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Master's Thesis

CLINICAL IMPACTS OF CYTOKINE LEVELS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL).

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To my father

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Summary

This study investigated the impact of cytokine levels on overall survival and their associations with clinical and genetic characteristics in a cohort of 100 patients (males 53 (53.0% and females 47 (47.0%)). The median age of the patients was 67.92 years. Key clinical parameters such as hemoglobin levels, lymphocyte counts, and platelet counts were measured, along with genetic markers including IGHV mutation status and chromosomal abnormalities. The study identified significant correlations between elevated cytokine levels and shorter overall survival. Notably, eight cytokines—Eotaxin, IL-2, IL-5, IL-6, IP-10, MIP-1α, MCP-1 (MCAF), and PDGF-β—were associated with shorter overall survival rates when their levels were elevated. Pathway analysis highlighted the involvement of several key signaling pathways, including cytokine-cytokine receptor interaction and the JAK-STAT pathway. Pearson's correlation analysis further revealed significant relationships between cytokine levels and clinical parameters, suggesting the potential of cytokines as prognostic biomarkers. The findings underscore the importance of cytokine profiling in understanding disease mechanisms and guiding therapeutic strategies.

1. Introduction

1.1 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world with an incidence rate of 4.2/100.000 new cases per year (Eichhorst *et al*., 2021) . The incidence of CLL rises to over 30/100.000 new cases per year in individuals aged over 80 years, with a median age at diagnosis of 72 years. Approximately only 10% of CLL cases occur in patients younger than 55 years (Eichhorst *et al*., 2021). The risk of developing CLL results higher in male than female (male:female ratio of 1.5-2:1) and this gender disparity appears consistent across all ethnic groups (Ou *et al*., 2022).

CLL is defined by the World Health Organization (WHO) as an hematological B-cell neoplasm characterized by clonal proliferation and accumulation of mature B lymphocytes within the bone marrow, peripheral blood, and lymphoid tissues (Alaggio *et al.*, 2022) (Swerdlow *et al*., 2016). CLL is characterized by the accumulation of dysfunctional mature B lymphocytes with a distinctive immunophenotype featuring CD5, CD23, and CD19 expression, alongside dim surface expression of immunoglobulin, CD20, CD22, and CD79b (Swerdlow *et al*., 2016).

Molecular studies of the B cell receptor (BCR) show that 60%-65% of CLL cases have immunoglobulin heavy-chain variable (IGHV) gene with somatic hypermutations in their variable regions, a process that occurs in the germinal center and potentially could alter BCR antigen affinity (Gaidano *et al*., 2012).

On the other hand, 35%-40% of CLL cases lack somatic mutations in the IGHV genes (Hamblin *et al*., 1999; Kipps, 1993). Mutated IGHV genes in some CLL cases (M-CLL) suggest origin from germinal center-experienced B cells, while others with unmutated IGHV genes (UM-CLL) arise from B cells differentiated independently of the germinal center (Figure 1.1) (Chiorazzi and Ferrarini, 2011; Küppers *et al*., 1999; Stevenson *et al*., 2001) .

