inclusion and exclusion criteria.

Inclusion Criteria

- Newly diagnosed patients with CLL from 1 September 2015 to 30 September 2020
- CD38 positive/CD49d negative patients on flow cytometry
- CD49 positive/CD38 negative patients on flow cytometry
- CD38 positive/CD49d positive patients on flow cytometry
- CD38 negative/CD49d negative patients on flow cytometry

Exclusion Criteria

- Patients diagnosed with CLL before 1 September 2015 and after 30 September 2020
- Patients not meeting the criteria for the diagnosis of CLL

5. POTENTIAL RISKS OR DISCOMFORT

5.1 Can the project have any potential risks or discomfort on participants, members of the public, researchers, field staff or the physical environment?	Yes		No	✓	

5.2 If "yes" to (6.1) indicate, for each study group/arm, the potential additional risks as follows: N/A

5.2.1 Biological risks
5.2.2 Psychological risks
5.2.3 Social Risks
5.2.4 Legal risks
5.2.5 Financial risks
5.2.6 Other risks

5.3 Please detail steps that will be taken to minimise the risks indicated above: N/A

5.3.1 Biological risks
5.3.2 Psychological risks
5.3.3 Social Risks
5.3.4 Legal risks
5.3.5 Financial risks
5.3.6 Other risks

6. INFORMED CONSENT: GIVEN TO PARTICIPANTS

See SAMPLE INFORMATION SHEET AND CONSENT FORM ON UKZN BREC WEBSITE at *http://research.ukzn.ac.za/Libraries/Notices2011/BREC_Informed_consent_form_sflb.sflb.ashx*

Other consent forms are acceptable provided that they contain at least the essential elements outlined in the current UKZN BREC Terms of Reference (ToR) and Standard Operating Procedures (SoP) available at *http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx*

If necessary, information sheets and consent forms, after ethics approval of the English version, must be translated into appropriate local languages and submitted to BREC for further approval prior to implementation, with a copy of the translator's certificate, and back translations if applicable.

The correct and complete contact details for the UKZN Biomedical Research Ethics Committee should be in the information sheets and consent forms as follows:

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office, Westville Campus
Govan Mbeki Building
University of KwaZulu-Natal
Private Bag X 54001, Durban, 4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2602486 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za

Appendix 2: The Guidelines for Authorship for the Journal selected for submission of the manuscript

Original Research full structure

Title
The article's full title should contain a maximum of 95 characters (including spaces).

Abstract
The abstract, written in English, should be no longer than 250 words and must be written in the past tense. The abstract should give a succinct account of the objectives, methods, results and significance of the matter. The structured abstract for an Original Research article should consist of six paragraphs labelled Background, Objective, Methods, Results, Conclusion and What this study adds.

- **Background:** *Why do we care about the problem*? State the context and purpose of the study. (What practical, scientific or theoretical gap is your research filling?)
- **Objective:** *What problem are you trying to solve*? What is the scope of your work (e.g. is it a generalized approach or for a specific situation)? Avoid using jargon that will not be readily understood by non-expert readers.
- **Methods:** *How did you go about solving or making progress on the problem*? Clearly state the basic design of the study, what data and/or samples were collected and name or briefly describe the key techniques without going into excessive detail.
- **Results:** *What is the answer*? Present the main, most important findings. Identify trends, relative change or differences in answers to questions.
- **Conclusion:** *What are the implications of your answer*? Briefly summarise any potential implications. What are the larger implications of your findings, especially for the problem or gap identified in your motivation?
- **What this study adds:** *What key insights into the research results and its future function are revealed? How do these insights link to the focus and scope of the journal? It should be a concise statement of the primary contribution of the manuscript; and how it fits within the scope of the journal.*

Do not cite references in the abstract.
Do not use abbreviations unless they are used at least 3 times in the abstract.

Main text
Introduction: The Introduction section must contain your argument for the social and scientific value of the study, as well as the aim and objectives. No structural sub-headings should be used. All text should be presented in narrative paragraphs; bulleted or numbered lists are not permitted.

