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INYUVESI YAKWAZULU-NATALI

T cell function and immune checkpoint inhibition in chronic lymphocytic leukaemia

By

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Preface

The study described in this dissertation was carried out by Miss Zekhethelo Mkhwanazi and has not been submitted in any other form to another University. This study was carried out in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from February 2020 to November 2021 under the supervision of Prof. Bongani Nkambule.

Zekhethelo Mkhwanazi: ..... Date: 18/01/2022

As a candidate's Supervisor I agree to the submission of this thesis.

Professor B. Nkambule: Date:18/01/2022

Declaration

I, Miss, Zekhethelo Alondwe Mkhwanazi declare that:

i. The research reported in this thesis, except where otherwise indicated, is my own original work.
ii. This thesis has not been submitted for any degree or examination at any other university.
iii. This thesis does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.
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    a) Their words have been re-written, but the general information attributed to them has been referenced.
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Signed: ..... Date: 18/01/2022

This work is dedicated to my daughter. You have given me a new lease in life. You have helped me grow, evolve and be better. I will travel the world and sail the seven seas for you.

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Abstract

Background: The global burden of lymphoid malignancies gradually increased over the past decade, with chronic lymphocytic leukaemia (CLL) accounting for a quarter of the burden of all haematological malignancies. Chemotherapy in combination with rituximab has been used as standard care for patients with CLL. This regimen has been characterized by variable responses, relapses, and remissions. The exploration of the predictive value of prognostic factors in patients on chemoimmunotherapy (CIT) is crucial, however, such studies on the multi-ethnic group remain scant.

Methods: This study consist of two phases, firstly we performed a systematic review and meta-analysis on the predictive value of protein biomarkers in patients with CLL on rituximab-based therapy. A total of 10 prognostic factors were identified and evaluated. In a subsequent prospective cross-sectional study comprising of treatment-naïve patients with CLL, we evaluated immune checkpoint profiles on T helper and cytotoxic T cells. The expression of selected inhibitory proteins (Programmed death 1; PD-1, programmed death ligand 1; PD-L1, cytotoxic T lymphocyte-associated antigen 4; CTLA-4 and CD56) were measured at baseline, and post-stimulation with phorbol 12-myristate 13-acetate (PMA), and following ex vivo blockade with monoclonal antibody therapy (anti-PD1 and anti-PDL1). Furthermore, correlations between beta-2-microglobulin (B2M), a marker of disease progression, and immune checkpoints was evaluated.

Results: In our meta-analysis, chemoimmunotherapy with rituximab improved progression-free survival (PFS) (HR= 0.58; 95% Cl 0.49 – 0.68; p <0.001) and overall survival (OS) (HR= 0.77; 95% Cl 0.63 – 0.91; p <0.001) in patients with CLL. The following prognostic factors were confirmed and associated with poor patient outcomes; deletion 17p (HR = 4.88), Immunoglobulin heavy chain variable region gene mutation status (HR = 0.96), WCC (HR = 1.27), β_2-microglobulin (HR = 0.96), and lactate dehydrogenase levels (HR = 1.20). Our results from the cross-sectional study demonstrated an increase in expression levels of PD-1, PD-L1 and CTLA-4 ($p > 0.05$) and no significant differences in expression levels of CD56 ($p = 0.4126$) on CD4+ T cells following stimulation. There were no statistically significant differences in the expression levels of PD-1 ($p = 0.1826$), while there were significant reductions in the expression levels of CTLA-4 and CD56 ($p > 0.05$), conversely, there was an increase in the expression levels of PD-L1 following CD8+ T cells stimulation. PD-1 and PD-L1 immune checkpoint blockade failed to reduce the expression levels of PD-1, PD-L1 and CTLA-4 on CD4+ and CD8+ T cells. Furthermore, the results showed novel positive correlation between B2M and soluble PD-1 ($r = 0.65$, $p = 0.022$), PD-L1 ($r = 0.60$, $p = 0.036$) and CD56 expression ($r = 0.63$, $p = 0.033$) on CD8+ T cells.

Conclusions: Prognostic factors such as deletion 17p and 11q, white cell count, LDH, and most importantly B2M retained predictive value in patients with CLL on rituximab-containing CIT. These factors should be included in future prognostic factors in CIT and chemotherapy-free era of patient

management. Our novel findings of the correlation between B2M and PD-1/PD-L1 suggests that monitoring B2M levels in patients with CLL may also be valuable in predicting patient responses to immunotherapy targeting the PD-1/PD-L1 axis.

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List of Abbreviations

APC Allophycocyanin

APC/Cy7 Allophycocyanin-cyanine 7

APRIL A proliferation-inducing ligand

ATM Ataxia-telangiectasia mutated

B2M Beta 2 microglobulin

BAFF B cell-activating factor

Bcl-2 B cell lymphoma 2

Bcl-XL B cell lymphoma extra-large

BIRC3 Baculoviral IAP repeat-containing protein 3

BCR B cell receptor

BKT Bruton Tyrosine Kinase

BLNK B-cell linker

BM Bone marrow

BV421 Brilliant violet 421

CCL C-C motif chemokine ligand

CD- Cluster differentiation

CD4+ cluster of differentiation-4 positive

CD8+ cluster of differentiation-8 positive

CIT Chemoimmunotherapy

CLL Chronic lymphocytic leukaemia

CTLA-4 Cytotoxic T lymphocyte-associate antigen 4

CXCL C-X-C motif chemokine ligand

CXCR C-X-C chemokine receptor type

ECOG Eastern cooperative oncology group

ELISA enzyme-linked immunosorbent assay

FBC Full blood count

FITC Flourescein isothiocynate

FSC Forward scatter

HR Hazard ratio

HSCs Hematopoietic stem cells

ICIs Immune checkpoint inhibitors

IgD immunoglobulin D

IGHV immunoglobulin heavy chain variable region gene

IgM immunoglobulin M

IFN-γ interferon-gamma

IL interleukin

ITAMs Immunoreceptor tyrosine-based activation motifs

LDH Lactate dehydrogenase

MAPKs mitogen-activated protein kinases

Mcl-1 Myeloid cell leukaemia 1

MSCs Mesenchymal-stromal cells

MBL Monoclonal B cell lymphocytosis

NFAT nuclear factor of activated T cells

NF- κ B nuclear factor-κB

NLCs nurse like cells

OS overall survival

PD-1 Programmed death 1

PD-L1 Programmed death ligand 1

PE Phycoerythrin

PE/Cy7 Phycoerythrin-cyanine 7

PerCP Peridinin chlorophyll protein

PFS Progression-free survival

PI3K phosphatidyl 3-kinase

PIP3 phosphatidylinositol-3,4,5-triphosphate

PKC Protein kinase c

PLCγ2 phospholipase Cγ2

SSC Side scatter

Syk Spleen tyrosine kinase

WCC white cell count

TNF-α tumour necrosis factor-α

TP53 Tumour protein 53

ZAP-70 Zeta-chain-associated protein kinase 70

THESIS STRUCTURE

This thesis is presented in the following manner**:**

Chapter 1: Introduction

Chapter 2: Background and literature review

Chapter 3: Manuscript 1: A systematic review and meta-analysis, **"The predictive value of protein biomarkers in patients with Chronic Lymphocytic Leukaemia on Rituximab-based therapy: A systematic review and meta-analysis of prognostic factors"**

Chapter 4: Manuscript 2: Experimental paper, **"T cell function and immune checkpoint inhibition in chronic lymphocytic leukaemia"**

Chapter 5: Synthesis

CHAPTER 1: INTRODUCTION

1.1 Background

The incidence of lymphocytic leukaemia or lymphoid leukaemia has increased over the past three decades (Dong et al., 2020). Treatment options available for the management of patients with these hematologic malignancies have evolved over time and novel immunotherapy approaches have improved patient outcomes (Hallek, 2019; Jaglowski and Jones, 2011; Terwilliger, 2017). However, chronic lymphocytic leukaemia (CLL) remains largely incurable and disparities in patient outcomes exist (Sharma and Rai, 2019). The exploration of novel therapeutic approaches, such as the advent of immunotherapies have prolonged the event-free survival of patients with lymphocytic leukaemia (Barbari et al., 2020; Waldman et al., 2020). A novel approach focusing on the resuscitation of the immune system by targeting immune checkpoint proteins is a breakthrough in the field of anti-cancer immunotherapy (Giannopoulos, 2019; Ma et al., 2019; Zhang and Zhang, 2020).

Tumour metastasis is mediated by various mechanisms, one which includes the activation of immune checkpoint pathways that suppress antitumor immune responses (Zappasodi et al., 2018). Immune checkpoint inhibitors (ICIs) target co-inhibitory signalling pathways and restore immune-mediated obliteration of tumorous cells, delaying disease progression (Darvin et al., 2018; Wierz et al., 2018). The two commonly targeted immune checkpoints are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the programmed death 1 (PD-1) receptor signalling (Darvin et al., 2018; Knaus et al., 2017; Zappasodi et al., 2018). These co-signalling receptors regulate T cell-driven immune responses (Pianko et al., 2017).

Several ongoing phase I, II and III/IV clinical trials are aimed at evaluating the efficacy of multiple ICIs as monotherapy or in combination (Darvin et al., 2018). Notably, Ipilimumab, which targets CTLA-4, was the first ICI approved for the treatment of patients with advanced melanoma (Hodi et al., 2010). While Nivolumab, which targets PD-1, was well tolerated in an independent cohort of patients with relapsed/refractory Hodgkin's lymphoma (Ansell et al., 2015). The efficacy of these ICIs has been reported in several clinical trials and when compared to traditional therapy, immune checkpoint inhibitors yield improved anti-tumour activity and patient outcomes.

1.2. Statement of research problem

Altered T cell function has been reported as a prognostic factor in CLL (Palma et al., 2017; Riches et al., 2013). Although CLL is a B cell malignancy, T cell dysfunction is well characterized in patients with CLL (Gassner et al., 2015; Mellstedt and Choudhury, 2006). Dysfunctional T cells alter cytokine profiles, impair cytotoxic T cell function, and exacerbate immune exhaustion (Blaeschke et al., 2020; Gassner et al., 2015). Exhausted T cells are characterized by an overexpression of immune checkpoints

(Wherry, 2011), which are expressed on the surface of activated T and B cells (Riches et al., 2013). These immune checkpoint proteins bind to their counter-receptors and these interactions result in tolerance and is mechanism of how tumorous cells evade immunosurveillance (Darvin et al., 2018; Egen and Allison, 2002; Woo et al., 2012).

Patients with lymphocytic leukaemia have variable treatment outcomes which remains a challenge in patient management and in the designing of coherent and functional therapy (Hallek, 2019; Terwilliger, 2017). Patients with relapsed or refractory lymphocytic leukaemia and particularly those with high-risk features such as the TP53 mutation, unmutated IGHV status and elevated B2M levels have poor outcomes (Stefaniuk et al., 2021; Stilgenbauer and Zenz, 2010).

Malignant cells can evade the tumour surveillance mechanisms of the immune system, by inhibiting T cell function (Darvin et al., 2018). The advanced understanding of this phenomenon has led to the development of a novel class of immunotherapies targeting various immune checkpoints (Waldman et al., 2020). These include PD-1/PD-L1 and CTLA-4 which have been shown to promote tumorigenesis and enhance anti-apoptotic microenvironment (Giannopoulos, 2019; Waldman et al., 2020). Studies elucidating mechanisms that drive immune exhaustion remains of noteworthy importance.

1.2 Aim of the study

To investigate T cell immune-checkpoint inhibitor profiles in treatment-naïve patients with chronic lymphocytic leukaemia.

1.3. Objectives of the study

This will be accomplished by the following objectives:

I. To determine the baseline levels of immune checkpoint inhibitor expression on circulating T lymphocytes in treatment-naïve patients with CLL and healthy controls.
II. To assess CD4 and CD8 T cell responses and changes in immune checkpoint profiles following protein kinase C mediated T cell activation in treatment naïve patients with CLL.
III. To evaluate correlations between the marker of disease progression Beta 2 microglobulin and soluble PD-1, and immune checkpoint expression (PD-1, PD-L1, CTLA-4, CD56) on T cells.

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CHAPTER 2: BACKGROUND AND LITERATURE REVIEW

2.1. Introduction

Chronic lymphocytic leukaemia (CLL) is one of the most commonly diagnosed adult leukaemia in the developed countries with an incidence rate of 4 – 5 per 100 000 population (Byrd et al., 2004; Hallek, 2019). The incidence rate of CLL is lower in Caribbean, African and Asian countries, suggesting the interplay of genetic factors in the epidemiology of CLL (Bosch and Dalla-Favera, 2019; Dores et al., 2007). CLL is prevalent in adults between the ages of 65 – 72 years and disproportionately affects males (Hallek, 2017; Kipps et al., 2017). In African countries, CLL has been described as uncommon and clinical studies exploring prognostic factors and patient survival patterns remain scant (Musaigwa et al., 2021). Briefly, CLL is characterized by the excessive accumulation of CD5+/CD23+ neoplastic B cells in the bone marrow, peripheral blood, and secondary lymphoid organs (Hallek, 2019; Zhang and Kipps, 2014). The aetiology of CLL remains unknown (Lanasa, 2010). Genetic predisposition is a well-recognised risk factor for CLL as family genome studies have indicated that 1st-degree relatives of individuals with CLL are at a higher risk and thrice as likely to develop the disease (Cuttner, 1992). Individuals diagnosed with this lymphoproliferative disorder present heterogeneous clinical course and extremely variable clinical outcomes (Rosenquist, 2017). Some patients survive for a lengthy periods of time without requiring treatment, whilst others present with a highly aggressive form of disease and poor patient outcomes despite extensive therapy (Darwiche et al., 2018; Gribben, 2010a; Kipps et al., 2017).

The presence of cytogenetic abnormalities and genetic mutations distinguish patients with an aggressive form of the disease (Bosch and Dalla-Favera, 2019). These genetic defects as well as the ability of the malignant cells to evade immune recognition and obliteration are the main causes of tumour progression in CLL (Griggio et al., 2020). The chemotherapeutic approach using fludarabine and cyclophosphamide in combination with a CD20-specific antibody rituximab (FCR) have been used as standard treatment for patients with CLL (Herishanu et al., 2019). This regimen has translated into improvements in patient responses to therapy with 95% overall response rates (ORR) (Hallek et al., 2010; Thompson et al., 2016). Despite these promising results with chemoimmunotherapy, not all patients with CLL are suited for FCR therapy, as elderly and a high-risk subgroup of patients, including those with chromosome 17p deletions demonstrate unacceptably low response rates (Riches et al., 2011). Novel immune-based therapeutic approaches that target molecular pathways have been introduced to the treatment armamentarium of CLL and are progressively replacing chemoimmunotherapy (Burger and O'Brien, 2018; Griggio et al., 2020; Sethi and Reddy, 2017). Nevertheless, CLL is still an incurable disease with only allogeneic hematopoietic stem cell transplantation (HSCT) demonstrating long term curative effects on a fraction of young patients (Griggio et al., 2020; Yosifov et al., 2019). The key to attaining

a long-term remission is identifying the prognosis aspects which are established at diagnosis prior to initiation of treatment (Ivanescu et al., 2012). This review discusses current knowledge on the diagnosis and the mechanisms influencing the differing survival outcomes observed in patients with CLL, pathophysiology and the biology of the malignant cells in CLL, the pro-survival pathways known to be relevant to CLL B-cell biology and the T cell function in CLL.

2.2. Pathogenesis and biology of malignant cells in CLL

The recognition of the cell of origin and malignant cells normal counterpart is crucial in understanding the biology and pathogenesis of hematologic malignancies (Darwiche et al., 2018). Although a number of hypotheses have been generated, the exact cell of origin of B-CLL is still unclear (Chiorazzi and Ferrarini, 2011). However, our knowledge of CLL biology has progressed over the last decade and the importance of the B cell receptor (BCR) signalling, along with the upregulation of antiapoptotic proteins in the growth and survival of malignant cell clones has been established (Yosifov et al., 2019).

The expression of cluster of differentiation 5 (CD5) antigen by B cells resulted to initial conclusions that CLL originates from CD5+ B cells (Caligaris-Cappio, 1996). Since then, there has been cumulative evidence showing that B-CLL originates from hematopoietic stem cells (HSCs) to immature B-cells in the bone marrow (BM), then progresses from transitional B cells to fully mature B cells in the periphery (Darwiche et al., 2018). HSCs differentiate into the pro-B cell, the first known cell type committed to the B cell lineage during early B-cell differentiation in the BM (Figure 1). These cells are subjected to clonal selection, which results in monoclonal B cell lymphocytosis (MBL) and eventually, CLL (Kikushige et al., 2011). The pro-B cell undergo a reshuffle of its immunoglobulin heavy-chain genes, featuring μ heavy chains to become pre-B cell. The recombination of the variable (V), diversity (D), and joining (J) gene segments in pro- and pre-B-cells permits the cells to progress into immature B-cells that express surface immunoglobulin M (IgM). BM immature B-cells begin to express surface immunoglobulin D (IgD) to grow into completely mature naive B-cells in secondary lymph organs. B-cell survival is aided by surface IgD, which reduces self-antigen-induced anergic B-cell responses (García-Muñoz et al., 2012).

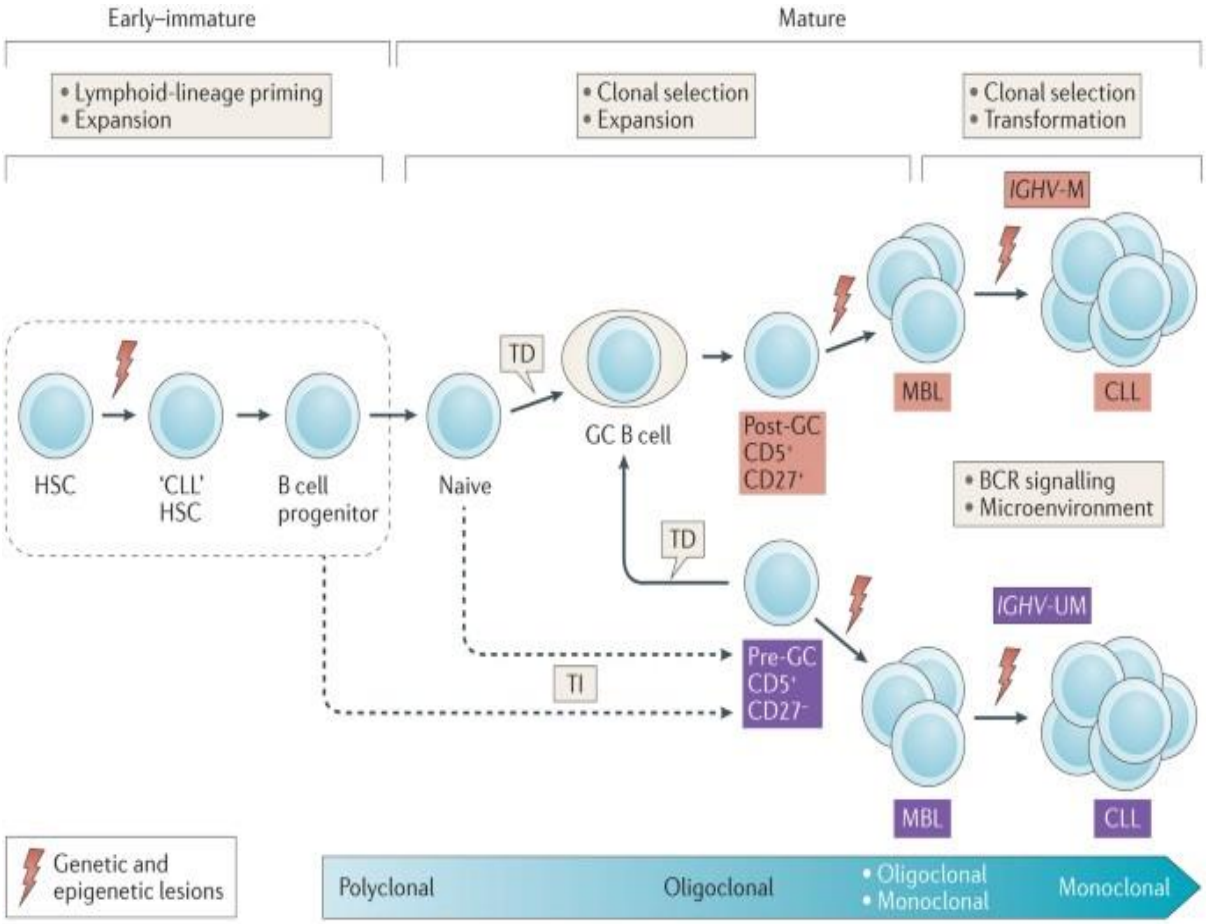

Figure 1: A representation of the cell of origin of CLL (adapted from Fabbri and Dalla-favera, 2016). Mutations contributing to the progression of CLL may occur at any phase of B cell development, including in HSCs. CLL cells derived from B cells that have experienced the germinal centre express unmutated Ig heavy-chain (IGHV-UM) genes, whereas CLL cells derived from B cells from post germinal centre (post-GC) express unmutated Ig heavy-chain (IGHV-M) genes (Seifert et al., 2012).

2.3. Immunoglobulin heavy chain gene mutation in B cell maturation

In CLL, the immunoglobulin heavy chain variable-region gene (IGHV) mutational status is a well-established prognostic marker that divides the disease into two prognostic categories (Damle et al., 1999; Rozovski et al., 2018). This characteristic is both biologically and clinically important with mutated IGHV gene (<98% sequence homology) demonstrating superior survival and the unmutated cases (30 – 40%), identified by having ≥ 98% sequence homology with the germline sequence present an aggressive disease with a significantly shorter time to initiation of first treatment, relapse after therapy and have worse patient outcomes (Rassenti et al., 2008). The Mutated IGHV comprises of cells believed to have progressed from germinal centre (GC) thereafter went through a final transformation, whereas cells in unmutated IGHV gene are derived from B cells stimulated by an antigen and did not acquire mutations and possibly went through final transformation ahead of entry into the GC (García-Muñoz et al., 2012). Chiorazzi *et al*, hypothesized that the absence of mutations in the latter case could

be a result of the kind of antigenic stimulation the cell experienced, for instance, T-independent or a consequence of when and where the transformation event occurred (pre-GC) (Chiorazzi and Ferrarini, 2011). This observation suggests that both cases of CLL derive from progenitors that includes marginal zone B cells and memory B cells, of which the IGHV genes could be mutated or unmutated (Klein et al., 1998; Martin and Kearney, 2002). These B cell subtypes have been advocated as cell of origin for CLL (Chiorazzi and Ferrarini, 2011). On the other hand, gene expression studies found only few variations between genes expressed on mutated-IGHV CLL (M-CLL) and unmutated-IGHV CLL (U-CLL), implying a one-cell originating model for CLL (Rosenwald et al., 2001).