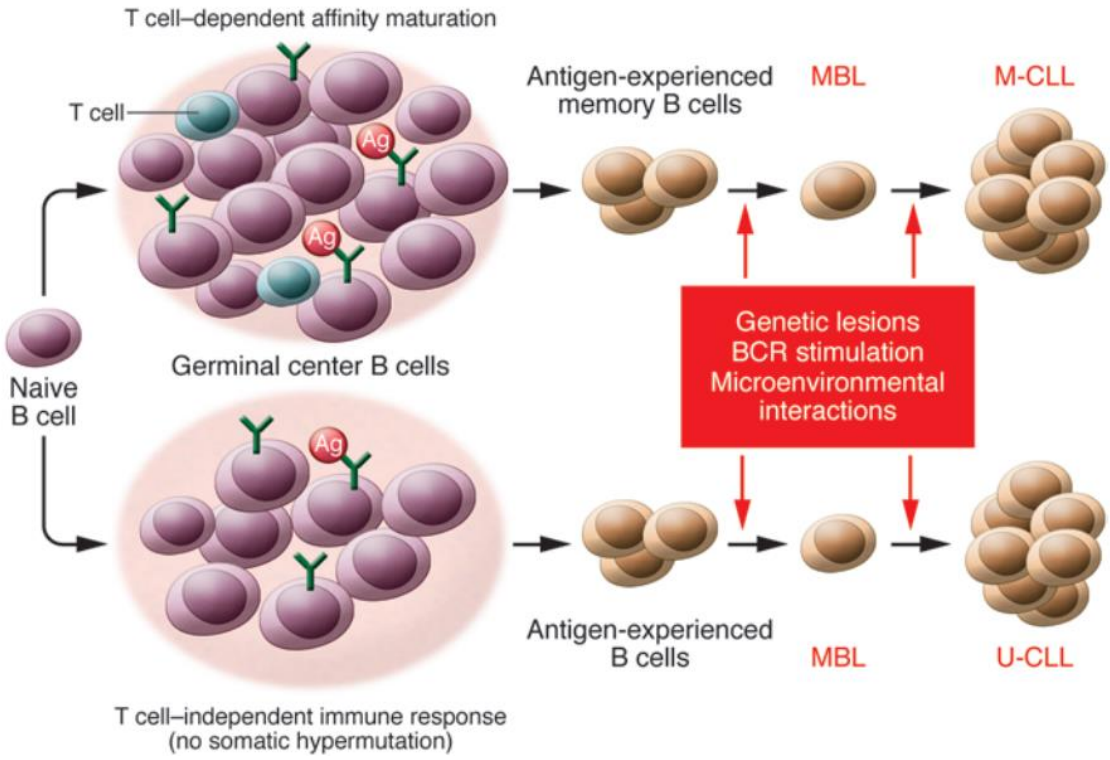

Figure 1.1 **Origin of CLL.** The figure illustrates the cellular origin of CLL, showing that it can arise from naive B cells following antigen exposure. This can occur via T cell-dependent reactions in germinal centers, leading to mutated IGHV genes in memory B cells (M-CLL), or through T cell-independent responses, resulting in unmutated IGHV genes (U-CLL). CLL's development is driven by genetic changes and interactions with antigens and the microenvironment, promoting growth and survival (Gaidano *et al*., 2012). MBL, monoclonal B-cell lymphocytosis; M-CLL, mutated CLL; U-CLL, unmutated CLL.

1.1.1 Pathogenesis

CLL is a multifactorial disease which exhibits marked heterogeneity from both biological and clinical perspectives (Gaidano and Rossi, 2017). Although the majority of CLL cases occur sporadically, a significant risk factor for CLL development is a familial history of CLL, observed in approximately 10% of the patients (Cerhan and Slager, 2015). The phenotype and the clinical outcome of familial CLL and sporadic CLL are indistinguishable, suggesting similar pathogenic mechanisms (Fabbri and Dalla-Favera, 2016).

Approximately 80% of the patients carry at least one of the most common chromosomal alterations, namely deletion of long arm of chromosome 13 and 11

[del(13q14) and del(11q22-23)], del short arm of chromosome 17 [del(17p13)] and trisomy 12 (Gaidano and Rossi, 2017). A hierarchical prognostic model was established identifying del(17p13) and del(11q22-23) as independent predictors of faster disease progression and inferior survival, whereas del(13q14) occurring as the sole abnormality is associated with a favourable outcome (Döhner *et al*., 2000).

Del13q is the most frequent cytogenetic abnormality, found in 55% of cases and it encompasses *DLEU2* gene, responsible for encoding miR-15a and miR-16-1. These miRNAs play crucial roles in regulating apoptosis and cell cycle arrest (Garding *et al*., 2013).

Del11q22-23 is the second most frequent aberration, found in 20% of CLL patients and in most cases affects the *ATM* gene, the deficiency of which leads to genomic instability and increases the risk of lymphoid malignancies when inherited (Goy *et al*., 2017; Rossi and Gaidano, 2012). In some instances of this deletion, the *ATM* gene remains unaffected, while the nearby *BIRC3* gene is impacted (Gaidano *et al*., 2012).