- Social value: The first part of the introduction should make a clear and logical argument for the importance or relevance of the study. Your argument should be supported by the use of evidence from the literature.
- Scientific value: The second part of the introduction should make a clear and logical argument for the originality of the study. This should include a summary of what is already known about the research question or specific topic and should clarify the knowledge gap that this study will address. Your argument should be supported by the use of evidence from the literature.

- Conceptual framework: In some research articles it will also be important to describe the underlying theoretical basis for the research and how these theories are linked together in a conceptual framework. The theoretical evidence used to construct the conceptual framework should be referenced from the literature.
- **Aim and objectives:** The introduction should conclude with a paragraph that clearly summarises the aim and objectives of the study.
- **Methods:** The Methods section must address the elements listed below. Structural sub-headings are encouraged and required as noted.
- **Ethical considerations** (required): For studies involving human or animal research, approval must have been obtained for all studies from the author's institution or other relevant ethics committee before the start of the study. Required information:
- Name of Institutional Review Board or ethical review committee
- Study approval number(s)
- Manner of consent (written, oral) for human participants
- Description of measures taken to maintain the confidentiality of data
- If the study was not human or animal research or the study was determined to be non-human subjects research or exempt, the authors must provide a statement with those details in this section
- Study design: An outline of the type of study design.
- Setting: A description of the setting for the study; for example, the type of community from which the participants came or the nature of the health system and services in which the study was conducted.
- Study population and sampling strategy: Describe the study population and any inclusion or exclusion criteria. Describe the intended sample size and your sample size calculation or justification. Describe the sampling strategy used. Describe in practical terms how this was implemented.
- Intervention (if appropriate): If there were intervention and comparison groups, describe the intervention in detail and what happened to the comparison groups. If a clinical trial, the trial registration number should be indicated.
- Sample and/or data collection: Describe what type of samples were collected (e.g. sputum, blood), the manner of sample collection (e.g. venous blood into EDTA tube), the volume of sample collected (e.g., two 4-ml samples from each patient) and how samples were stored (e.g. in -20°C freezer) and/or transported after collection (e.g. in cooler with ice packs). Define any data collection tools that were used (e.g. worksheets, questionnaires, etc.); briefly describe how questionnaires were validated for the study population. Describe in practical terms how data were collected (e.g., in-person interviews) and any key issues involved (e.g. language barriers). Provide manufacturer information for all supplies and equipment (required: name and location). Cite references for established data collection tools and their validation studies.
- Laboratory analyses: Describe sample preparation and details about all laboratory tests, analyses and/or assays that were conducted. Provide manufacturer information for all supplies and equipment (required: name and location). Cite references for established methods and explain any deviations from these.
- **Data analysis** (required): Describe how data were captured, checked and cleaned. Describe the analysis process, for example, the statistical tests used or steps followed in qualitative data analysis. Provide manufacturer information for all software used (required: name and location).
- **Results:** Present the results of your study in a logical sequence that addresses the aim and objectives of your study. Use sub-headings, if needed, to organize the presentation of the study findings. Use tables and figures as required to present your findings. Tables should not contain vertical lines. Each table and figure should have a legend. Use quotations as required to establish your interpretation of qualitative

data. All units should conform to the **SI convention** and be abbreviated accordingly. Biomolecular sequence or structure data and datasets must be submitted to appropriate publicly available databases and their accession numbers should be cited in the results section. Metric units and their international symbols must be used throughout, as is the decimal point (not the decimal comma).

- Authors should ensure that:
    - The Results section does not contain descriptions of methods used that should be presented in the Methods section
    - All results presented in the Results section have any associated data/sample collection and assay or analysis methods presented in the Methods section.

Discussion: The discussion section should address the elements listed below. Other than the required sub-headings listed (bold, italics), sub-headings are not expected but may be helpful to readers.

- Key findings: In the first paragraph, summarize the key findings without reiterating details of the results.
- Discussion of key findings: Explain how the key findings relate to previous research or existing knowledge, practice or policy.
- Implications or recommendations: State the implications of your study or recommendations for future research (questions that remain unanswered), policy or practice. Make sure that the recommendations flow directly from your findings.
- **Limitations** (required second-to-last sub-heading): Describe all inherent weaknesses of the study's design and/or implementation, how they may have affected your findings, and what you did (or could not do) to mitigate their effect on your findings. Mention any other factors that the reader should take into account when interpreting your results.
- **Conclusion** (required final sub-heading): Provide a brief conclusion that summarizes the results and their meaning or significance to each objective of the study.