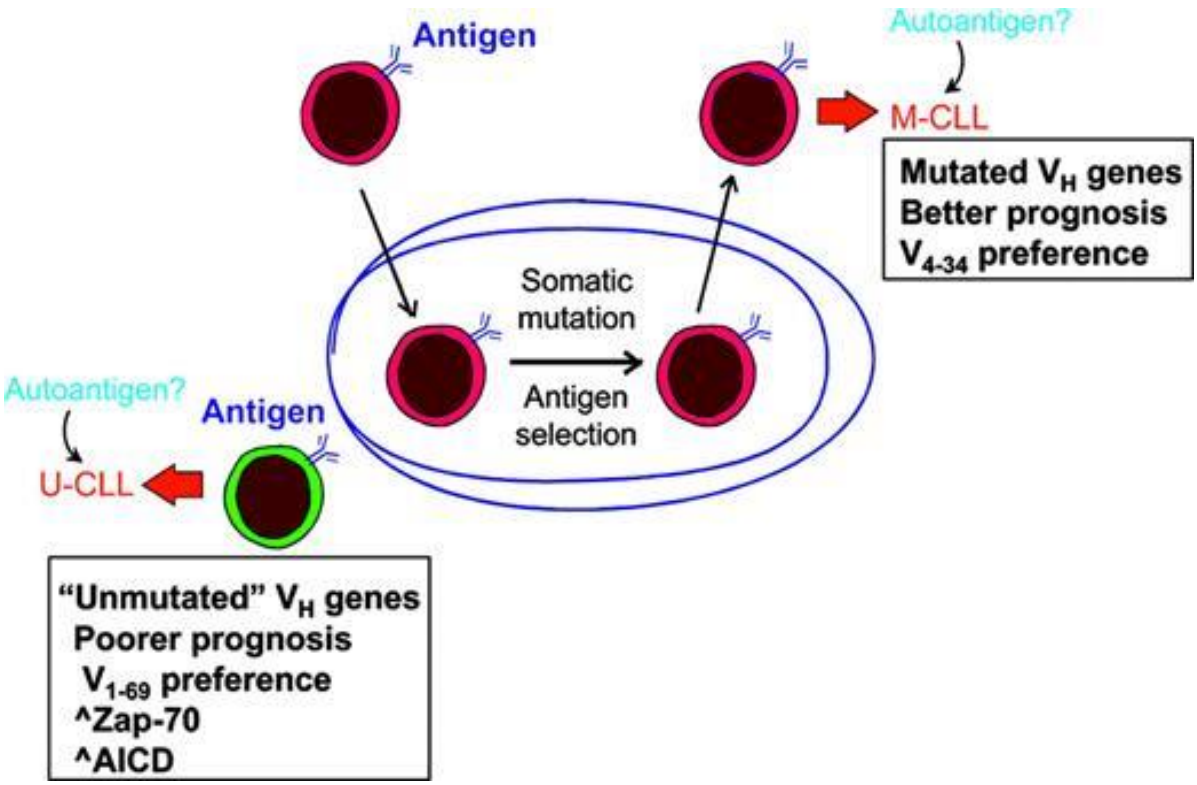

Figure 2: Origin and characteristics of the two subsets of CLL (Stevenson and Caligaris-Cappio, 2004)**.** The evolution of unmutated-IGHV CLL (U-CLL) probably originate from naive B cells that have been exposed to an antigen but has not been stimulated enough to develop a germinal centre (GC). This subtype has a worse prognosis and often expresses ZAP-70. Contrarily, mutated-IGHV CLL (M-CLL) arises from a cell which has experienced somatic mutation following antigen encounter, and possibly antigen selection in the GC. The complete neoplastic event presumably took place after exiting the GC. This subtype has superior prognosis and rarely expresses ZAP-70.

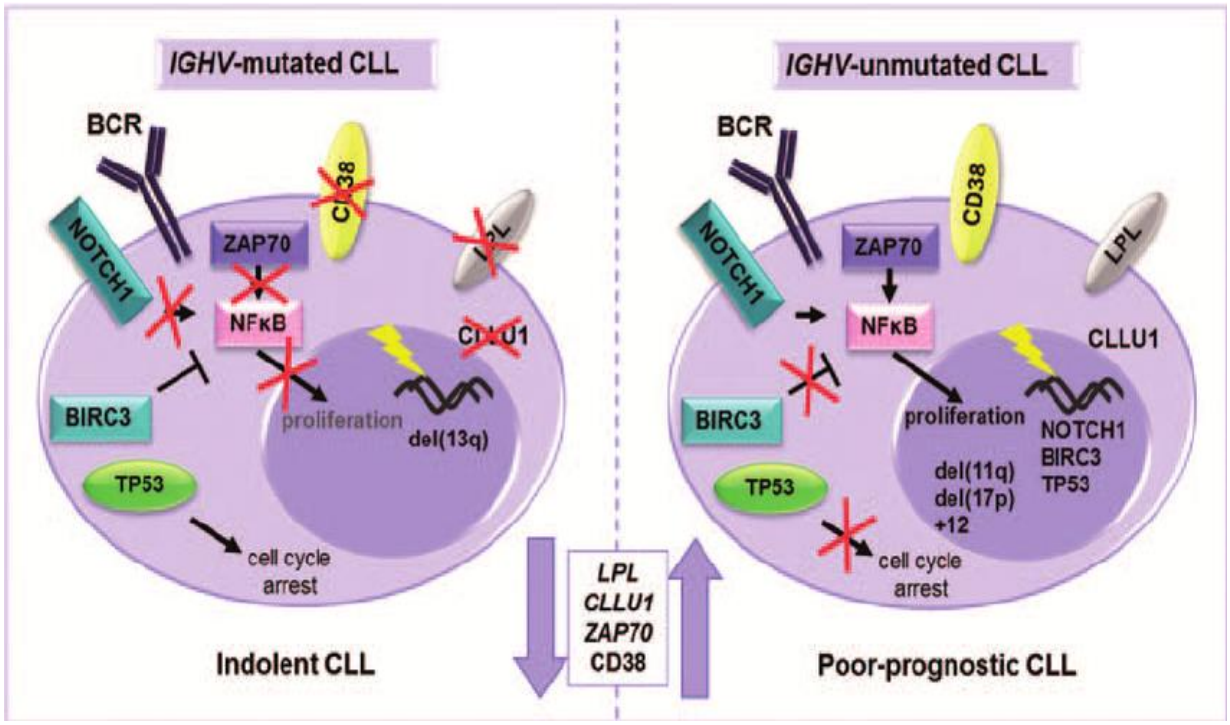

Figure 3: Different pathogenic mechanisms involved in mutated-IGHV and unmutated-IGHV CLL (Rosenquist et al., 2014)**.** In unmutated-IGHV CLL, NOTCH1 activating mutations and BIRC3 disruptive mutations result in increased NF- κ B signalling and proliferation. The overexpression of ZAP70 involved in BCR signalling leads to increased proliferation via NF- κ B signalling, for example. Unmutated-IGHV CLL has a higher rate of TP53 disruption due to deletion and/or mutation, which results in a dysfunction cell cycle arrest and increased cell survival.

2.4. Diagnosis and differentiation of CLL from other leukaemia/lymphoma

The World Health Organisation (WHO) classifies CLL as a mature B cell neoplasm, lymphoid leukaemia (Swerdlow, 2008; Swerdlow et al., 2016). The identifying characteristic of B-CLL is the co-expression of CD5, CD19 and CD23 with low levels of surface immunoglobulin (Ig) - CD79b and CD20 as compared to those on normal B cells (García-Muñoz et al., 2012) making it distinguishable from other B cell neoplasm like mantle cell lymphoma which usually mimics CLL (Matutes et al., 1994). According to the 2008 International Workshop on CLL (IWCLL) guidelines, CLL is diagnosed at the presence of $\geq 5 \times 10^9$/L monoclonal B cells in peripheral blood confirmed by peripheral blood flow cytometry on circulating B cells, with a phenotype of mature, activated B lymphocytes (Fabbri and Dalla-favera, 2016; Hallek et al., 2012; Kipps et al., 2017). The presence of $<5 \times 10^9$/L clonal B lymphocytes in an absence of cytopenia, organomegaly or lymphadenopathy denotes monoclonal B lymphocytosis (MBL), a condition that leads to the genesis of CLL at a rate of roughly 1% per year (Hallek et al., 2018; Rawstron et al., 2008; Swerdlow et al., 2016). In some individuals, the tumour is restricted to lymph nodes and other tissues without bone marrow or blood involvement. In these cases, the disorder is termed small lymphocytic leukaemia (Swerdlow et al., 2016). CLL diagnosis is based

on laboratory findings, that is immunophenotyping, morphology and blood count (Hallek, 2019). In addition, even though it is optional for the initial diagnosis of CLL, a bone marrow test is also routinely used to demonstrate a baseline for measuring response to therapy and establish the growth in the amount of B lymphocytes and reduction in the amount of normal marrow cells (Hallek et al., 2018).

Table 1: Diagnosis of CLL as outlined by the International Workshop on CLL (iwCLL) (compiled by author based on work by Gribben, 2010)

Clonal growth of abnormal B lymphocytes in the blood
CD5 surface antigen
No less than 5 x 10^9 B lymphocytes/L
B-cell surface antigens (CD23, CD19, $CD20^{dim}$)
≤55% immature lymphoid cells
Low density of surface Ig expression (IgM or IgD) with kappa or lambda light chains

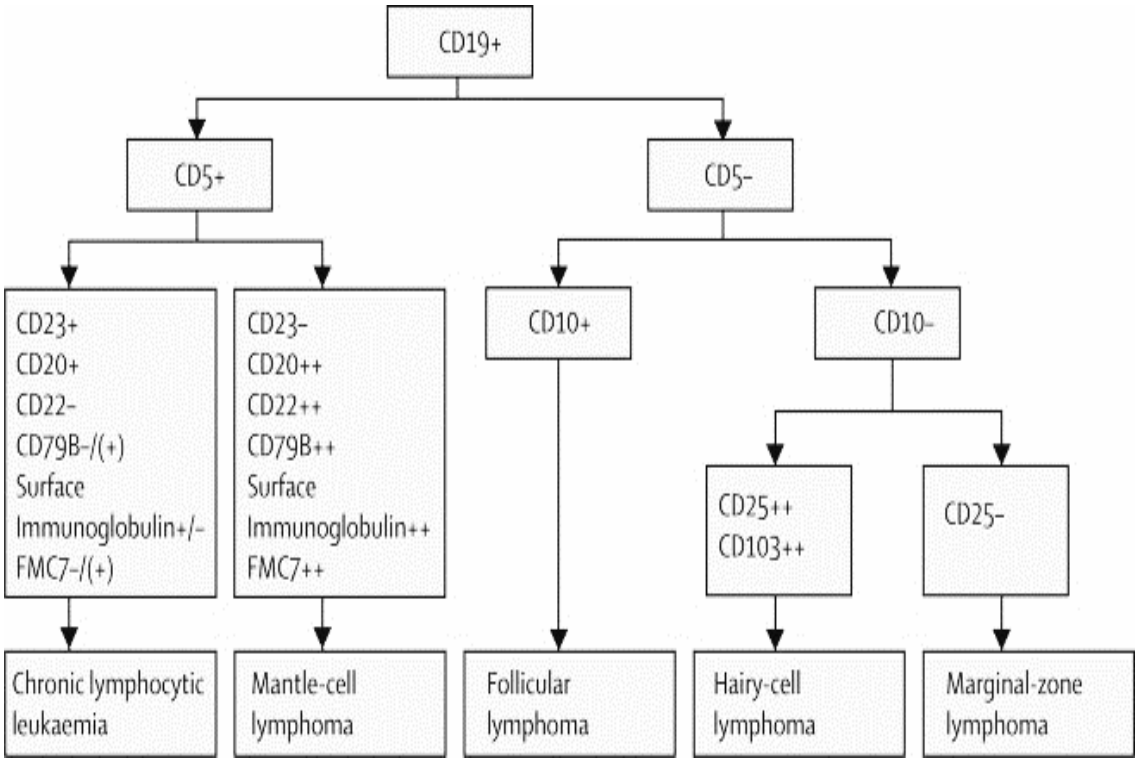

Figure 4: Immunophenotype of chronic lymphocytic leukaemia (CLL) (Dighiero and Hamblin, 2008). Panel of cell surface markers to distinguish CLL from other entities includes CD5, CD23, CD20, CD23, and surface immunoglobulin with light chain restriction.

2.5. Clinical staging and prognosis of CLL

The two currently used staging systems for CLL, independently developed by Rai and colleagues (Rai et al., 1975) and Binet and colleagues (Binet et al., 1981) have been shown to be highly predictive and useful in clinical practice (Leporrier, 2006). Both systems classify patients with CLL into three broad prognostic subgroups identifying low, intermediate, and high-risk patients and have proven usefulness for estimating patient outcomes (Rosenquist et al., 2014). Each of the staging systems emphasizes the significance of bone marrow function and describe high-risk CLL by the presence of excessive thrombocytopenia or anaemia (Kipps et al., 2017). Patients with Rai stage 0 or Binet stage A demonstrate prolonged overall survival (OS) (median survival surpassing 10 years). Whereas patients with Rai stage I/II or Binet stage B demonstrate an intermediate median survival of about 7 years. Contrarily, patients with Rai stage III/IV or Binet stage C are classified as a high-risk group and have a markedly shorter survival of <4 years (Hamblin, 2007). While these staging systems provide a useful tool for assessing prognosis in CLL, they fail to recognize subgroups of patients who may or may not respond to treatment (Rosenquist et al., 2014).

Table 2: Staging systems in CLL (adapted from Gribben, 2010b)

System	Clinical features	Median survival, y
Rai stage (simplified 3-stage)		
0 (low risk)	Lymphocytosis in blood and marrow only	> 10
I and II (intermediate risk)	Lymphadenopathy, splenomegaly +/− hepatomegaly	7
III and IV (high risk)	Anemia, thrombocytopenia	0.75-4
Binet group		
A	Fewer than 3 areas of lymphadenopathy; no anemia or thrombocytopenia	12
B	More than 3 involved node areas; no anemia or thrombocytopenia	7
C	Hemoglobin < 100 g/L platelets < 100 × 10 g/L	2-4

Several factors complementary to the classical staging systems that have proven to negatively influence the evolution of CLL have been identified and validated (Ivanescu et al., 2012). Biological and clinical parameters, such as Beta-2-microglobulin (β2M), age, gender, European cooperative oncology group (ECOG) status, ZAP-70 and/or CD38 expression, elevated serum levels of soluble CD23 (sCD23) and thymidine kinase demonstrate prognostic relevance in CLL (Sagatys and Zhang, 2012). Cytogenetic and genomic aberrations such as IGHV mutational status and chromosomal mutations i.e., del(11q), del(13q), del(17p) and trisomy 12 involved in the prognosis of CLL will be discussed separately in this review.

Serum markers prognostic value in patients with CLL

Elevated β2M levels denote patients with CLL with increased tumour burden and a more advanced clinical stage (Hallek et al., 1996). Moreover, patients with lower risk CLL, that is Binet stage A, with elevated β2M demonstrate shorter progression-free survival compared to patients with lower levels of β2M in Binet stage A, thus suggesting that β2M provides additional prognostic data in CLL patients (Gentile et al., 2009).

Serum lactate dehydrogenase (LDH) is known as a marker for cell turnover that is frequently elevated in patients with haematological malignancies and several other neoplastic conditions (Schwartz, 1991). Elevated LDH levels are linked to a shorter survival time in CLL patients (Autore et al., 2019). Various membrane proteins can be secreted into the blood, and their concentrations can be used as CLL tumour indicators. In CLL patients, the serum levels of sCD23 is a prognostic indication, and in patients with Binet stage A, it predicts disease progression (Sarfati et al., 1996).

2.6. Molecular genetics and cytogenetic abnormalities in CLL

Understanding molecular genetics of CLL have contributed greatly in patient management and in designing of coherent and functional therapy for patients with CLL by providing significant diagnostic, prognostic and clinical information (Foà et al., 2013; Wu et al., 2017). Several prognostic markers are currently being used for risk stratification prior to therapy in CLL (Rosenquist, 2017). These are factors that predict variances in time to first treatment, time to disease progression and patient response to therapy (Döhner et al., 2000; Gowda and Byrd, 2006). Detection of chromosomal translocations by fluorescence in situ hybridization (FISH) including 11q, 13q, 17p deletions and trisomy 12 provides crucial prognostic information and explain the differing survival outcomes observed in CLL. Patients with del(17p) have the poorest prognosis, followed by del(11q), whereas those with del(13q) and trisomy 12 have much better outcomes (Wu et al., 2017). Other infrequent chromosomal abnormalities that have also been discovered are gain of chromosome 2p or 8q, and deletions in chromosomes 8p and 15q (Lazarian et al., 2017).

Apart from mutations in the TP53 gene, the introduction of next-generation sequencing technologies and gene copy-number analysis have revealed other gene mutations associated with the progress of high-risk disease such as mutations found in ATM (ataxia–telangiectasia mutated), NOTCH1 (neurogenic locus notch homolog protein 1), BIRC3 (baculoviral IAP repeat-containing protein 3) and SF3B1 (splicing factor 3B subunit 1) genes (Nadeu et al., 2016). Such mutations could be exploited as possible treatment targets or indicators to differentiate between patients with varying clinical outcomes (Nadeu et al., 2016; Puente et al., 2012). In fact, it has been recently proposed that mutations in these genes be incorporated in the prognostic evaluation, which now only includes known recurring aberrations and TP53 mutations (Mansouri et al., 2013).

2.6.1 TP53 aberrations in patients with CLL

Patients with TP53 aberrations in particular, that is TP53 mutations and/or the deletion of the TP53 locus on chromosome 17 (del17p13.1) have the poorest survival outcomes of all CLL patients and almost uniformly require treatment (Foà et al., 2013). TP53 is a gene that encodes the tumour-suppressor protein p53, a critical cell-cycle regulator that can activate apoptosis or G1 cell-cycle arrest in apropos to DNA damage (Bieging, 2014; Pfister and Prives, 2017). TP53 mutations are associated with the worse prognosis independent of the deletion of 17p (Zenz et al., 2008). The most recurrent TP53 mutations are missense mutations found in TP53 coding region, which precedes an amino acid alteration in the p53 protein accounting for ~75% of TP53 mutations (Pfister and Prives, 2017). These genetic aberrations are reported in 5 -12% of CLL cases at first-line therapy with increasing prevalence in advanced disease and previously treated patients (Campo et al., 2018). Mutations in the TP53 gene are associated with decreased overall survival (OS) (less than 7 years) in newly diagnosed patients with

CLL (Döhner et al., 2000). The prognostic role of Del(17p) has been confirmed in multivariate analysis of randomised controlled trials (Catovsky et al., 2007; Stilgenbauer et al., 2005)

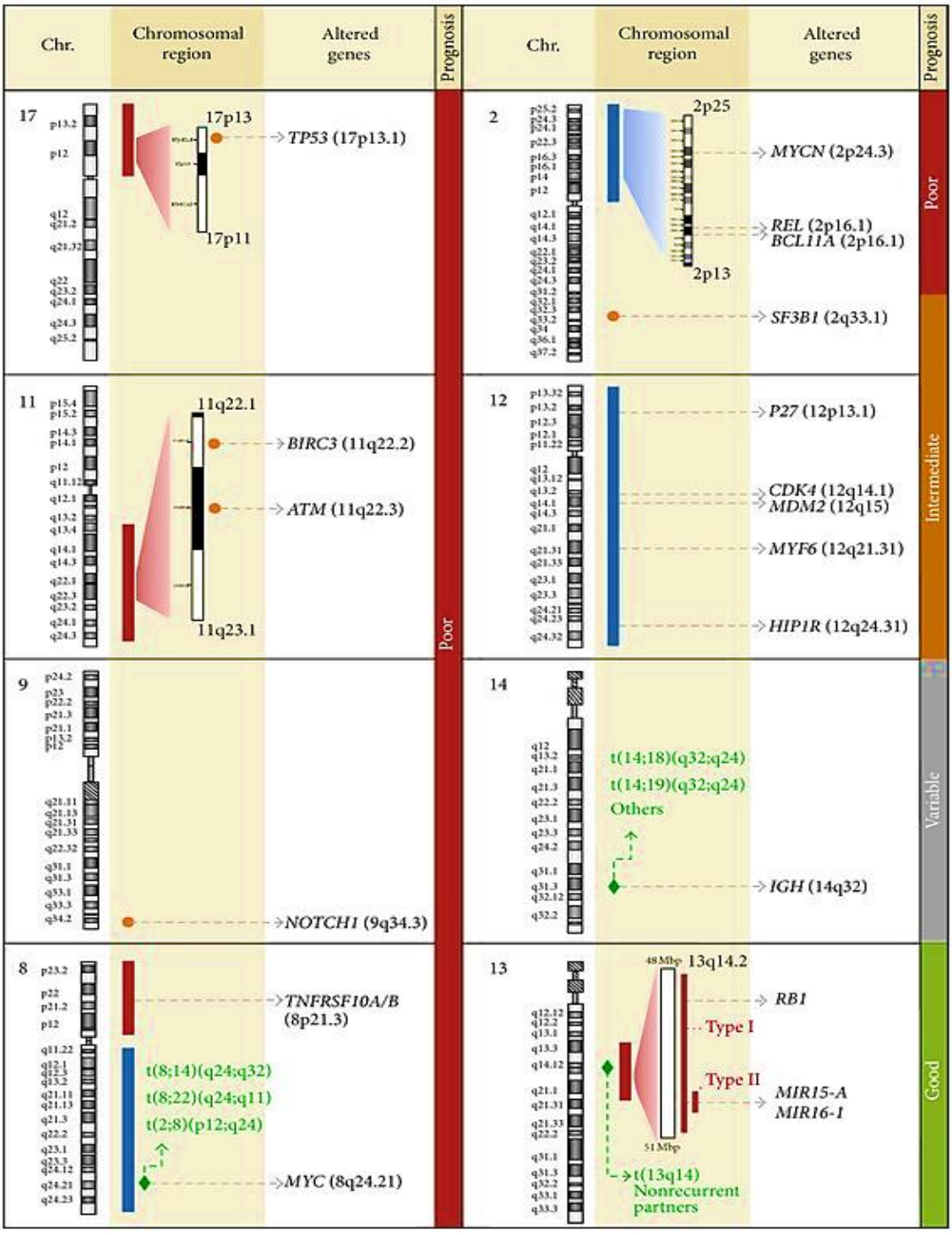

Figure 5: Genetic mutations in CLL (Puiggros et al., 2014). The most prevalent genetic anomalies in CLL that have been linked to poor prognosis. Chromosomes are used to categorize genetic disorders (Chr.). Losses and gains are indicated as red and blue bars, respectively, in the chromosomal region section; translocation breakpoints are depicted as green diamonds, and loci where recurrently mutant genes are situated are depicted as orange circles.