Currently, only a limited number of target genes for these recurrent lesions have been elucidated. The adverse prognosis associated with del(17p13) is attributed to the alteration of the tumor suppressor gene *TP53* (Gaidano *et al*., 1991). In patients with del(17p), the non-deleted *TP53* allele frequently undergoes a loss-of-function mutation, resulting in biallelic gene inactivation (80% of cases) (Edelmann *et al.*, 2012; Rossi *et al*., 2014).

Trisomy 12 is observed in 10–20% of CLL patients, yet the specific genes implicated in the pathogenesis of CLL with trisomy 12 remain largely unidentified. Additionally, the prognostic significance of trisomy 12 remains contentious (Seiffert *et al*., 2012).

Genomic studies have refined the understanding of CLL, pinpointing key driver genes and revealing that 5%-10% of untreated cases involve a *TP53* gene disruption. Recent whole exome sequencing has uncovered consistent genetic alterations in genes associated with various biological pathways, which may be crucial for understanding the pathogenesis of CLL. These genes include *NOTCH1*, *SF3B1*, *BIRC3*, and *MYD88.* About 10%-15% of CLL patients develop Richter Syndrome (RS) within 5-10 years, with *TP53, NOTCH1*, and *MYC* being common genetic alterations (Gaidano *et al*., 2012).

Additionally, the *XPO1* gene, coding for a crucial protein in cellular transport, often undergoes gain of function mutation in CLL (Moia *et al*., 2023; Moia and Gaidano, 2024).

1.1.2 Diagnosis and Staging

The diagnosis of CLL requires the presence of ≥5000 B-lymphocytes/µL in the peripheral blood consistently for at least 3 months (Hallek and Al-Sawaf, 2021). A flow cytometry test of peripheral blood is typically necessary and often sufficient for establishing a diagnosis of CLL (Hallek *et al*., 2018).

Typical immunophenotype of CLL cells includes expression of CD5, CD23, and CD19 and dim surface expression of immunoglobulin, CD20, CD22, and CD79b (Alaggio *et al*., 2022). Additionally, each clone of leukemic cells exclusively expresses either kappa or lambda immunoglobulin light chains (Campo *et al.,* 2022; Rawstron *et al.,* 2018).

CLL can manifest with a spectrum of clinical presentations; approximately 70% of CLL patients are asymptomatic at diagnosis, often detected incidentally due to unexplained lymphocytosis (Abrisqueta *et al*., 2009). Among symptomatic CLL patients, around 50% exhibit symptoms related to lymphadenopathy, while hepatosplenomegaly accounts for symptoms in approximately 20% to 50% of cases. Additionally, 5% to 10% of patients experience significant weight loss along with fever, night sweats, or extreme fatigue (B symptoms). Cytopenia may occur due to bone marrow infiltration or immune-mediated complications such as autoimmune hemolytic anemia (Nabhan and Rosen, 2014).

Two staging systems, Rai and Binet staging systems, are currently applied to CLL patients to define disease burden and treatment indication after diagnosis (Kipps *et al*., 2017).

The Rai staging system classifies patients based on lymphocytosis, lymphadenopathy, organomegaly and cytopenia (anemia and thrombocytopenia). The original 1975 classification includes five stages, but in 1987, a modification was proposed, condensing it into three stages, with stage 0 corresponding to low risk, stages I and II to intermediate risk, and stages IV and V to high risk. Low-risk disease is characterized by lymphocytosis with leukemic cells in the blood and/or bone marrow (lymphoid cells >30%) (former Rai stage 0). Intermediate-risk disease (formerly considered Rai stage I or stage II) encompasses patients with lymphocytosis, enlarged nodes in any site, and splenomegaly and/or hepatomegaly (lymph nodes being palpable or not). High-risk disease involves patients with disease-related anemia (Hemoglobin [Hb] level <11 g/dL) (formerly stage III) or thrombocytopenia (platelet count <100 x 10^9/L) (formerly stage IV) (Rai *et al*., 1975).

Conversely, Binet staging system, published in 1981, classifies patients based on enlarged lymph nodes (>1 cm) and organomegaly, along with anemia or thrombocytopenia. Binet staging system defines stage A (lymphocytosis and ≤2 involved lymph node areas); stage B (lymphocytosis and ≥3 involved lymph node areas); and stage C (lymphocytosis, Hb<10 g/dL and/or platelet count <100 x 10^9 /L (Hallek and Al-Sawaf, 2021).