Acknowledgements: Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution. Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named. Refer to the acknowledgement structure guide on our *Formatting Requirements* page.

Also provide the following, each under their own heading:

- Competing interests: This section should list specific competing interests associated with any of the authors. If authors declare that no competing interests exist, the article will include a statement to this effect: *The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.* Read our **policy on competing interests**.
- Author contributions: All authors must meet the criteria for authorship as outlined in the **authorship** policy and **author contribution** statement policies.
- Sources of support: Provide information on funding if relevant.
- Data availability: All research articles are encouraged to have a data availability statement.
- Disclaimer: A statement that the views expressed in the submitted article are his or her own and not an official position of the institution or funder.

References: Authors should provide direct references to original research sources whenever possible. References should not be used by authors, editors, or peer reviewers to promote self-interests. Refer to the journal referencing style downloadable on our *Formatting Requirements* page.

v

Appendix 3: Ethical approvals

Included hospital and provincial approvals as well as the BREC approval (or waiver if appropriate).

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

18 August 2022

Dr Siyabonga Eric Voxeka (216075863)
School of Lab Med & Medical Sc
Medical School

Dear Dr Voxeka,

Protocol reference number: BREC/00004494/2022
Project title: The incidence and significance of CD38 AND CD49d expression in patients with Chronic Lymphocytic Leukemia: A 5 year retrospective study
Degree: MMed

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 18 August 2022. Please ensur that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site

This approval is valid for one year from 18 August 2022. To ensure uninterrupted approval of this study beyond the approva expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before th expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior t implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015) South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements a contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available a http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office fc Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 13 September 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

BREC application (The incidence and significance of CD38 AND CD49d expression in patients with Chronic Lymphocytic Leukemia: A 5 year retrospective study, (BREC/00004494/2022)) Voxeka, Siyabonga Eric (216075863)

NB:
Please click on **Edit** on the top right of the screen to view the full information and make changes to the application. If there is no edit button visible to you, you may be unable to edit the application as it is may be with someone else at the moment.
However, you can view the application by clicking on Ethics Applications on the left menu. If there are many applications displayed, you can filter for the application you are looking for.

If you require more help, you can find Ethics User Guides here, OR you can contact the Ethics Office here OR using the Ethics Office contact details here.

Type of ethics review: BREC application

Title: The incidence and significance of CD38 AND CD49d expression in patients with Chronic Lymphocytic Leukemia: A 5 year retrospective study

Date of approval: 18/08/2022

Summary of the proposed research methodology (maximum 100 words): The data will be collected using laboratory information system (LIS) on National Health Laboratory Services (NHLS) database identifying all newly diagnosed patients with Chronic Lymphocytic Leukemia (CLL) from 1 September 2015 to 30 September 2020. Data with specific prognostic markers of CLL will be extracted. Other documented parameters (haemoglobin levels, platelet counts, white cell counts, lymphocyte count, β2 micro-globulin levels, bone marrow aspirate and trephine and FISH molecular studies will also be obtained to investigate correlation with CD38 and CD49d markers which are used for prognostication of CLL patients.

Principal Investigator:

Voxeka, Siyabonga Eric (216075863) - School Of Lab Med & Medical Sc (Active)

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

22 February 2022

Dr N Rapiti
School of School of Laboratory Medicine and Medical Sciences
College of Health Sciences
rapitin@ukzn.ac.za

Dear Dr Rapiti

PROTOCOL: Heamatology Patients Treated at King Edward V111 Hospital and Haematology Laboratory KZN academic complex
Degree: Non-Degree
BREC Ref No: BCA608/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**24 January 2022**
Expiration of Ethical Approval:	**23 January 2023**

I wish to advise you that your application for Recertification received on 10 February 2022 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 08 March 2022.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Appendix 4: Data collection tools (for example)

THE INCIDENCE AND SIGNIFICANCE OF CD38 AND CD49d EXPRESSION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A RETROSPECTIVE 5 YEAR STUDY