2.7 . Apoptotic pathways relevant to CLL B-cell biology

2.7.1 B-Cell antigen receptor and signalling

The B-cell receptor (BCR) is the result of an assemblage of Igα (CD79A) and Igβ (CD79B) heterodimer (Dal Porto et al., 2004; Riches et al., 2011). Its expression on CLL malignant cells is crucial in CLL pathogenesis as it provides survival and proliferation signals, and the signal transduction pathway triggered by BCR interaction is now the focus of various treatment methods in CLL (Delgado et al., 2020; Slupsky, 2014). The BCR is linked to a complex system of phosphatases and kinases that control and enhance its activation (Seda and Mraz, 2015).

In normal B cells, interaction of the BCR with an antigen incite immunoreceptor tyrosine-based activation motifs (ITAMs) phosphorylation which is mediated by the Src family kinase Lyn. This results to engagement and Syk tyrosine kinase activation, which forms a membrane-associated system with several other tyrosine kinases like Bruton's tyrosine kinase (BTK), Lyn, and adapter molecules including B-cell linker (BLNK) protein (Stevenson et al., 2011) This complex brings about activation of the downstream signalling pathways including phospholipase Cγ2 (PLCγ2) and phosphatidyl 3-kinase (PI3K). PI3K triggers phosphatidylinositol-3,4,5-triphosphate (PIP3), a second messenger that recruit molecules like kinase Akt (Carnero and Paramio, 2014; Woyach et al., 2012). PLCγ2 activation leads to consequent activation of protein kinase C (PKC). As a results, nuclear factor of activated T cells (NFAT), mitogen-activated protein kinases (MAPK), transcription factor nuclear factor-κB (NFκB) expression and RAS pathways, which promotes the growth and survival of normal and malignant B cells are activated (Figure 6) (Dal Porto et al., 2004; Riches et al., 2011).

In CLL, the activation of BCR induces growth of the malignant clone (Stevenson and Caligaris-Cappio, 2004). Inhibitors of BCR or chemokine receptor signalling have the potential to provide significant therapeutic benefit to patients with CLL (Figure 7) (Zhang and Kipps, 2014). Several prognostic markers that are clinically relevant in CLL, including the unmutated IGHV gene and the zeta-chain associated protein kinase 70 (ZAP-70) expression are associated with function and antigen binding in BCR suggesting a close link between increased BCR signalling and poor prognosis (Damle et al., 1999; Riches et al., 2011; Sivina et al., 2011). ZAP-70 can stimulate BCR signalling in CLL independent of its kinase activity, by aiding recruitment of other kinases, like SYK (Vladimirova et al., 2015). Novel agents that can inhibit BCR signalling, such as idelalisib (CAL-101), ibrutinib (PCI-32765) and fosamatinib have demonstrated significant clinical activity in CLL patients (Woyach et al., 2012).

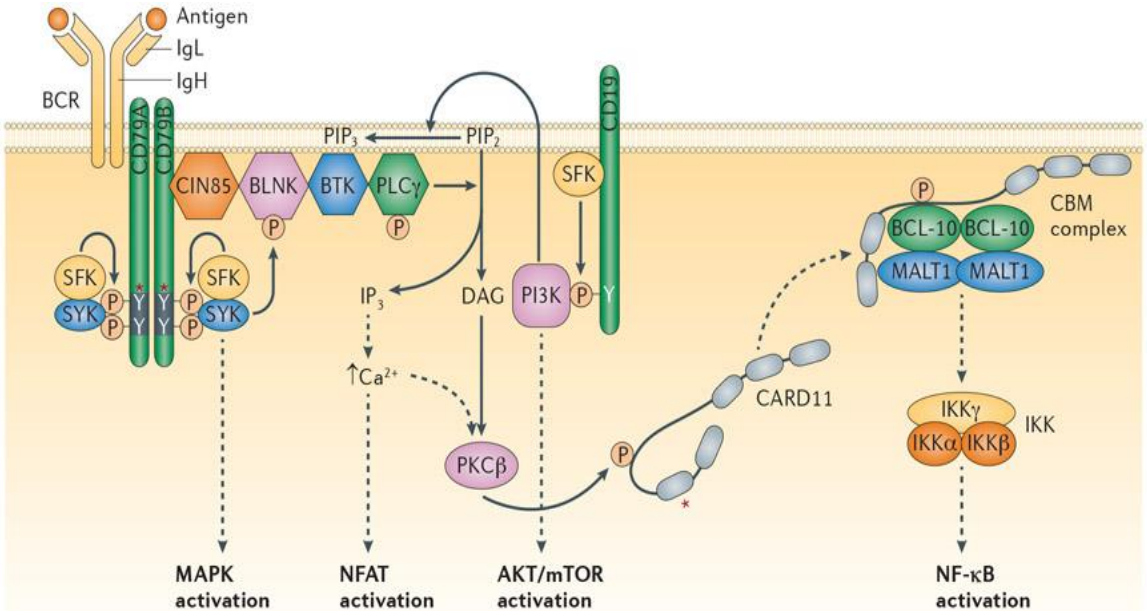

Figure 6: BCR signalling pathways (Dal Porto et al., 2004). BCR signalling is triggered by the identification of an antigen or by self-binding. BCR binding sets off signalling whereby Igα and Igβ are phosphorylated by Lyn or different Src family kinases. Thus activating BTK, PI3K and SYK, regulating distinct pathways impacting the survival, expansion, and migration of the CLL cells.

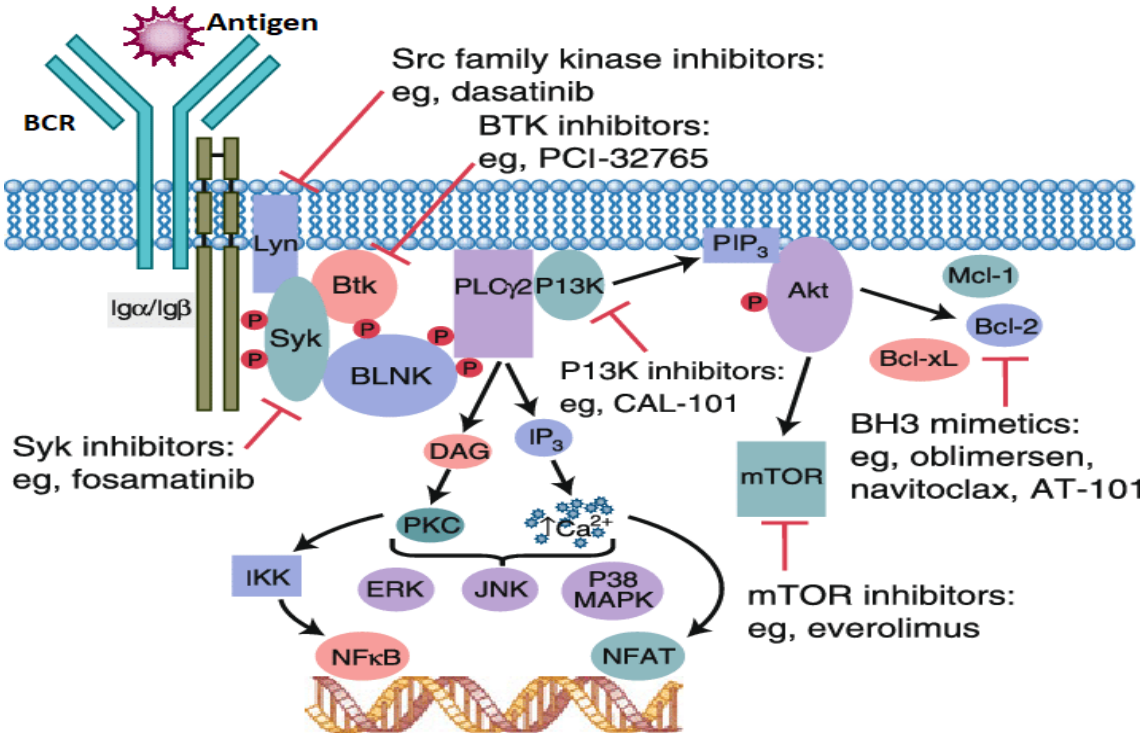

Figure 7: Target agents that blocks Syk, BTK and Pl3K (Riches et al., 2011)**.** BCR signalling kinases can be targeted by Src family kinase inhibitor dasatinib, BTK inhibitor e.g., ibrutinib, Syk inhibitor fosamatinib and the inhibitor for PI3K, idelalisib.

2.7.2 Targeting BCR signalling and pro-inflammatory pathways in CLL

According to the detection of several proteins significant for signal transduction, it was discovered that a number of these mechanisms are aberrantly activated in CLL (Riches et al., 2011). This understanding has resulted to the development of BCR pathway inhibitors, which have shown encouraging early therapeutic activity in patients with refractory CLL (Woyach et al., 2012). BCR signalling in B-CLL is characterized by low expression of IgM, tonic stimulation of antiapoptotic signalling pathways and variable reaction to antigen stimulation (Riches et al., 2011). The constantly active phosphorylation of specific kinases and a varied response to IgM activation denote BCR signalling pathway dysregulation in CLL (Woyach et al., 2012).

The importance of constitutively activated Syk in CLL B-cells in BCR signalling have made it a popular target for the evolution of novel CLL therapies as BCR-induced Akt activation and Myeloid cell leukemia-1 (Mcl-1) overexpression are reduced when it is inhibited (Gobessi et al., 2009). Syk inhibitor fostamatinib demonstrated high response rates with partial remission (PR) in 55% of patients with CLL (Friedberg et al., 2010). Ibrutinib, a molecule that irreversibly inhibit BTK by covalently bonding with Cysteine 481 in the catalytic region of BTK, inhibiting its phosphorylation (Davids and Brown, 2014; Honigberg et al., 2010). This novel agent has demonstrated improved outcomes in patients with del(17p) and relapsed/refractory CLL (Burger et al., 2014; O'Brien et al., 2016), remarkable ORR in patients previously treated with high-risk CLL and improved 2-year progression-free survival (PFS) (Byrd et al., 2013). Patient relapse on ibrutinib treatment is predominantly as a result of mutations on BTK, that decrease the ibrutinib-BTK binding affinity thus promoting reversible inhibition (Maddocks et al., 2015; Woyach, 2017; Woyach et al., 2014). Similarly, exploring inhibitors of antiapoptotic B-cell lymphoma 2 (Bcl-2) family members, B cell lymphoma-extra-large (Bcl-xL), and myeloid cell leukaemia 1 (Mcl-1) as treatment for CLL has sparked interest.

Chronic inflammation has been shown to contribute to tumour progression and even lead to several other types of malignancies (Elinav et al., 2013; Schulz et al., 2011). Inflammatory cytokines such as interleukin 6 (IL-6) and chemokines e.g. CCL2 (C-C motif ligand 2) were found to be connected with tumour growth and metastasis through generating a tumour-supportive milieu that promotes survival and differentiation of monocytes to tumour-associated microphages (Roca et al., 2009). In CLL cells, activation of pro-inflammatory signalling pathway allow cells to proliferate and survive while also inducing the release of inflammatory cytokines (Rozovski et al., 2013). Novel agents that target these pathways result in significant reduction in disease load aiding fractional restoration of immune-response in patients with CLL (Rozovski et al., 2013).

2.7.3 The microenvironment in CLL pathogenesis

The growth and survival of neoplastic CLL cells depends on their interactions with the microenvironment (Vladimirova et al., 2015). Monocyte-derived nurse like cells (NLCs),

mesenchymal-stromal cells (MSCs), natural killer (NK) cells and T cells are major components of the microenvironment, all which converse with CLL cells via a complex system of adhesion molecules, soluble factors, chemokine receptors, and members of tumour necrosis factor (TNF) family (ten Hacken and Burger, 2016). The microenvironment in patients with CLL is increasingly becoming a therapeutic target with lenalidomide, an immunomodulatory drug that works by interfering with a range of components in the CLL microenvironment (Ferrer and Montserrat, 2018). Lenalidomide upregulates B cell activation receptors on CLL cells while also improving the host immunological response, resulting in malignant cell recognition (Chen et al., 2011).

The interconnection between malignant cells and the T cells is essential for the growth and development of the malignant CLL clone (Pedersen and Reed, 2004). Interactions through CD40 antigen expressed on CLL cells, and its ligand (CD154) along with cytokines (CCL3, CCL4, IFNγ, TNFα, IL4), play a crucial part in the CLL-T cell cross-talk. Malignant cells adhere and reside in tissue microenvironments, especially the bone marrow and secondary lymphatic tissues by chemokines that are constitutively released by tissue MSCs and monocyte-derived NLCs (Okkenhaug and Burger, 2015). These stromal cells generate chemokine gradients, including those of CXCL12 and CXCL13 which engage CLL cells through the correlating chemokine receptors, CXCR4 and CXCR5 (Vladimirova et al., 2015). Adhesion molecules on malignant cells, including CD49d, collaborate with chemokine receptors throughout this mechanism (Buggins et al., 2011; Vladimirova et al., 2015). NLC also express B cell-activating factor (BAFF) and A proliferation-inducing ligand (APRIL), along with CD31 thus activating respective ligands on CLL cells, encouraging their expansion and survival (Herishanu et al., 2013; Okkenhaug and Burger, 2015).The dysfunctional apoptosis of CLL B-cells is attributed not only by intrinsic neoplastic cell abnormalities, but also by extrinsic stimuli that affect their activity in the tissue microenvironment (Pedersen and Reed, 2004).

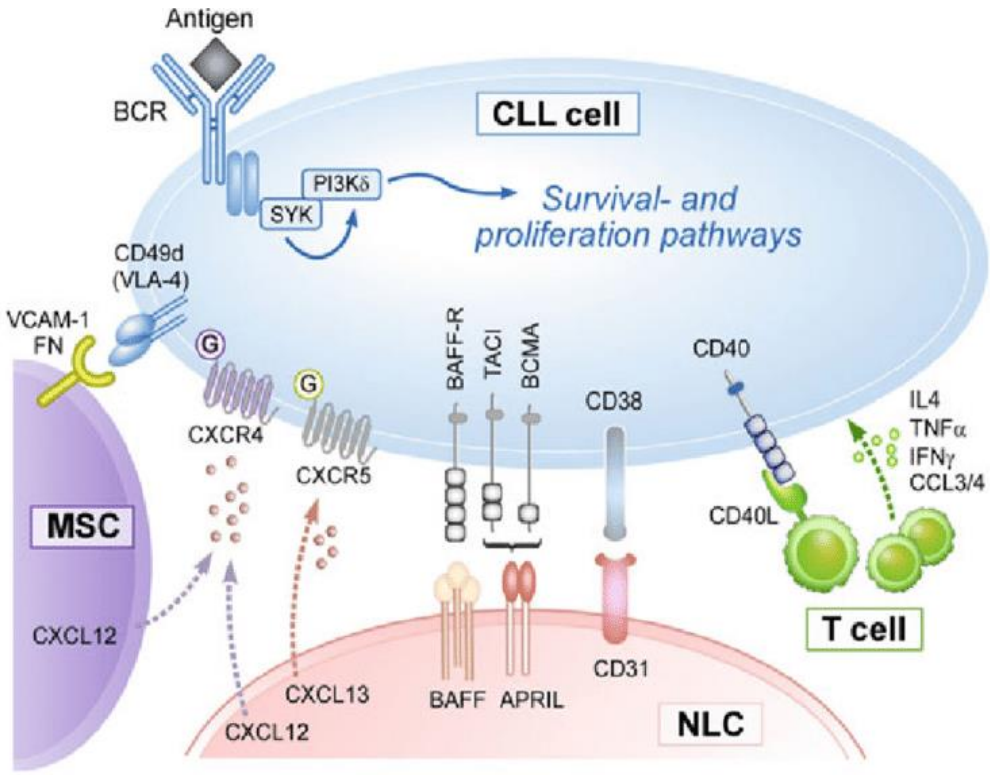

Figure 8: B-CLL microenvironment (Okkenhaug and Burger, 2015)**.** Mesenchymal-stromal cells (MSCs), Monocyte-derived nurse like cells (NLCs), and T cells are major components of the microenvironment, all which communicate with CLL cells.

2.8. T cell function in CLL

An aberrant T cell compartment in peripheral blood of patients with CLL have been reported in the past, thus enhancing our understanding of the nature of T cells in CLL (Catovsky et al., 1974; Tötterman et al., 1989). However, it is still argued whether T cells operate as pro-tumoral bystander cells or have anti-tumor activity (Bagnara et al., 2011; Os et al., 2013). Patients with CLL have increased numbers of CD4+ T helper cells, and T helper cytokines have been shown to invigorate CLL cell survival and growth in vitro indicating that T cells are tumor-supportive (Elston et al., 2020; Roessner and Seiffert, 2020). In recent years, studies have shown that CD8+ cytotoxic T cells control CLL in mouse model, suggesting that these cells recognize CLL-specific antigens and employ anti-leukaemia function (Hanna et al., 2019). T cells in CLL express high levels of several inhibitory molecules, including programmed death-1 (PD-1) (Elston et al., 2020; Palma et al., 2017; Riches et al., 2013) and cytotoxic T lymphocyte associated-protein 4 (CTLA-4) (Motta et al., 2005; Palma et al., 2017). Persistent antigen-driven activation of CD8+ T cells results to an upregulation of inhibitory molecules such as PD-1, lymphocyte activation gene 3 (LAG3), CD160 leading to a phenomenon called T cell exhaustion (Wherry, 2011). Exhausted T cells gradually loses their ability to proliferate and produce cytokines such as IL-2, TNFα, and IFNγ (Wherry, 2011; Wherry and Kurachi, 2015). Elucidating T cell dysfunction in CLL

and identifying processes driving immune suppression is critical to reinvigorate the immune system (Griggio et al., 2020). T cell and other immune-based therapeutic approaches with novel agents known as immune checkpoint inhibitors are actively being researched for CLL, with the potential to complement chemoimmunotherapy. The blockade of the interaction between PD-1 and CTLA-4 with their ligands are the most studied (Griggio et al., 2020). In research evaluating PD-1/PD-L1 axis disruption, promising preclinical results have been obtained (McClanahan et al., 2015; Wierz et al., 2018).

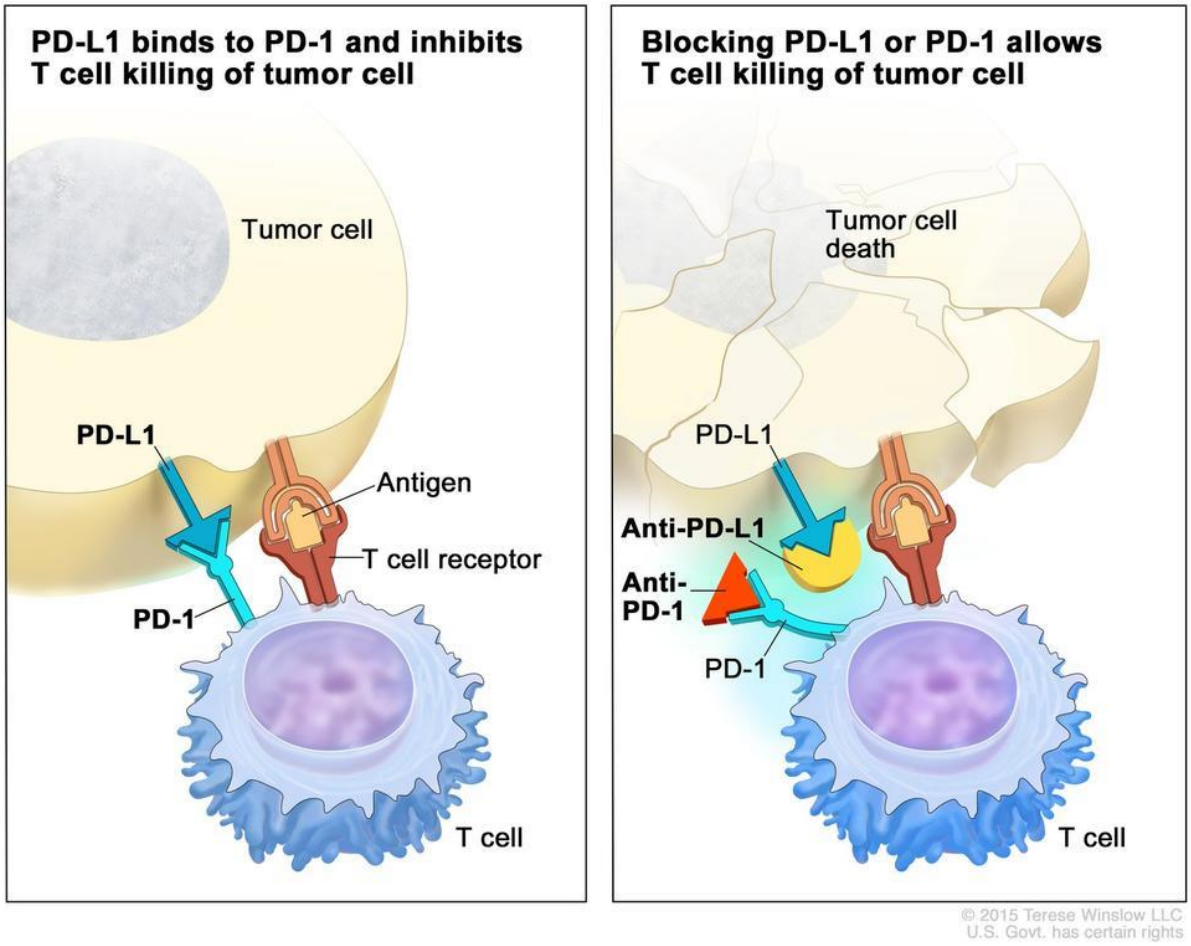

Figure 9: Blockade of PD-1/PD-L1 axis in cancer. Immune checkpoint proteins, such as PD-L1 on tumor cells and PD-1 on T cells, aid in the regulation of immune responses. T lymphocytes are prevented from destroying tumor cells in the body when PD-L1 binds to PD-1 (left). T cells can destroy tumor cells by blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor (anti-PD-L1 or anti-PD-1) (right). (Credit: National cancer institute. https://www.cancer.gov/about-cancer/treatment/types/immunotherapy/checkpoint-inhibitors)

To summarize, more research is needed to investigate the potential of immune checkpoint blockage in CLL and to design rational combination techniques to overcome T-cell exhaustion and immunological escape of CLL.