Variable	**Code**
Study no.	
[1] Episode No	
[2] Age	
[3] Gender	Not documented = 0 Male=1 Female =2
[4] Flow cytometry	CD38 positive (> 30%) = 1 CD49d positive (>30%) = 2 CD38 & CD49d positive (> 30% each) = 3 CD38 & CD49d negative (<30% each) = 4
[5] Haemoglobin concentration (g/dL)	Not documented = 0 > 10 = 1 < 10 = 2
[6] Platelet count (X 10^9/L)	Not documented = 0 > 100 = 1 < 100 = 2
[7] Molecular studies	Not documented = 0 13q deletion = 1 Trisomy 12 = 2 11q deletion = 3 17p deletion = 4 13q and 11q deletions=5 13q,trisomy12 and 11q deletions=6

	13q,trisomy12,11q and 17p deletions=7 All negative=8

THE INCIDENCE AND SIGNIFICANCE OF CD38 AND CD49d EXPRESSION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A RETROSPECTIVE 5 YEAR STUDY

1. **STUDY NUMBER**

2. **EPISODE NUMBER**

3. **AGE**

4. **GENDER**

5. **FLOW CYTOMETRY MARKERS**

(i) CD38 %

(ii) CD49d %

6. **HAEMOGLOBIN CONCENTRATION (g/dL)**

7. **PLATELET COUNT (X 10^9/L)**

8. **WHITE CELL COUNT (X 10^9/L)**

9. **LYMPHOCYTE COUNT(X 10^9/L)**

10. β2 MICROGLOBULIN (mg/L)

11. BONE MARROW ASPIRATE AND TREPHINE

Involved		Not involved		Failed		Not done	

12. MOLECULAR STUDIES

(i) 11q deletion

Positive		Negative		Unknown	

(ii) Trisomy 12

Positive		Negative		Unknown	

(iii) 13q deletion

Positive		Negative		Unknown	

(iv) 17p deletion

Positive		Negative		Unknown	

Appendix 5: Raw data

Study No	Age (years)	Gender	Flow cytometry markers	Haemoglobin levels (g/L)	Platelet count (X 1	First FBC	Last FBC	Molecular studies
1	81	2	1	2	1	2015/09/15	2015/09/15	0
2	76	1	1	2	2	2015/09/17	2020/05/27	5
3	81	2	4	2	1	2015/11/16	2015/11/16	0
4	70	1	4	2	1	2015/11/19	2015/11/19	0
5	68	1	1	1	1	2015/12/02	2019/12/20	0
6	82	1	1	1	1	2015/11/11	2020/09/02	0
7	68	2	1	1	2	2016/01/19	2016/03/07	8
8	68	1	1	1	1	2016/02/17	2020/01/17	0
9	57	2	4	1	1	2016/02/11	2018/04/25	1
10	75	2	4	1	1	2016/03/14	2022/01/17	2
11	47	2	1	1	1	2016/03/17	2022/10/07	2
12	78	1	1	1	1	2016/03/07	2018/04/30	0
13	49	1	3	2	2	2016/01/21	2016/04/14	0
14	81	2	1	2	1	2016/04/18	2016/05/17	0
15	67	2	4	2	2	2016/04/12	2016/04/12	5
16	63	1	4	1	1	2016/05/25	2017/10/04	0
17	80	1	2	2	1	2016/04/21	2016/06/13	0
18	77	2	3	1	1	2016/05/23	2016/05/23	0
19	67	2	1	2	1	2016/05/11	2016/05/11	0
20	74	2	1	1	1	2016/04/25	2016/05/12	0
21	76	2	1	2	1	2016/05/11	2016/06/08	3
22	74	1	2	2	2	2016/05/31	2016/06/17	0
23	75	2	1	2	1	2016/07/06	2017/10/25	0
24	75	1	4	1	1	2016/06/06	2016/06/06	3
25	65	1	4	1	1	2016/07/13	2016/07/13	0
26	78	2	3	2	1	2016/07/11	2020/02/26	2
27	55	1	1	1	1	2016/07/20	2017/05/29	0
28	46	1	3	2	2	2016/08/23	2016/08/23	8
29	70	2	4	2	1	2016/08/29	2022/01/12	8
30	70	1	3	1	1	2016/08/16	2016/08/16	2
31	72	1	4	2	1	2016/08/29	2016/08/29	0
32	71	1	1	1	1	2016/08/17	2016/08/17	0
33	67	2	4	2	1	2016/09/08	2016/09/08	0
34	70	2	4	1	1	2016/09/26	2016/09/26	5
35	63	2	4	2	1	2016/10/17	2019/11/13	0
36	67	1	4	1	1	2016/10/26	2020/05/22	8
37	67	2	3	2	2	2016/11/02	2020/10/02	2
38	68	2	4	2	2	2016/10/13	2016/10/13	0
39	64	2	3	1	1	2016/10/10	2016/10/10	2
40	68	1	1	2	2	2016/10/03	2018/01/10	1
41	62	1	3	1	1	2016/11/21	2017/01/04	0
42	64	2	2	2	2	2016/11/18	2016/11/18	0
43	43	1	1	1	1	2016/11/18	2018/05/03	0
44	45	2	1	1	1	2016/12/12	2019/09/14	1
45	41	1	4	2	2	2016/12/14	2016/12/14	8
46	71	1	1	2	2	2016/12/29	2017/01/03	0
47	87	1	4	2	1	2016/01/18	2017/04/26	0
48	81	2	3	2	2	2017/05/10	2017/10/11	0
49	75	1	2	2	1	2017/02/06	2017/02/06	0
50	55	1	1	1	1	2017/03/14	2017/03/14	0
51	74	2	3	1	1	2017/04/03	2020/04/08	0
52	74	2	2	2	2	2017/05/08	2017/07/24	6
53	74	2	4	2	1	2017/05/10	2017/07/27	0
54	54	1	4	2	2	2017/05/24	2017/05/24	0
55	62	2	1	1	2	2018/02/21	2018/05/04	0
56	63	2	1	1	1	2017/05/03	2022/06/24	1
57	88	2	4	2	1	2017/06/22	2017/06/22	0
58	69	1	4	1	1	2017/06/09	2018/09/05	0
59	75	1	2	2	1	2017/06/19	2017/06/19	0
60	74	2	4	2	2	2017/06/26	2017/09/07	8
61	46	2	1	2	2	2017/08/28	2017/08/28	0
62	88	1	4	1	1	2017/07/03	2017/07/03	0
63	63	1	3	2	2	2017/11/15	2017/11/15	0
64	59	2	1	2	1	2018/01/24	2018/02/20	1
65	64	2	2	1	1	2018/01/09	2018/01/09	0
66	67	1	4	2	2	2018/03/19	2019/09/10	1
67	67	1	1	2	1	2018/04/18	2018/06/13	8
68	71	1	3	2	1	2018/04/03	2018/04/03	0
69	57	1	4	1	2	2018/06/27	2019/05/13	7
70	63	1	4	2	2	2018/07/02	2021/10/12	2
71	83	2	2	2	2	2018/09/03	2020/06/17	0
72	75	2	4	2	2	2018/10/08	2018/10/08	0
73	72	1	4	2	1	2018/01/07	2019/03/04	0
74	73	2	2	1	1	2019/04/03	2022/10/03	0
75	76	1	2	1	1	2019/03/11	2019/10/07	2
76	68	1	4	1	1	2019/04/16	2019/04/16	0
77	81	2	4	1	2	2019/05/06	2019/05/06	8
78	64	1	2	2	2	2019/07/23	2021/03/11	1
79	74	1	2	1	1	2020/01/17	2020/06/29	2
80	77	2	4	2	2	2020/01/15	2020/01/15	0
81	65	1	4	1	1	2020/04/16	2022/09/14	0
82	64	2	4	2	1	2020/07/14	2020/07/23	0
83	78	1	2	1	1	2020/08/17	2021/08/20	0
84	60	2	4	1	1	2020/08/27	2022/06/21	0
85	36	1	4	1	1	2020/09/28	2021/05/17	8
86	63	1	3	2	2	2020/09/21	2022/09/23	2
TOTAL								