2.9. Conclusions

In the previous two decades, our understanding of B-CLL biology has rapidly advanced, paving a way for novel therapeutic molecules that target specific disease pathways and immune checkpoint inhibitors. These drugs are effective in disease settings such as relapsed/refractory and high-risk chromosomal aberrations (del(17p), where traditional chemotherapy is ineffective. As a result, CLL management algorithms are evolving and the introduction of these novel agents and monoclonal antibodies, however, does not mean the end of chemotherapy, but provides complementary treatment strategy for the management of patients with aggressive CLL. Modulating the B-CLL microenvironment could also lead to novel techniques for restoring apoptosis susceptibility and improving patient outcomes in CLL.

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CHAPTER 3: MANUSCRIPT 1 - SYSTEMATIC REVIEW

Title: The predictive value of protein biomarkers in patients with chronic lymphocytic leukaemia on rituximab-based therapy: A systematic review and meta-analysis of prognostic factors.

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Abstract

Combination immunotherapy consisting of rituximab has improved the progression-free survival (PFS) and overall survival (OS) of patients with chronic lymphocytic leukaemia. We performed a comprehensive synthesis of prognostic factors to examine the efficacy of combined chemoimmunotherapy (CIT) with rituximab compared with a standard chemotherapy alone. We searched the MEDLINE and academic search complete electronic databases from inception up to 31 August 2021. The risk of bias and the quality of evidence were independently assessed using the quality in prognostic studies tool (QUIPS). We included 3633 patients with CLL on CIT reported in 10 randomized controlled studies. A total of 10 prognostic factors were identified and evaluated in patients with CLL on rituximab containing CIT. The predictive value of the following prognostic factors was confirmed and associated with poor patient outcomes; deletion 17p (HR = 4.88), Immunoglobulin heavy chain variable region gene mutation status (HR = 0.96), WCC (HR = 1.27), β_2-microglobulin (HR = 0.96), and lactate dehydrogenase levels (HR = 1.20). These conventional prognostic factors may have retained prognostic value and could be useful in the stratification of patients who may be non-responsive to CIT. This review was not funded, and the study review protocol was prospectively registered on the International Prospective Register of Systematic Reviews (PROSPERO) registry (CRD42021218997).

KEYWORDS: Chronic lymphocytic leukaemia, prognosis, chemoimmunotherapy, rituximab.

1. Introduction

The prevalence of chronic lymphocytic leukaemia (CLL) in adults over the age of 65 has gradually increased in high income countries (Gribben, 2010; Siegel et al., 2013). In comparison, CLL is uncommon in low to lower-middle income countries including India and Sub-Saharan African regions (Lad et al., 2018; Musaigwa et al., 2021). Reasons for these differences are not well understood. There is paucity of clinical and laboratory data on CLL in low resource settings. CLL disproportionately affects males, and an inferior survival rate in males has been reported in several studies (Catovsky et al., 2014; Kristinsson et al., 2009; Molica et al., 2005).

Over the last two decades, novel clinical and genetic-based prognostic factors have been identified in patients with CLL (Cohen et al., 2020). These include age, gender, immunoglobulin heavy chain variable region gene (*IGHV*) mutation status and cytogenetic abnormalities (Pflug et al., 2014; Rosenquist et al., 2013), the aberrant expression of CD38 and ZAP70 (Rassenti et al., 2008), *TP53* mutation (Landau et al., 2015), β_2-microglobulin (Hallek et al., 1996), and the Eastern Cooperative Oncology Group (ECOG) performance status (Cohen et al., 2020; Rosenquist et al., 2013). The development and implementation of prediction models have allowed for the risk-stratification of patients with CLL based on genetic traits (Gaidano and Rossi, 2017).

In patients with CLL, the conventional therapy consisting of ibrutinib (Burger et al., 2019; Woyach et al., 2018a), chlorambucil (Goede et al., 2014), fludarabine and cyclophosphamide (Hallek et al., 2010; Robak et al., 2010) yielded low overall response rates (ORR), with treated patients having an estimated 5-year overall survival (OS) of <40% (Gökbuget et al., 2016; Jaglowski and Jones, 2011). These clinical outcomes in patients with CLL led to a shift towards novel antibody-based therapies in the last decade. These include rituximab, an anti-CD20 monoclonal antibody which when administered in combination with standard chemotherapy, improves the patient response rates and is associated with complete remission (CR) in patients with CLL (Keating et al., 2005; Lee et al., 2018; Wierda et al., 2006). However, despite the benefit of chemoimmunotherapy (CIT) with rituximab, patient outcomes are highly variable (Brown et al., 2021). The efficacy of rituximab-based CIT has been demonstrated in cohorts of patients without the associated genetic aberrations such as Del(17p) and TP53 mutations (Brown et al., 2018).

The advances and refinement of prognostic risk scores has led to improved risk stratification of patients with CLL. The cornerstone of these risk scores, are the revised Rai (Rai et al., 1975) and Binet (Binet et al., 1981) staging systems, and novel prognostic indices such as CLL International Prognostic Index (CLL-IPI) (International, 2016) which allow for a precise risk stratification. Pertinent challenges in the risk stratification of patients with CLL on CIT include the lack of cumulative evidence on the predictive value of integrated cell and genetic based prognostic models (Kreuzberger et al., 2020). Moreover, the

lack of diverse multi-ethnic cohorts and prevalent risk factors (Parikh, 2018) also contribute to the imprecision of these predictive models (Yun et al., 2020). Therefore, the current systematic review and meta-analysis sought to identify and evaluate studies reporting on the prognostic factors in patients on CIT. Moreover, we aimed at providing a comprehensive synthesis and confirmation of prognostic factors associated with poor clinical outcomes in patients with CLL on CIT.

2. Methods

2.1. Eligibility criteria

The eligibility criteria was based on the Population, Index prognostic factor, Comparator prognostic factors, Outcome, Timing and Setting (PICTOS) guidelines (Hayden et al., 2006). We included randomised controlled trials reporting on patients with CLL on CIT, and the prognostic factors associated with overall 5-year survival or disease-free progression. We also included studies that aimed at developing or validating predictive models for mortality in CIT-treated patients with CLL. In addition, we included studies reporting on predictive measures at any time point and setting. Reviews, letters, and case-studies were excluded. In this systematic review, predictive models were considered as multivariable models used to predict survival in patients with CLL using selected predictors. We considered index prognostic factors derived from the CLL International Prognostic Index (CLL-IPI) (International, 2016), the German CLL Study Group (GCLLSG) (Tam and Seymour, 2014), and the MD Anderson Cancer Centre (MDACC) nomogram predictive models (Munk Pedersen and Reed, 2004).

2.2. Search strategy and selection process

A systematic literature search was performed by two independent reviewers (ZAM and BBN) on the MEDLINE, MasterFILE premier, Health source: Nursing/Academic edition, and clinical trials.gov. We made use of Medical Subject Headings (MeSH) and related synonyms which included, chronic lymphocytic leukaemia, rituximab, and prognosis. All electronic databases were searched from inception to the 31st of August 2021. A detailed search strategy is presented in Supplementary Table 1. To augment the database search, we screened the bibliographies of relevant reviews and included studies.

2.3. Data extraction

Two reviewers (ZAM and BBN) independently extracted data items from the included studies defined in the critical Appraisal and data extraction for systematic Reviews of prediction Modelling Studies for Prognostic factors CHARMS-PF checklist (Moons et al., 2014). The extracted study characteristics included, source of data, participant description, sample size, outcomes to be predicted, candidate predictors, type of model.

2.4. Risk of Bias and quality assessment

The certainty and strength of the evidence was assessed by two independent reviewers (ZAM, SAM) using the Quality In Prognostic Studies (QUIPS) tool (Hayden et al., 2006). The tool consists of six domains used to appraise studies of prognostic factors (Supplementary file 2). A third reviewer (BBN) was consulted for arbitration.

2.5. Statistical analysis

The Cohen's kappa was used to assess the inter-rater reliability for the study selection and the study quality and risk of bias assessments (Landis and Koch, 1977). The hazard ratios (HR) or odds ratios (OR) and 95% confidence interval (CI) were pooled to estimate the survival increases in OS and PFS. The effect estimates of studies were pooled using a random-effects model (Schroll et al., 2011). The I^2 and Chi squared statistical tests were used to assess the levels of statistical heterogeneity (Higgins et al., 2011). An I^2 value of >50% was considered as substantial (Schroll et al., 2011). All data analysis was performed using STATA 16.0 (StataCorp LP, TX, USA).

2.6. Subgroup and sensitivity analyses

To explore the sources of heterogeneity amongst the included studies, we performed a sensitivity analysis based on the study design and risk of bias.

2.7. Confirmation of predictive factors

The reported prognostic factors were confirmed based on the robustness of the overall direction of the effect across all eligible studies. Moreover, adjusted effect estimates that remained statistically significant ($p<0.05$) after adjusting for covariates in the multivariate analysis were considered as confirmed.

3. Results

3.1. Included studies

We retrieved a total of 506 citations through the database search, and only 391 studies were eligible for screening. Amongst these, 315 studies were ineligible and excluded during the abstract screening phase. A total of 63 citations were retrieved and the full-texts were assessed for eligibility.

Randomised controlled clinical trials including patients with CLL

A total of 63 full-text articles reporting on patients with CLL enrolled in RCTs were screened, and 53 studies were excluded due to the study outcomes (n = 8), illegible comparator groups (n = 4), were non-randomised controlled trials (n = 30) and were irrelevant (n = 11). In all, 10 studies met the inclusion

criteria and were included in the qualitative and quantitative analysis (Burger et al., 2019; Byrd et al., 2005; Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Mato et al., 2019; Robak et al., Robak et al., 2018, 2010; Woyach et al., 2018) (Figure 1). The overall reviewer agreement for study selection was 89% (kappa = 0.82).

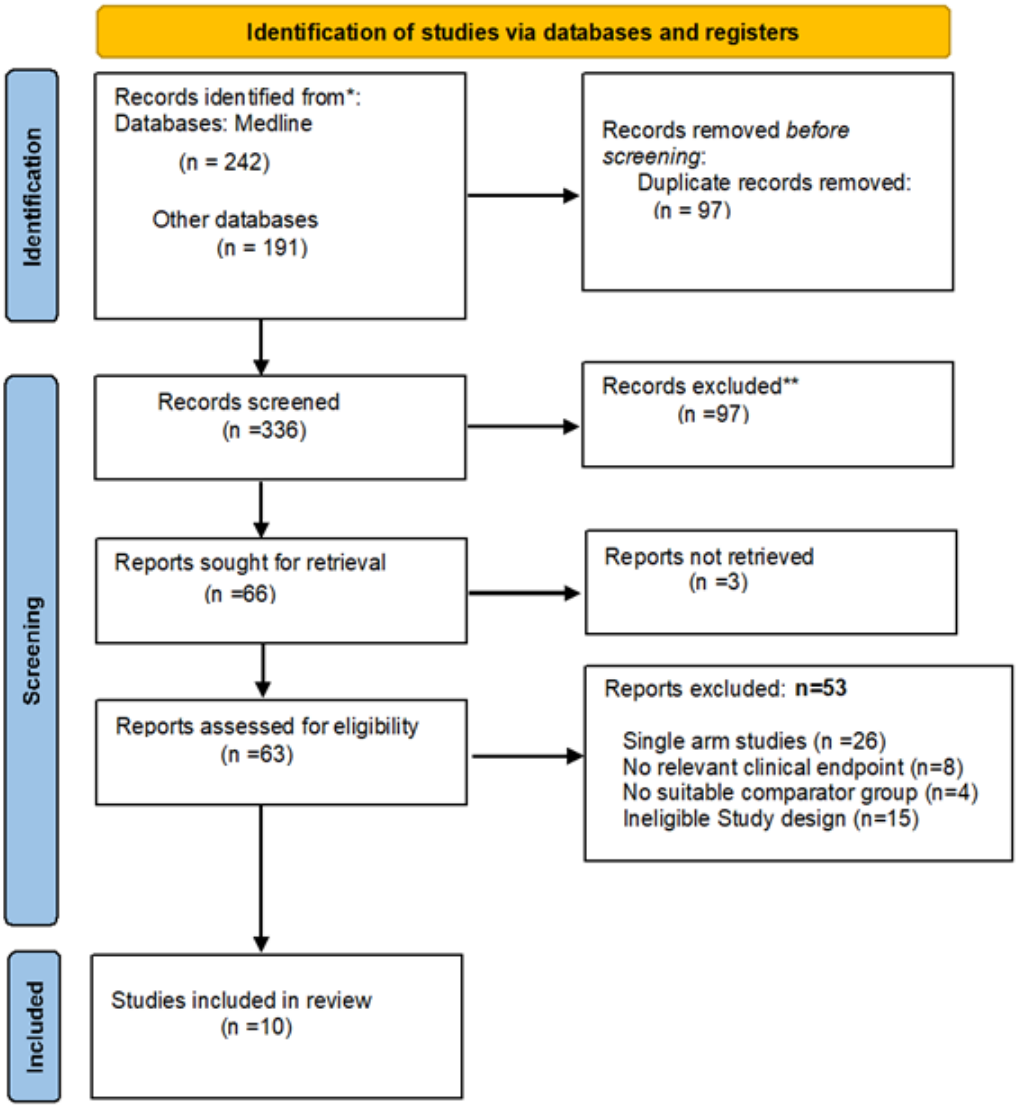


Figure 1: PRISMA flow diagram showing the study selection process.

3.2. Characteristics of included studies

The 10 included studies were published between 2005 and 2019 comprising of a total of 3633 patients with CLL (Table 1). Most of the included trials were multicentre studies (n = 9) (90%) and the individual study sample size varied from 66 to 817 patients (Median 336, IQR, 263 - 409). The age of enrolled participants ranged from 24 – 91 years.

The geographic distribution of the included studies consisted of Europe (n = 6, 60%) (Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2018, Robak et al., 2010), Americas (n = 3; 30%) (Burger et al., 2019; Byrd et al., 2005; Woyach et al., 2018), in both Europe and America (n = 1; 10%) (Mato et al., 2019), (Table 1). The included studies comprised of 64% (n = 2316) patients who were treatment-naïve, 22% (n = 815) of patients who were previously treated and 14% (n = 502) who were relapsed/refractory. In addition, 50% (n = 5) of the included studies reported on the RAI staging whereas 40% (n = 4) reported on Binet staging system. One study did not specify the staging system used (Mato et al., 2019).

Table 1: Characteristics and outcomes of included CLL studies (n = 10)

Study	Geographic region	Aim	Staging	Model performance	Outcome; Adjusted effect estimate	Main Findings
Byrd et al 2005	Americas	To compare the survival outcomes of patients with CLL treated with fludarabine in combination with rituximab with chemotherapy alone.	RAI stage III/IV or I/II	No	PFS; HR: 2.89, OS; HR: 2.59	The addition of rituximab to fludarabine significantly improved a 2-year PFS in patients with B-CLL. The treatment effect remained unchanged across prognostic factors examined.
Robak et al 2010	Europe	To compare CIT with fludarabine, cyclophosphamide and rituximab (FCR) with standard chemotherapy (FC) in patients with previously treated CLL.	Binet Stage A, B and C	No	PFS; HR: 0.65, OS; HR: 0.83	CIT with rituximab improved a 2-year PFS. Patients with poor prognostic factors such as del11q, unmutated *IGHV*, or positive ZAP-70 benefited from FCR.
Hallek et al 2010	Europe	To investigate whether adding rituximab to chemotherapy with FC would improve the survival outcomes of treatment-naïve, physically fit patients with $CD20^+$ CLL.	Binet Stage A, B and C	No	PFS; HR: 0.56, OS; HR: 0.67	The addition of rituximab to chemotherapy improved 3-year PFS and OS and resulted in significantly higher PFS in most genetic subgroups including del(17p), del(11q), del(13q) and trisomy 12. An improvement in PFS was noted in all disease stages.
Goede et al 2014	Europe	To determine whether CIT with rituximab would be beneficial in previously untreated patients with CLL and comorbidities.	Binet stage C, symptomatic disease	No	PFS; HR: 0.44, OS; HR: 0.66	CIT with rituximab resulted in a better response and prolongation of a 2-year PFS as compared to treatment with chlorambucil alone.
Greil et al 2016	Europe	To investigate the potential of rituximab maintenance therapy to improve survival outcomes in patients with CLL who respond to rituximab-containing induction regimen	Rai stage 0/I/II or stage III/IV	Yes	PFS; HR: 0.50 OS; HR: 0.77	Rituximab maintenance therapy prolonged a 3-year PFS. The effect of rituximab on PFS was comparable across prognostic factors analysed. OS was not reached in both the rituximab and observation group due to shorter follow-up time.
Dartigeas et al 2017	Europe	To compare maintenance treatment with rituximab vs. no further treatment to prolong PFS in treatment-naïve, elderly fit patients with CLL.	Binet stage B or C	Yes	PFS; HR: 0.55, OS; HR: 0.89	Maintenance therapy with rituximab improved 3-year PFS as compared to observation. OS was not reached in both groups at the time of analysis.

Robak et al 2018	Europe	To assess the effect of maintenance treatment with rituximab vs. no further treatment in previously untreated patients with progressive CLL.	Rai stage I-IV	No	PFS; HR: 0.418	A 3-year PFS was significantly longer in the maintenance arm compared to the observation arm.
Woyach et al 2018	Americas	To evaluate the efficacy of ibrutinib, either alone or in combination with rituximab in older patients with untreated CLL.	Intermediate to high-risk modified Rai stage disease	No	PFS/OS; HR: 1.06	There was no significant difference in 2-year PFS and OS between the two arms. Interactions between cytogenetics and effect of treatment on PFS were observed.
Burger et al 2019	Americas	To determine whether addition of rituximab to ibrutinib therapy improves PFS in treatment naïve, high-risk disease relapsed CLL.	Rai stage III-IV	No	PFS; HR: 1.16, OS; HR: 0.75	Combination of rituximab with ibrutinib showed no improvements in estimated 2-year PFS and OS as compared to ibrutinib alone
Mato et al 2019	Europe and Americas	To compare survival outcomes among patients with relapsed/refractory CLL treated with venetoclax in combination with anti-CD20.	Not stated	No	PFS; HR: 1.0, OS; HR: 1.2	No significant differences in 1-year PFS and OS were observed between the two groups. Moreover, the addition of rituximab to venetoclax did not impact PFS.

Abbreviations: CLL, chronic lymphocytic leukaemia, HR, hazard ratio, OR, odd ratio, PFS, progression-free survival, OS, overall survival, Del-, deletion, FCR, fludarabine, cyclophosphamide and rituximab, CIT, chemoimmunotherapy, EFS, event-free survival, ORR, overall response rate, RCC, rituximab, cladribine and cyclophosphamide, IGHV, immunoglobulin heavy chain variable region gene, CD-, cluster differentiation.

3.2.1. Prognostic factors in patients with CLL

In the included studies, prognostic factors were analysed before the start of treatment (Table 2). Overall, the studies comprised of 23% (n = 832) of patients who were 70 years or older, 34% (n = 1232) of patients with an unmutated *IGHV* status, 15% (n = 533) with del11q, 14% (n = 513) with a del17p, and 2% (n = 68) had both the del11q and del17p. Notably, 23% of the patients (n = 824) had del13q, and 5% (n = 168) had TP53 mutation. In the reported cell-based prognostic factors the included studies reported on ZAP-70 expression in 17% (n = 607) of the patients, and CD38 expression was reported in 24% (n = 863) of the included patients. In all, 36% of the included patients with CLL were in the advanced stage of the disease.

Table 2: Treatment arms and confirmed prognostic factors in studies included in the meta-analysis (n = 10)

Author, year	Study arms	Confirmed prognostic factors
Byrd 2005	FR vs F	Age, high WCC, LDH
Robak 2010	FCR vs FC	None
Hallek 2010	FCR vs FC	Del(17p), B2M, WCC, unmutated *IGHV*
Goede 2014	R-Chl vs Chl	None
Greil 2016	MR vs OBS	None
Dartigeas 2017	MR vs OBS	None
Robak 2018	MR vs OBS	Del(17p), Del(11q), elevated B2M
Woyach 2018	IR vs Ibr	Age, Del(17p), LDH
Burger 2019	IR vs Ibr	None
Mato 2019	VenR vs Ven	None

Abbreviations: FR, fludarabine plus rituximab; F, fludarabine only; FCR, fludarabine, cyclophosphamide plus rituximab; Chl, chlorambucil; MR, rituximab maintenance; OBS, observation; IR, Ibrutinib plus rituximab; Ibr, ibrutinib; VenR, venetoclax plus rituximab; ven, venetoclax, WCC, white cell count, LDH, lactate dehydrogenate, *IGHV*, immunoglobulin heavy chain variable region gene, B2M, beta-2-microglobulin, Del-, deletion.

Table 3: Characteristics of studies reporting on PFS/OS in patients with CLL on rituximab-containing regimens (n=10).

Study	Source of data	Participant description	Sample size	Candidate predictors	Type of model	Model selection: stepwise selection, univariate p-values, no selection	Handling of continuous variables: retained as linear, categorised, dichotomised
Byrd et al 2005	Retrospective Cohort	All patients had received no prior therapy. CALGB 9712 open to accrual from 1997 to 1999 received rituximab either concurrently or sequentially with fludarabine. CALGB 9011, open to accrual from 1990 to 1994 received fludarabine.	N= 282 (FR: n=104, F: n=178) Males: 63%	sex, age, WCC, LDH, stage (I/II versus III/IV), splenomegaly.	Logistic regression (response rates) Proportional hazards model (PFS and OS)	Not stated	Not stated
Robak et al 2010	RCT	International, multicentre study conducted in Europe (88 centres, 17 countries). Patients ≥18 years with CD20+ CLL. Received prior treatment.	N = 552 (FCR: n=276, FC: n = 276) Males: 67%	age, disease stage, creatinine clearance, and lymphocyte count	Cox regression (response rates) Logistic regression (prognostic factors)	Not stated	Not stated
Hallek et al 2010	RCT	Previously untreated patients with CLL (30-81 years) in 190 centres and 11 countries in Europe, enrolled between July 2003 - March 2006	N = 817 (FCR: n = 408, FC: n = 409) Males: 74%	sex, age, disease stage, creatinine clearance, B2M, thymidine kinase, genomic aberrations, and *IGHV* status	Cox proportional hazard model	Stepwise backward selection	Categorised
Goede et al 2014	RCT	Multinational trial. CD20+ CLL untreated patients requiring treatment with coexisting conditions. Age ≥18 years.	N= 351 (R-Clb: n = 233, Clb: n = 118) Males: 62%	Genomic aberrations, *IGHV* mutational status	Not stated	Not stated	Categorised
Greil et al 2016	RCT	International trial conducted between April 2010 - Dec 2013 Patients ≥18 years following	N = 263 (MR: n = 134, OBS: n = 129)	Sex, cytogenetic risk group, *IGHV* mutation status, and CD38 expression	Cox regression model	Univariate p values	Categorized

		previous first-/second-line rituximab-containing CIT.	Males: 71%				
Dartigeas et al 2017	RCT	Multicentre phase 3 trial conducted between Dec 2007 - Feb 2014. Fit, treatment naïve CLL patients aged ≥65 years requiring treatment.	N = 409 (MR: n = 202, OBS: n = 207) Males: 66%	Age, sex, del(11q), Binet stage, *IGHV* mutational status, response to FCR.	Cox regression model	Not stated	Not stated
Robak et al 2018	RCT	International multicentre trial conducted between Jul 2009 - Dec 2011. Patients ≥18 years old with previously untreated, progressive CLL.	N = 66 (MR: n = 33, OBS: n = 33) Males: 68%	Age, sex, Rai stage, B2M level, ZAP-70, CD38 expression	Multivariate Cox's proportional hazards regression model	Not stated	Categorized
Woyach et al 2018	RCT	Multicentre trial conducted between Dec 2013 - May 2016. Patients ≥65 years, untreated CLL.	N = 364 (Ibr: n = 182, IR: n = 182) Males: 67%	ZAP-70 methylation status, Rai stage, del(17p13.1) or del(11q22.3)	Not stated	Not stated	Not stated
Burger et al 2019	RCT	Single centre trial conducted between Dec 2013 – Oct 2017, Included relapsed (87%) and treatment naïve CLL with high risk cytogenetics (13%) (17p- and TP53 mutation).	N = 208 (Ibr: n = 104, IR: n=104) Males: 70%	Cytogenetic risk factors (TP53 mutation/del17p, del11q without del17p or TP53 mutation), ECOG performance status.	Not stated	Not stated	Not stated
Mato et al 2019	Retrospective cohort	Multicentre, international study comparing outcomes of Relapsed/refractory CLL patients across 24 US and 42 UK centres.	N=321 (Ven: n = 270; [VenCombo – ven + R: n = 38, Ven + G: n = 13])	Del17p and Del11q status, BTK inhibitor exposure in a prior therapy, number of prior therapies, age, and complex karyotype	Cox regression	Not stated	Not stated

Abbreviations: RCT, randomised control trials, WCC, white-cell count; LDH, lactate dehydrogenate; CLL, Chronic lymphocytic leukaemia; PFS, Progression-free survival; HR, Hazard ratio; CIT, chemoimmunotherapy; R, rituximab; FCR, fludarabine, cyclophosphamide and rituximab; R-Clb, rituximab plus chlorambucil; G-Clb, Obinutuzumab plus Chlorambucil; MR, rituximab maintenance; OBS, observation arm; Ibr, ibrutinib; Ven, Venetoclax, B2M, Beta-2-microglobulin, Del-, deletion, *IGHV*, immunoglobulin heavy variable region gene, Del-, deletion, BTK, Bruton tyrosine kinase, ECOG, Eastern cooperative oncology group.

3.3. Risk of bias and quality assessment

We assessed the quality of all included studies using the QUIPS tool for assessing risk of bias in prognostic factor studies (Hayden et al., 2006). The study-level risk of bias assessment is presented in supplementary table S1. Briefly, two studies were scored as high-risk (Byrd et al., 2005; Mato et al., 2019), two as moderate risk (Greil et al., 2016; Woyach et al., 2018a), whilst the rest were deemed to be at low risk of bias (Burger et al., 2019; Dartigeas et al., 2017; Goede et al., 2014; Hallek et al., 2010; Robak et al., 2018, 2010). Overall, the included studies were scored as moderate for study participation (k = 0.86), good for study attrition (k = 0.93), excellent for prognostic factor measurement (k = 0.90), excellent for outcome measurement (k = 1), moderate for confounding measurement (k = 0.64), and excellent for statistical analysis and reporting (k = 0.64) (figure 2).

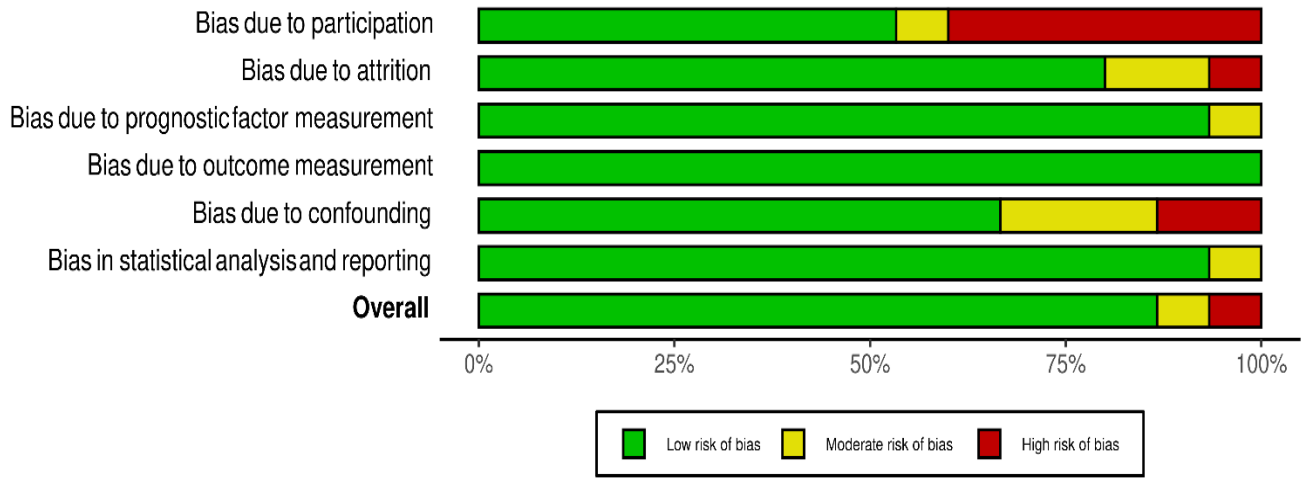


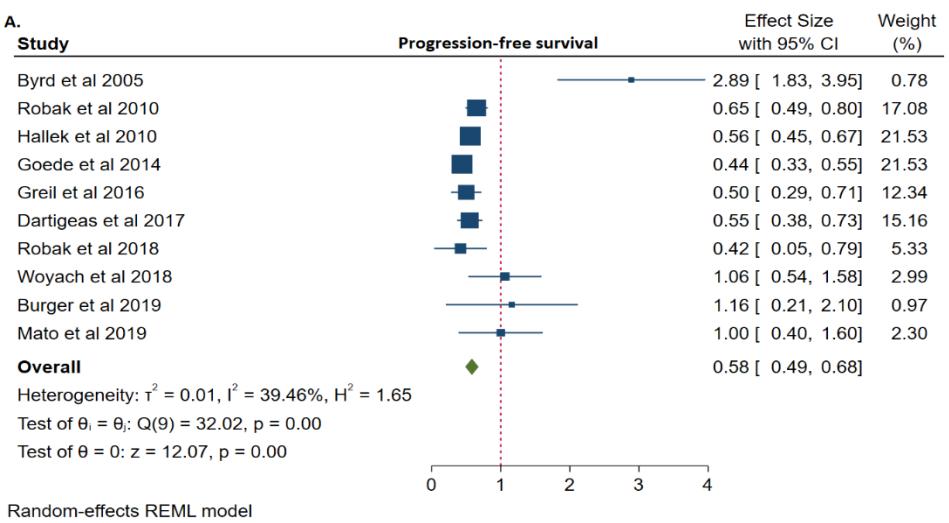
Figure 2. Risk of bias assessment of the prognostic factor studies.

3.4. Primary outcomes

3.4.1. PFS and OS in patients with CLL receiving chemoimmunotherapy with rituximab

A total of 6 studies (Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2018, 2010) reported on an improved PFS in patients with CLL, when rituximab was concurrently used with standard chemotherapy. In two studies (Burger et al., 2019; Woyach et al., 2018a), rituximab showed no therapeutic benefit in patients with CLL who were classified as having a high-risk cytogenetic profile and those who had relapsed. One study (Mato et al., 2019) reported no differences in PFS with the use of rituximab in combination with venetoclax in relapsed/refractory CLL patients.

The OS was reported in 8 studies, and in these studies, comparable survival rates were reported in RCTs (Burger et al., 2019; Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2010). There were no differences in OS in relapsed/refractory CLL patients treated with rituximab in combination with venetoclax (Mato et al., 2019). There were no substantial levels of heterogeneity among trials (I^2 = 39,46%) (Figure 3A). CIT with rituximab was associated with improved PFS (HR = 0.58 Cl [0.49-0.68], $p < 0.001$) (Figure 3A) and OS (HR = 0.77 Cl [0.63-0.91], $p < 0.001$) (Figure 3B). Notably, in one retrospective cohort study, reported on a higher OS in patients with CLL (Byrd et al., 2005). To explore whether this study modified the findings, we performed a sensitivity analysis based on study design (see supplementary table S3).



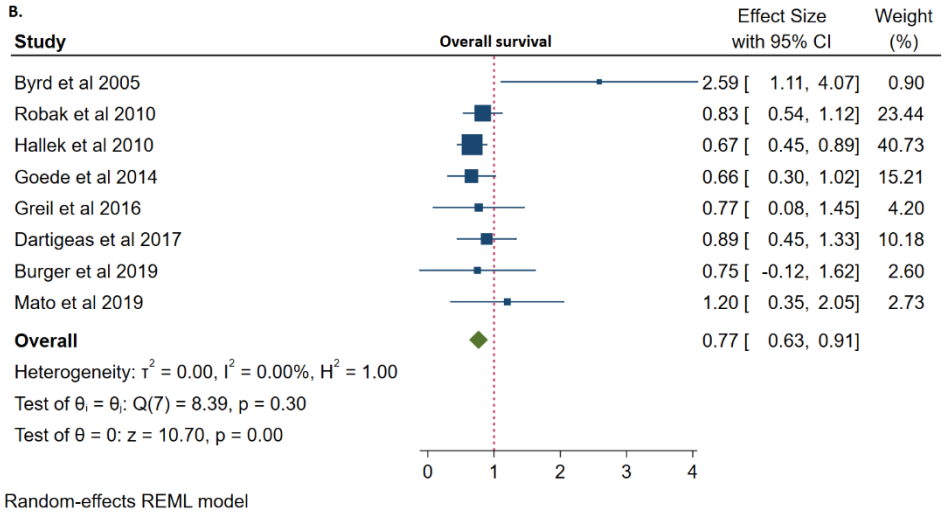


Figure 3: A. Meta-analysis of the hazards ratios (HR) for PFS for CLL patients treated with rituximab-containing CIT and standard chemotherapy alone. **B.** OS in rituximab-containing CIT and chemotherapy treated CLL patients.

3.4.2. Prognostic factors associated with poor patient outcomes in CLL patients

Prognostic markers ranged from host factors, such as age (Byrd et al., 2005) and cytogenetics, whereby six (60%) studies reported Del(17p) as a prognostic factor for PFS (Burger et al., 2019; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2018; Woyach et al., 2018a). One study (10%) excluded patients with Del(17p) (Dartigeas et al., 2017) and in separate studies, del(17p) and del (11q) did not impact PFS, (Greil et al., 2016; Mato et al., 2019)), whereas two studies reported unmutated *IGHV* as a prognostic factor (Greil et al., 2016; Hallek et al., 2010). Other factors included haematological parameters, such as increased WCC (of at least 50 x 10^9/L), which was reported in 20% (n = 2) of the included studies (Byrd et al., 2005; Hallek et al., 2010).

The reported prognostic factors associated with early disease progression included elevated LDH levels (Byrd et al., 2005), elevated B2M levels (levels of ≥3.5 mg/L) (Hallek et al., 2010; Robak et al., 2018), thymidine kinase (concentration of 10 μ/L) (Hallek et al., 2010) and advanced disease stage III/IV (Hallek et al., 2010; Robak et al., 2018). After adjusting for covariates, Del(17p), unmutated IGVH status, elevated B2M and LDH levels retained their predictive value (Table 4).

Table 4. Overview of confirmed prognostic factor included in the meta-analysis

Prognostic factors	Studies	Pooled HR[a]	Lower limit	Upper limit	I^2(%)	References
Cytogenetic						
Deletion 17p	3	4.88	0.14	9.62	72.45	(Hallek et al., 2010; Robak et al., 2018; Woyach et al., 2018b)
IGHV status	2	0.96	-0.06	1.99	94.02	(Dartigeas et al., 2018; Hallek et al., 2010)
Haematological						
White cell count	2	1.27	1.13	1.42	0.00	(Byrd et al., 2005; Hallek et al., 2010)
Protein factors						
β_2 microglobulin	2	0.96	-0.07	2.00	94.02	(Hallek et al., 2010; Robak et al., 2010)
LDH	2	1.20	1.05	1.35	4.41	(Byrd et al., 2005; Woyach et al., 2018b)

IGHV: Immunoglobulin heavy variable gene; LDH: Lactate Dehydrogenase; B2M: β_2-microglobulin

4. Discussion

We conducted a systematic review and meta-analysis of prognostic factors associated with poor survival in patients with lymphocytic leukemias on CIT. The available data on the use of ICIs in the management of lymphocytic leukemias is limited to predominantly European and American populations (Table 1). The current study also highlights the lack of multi-ethnic RCTs with diverse population with CLL. The

included studies reported on various candidate predictors of survival in patients with CLL on CIT (Table 3).

Amongst the reported prognostic factors only two protein factors (β_2-microglobulin and LDH) retained predictive value in patients with CLL on rituximab-containing CIT, after multivariable analysis. Only two other prognostic factors met our criteria for confirmed prognostic factors and these included, cytogenetic factors (deletion 17p, IGHV status) and hematological factors (white cell count $\geq 50x10^9$/L). Notably, in our meta-analysis we pooled studies that reported on adjusted estimates and the levels of statistical heterogeneity were high (I^2 >70%) for the confirmed cytogenetic factors and for β_2-microglobulin (Table 4). Interestingly, the value of β_2-microglobulin as an independent prognostic marker has not been extensively assessed in patients with CLL on CIT, although in a previous study its predictive value for treatment-free survival was retained after adjusting for factors such as CD38 expression and IGHV mutation status (Delgado et al., 2009).

The cut-off levels of B2M associated with poor prognosis remain unclear and in untreated CLL patients a value of 2 mg/l (A. M. Tsimberidou et al., 2007) while in our analysis B2M levels of ≥3.5 mg/L (Hallek et al., 2010; Robak et al., 2018) were associated disease progression in treated patients with CLL. Notably in the current analysis, we report on the retained predictive value of B2M in CLL patients on Rituximab-containing CIT. Future studies comprised of diverse patient populations are needed especially in minority ethnic groups to allow for validation of this prognostic marker in the era of CIT. In the era of CIT, and chemotherapy-free CLL management, future studies evaluating the correlations between B2M levels and expression of CD20 and other immune checkpoints in patients with CLL, may assist in the stratification of patients who are most responsive to immunotherapy.

The prognostic value of LDH has been reported in several cohorts of untreated patients with CLL (Autore et al., 2019; Li et al., 2017). In our analysis which comprised of treated patients with CLL, LDH levels retained predictive value in patients on CIT (Byrd et al., 2005; Woyach et al., 2018a). The levels of heterogeneity were low between the studies reporting on LDH levels in patients on CIT containing rituximab. Moreover, in these studies the incorporation of rituximab showed no clinical benefit in these cohort of patients with CLL. Future studies evaluating the correlations between B2M and LDH levels, CD20 expression and other immune checkpoints in patients with CLL are warranted in the chemotherapy-free era of CLL management. This will assist in validating the predictive value of this protein biomarker in patients on immunotherapy regimens.

To the best of our knowledge, this systematic review and meta-analysis provides the first analysis of prognostic factors in rituximab containing CIT. The current study has several limitations, firstly these findings are mainly derived from American and European populations. This limits the extrapolation of these findings into other low-to-middle income countries. Lastly, due to the low number of studies reporting on these prognostic factors in patients with CLL on CIT, we could not explore the sources of

heterogeneity in a subgroup analysis based on the potential differences in disease stage and duration of follow-up.

5. Conclusion

A plethora of novel prognostic factors have been described in untreated patients with CLL. However, in the era of CIT there is a lack of adequate studies exploring the predictive value of the conventional and novel prognostic factors in a multi-ethnic cohort of patients with CLL. In this systematic review and meta-analysis of prognostic factors, classical cytogenetic factors such as deletion 17p and 11q retained predictive value in patients with CLL on CIT. Lastly, the white cell count and conventional prognostic markers such as B2M and LDH levels were also regarded as confirmed prognostic factors in patients with CLL on rituximab containing CIT. These factors should be included in future prognostic factors in the era of CIT and chemotherapy-free era of CLL patient management.

Conflicts of interests

The authors declare that they have no known competing financial or academic interests that could have appeared to influence the work reported in this paper.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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Authors' contributions

ZAM, SAM and BBN conceptualised and designed the study. ZAM was responsible for the writing of the original draft and ZAM, BBN, TMN reviewed, edited, and approved the final manuscript. BBN is the guarantor of the study.

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CHAPTER 4: MANUSCRIPT 2 - EXPERIMENTAL PAPER

T cell function and immune checkpoint inhibition in chronic lymphocytic leukaemia

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(Under review in Leukaemia research)

Abstract

The aberrant expression of co-inhibitory proteins has been reported in patients with chronic lymphocytic leukaemia (CLL). Patient outcomes vary, with poorer prognosis in patients with advanced disease stage, unfavourable disease features and older age. Evaluating checkpoint inhibitor profiles may be useful to enhance immunologic activity against tumours in attempt to improve clinical outcomes in patients with CLL. Therefore, this study aimed at investigating T cell immune checkpoint inhibitor profiles in CLL. Furthermore, we investigate the correlation between immune checkpoint expression on T cells and beta-2-microglobulin (B2M) levels. This is a prospective cross-sectional study involving untreated patients with CLL. We evaluated immune checkpoint expression on T helper and cytotoxic T cells using a flow cytometry. In addition, baseline B2M and soluble PD-1 levels were also measured. After adjusting for age and sex, B2M levels strongly correlated with soluble PD-1 ($r = 0.65$, $p = 0.022$), and the surface expression of PD-L1 ($r = 0.60$, $p = 0.036$) and CD56 ($r = 0.63$, $p = 0.033$) on CD8+ T cells. The expression of PD-1, PD-L1 and CTLA-4 were increased following CD4+ T cell activation with PMA ($p<0.05$). There were no significant differences in the expression of PD-1 following CD8+ T cell activation ($p = 0.1826$), while the levels of CTLA-4 and CD56 were reduced ($p<0.05$). Conversely the expression of PD-L1 on CD8+ T cells was increased following activation ($p<0.05$). The blockade of PD-1 and PD-L1 axis on CD4+ and CD8+ T cells showed no effect in inhibiting expression levels of PD-1, PD-L1 and CTLA-4. Immune checkpoint profiling in patients with CLL and association between B2M and the PD1/PD-L1-axis may be useful in identifying patients that may benefit from PD-1/PD-L1 checkpoint based therapies.

KEYWORDS: Immune checkpoint inhibition, β2 microglobulin, T cell function, CD4 T helper cells, CD8 cytotoxic T cells

1. Introduction

The incidence of lymphoid leukaemia varies across ethnic groups (Dong et al., 2020). Chronic lymphocytic leukaemia (CLL) is the most prevalent type of adult leukaemia with an annual estimate of 21 250 new reported cases and accounts for almost 4 320 deaths in the Unites States (Siegel et al., 2021). There has also been an increase cancer burden in Sub-Saharan Africa with haematological malignancies including Hodgkin lymphoma (HL), Non-Hodgkin lymphoma (NHL) and leukaemia accounting for approximately 10% of reported cases (Gopal et al., 2016) . Although over the past decade there has been considerable advances in the diagnosis and prognosis, there is still a need to identify and validate biomarkers associated with poor prognosis in patients with CLL have been proposed (Lee and Wang, 2020). T lymphocytes play a central role in immune surveillance (Griggio et al., 2020) and the aberrant expression of immune checkpoints impair immune surveillance in patients with CLL (Griggio et al., 2020).

The CTLA-4 and PD-1/PD-L1 axis is amongst the most studied regulatory pathway in patients with CLL (Griggio et al., 2020; Waldman et al., 2020). Previous studies demonstrate reduced progression and prolonged survival following the inhibition of the immune checkpoints in B-cell leukaemia (McClanahan et al., 2015; Motta et al., 2005; Wierz et al., 2018). The shift towards modelling of immune checkpoint blockade under various immune activation and exhaustion conditions may assist in the stratification of patients with CLL who may benefit from immunotherapy (Roberts et al., 2021). β_2-microglobulin (B2M) levels remain the strongest predictor for overall survival and treatment-free survival (Delgado et al., 2009). Elevated levels of B2M in patients with CLL is linked with advanced clinical stage and increased tumour burden (Di Giovanni et al., 1989; Hallek et al., 1996; Molica et al., 1999; Robak et al., 2010; Tsimberidou et al., 2007; Wierda et al., 2007). The aim of this study was to investigate T cell immune checkpoint inhibitor profiles in treatment-naive patients with CLL. Furthermore, we investigate the correlation between immune checkpoint expression on T cells and β_2 microglobulin levels.

2. Methods

2.1 Study design and participant recruitment

This study comprised of 18 newly diagnosed patients with CLL who were recruited from the haematology clinic at King Edward Regional Hospital in Durban, South Africa (between April 2019 - November 2021), as well as nine (9) age- and sex-matched healthy volunteers. The study protocol was approved by the University of KwaZulu-Natal, Biomedical Research Ethics Committee (study approval no. BE456/18) and written informed consent was obtained from participants. We included patients with CLL who were newly diagnosed, and not on chemotherapy. The leukaemia diagnoses that were established and subtyped to identify CLL were performed using the 2008 WHO classification of

tumours of haematopoietic and lymphoid organs guidelines (Swerdlow, 2008) by qualified pathologists and clinicians. The diagnostic process involved investigations on the patient's full blood counts. In addition, morphological examinations of peripheral blood films under a microscope were conducted followed by flow cytometry immunophenotyping.

2.2. Haematological analysis

Peripheral blood from each participant was collected in ethylenediamine tetra-acetic acid tubes and processed within 1-2 hours of collection. Full blood counts (FBC) were performed by the automated Coulter AcT Diff haematology analyser (Beckman Coulter Inc., California, United States). Due to an important role in CLL diagnosis and progress, several parameters of FBC were selected, including white cell count (WCC), platelet count (PLT), haemoglobin (Hb), Haematocrit (HCT) and red blood cell (RCB).

2.3 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated using the density gradient centrifugation method. Briefly, 4 mL of the fresh whole blood samples were gently overlayed on 3 mL of the Ficoll-paque PLUS (Amersham, Biosciences, Uppsala, Sweden) on a polystyrene tube as previously described (Jaatinen and Laine, 2007). The samples were then centrifuged at 400 x g for 40 minutes without break, at 18 - 20°C. The PBMCs layer was transferred to Eppendorf conical tube and stored at -80°C.

2.4. T cell enrichment and isolation

T cells were negatively selected using the BD IMag isolation system (BD Bioscience. USA). Briefly, 50 µl of Biotinylated human T lymphocyte enrichment cocktail containing anti-human CD11b/Mac-1 (CR3), clone ICRF44, anti-CD16, clone 3G8, anti-CD19, clone HIB19, anti-CD36, clone CB38 (NL07), anti-CD41a, clone HIP8, anti-CD56, clone B159, anti-CD235a (Glycophorin A), clone GA-R2 (HIR2) was added to 50 uL PBMCs. The samples were then gently vortexed and incubated for 15 minutes at room temperature. To isolate T cells, we followed a negative selection method, whereby 50 µl of the BD IMag™ Streptavidin Particles Plus - DM (BD Bioscience. USA) were added to each sample, vortexed and incubated for 30 minutes at room temperature. The samples were then reconstituted into 1 mL of BD IMag buffer solution and then stored at -80 ºC.

2.5. Culture conditions and T cell stimulation assays

To determine immune checkpoint expression and inhibition in patients with CLL we performed T-cell stimulation and inhibition assays. Briefly isolated cells were cultured using media containing RPMI 1640 supplemented with 5% foetal bovine serum (FBS) and 1% liquid penicillin/streptomyosin (Biowest, USA) at 37° C and 5% CO_2. Saturated cells were split 1:2 at every 3 days.

To explore the differences in T cell responses in B cell leukaemia, we stimulated T cells isolated from patients with CLL. Briefly isolated cells (1 x 10^6) were incubated for 12 hrs with phorbol 12-myristate 13-acetate (12,5 ng/mL) (Cayman chemical, Michigan, USA). This concentration was chosen following EC50 calculations with varying concentrations of PMA (6.25 – 50 ng/mL) in 12 h and 24 h incubation period prior to the stimulation procedure. For inhibition assays, we incubated stimulated cells for 1 hr using purified anti-human CD279/PD-1 and anti-human CD274/PDL-1 antibodies (Elabscience, USA) at concentrations of 10 ug/mL (Ioannou et al., 2021; Stecher et al., 2017; Zelba et al., 2019). For the stimulation assays the following co-culture conditions were used; (1) Isolated T-cells; (2) Isolated T cells and anti-CD279 (3) Isolated T cells and anti-CD274.

2.6. Flow cytometry analysis of cell purity and viability

The purity of isolated T cells and viability of cultured cells was determined using the Zombie Aqua Fixable Viability Kit dye (Biolegend, San Diego, CA, USA). All data analysis was performed using De Novo analysis software version 7.10 (Pasadena, CA, USA).

2.7. Immunophenotyping of isolated T cells

Antibody titration assays were performed to determine the optimal antibody concentrations as previously described (Hulspas, 2010). Details of the final volumes used, source, and specificity of the monoclonal antibodies are provided in the supplementary File 2. Fifty microlitres of samples (1x10^6 cells) were stained with 5 µL of titrated volumes of the antibody cocktail containing CD3-PerCP, CD4-FITC, CD8-APC/Cy7, CD56-PE/Cy7, CD152-PE, CD279-BV421 and CD274-APC (BioLegend, San Diego, CA, USA). Cell viability was assessed using the Zombie Aqua Fixable Viability Kit (BioLegend). The samples were then gently vortexed and incubated in the dark for 15 minutes at room temperature. Lastly, the samples were reconstituted using 500 µl of staining buffer and analysed using the BD FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA). The De Novo analysis software version 7.10 (Pasadena, CA, USA) was used for data analysis.

2.8. Gating strategy

We identified viable T cells as Zombie Aqua negative CD3 positive events (Figure 1A). An unstained sample was used as a negative control and to correct for autofluorescence. T-helper and T-cytotoxic cells were identified as $CD4^+CD8^-$ and $CD3^+CD8^+$ respectively (Figure 1B-C) and the expression and CD279-BV421, CD274-APC, CD152-PE and CD56-PE/Cy7 on T-helper and cytotoxic T-cells was measured. At least 2 000 total events were acquired per sample at a medium flow rate.

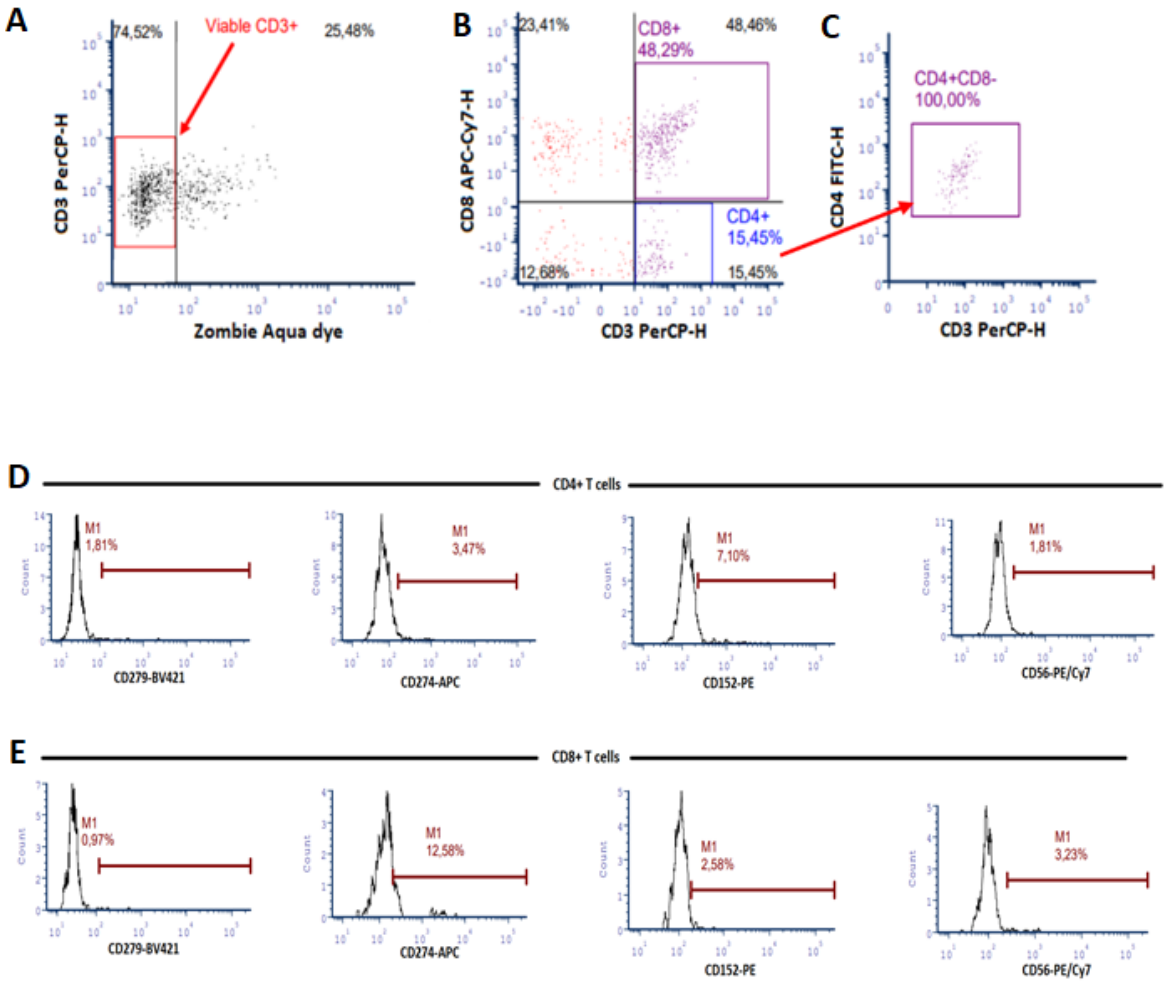

Figure 1: Gating strategy. Plot **A)** Illustrates the primary gate for viable T lymphocytes using the Zombie Aqua dye and CD3+ events. Plot **B)** shows further sub-classification of T-cells into T-helper cells (CD3+CD4+) and cytotoxic T-cells (CD3+CD8+). Panel **D**) demonstrates the measurements of Immune checkpoints on CD4+ T cells while panel **E)** shows the measures of immune checkpoints on CD8+ T cells.

2.9. Quantitative determination of soluble immune checkpoint inhibitors

2.9.1 Measurements of soluble PD-1 (sPD-1) and soluble beta-2-microglobulin levels

The serum levels of sPD-1 were measured using enzyme-linked immunosorbent assay kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instruction. Serum levels of sPD-1 were measured using enzyme-linked immunosorbent assay kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instruction.

Statistical considerations

Sample size estimation

We determined the minimum number of patients with CLL required to detect a large effect size (d) in the expression of immune checkpoints (CD274, CD279, CTLA4, CD56), following protein kinase C (PKC)-mediated T lymphocyte activation. The primary effect measure was the levels of immune checkpoint expression on T cells (CD4 T cells and CD8 T cells) post-stimulation (PMA) and treatment with immune checkpoint inhibitors (Anti-CD274 and anti-CD279). Based on our sample size estimation, a minimum of seventeen patients (n = 17) were required to detect a large effect size of 0.85 between the two dependent means using a repeated measures test (paired t-test), at 95.6% power and the alpha (α) set at 0.05. All sample size estimations were performed using the GPower 3.1.94 software (Universität, Germany).

All statistical analyses were performed using the GraphPad Prism software 6.0 (GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and $p<0.05$ was considered statistically significant. For normality testing, the Kolmogorov-Smirnov (KS) normality test was performed. The Mann-Whitney U test was used to compare non-parametric data, reported as median and interquartile range. For parametric data, an unpaired student t test was performed, and data was reported as mean and standard deviation. In functional studies, a repeated measures one-way ANOVA was used for analysis of differences between groups with Dunnett's as a post hoc test for multiple comparisons. Correlations between our marker for disease progression, B2M and immune checkpoint inhibitors was measured using Pearson correlation coefficient.

Results

Baseline characteristics and immune checkpoint expression on T lymphocytes in patients with CLL.

A Total of 27 participants were included in this study, 18 patients newly diagnosed with CLL as well as nine age- and sex-matched healthy controls. The demographics and characteristics of the control and CLL groups are illustrated in Table 1. The groups had similar age and sex distributions and shared similar socio-economic and ethnic origin. Increased expression of inhibitory receptors and ligands are key features of T cell exhaustion (Agresta et al., 2018; Dong et al., 2019; Wherry, 2011). Thus we measured expression levels of PD-1, PD-L1, CTLA-4 and CD56 from peripheral blood CD4+ and CD8+ T cells from patients with CLL compared with healthy controls, to determine whether they express these inhibitory markers. Consistent with previous reports (Brusa et al., 2013; Kondo et al., 2018; Riches et al., 2013), there was a significant increase in percentage expression of PD-1 and PD-L1 on CD4+ and CD8+ T cells from patients with CLL compared to healthy controls. There was also an increase in the percentage expression of CD56 on CD8+ T cells from patients with CLL compared to controls, as shown in Table 1.

Table 1: Baseline characteristics and immune checkpoint values of the participants

Parameters	Control group (n=9)	Patients with CLL (n = 18)	P-value
Age, years (IQR)	56.5 (62.75-50.50)	57 (68-55)	0.3904
Female: male	1 : 3	1: 1.8	
RBC (10^6/µL)	5.038 ± 0.7132	2.664 ± 0.6677	**<0.0001**
HCT (%)	44.80 (48.65-41.10)	29.70 (34.15-24.15)	**0.0010**
Hgb (g/dL)	15.41 ± 2.828	8.65 ± 1.86	**<0.0001**
WCC (10^3/µL)	4.833 ± 1.309	173.8 ± 132.70	**0.0012**
PLT (10^3/µL)	210.0 (263.5-154.5)	107.5 (175.0-75.75)	0.0519
%PD-1 on CD4	3.306 ± 1.627	6.978 ± 5.111	**0.0355**
%PD-L1 on CD4	3.558 ± 2.337	23.03.84 ± 7.963	**<0.0001**
%CTLA-4 on CD4	14.38 (37.40-10.52)	37.03 (44.34-26.40)	0.0757
%CD56 on CD4	3.110 (8.420-2.060)	3.645 (4.248-1.523)	0.4632
%PD-1 on CD8	0.900 (1.135-0.355)	4.165 (9.298-1.728)	**<0.0001**
%PD-L1 on CD8	10.24 ± 3.739	38.73 ± 13.49	**<0.0001**
%CTLA-4 on CD8	11.30 (18.15-5.940)	12.33 (20.10-5.265)	0.9799
%CD56 on CD8	2.910 (5.310-1.230)	9.240 (12.61-6.145)	**0.0009**

Abbreviations: IQR: interquartile rang; RBC; red blood cell; HCT: haematocrit; Hgb: haemoglobin; WCC: white cell count; PTL: Platelets; PD1: programmed cell death 1; PDL1: programmed cell death ligand 1; CTLA-4: cytotoxic T-lymphocyte-associated antigen 4; CD-: cluster of differentiation; s-PD1: soluble programmed death 1; B2M: beta-2-microglobulin.
Parametric data is presented as mean ± standard deviation (SD) and non-parametric data is presented as median (IQR).
Significant values are shown in boldface

β_2 microglobulin levels are associated with increased soluble PD-1 and PD-L1 expression on $CD4^+$, and $CD8^+$ T cells

The levels of B2M are independently associated with disease progression in patients with CLL (Hallek et al., 2010, 1996; Keating et al., 1993; Molica et al., 1999; Robak et al., 2018; Rossi et al., 2010; Tsimberidou et al., 2007; Wierda et al., 2009, 2007). Therefore, we assessed the relationship between immune checkpoint expression and basal B2M levels in patients with CLL. The levels of soluble PD-1 were associated with increasing B2M levels on patients with CLL (Figure 2A). Moreover, B2M levels were directly associated with the surface expression of PD-L1 on T helper cells ($r = 0.50$, $p = 0.035$) (Figure 2B) and cytotoxic T cells ($r = 0.52$, $p = 0.028$) (Figure 2C). After adjusting for age and sex, the correlation between %CD56 on CD8 T cells was found to be significant ($r = 0.63$, $p = 0.033$).

Table 2. Association between the levels of β_2 microglobulin and soluble PD-1, and immune checkpoint expression on T lymphocytes of untreated patients with CLL.

	N	Crude β	p-value	Adjusted β*	p-value
sPD1-1	18	0.57	**0.014**	0.65	**0.022**
T helper cells					
%PD-1	18	0.32	0.190	0.43	0.153
%PD-L1	18	0.50	**0.035**	0.60	0.053
%CTLA-4	18	0.39	0.114	0.58	0.056
%CD56	18	0.44	0.068	0.44	0.139
Cytotoxic T cells					
%PD-1	18	0.36	0.144	0.51	0.098
%PD-L1	18	0.52	**0.028**	0.60	**0.036**
%CTLA-4	18	0.35	0.145	0.55	0.079
%CD56	18	0.45	0.059	0.63	**0.033**

*Adjusted for Age and Sex. sPD-1, soluble programmed death 1; PD-1, programmed death 1; PD-L1, programmed death ligand 1; CTLA-4, cytotoxic T lymphocyte antigen 4, CD-, cluster differentiation.

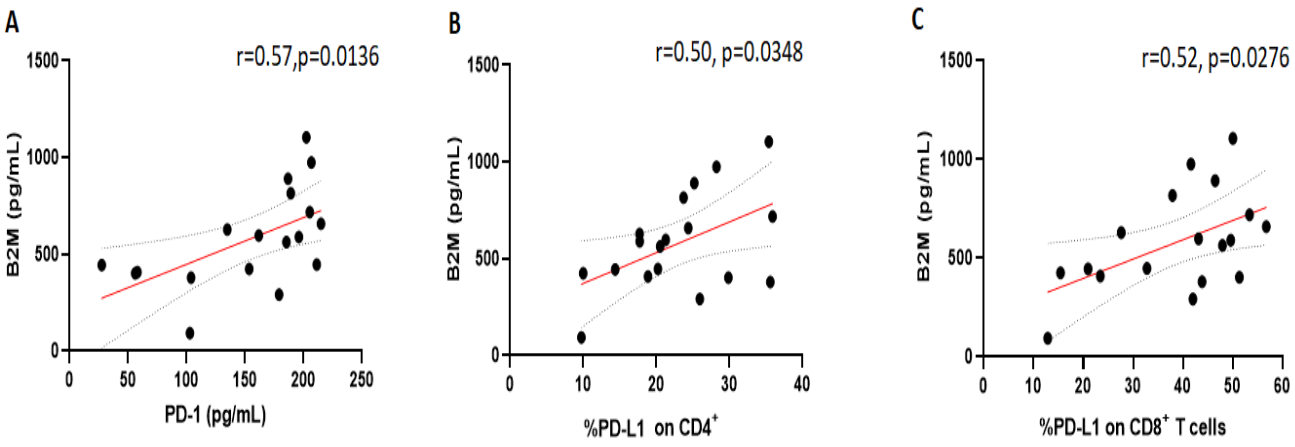

Figure 2: Expression of B2M correlated with **(a)** sPD-1 expression, **(b)** PD-L1 expression on $CD4^+$ T cells and **(c)** PD-L1 expression on $CD8^+$ T cells.

Immune checkpoint expression on CD4+ T-cells following protein kinase c activation and inhibition

Upon activation, T cells upregulate the surface expression of immune checkpoints (Keir et al., 2008; Walker and Sansom, 2011). We assessed the changes in expression of immune checkpoint proteins (CD56, CTLA-4, PD-1, and PD-L1) in patients with CLL post stimulation with phorbol 12-myristate 13-acetate (12,5 ng/mL) for 12hr and inhibition with anti-(PD-1 and PD-L1) monoclonal antibodies. Notably, there were significant changes in CD4+ T helper cells expressing PD-1 ($F_{(2.477, 42.12)} = 7.860$, $p = 0.0006$), PD-L1 ($F_{(2.669, 45.37)} = 28.61$, $p < 0.0001$) and CTLA-4 ($F_{(3, 67)} = 11.04$, $p < 0.0001$) following activation and inhibition with PD-1/PD-L1 blocking antibodies. As expected, the levels of PD-1 expression (baseline 0.79 ± 1.051 vs stimulation 6.978 ± 5.111; $p = 0.0003$), PD-L1 (baseline 5.466 ± 5.150 vs stimulation 23.03 ± 7.963; $p < 0.0001$) and CTLA-4 (Baseline 15.77 ± 14.12 vs stimulation 35.25 ±- 16.14; $p = 0.0039$) were increased following CD4 T cell activation (Figure 2A-C).

Furthermore, we investigated the effect of immune checkpoint inhibition on CD4+ T cells using anti-PD1 and anti-PDL1 at concentrations of 10 µg/mL. Interestingly, there was an increase in the levels of PD-1 ($p = 0.0016$), PD-L1 ($p < 0.0001$) and CTLA-4 ($p < 0.0001$) expression on CD4+ T cells following PD-1 immune checkpoint blockade. Similarly, there was an increase in the expression levels of PD-1 ($p=0.0009$), PD-L1 ($p <0.0001$) and CTLA-4 ($p = 0.0001$) on CD4+ T cells following PD-L1 blockade (Figure 2A-C).

Immune checkpoint profiles on CD8+ T cells following activation and PD-1/PD-L1 blockade in patients with CLL

To assess changes in immune checkpoint profiles of activated $CD8^+$ T cells in patients with CLL, we measured the expression of PD-1, PD-L1, CD56, and CTLA-4 on isolated $CD8^+$ T cells following PKC activation and PD-1/PD-L1 blockade. There were no statistically significant differences in PD-1 expression on $CD8^+$ T cells ($F_{(1.765,\ 30.01)} = 2.463$, $p = 0.1079$) following activation and PD-1/PD-L1 blockade (Figure 3E).

There were significant changes in the expression of PD-L1 on $CD8^+$ T cells ($F_{(2.529,\ 42.99)} = 11.53$, $p < 0.0001$). As expected, there was an increase in expression levels of PD-L1 following stimulation with PMA (baseline 23.04 ± 11.26 vs stimulation 38.73 ± 13.46, $p = 0.0057$). Unexpectedly, the same trend was observed following PD-1 ($p = 0.0003$) and PD-L1 blockade ($p = 0.0045$) (Figure 3F). Notably, immune checkpoint blockade was not effective at reducing PD-L1 expression on CD8+ T cells.

CTLA-4 expression on CD8+ T cells following PD-1 and PD-L1 blockade

There were statistically significant changes in the expression levels of CTLA-4 following CD8+ T cell activation and immune checkpoint blockade with PD-1/PD-L1 blocking antibodies ($F_{(2.286,\ 38.87)} = 6.062$, $p = 0.0037$). Moreover, there was a significant reduction in expression levels of CTLA-4 (baseline 47.10 ± 19.75 vs stimulation 16.85 ± 16.57; $p = 0.0007$) following activation. There were no statistically significant differences in expression levels of CTLA-4 following PD-1 ($p = 0.3810$) and PD-L1 ($p = 0.2307$) immune checkpoint blockade (Figure 3G).

CD56 expression is downregulated by PKC activation on CD8+ cytotoxic T cells

There were no significant differences in the expression of CD56 on CD4+ T-helper cells ($F_{(1.827,\ 31.06)} = 1.154$, $p = 0.3246$) following activation and PD-1/PD-L1 blockade (Figure 3D). There were statistically significant differences in CD56 expression on CD8+ T cells ($F_{(2.210,\ 50.09)} = 4.359$, $p = 0.0152$). The expression levels of CD56 were significantly reduced following stimulation with PMA (baseline 25.45 ± 13.59 vs stimulation 10.60 ± 7.035; $p = 0.0041$). We found no statistically significant differences in the expression levels of CD56 on CD8+ T cells following PD-1 ($p = 0.5809$) and PD-L1 ($p = 0.4614$) immune checkpoint blockade (Figure 3H).

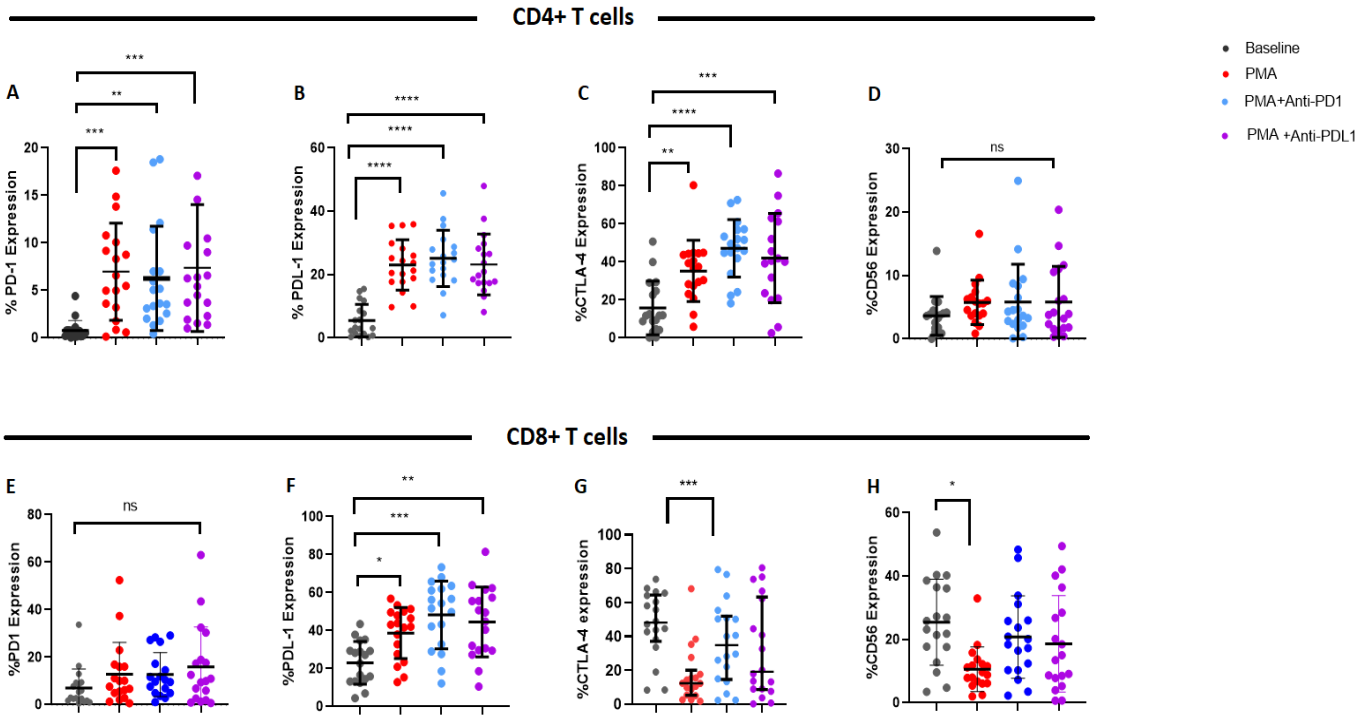

Figure 3: Immune checkpoint expression following PKC activation and immune checkpoint inhibition. **A-D** % immune checkpoint expression in T-helper cells. **E-H** % immune checkpoint expression in T-cytotoxic cells. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$, ns: not significant.

Discussion

The aim of this study was to assess the immune checkpoint profiles of untreated patients following T cell activation. We further explored the expression of immune checkpoint proteins in T-helper and T-cytotoxic cells following the ex vivo inhibition of PD-1 and PD-L1 pathway. In addition, we assessed the correlation between the selected immune checkpoints (CD56, PD-1, PD-L1 and CTLA-4) and an independent marker of disease progression (B2M) (Di Giovanni et al., 1989; Gentile et al., 2009; Hallek et al., 1996; Sagatys and Zhang, 2012). Elevated B2M levels are a prognostic factor in CLL (International, 2016). In the present study, B2M levels were directly proportional with s-PD1 after adjusting for age and sex. B2M concentrations were also correlated with PD-L1 expression on CD4+ and CD8+ T cells. The association between B2M and PD-L1 on CD4+ T helper cells was attenuated after adjusting for age and sex and the association between B2M and %CD56 on CD8+ T cells was significant (Table 2, Figure 2). These associations confirm a close relationship between PD-L1, CD56 expression on CD8+ T cells and poor prognosis and also suggests that B2M may determine the response to immune checkpoint-based immunotherapy in CLL.

β2M forms part of the major histocompatibility complex (MHC) class I antigen, also known as human leukocyte antigen (HLA) which, unlike MHC class II, is present on the surface of all nucleated cells including malignant cells (Nomura et al., 2014). A high tumour burden correlates with the levels of β2M making it a very useful marker for measuring the proliferation of leukemic cells and disease progression (Simonsson et al., 1980). Elevated β2M levels are also a hallmark of chronic inflammatory diseases correlating with poor prognosis, tumour burden and disease stage in CLL (Ali et al., 2020; Gentile et al., 2009; Wierda et al., 2009) and is associated with increased secretion of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) (Rozovski et al., 2013). In these bases, β2M inhibits the ability of immune cells to initiate a T cell response (Xie, 2003). High β2M levels observed in our cohort may also possibly reflect increased B2M synthesis as a consequence of T lymphocytes activation.

We found no statistically significant correlation between B2M and the expression levels of PD-1 on T helper and T Cytotoxic T cells (Table 2). Similarly, Rusak et al., also reported no significant correlation between B2M and the number of CD4+ T cells expressing PD-1 in newly diagnosed patients with CLL ($r = 0.3137$, $p = 0.1041$) (Rusak et al., 2015). On the other hand, it should be noted that our null findings suggest that B2M is expressed independently of PD-1 and does not regulate immunosuppression of the PD-1 axis in the tumour microenvironment. However, when evaluating PD-1 blockade therapy, B2M expression should be analysed since its upregulation can limit the efficacy of T cell mediated responses to checkpoint therapy (Cao et al., 2018).

In our study, the levels of PD-1, PD-L1 and CTLA-4 on CD4+ T-helper cells were increased following stimulation with PMA. The prognostic significance of PD-1 expression in CD4+ T cells have been studied on newly diagnosed patients with CLL at different disease stages (Rusak et al., 2015). Patients with advanced stage (Rai III/IV) had significantly higher frequency of CD4+ T cells expressing PD-1 compared to patients with low-grade disease (Rusak et al., 2015). In treatment-naïve patients, elevated levels of PD-1 (≥ 15.79%) on CD4+ T cells were associated with rapid disease progression and a need for earlier initiation of treatment (Rusak et al., 2015). In our study, 28% of treatment-naive patients with CLL did not express PD-1 on CD4+ T cells at baseline. The reported mean of all baseline values in our entire cohort was much lower than that reported previously (Rusak et al., 2015). The mean age of the patient cohort is comparable between studies, however, the patents in Rusak et al study are non-African and those with progressive disease were in receipt of immune chemotherapy which could explain these discordant findings. The present findings suggest that CLL patients with low levels of PD-1 expression at baseline, may not be at higher risk of disease progression. Low levels of PD-L1 expression were observed in treated patients with CLL and elevated PD-L1 levels (≥5%) were associated with complete response to treatment and durable overall survival in diffuse large B cell lymphoma and Richter's transformation (Younes et al., 2019).

The blockade of PD-1/PD-L1 or CTLA-4 immune checkpoints has revolutionized cancer immunotherapy (Griggio et al., 2020). High response rates and the safety profile of ICIs has been reported in treated patients with classical Hodgkin lymphoma (Younes et al., 2017). Although, controversial findings have been reported in patients with CLL (Ding et al., 2017; Jain et al., 2016; Younes et al., 2019). In previous studies, a reduction in the expression of CTLA-4 and PD-1/PD-L1 on T cells following ICI therapy has been reported (Hanna et al., 2021; Roberts et al., 2021; Rosskopf et al., 2019). In our functional experiments, we assessed the impact of PD-1 and PD-L1 blockade therapy on the expression of PD-1, PD-L1, CTLA-4, CD56 on CD4+ and CD8+ T cells. The blockade of PD-1 and PD-L1 axis showed no effect in inhibiting expression levels of PD-1, PD-L1 and CTLA-4 on CD4+ T cells. T helper cells in patients with CLL upregulated immune checkpoints even after immune checkpoint blockade, the plausible explanations for these findings could be that the concentration of blocking antibody used provided partial inhibition. The upregulation of immune checkpoints following blockade have not been reported elsewhere. In animal models, the expression of PD-1 and PD-L1 in mice treated with PD-L1 blocking antibody were significantly reduced as compared to isotype-treated mice (McClanahan et al., 2015). The blockade of PD-1/PD-L1 axis resulted in delayed disease progression and restored T cell function in CLL (Hanna et al., 2021). Patients with relapsed/refractory CLL had no confirmed response to immune checkpoint blockade by PD-1 specific antibody Pembrolizumab, meanwhile CLL patients with Richter's transformation with high PD-1 expression were responsive to PD-1 blockade (Ding et al., 2017).

In CD8+ T-cytotoxic cells, PD-L1 expression was increased following stimulation and PD-1/PD-L1 blockade. We did not observe significant differences in PD-1 expression on $CD8^+$ T cells post stimulation. In contrast, previous studies have reported on increased PD-1 levels on $CD8^+$ T cells from both newly diagnosed untreated patients and patients with relapsed disease (Brusa et al., 2013; Novák et al., 2015; Riches et al., 2013). In addition, Novák et al., reported a high proportion of CD8+ T cells expressing PD-1 in relapsed/refractory patients as compared to previously untreated patients with CLL (Novák et al., 2015). Notably, PD-1 expression was decreased following T cell activation on previously untreated patients with CLL when compared to healthy controls (Tonino et al., 2012). The upregulation of PD-1 on different subpopulation of the CD4+ and the CD8+ T cell subsets is widely described in patients with CLL, and elevated PD-1 levels are associated with poor patient outcomes (Arruga et al., 2020). Moreover, the upregulation of PD1/PDL1 on leukemic cells is associated with poor prognosis and advanced clinical stage in CLL (Taghiloo et al., 2017). In several malignancies PD-L1 expression is strongly correlated with PD-1 blocking antibodies (Grosso et al., 2013; Muenst et al., 2014; Zeng et al., 2011). Prior studies have observed a correlation between the expression of PD-1 and PD-L1 (Grzywnowicz et al., 2015). In our study, PD-L1 blockade did not regulate PD-1/PD-L1 expression on $CD8^+$ T cells. Interestingly, the dual blockade of LAG-3 and PD-1 enhances the effector function of $CD8^+$ T cell when compared to the sole blockade of PD-1 (Zelba et al., 2019) and also reduces tumour

growth (Wierz et al., 2018). While PD-L1 blockade in combination with ibrutinib targeted therapy regulates $CD8^+$ T cell function and aids disease control in CLL (Hanna et al., 2021).

The expression of CTLA-4 (CD152) on activated T cells downmodulates T cell responses (Pavkovic et al., 2003). We found no statistically significant correlation between B2M and the expression of CTLA-4 on CD4+ and CD8+ T cells. These findings may suggest that there is no direct association between CTLA-4 expression levels and disease progression in CLL. An increase in CTLA-4 expression on CD4+ T cells in our study is in agreement with previous findings on treatment-naïve patients with CLL (Motta et al., 2005). Despite prior evidence (Karabon et al., 2020; Motta et al., 2005), we observed a significant reduction in CTLA-4 expression on $CD8^+$ T cells following T cell activation in patients with CLL. The mean age of the participants in these cohorts is lower than that of the present study. The discrepancy in our findings may also be attributed to the stimulant used as CTLA-4 expression is dependent on the potency of T cell agonist and CD28-mediated signalling (Egen and Allison, 2002). Notably , Ciszak et al., showed a significant decrease in CTLA-4 expression on leukemic B cells from untreated patients with CLL (Ciszak et al., 2016). Increased levels of CTLA-4 are associated with poor prognosis, early disease progression (Kosmaczewska et al., 2005) and an advanced Rai stage in untreated patients with CLL (Motta et al., 2005). In our study the blockade of the PD-1/PD-L1 axis had no effect on the expression of CTLA-4 in patients with CLL.

In our study, CD56 expression was decreased following stimulation of CD8+ T cells. CD56 is a lineage marker for natural killer (NK) cells and terminally differentiated CD8+ cytotoxic T cells (Dorwal et al., 2015). In a recent study, elevated levels of CD56 in the bone marrow was associated with adverse patient outcomes (Sun et al., 2021). Studies on the prognostic significance of CD56 expression on CLL have been relatively scanty. In our study, we found no significant difference in CD56 expression following PD-1 and PD-L1 blockade. Our novel findings on the association between sPD-1, PD-L1 and B2M levels in untreated patients with CLL, may be useful in stratification of patients at risk of early disease progression and who may benefit from the early initiation of immunotherapy. B2M levels are strongly correlated with rapid proliferation and tumour burden (Huang et al., 2006; Tsimberidou et al., 2007). Lastly, although several prognostic markers have been described in patients with CLL, monitoring B2M levels in patients with CLL may also be valuable in predicting patient responses to immunotherapy targeting the PD-1/PD-L1 axis. A limitation of the present study is the lack of patient follow-up which limits our findings and provide no insight on the associations between the reported correlations and patient outcomes. Lastly, we did not measure cytokine profiles to assess T cell function.

Conclusions

Immune checkpoint blockade of PD-1 and PD-L1 axis was not effective in inhibiting the expression of PD-1, PD-L1 and CTLA-4 checkpoint molecules on T helper and cytotoxic T cells from treatment-naïve patients with CLL. However, there were novel associations between soluble PD-1 and PD-L1 and a marker for disease progression, B2M. Further studies should continue to investigate immune checkpoint inhibitor profiles of PD-1/PD-L1 axis taking into consideration the close relationship between the PD-1/PD-L1 axis and B2M which may be responsible for resistance to blockade therapy.

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Disclosure

The authors declare that there are no financial, personal, or professional competing interests that may interfere with this work.

Ethics

Ethical approval for this study was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (study approval no. BE456/18).

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Author's contributions

ZAM, BBN conceptualized, designed the study, and drafted the manuscript. ZAM, AV performed data curation. ZAM was responsible for the investigation, acquisition, analysis, or interpretation of data and writing of the original draft. BBN, TMN reviewed and edited the manuscript. BBN was responsible for supervision. All authors approved the final manuscript.

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CHAPTER 5: SYNTHESIS

The era of CIT and immune checkpoint inhibitors has transformed patient management strategies for chronic lymphocytic leukaemia (Knauf et al., 2015; Patel and Pagel, 2021; Seidel et al., 2018). The use of novel monoclonal antibodies such as rituximab, ibrutinib and immune checkpoint inhibitors such as PD-1 and PD-L1 blocking antibodies is however associate with variable patient responses and severe adverse events, resistance to treatment. (Huhn et al., 2001; Younes et al., 2019; Zhou et al., 2021) There is a need of systematic reviews and meta-analysis (SRMA) of prognostic factors which are crucial in identifying prognostic factors in multi-ethnic cohorts which would improve the risk stratification of patients with CLL. In the available prognostic factor studies, findings are inconsistent and there is a lack of multi-ethnic RCTs, and it remains unclear if ethnicity is a covariate that should be considered in establishing cut-off values for normal basal immune checkpoint expression levels.

Our meta-analysis of 3633 patients with CLL on rituximab-containing CIT, showed improved progression-free and overall survival in CLL patients on CIT as compared to standard chemotherapy. We also found that addition of rituximab to chemotherapy was a strong independent predictor for PFS or OS in patients with CLL. Our study demonstrated the benefit of adding rituximab to chemotherapy and adds further evidence of the efficacy of rituximab-containing CIT in a real-world setting. To date, the most comprehensive analysis which included three RCTs evaluating the efficacy of rituximab-containing CIT on 1421 patients with untreated and relapsed CLL showed that patients in the rituximab arm had improved ORR, CRR, OS and PFS when compared to patients on chemotherapy alone (Bauer et al., 2012). Rituximab-containing CIT have also demonstrated superior efficacy in terms of overall response, PFS/OS and disease control in patients with advanced stage indolent and mantle cell lymphomas (Schulz et al., 2007).

Prognostication at diagnosis is crucial in the stratification of patients into different risk groups to facilitate patient management and treatment decisions (Lee and Wang, 2020). In our meta-analysis we confirmed the prognostic value of known prognostic factors in patients with CLL on CIT which include, del(17p) (Hallek et al., 2010; Robak et al., 2018; Woyach et al., 2018a), unmutated IGHV status (Dartigeas et al., 2017; Hallek et al., 2010), WCC (Byrd et al., 2005; Hallek et al., 2010), LDH (Byrd et al., 2005; Woyach et al., 2018a) and B2M (Hallek et al., 2010; Robak et al., 2018)..

In our experimental study, we evaluated changes in T cell immune checkpoints following T cell activation and immune checkpoint inhibition in patients with CLL. Inhibitory molecules, prominently the PD-1/PD-L1 and CTLA-4 were upregulated on T cells in untreated patients with CLL following PKC activation. As previously described, PD-1, PD-L1 and CTLA-4 levels are significantly upregulated on CLL cells (McClanahan et al., 2015; Motta et al., 2005; Palma et al., 2017; Riches et al., 2013; Rusak et al., 2015; Scrivener et al., 2001). The aberrant expression of these inhibitory

molecules results in T cell exhaustion which plays a critical role in anti-tumour responses (Mckinney and Smith, 2016). Furthermore, we analysed PD-1, PD-L1, CTLA-4 and CD56 expression following PD-1 and PD-L1 blockade. Immune checkpoint blockade with PD-1 and PD-L1 blocking antibodies showed no effect on expression of PD-1, PD-L1, CTLA-4 on CD4+ T cells and expression of PD-L1 and CTLA-4 on CD8+ T cells. The divergent findings may be due the differences in stimulants used in previous studies and the concentration of inhibitors used which may have led to incomplete inhibition in our study. The immunophenotype and profiling of inhibitory molecules on T cells may be important in the management of patients with CLL and prediction of responses to immunotherapy.

Lastly, we evaluated the correlation between B2M and immune checkpoints. We report on a positive correlation between B2M and soluble PD-1, PD-L1 and CD56 expression on $CD8^+$ T cells after adjusting for age and sex. B2M levels are associated with tumour burden and advanced stage in CLL and patients with elevated B2M levels have inferior survival (Hallek et al., 2010; Robak et al., 2018; Stilgenbauer et al., 2013). In all, our findings suggest that targeted and guided treatment approach could be useful for patients with CLL with elevated B2M levels. Overall, cell-based prognostic markers and immune checkpoints are required to predict response to treatment, the novel finding of a direct association between B2M and the PD1/PD-L1-axis may be useful in identifying patients that may benefit from CIT.

This study provides important basal data from a South African cohort of patients with CLL and may be useful in guiding the management and treatment of patients with CLL. Moreover, future longitudinal studies are required to provide the needed clinical significance of these findings and the value of immune checkpoint profiling in patients with CLL at diagnosis. This could potentially improve the clinical outcomes of patients with CLL by guiding treatment choices and accurately predicting response to treatment.

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APPENDICES

APPENDIX A: PUBLISHED MANUSCRIPT – PROTOCOL FOR A SYSTEMATIC REVIEW

Study Protocol Systematic Review

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Prognostic value of CD20 antigen mediated immune checkpoint inhibition in patients with acute or chronic lymphocytic leukemia

A protocol for systematic review

Zekhethelo A. Mkhwanazi, BSc, Snenhlanhla A. Mfusi, BSc, Bongani B. Nkambule, PhD*

Abstract

Background: The addition of rituximab to standard chemotherapy has been shown to improve response rates in patients with acute or chronic lymphocytic leukemia. However, the prognostic factors associated with progression-free survival in rituximab treated patients with lymphocytic leukemias remains unclear. We will perform a comprehensive systematic review and meta-analysis on available data on prognostic factors associated with the clinical outcomes of patients with acute and chronic lymphocytic leukemia.

Methods and analysis: This protocol for a systematic review and meta-analysis of prognostic factors has been prepared following the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) 2015 guidelines. Electronic databases will be searched using keywords related to the objectives of this review. This systematic review and meta-analysis will include published randomized clinical trials, observational, prospective, and retrospective comparative cohorts. Two reviewers (ZAM and SAM) will independently screen studies, with a third reviewer consulted in cases of disagreements using a defined inclusion and exclusion criteria. Data items will be extracted using a predefined data extraction sheet. Moreover, the risk of bias and the quality of evidence were independently assessed using the quality in prognostic studies tool (QUIPS). The I2 and chi squared statistical tests will be used to analyze statistical heterogeneity across studies. An I2 values of > 50% will be considered substantial. All data analysis will be performed using STATA 16.0 (StataCorp LP, TX, USA). The outcomes examined will be progression-free and overall survival.

Ethics and dissemination: No ethical approval will be required and the findings of this meta-analysis will be published in a peer-reviewed journal.

Systematic review registration: International prospective Register of Systematic Reviews (PROSERO) number: CRD42021218997.

Abbreviations: CLL = chronic lymphocytic leukemia; R-chemo = rituximab plus chemotherapy.

Keywords: acute lymphoblastic leukemia, chemoimmunotherapy, chronic lymphocytic leukemia, rituximab

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Consent for publication: None.

Ethics and dissemination: This will be a review of existing studies and will not require ethical approval. The findings will be published in peer-reviewed and open access journals and presented at local and international conferences.

The authors have no conflicts of interest to disclose.

Supplemental Digital Content is available for this article.

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Key Points

- This systematic review and meta-analysis will be the first to synthesise the prognostic factors associated with Rituximab and the effectiveness of rituximab-based therapy in patients with acute and chronic lymphocytic leukemia.
- To our knowledge, this systematic review will offer a robust assessment of the evidence and quality of clinical and cell-based traditional and novel prognostic factors.
- The various disease stage and the severity of the disease in CLL/ALL patients included in the studies will be one of the limitations of this systematic review and meta-analysis.

1. Introduction

Chronic lymphocytic leukemia (CLL) is characterized by progressive proliferation and accumulation of functionally incompetent lymphocytes in the peripheral blood, bone marrow, lymph nodes, and spleen.[1,2] CLL is predominantly due to

profound defects in B lymphocytes and is also characterized by T-cell exhaustion,[3] whereas acute lymphocytic leukemia (ALL) involves aggressive accumulation of blasts in the bone marrow and has been the primary cause of cancer-related mortalities in children and adolescents.[2,4] CLL is considerably more severe in adult patients over the age of 65 years[5] and men are disproportionally affected, with a higher incidence than women.[6–9] This can be attributed to gender-specific hormonal differences or the variance in IGVH gene usage and mutational status.[10]

Several clinical and genetic-based prognostic markers have been established. In fact, the CLL international prognostic index (CLL-IPI) includes validated clinical, genetic, and laboratory features in the prognostication of patients with CLL.[11] The incidence of CLL varies widely across geographic locations, with a high distribution of chronic lymphocytic leukemia in most European and North American countries.[9] In comparison, ALL cases remain relatively high in North America, Northern and Western Europe, while lower rates are apparent in Asian and African populations[12] Notably, there is minimal data on the incidence or prevalence of lymphocytic leukemia in African countries. Based on currently available data on the prevalence of lymphocytic leukemia in multi-ethnic studies, CLL seems to affect Europeans more than Africans. The discrepancy in patient outcomes across racial and ethnic groups have been presented in some studies.[12–19] In studies including ethnic minorities, a poor overall survival rate was reported when compared with their white counterparts. Disparities could be partly attributed to demographic variables, varied biological responses, environmental and genetic factors as well as socioeconomic status between these racial groups.[20,21] Patient outcomes have improved remarkably over the years due to the adoption of risk-based therapy that is determined by patient characteristics and leukemia phenotype at diagnosis.[17] For several decades, high-dose standard chemotherapy has been the primary treatment, however, this regimen has no demonstrable improvement in overall survival.[22]

Recently, a novel approach using monoclonal antibodies aimed at restoring the immune system by targeting immune checkpoints proteins has provided effective and complementary treatments.[23] The use of fludarabine, cyclophosphamide, and rituximab (FCR) therapy significantly improves the progression-free and overall survival of patients with CLL.[24] To date, there are currently no published systematic review and meta-analysis providing cumulative evidence on the predictors of mortality or poor patient outcomes of patients with CLL on rituximab. This systematic review and meta-analysis will assess the available studies reporting on the prognostic factors and efficacy of combined chemoimmunotherapy containing rituximab. We will further synthesize the novel prognostic factors associated with poor clinical outcomes.

1.1. Research question

Does combining rituximab with standard chemotherapy improve progression-free survival of patients with CLL?

Which clinical and laboratory features are associated with poor prognosis and variable patient outcomes following rituximab therapy?

1.2. Objectives

To assess progression-free and overall survival in patients with CLL on rituximab or immunochemotherapy containing rituximab. Furthermore, to determine novel prognostic factors (cell-based proteins: CD38, ZAP70, CD49D; Serology: β2M, thymidine kinase, lactate dehydrogenase, Interleukin 8) associated with poor patient outcomes.

2. Methods

This protocol for a systematic review and meta-analysis has been prepared in accordance with Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols 2015 (PRISMA-P) guidelines. The protocol was registered with the online PROSPERO registry (CRD42021218997).

2.1. Study design

In this review, we will include randomized control trials, prospective and retrospective comparative cohorts.

2.1.1. Inclusion criteria. Only primary studies assessing the prognosis (progression-free and overall survival) of patients with CLL on rituximab-based therapy will be included. The search will be restricted to full-text human studies written in English.

2.1.2. Exclusion criteria. Cross-sectional and case-control studies will be excluded. In addition, review articles, letters, and editorials will be excluded.

2.2. Population

Patients with CLL on rituximab-based therapy, will be included.

2.3. Index prognostic factor

We will consider the predictive factors included in the widely used CLL International Prognostic Index (CLL-IPI).[25] We will also consider the predictive factors used in the German CLL Study Group (GCLLSG),[26] the MD Anderson Cancer Center (MDACC) nomogram[27] predictive models.

2.4. Comparators

The comparators will include patients receiving standard therapy or usual care.

2.5. Outcomes

The primary outcome will be the overall 5-year overall survival, and the secondary outcome will include 2- to 4-year progression-free survival.

2.6. Timing and setting

The predictive information and measurements at diagnosis and initiation of treatment will be considered. Moreover, we will include studies reporting on inpatient and outpatient cohorts.

2.6.1. Search strategy and study selection. The search strategy will be developed using medical subject headings (MeSH) for MEDLINE, and this will be adapted to EBSCOhost search headings terms. We will search the databases from inception to February 28, 2021. The search strategy will consist of search terms that include chronic lymphocytic leukemia, acute lymphoblastic leukemia, rituximab (Supplementary file 1, http://links.lww.com/MD/G614).

2.7. Data management

2.7.1. Data collection process. The reviewers (ZAM and SAM) will develop a structured data extraction form that will be used in the data extraction process. Mendeley referencing manager will be used in this systematic review. The screening of the articles will be independently assessed by 2 reviewers (ZAM and SAM).

2.7.2. Data items. The 2 reviewers (ZAM and SAM) will extract data items using the data items defined in the checklist for critical appraisal and data extraction for systematic reviews of prediction modelling studies for prognostic factors (CHARMS-PF).[28] This will include the following information: source of data, participant description, the study dates, sample size, predicted outcome including outcome measures (hazards, odds ratio), candidate predictors, handling of missing data, modeling method, and model performance.

2.7.3. Data simplification. Studies will be grouped according to the type of lymphocytic leukemia (CLL or ALL). In addition, studies will be grouped based on the, gender ratio, age of participants and chemotherapy regimen used (e.g., fludarabine, cyclophosphamide, ibrutinib), duration of intervention and follow-up.

2.7.4. Risk of bias in individual studies. To assess the potential risk of bias in the included studies the quality in prognostic studies (QUIPS) tool will be used.[29] Two authors (ZAM and SAM) will independently assess the included studies based on the 6 domains of the tool. In a case of disagreements, a third reviewer (BBN) will be consulted for arbitration.

2.8. Data synthesis

A summary of findings table (SoF) will be used to provide a synthesis of the main outcomes of included studies. Furthermore, if the included studies are homogeneous in terms of the type of lymphocytic leukemia treated, therapy used, and participant characteristics, data will be analyzed using a fixed-effects model. All data analysis will be performed using R statistical software (The R foundation for statistical computing, Vienna, Austria). The I^2 and chi-squared statistical tests will be used to analyze statistical heterogeneity between studies.[30,31] An I^2 value of >50% will be considered substantial heterogeneity.[32]

2.9. Subgroup analysis

To explore the sources of heterogeneity within the included studies, we will perform a subgroup analysis based on the study-level characteristics, including the risk of bias of the included studies, geographic location, intervention type (rituximab plus chemotherapy [R-Chemo] and chemotherapy regimens). Lastly, the reported measure of progression-free and overall survival will also be considered in the subgroup analysis.

2.10. Confirmation of predictive factors

The prognostic factors will be confirmed based on the consistency of the overall direction of the effect across the included studies. In addition, adjusted effect sizes that remain statistically significant ($P < .05$) after adjusting for covariates and multivariate analysis will be considered as confirmed.

2.10.1. Quality assessment of the cumulative evidence. The quality and strength of the evidence of the confirmed prognostic factors will be evaluated by 2 independent reviewers (ZAM, SAM) using the Grading of Recommendations Assessment Development and Evaluation approach (GRADE).[33]

2.11. Patient and public involvement

There is no patient or public involvement in the process of conducting this study.

3. Discussion

Rituximab-based therapy has demonstrated therapeutic benefits in the treatment of patients with lymphocytic leukemia. This systematic review and meta-analysis of prognostic factors will provide a comprehensive synthesis of studies reporting on progression-free survival in patients with CLL on a rituximab-based regimen compared with standard chemotherapy. Moreover, this review will provide a synthesis of traditional and novel cell-based prognostic factors. To our knowledge, this will be the first meta-analysis to evaluate the efficacy of R-chemo and predictive factors associated with progression-free survival in patients with ALL and CLL compared with chemotherapy alone. Findings from this study will provide insight into the prognostic factors in patients with CLL following R-chemo and will assist in the patient management and prognostication.

Author contributions

Zekhethelo A. Mkhwanazi, Snenhlanhla A. Mfusi, Bongani B. Nkambule conceptualized, designed the study, and drafted the protocol. Zekhethelo A. Mkhwanazi and Bongani B. Nkambule wrote the first draft. All authors approved the final manuscript. Bongani B. Nkambule provided supervision and is the guarantor of the review.

Conceptualization: Zekhethelo A. Mkhwanazi, Bongani B. Nkambule.
Data curation: Zekhethelo A. Mkhwanazi.
Methodology: Bongani B. Nkambule.
Supervision: Bongani B. Nkambule.
Writing – original draft: Zekhethelo A. Mkhwanazi.
Writing – review & editing: Snenhlanhla A. Mfusi, Bongani B. Nkambule.

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APPENNDIX B: SEARCH STRATERGY

Table S1: Supplementary Search strategy

Database	**Search terms**	**Synonyms/associated terms**	**Hits**
Medline	Concept 1 "Chronic lymphocytic leukemia"[MeSH]	CLL	23 862
	Concept 2 “Prognosis” [MeSH]	Prognostication, prognostic, prediction	794,891
	Concept 3 “Rituximab” [MeSH]	Rituxan, Mabthera, Rixathon, truxima	25,346

Combine concept 1, 2 and 3

Concept 1 AND 3 AND 4 = **242**

APPENDIX C: RISK OF BIAS ASSESSMENT

Table S2: Risk of bias assessment of individual studies using the QUIPS tool.

References	Study participation	Study attrition	Prognostic factor measurement	Outcome measurement	Study confounding	Statistical analysis & reporting	Overall
Byrd 2005	Low	Moderate	Low	Low	High	Low	High
Robak 2010	Low	Low	Low	Low	Moderate	Low	Low
Hallek 2010	Low	Low	Low	Low	Low	Low	Low
Goede 2014	Low	Low	Moderate	Low	Low	Low	Low
Greil 2016	Moderate	Low	Moderate	Low	Low	Low	Moderate
Dartigeas 2017	Low	Low	Moderate	Low	Low	Low	Low
Robak 2018	Moderate	Low	Low	Low	Low	Low	Low
Woyach 2018	Low	Low	Low	Low	Moderate	Moderate	Moderate
Burger 2019	Low	Low	Low	Low	Low	Low	Low
Mato 2019	High	High	Low	Low	Moderate	Low	High

Low = All domains were classified as having Low RoB, or up to one moderate RoB.

Moderate = mainly Low RoB-domains and up to two moderate RoB.

High = ≥ one domain with high RoB or ≥ three moderate RoB

APPENDIX D: SENSITIVITY ANALYSIS

Table S3: Sensitivity analysis of all included studies that reported on PFS/OS in patients with CLL with respect to the design of the trials**.**

Outcomes	Number of studies	Omitted studies	HR [95%CI]	I^2 (%)	Overall effect: Z, p-value
Progression-free survival (PFS)	**8** (Burger et al., 2019; Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2018, 2010; Woyach et al., 2018a)	**2** (Byrd et al., 2005; Mato et al., 2019)	0.55 [0.47, 0.62]	22.67	13.93, p < 0.0001
Overall survival (OS)	**6** (Burger et al., 2019; Dartigeas et al., 2017; Goede et al., 2014; Greil et al., 2016; Hallek et al., 2010; Robak et al., 2010)	**2** ((Byrd et al., 2005; Mato et al., 2019)	0.74 [0.59, 0.88]	0.00	10.10, p < 0.0001

APPENDIX E: PMA OPTIMIZATION

Fifty microliters (50 µL) of isolated T cells were added to 5 µL of four different PMA concentrations (6.25, 12.5, 25, and 50 ng/mL). The samples were then incubated with media at two time intervals (12h and 24h). After each time interval the samples were stained with Zombie Aqua Fixable Viability Kit dye (Biolegend, San Diego, CA, USA) and acquired using the BD FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA). The De Novo analysis software version 7.10 (Pasadena, CA, USA) was used for data analysis. The percentage of viable and dead cells was obtained. EC50 calculations were performed using GraphPad Prism software 6.0 (GraphPad Software, La Jolla, CA, USA) to determine PMA concentration and incubation time eliciting 50% of maximum stimulation (Figure 1).

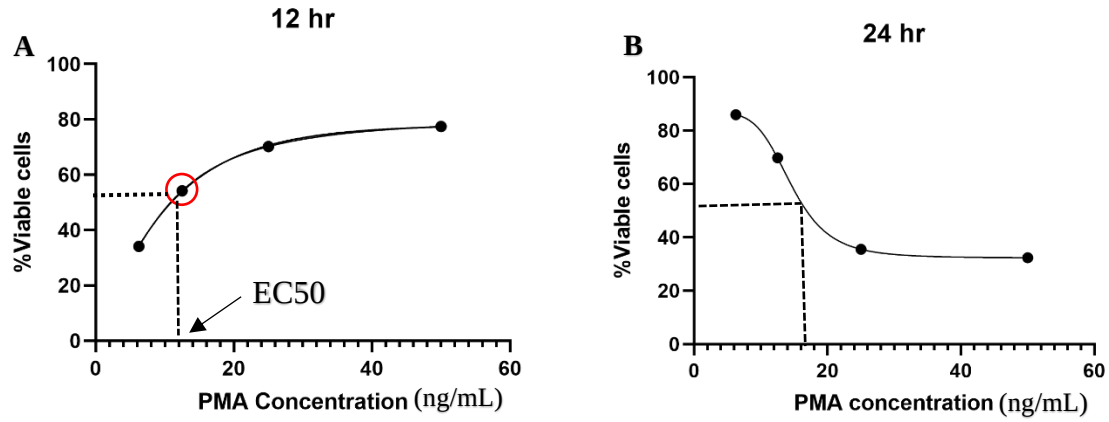

Figure 1: Viable cells at different PMA concentrations. **A)** illustrates viable cells at four different PMA concentrations at 12 h, **B)** shows viable cells at four different PMA concentrations at 24 h time period. 50% of maximum concentration was obtained at a concentration of 12,5 ng/mL in 12 h, hence this concentration and incubation time was used in functional experiments protocol.

APPENDIX F: FLOW OPTIMIZATION AND TITRATION

Compensation beads (VersaComp Antibody Capture Bead Kit from Beckman Coulter) were used to determine the percentage of spectral overlap and to perform colour compensation. Antibody titration assays were then performed to detect optimal antibody concentrations to be used when making up mAb cocktail to avoid nonspecific binding. The monoclonal antibody titration was performed as previous described (Hulpas 2010). Briefly, 5 tubes per mAb were prepared using a range (1 - 5 µL) antibody volume. For each tube, 50 µL of isolated T cells were stained with varying range of antibody volume. To ensure equal volume in all tube, PBS was added. The samples were then vortexed for a minute and incubated in the dark at room temperature for 15 minutes and acquired.

Kaluza version 1.2 (Beckman coulter, Inc Brea, CA) were used to analyse FCS file. Signal to noise ratios were then calculated using the formula below and the volume with the highest signal to noise ratio was chosen as the optimum antibody volume titration for each specific mAb used in a specific panel (Figure 1).

Formula: Signal-to-noise ratio = $\frac{Positive\ MFI}{Negative\ MFI}$ x 100

This process was implemented on all the mAbs that were used in this study. Figure 1 below illustrates how the optimum volume of CTLA-4 (CD152) was determined.

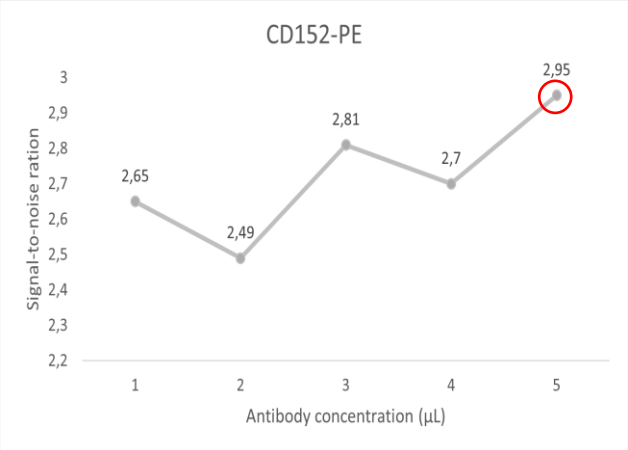

Figure 1: CD152-PE antibody titration. The positive and negative MFIs were used to calculate signal to noise ratio using the formula above.

APPENDIX G: IMMUNOPHENOTYPING

Table S4: Immunofluorescence staining and Flow cytometric analysis of lymphocytes subsets

Antigens	Fluorochromes	Manufacturer	Clone	Titrated volumes
CD3	PerCP	BioLegend	Clone HIT3a	5 µL
CD4	FITC	BioLegend	clone Sk3	5 µL
CD8	APC/Cy7	BioLegend	clone Sk1	5 µL
CD56	PE/Cy7	BioLegend	clone 5.1H11	5 µL
CD152	PE	BioLegend	clone L3D10	5 µL
CD274	APC	BioLegend	MIH1	3 µL
CD279	BV421	BioLegend	clone NAT105	3 µL

APPENDIX H: ETHICAL CLEARANCE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 October 2021

Mrs V Nundkissor (201267498)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Venishree@mut.ac.za

Dear Mrs Nundkissor

Protocol: The effect of immune check point inhibitors on Chronic Lymphocytic Leukaemia patients in KwaZulu-Natal.
Degree: MMedSc
BREC Ref No: BE456/18
New Title*: *The effect of immune check point inhibitors on lymphocytic leukaemias

We wish to advise you that your application for amendments listed below received 21 September 2021 for the above study has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee.

Amendments noted and approved:

1. Change of title to the new title above.
2. Additional co-investigators:
   Mr. Aviwe Ntsethe (213512600)
   Miss. Zekhethelo Mkhwanazi (216015946)

The committee will be notified of the above approval at its next meeting to be held on 09 November 2021.

Yours sincerely

[redacted]

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

APPENDIX I: TURNITIN DIGITAL REPORT

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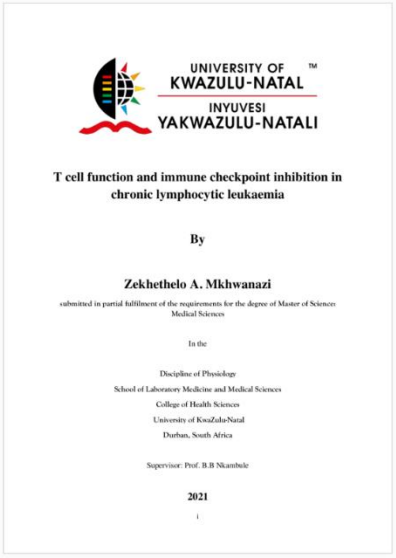

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T cell function and immune checkpoint inhibition in chronic lymphocytic leukaemia

By

Zekhethelo A. Mkhwanazi

submitted in partial fulfilment of the requirements for the degree of Master of Sciences Medical Sciences

In the

Discipline of Physiology
School of Laboratory Medicine and Medical Sciences
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