Due to advancements in CLL therapy, the two clinical staging systems have become insufficient to distinguish prognostic sub-groups (Pflug *et al*., 2014). The currently most relevant prognostic score is the CLL International Prognostic Index (CLL-IPI) (The International CLL-IPI working group, 2016). It uses a weighted grading of five independent prognostic factors: *TP53* deletion and/or mutation (collectively called *TP53* dysfunction), IGHV mutational status, serum β2-microglobulin, clinical stage, and age. The CLL-IPI separates four groups with different survival at 5 years. The prognostic value of the CLL-IPI is currently revisited for the use of targeted agents (Hallek and Al-Sawaf, 2021).

1.1.3 Prognostic markers in early-stage CLL

CLL's heterogeneity necessitates prognostic markers to identify high-risk patients for tailored treatment strategies, including early intervention and response to Bruton tyrosine kinase inhibitor (BTK) or B-cell lymphoma 2 (BCL2) inhibitors (BTKi, BCL2i). Initially based on IGHV mutation and FISH karyotype, the prognostic landscape now includes various clinical and molecular markers, crucial for precision medicine and patient management (Moia and Gaidano, 2024).

Some patients may remain untreated throughout their lives, while others face aggressive disease progression with limited therapeutic response. This diversity in prognosis is partly attributed to the mutational status of the IGHV, distinguishing between IGHV-mutated (M-CLL) and IGHV-unmutated (UM-CLL) subtypes. Traditionally, IGHV-mutated CLL is associated with a more favorable prognosis, whereas the IGHV-unmutated subtype tends to indicate a poorer prognosis, likely due to differences in genetic lesions, degree of clonal evolution, epigenetic alterations, activated signaling pathways, and interactions with the microenvironment in lymph nodes or bone marrow (Fabbri and Dalla-Favera, 2016).

International Prognostic Score for Early stage CLL (IPS-E) is a simple and robust prognostic model that predicts the likelihood of treatment requirement in patients with early-stage CLL. The IPS-E was created using data from 4933 patients, with TTFT as the main outcome. It considers three factors: unmutated IGHV status, lymphocyte count over 15×10^9/L, and detectable lymph nodes, each correlating with a quicker approach to treatment. The model categorizes patients into low (score 0), medium (score 1), or high risk (score 2-3), with 5-year TTFT rates varying significantly from 61.2% in low-risk to 8.4% in high-risk groups. (Condoluci *et al*., 2020).

Some patients exhibit an indolent form that defers the need for treatment, while others present with a rapidly advancing condition necessitating immediate intervention, which may undergo histologic transformation and evolve into a more aggressive disease termed as Richter syndrome (RS), which morphologically mimics diffuse large B cell lymphoma (DLBCL) (Gaidano *et al*., 2012).

Disruptions in *TP53* (50%–60%), activations of *NOTCH1* (30%), and abnormalities in *MYC* (20%) are the predominant recurring genetic alterations observed in RS. These genetic changes often arise during the transformation phase, highlighting their pathogenic relevance in the development of the DLBCL phenotype (Fabbri *et al*., 2011; Rossi *et al*., 2011). In RS, *NOTCH1* mutations typically occur without concurrent *MYC* oncogenic activation (Fabbri *et al*., 2011; Weng *et al*., 2006). Moreover, *NOTCH1* mutation and *MYC* deregulation frequently coincide with *TP53* inactivation in tumors, thus contributing to a dual-hit genetic mechanism of transformation (Fabbri *et al*., 2011). Genetic lesions of *TP53*, *NOTCH1*, and *MYC* account for many (∼60%), though not all, cases of RS (Fabbri *et al*., 2011; Rossi *et al*., 2011).

1.1.4 Treatment

Over the past decade, the treatment landscape of CLL has undergone significant changes. Initially, chemoimmunotherapy was the standard for all patients, but its use has been refined to young, fit patients without adverse prognostic markers. Most recently, there has been a move towards continuous single-target drugs and, lastly, to fixed-duration (FD) combination therapy (Visentin *et al*., 2024). In general practice, patients with

asymptomatic early-stage disease (Rai 0, Binet A) do not require intervention and are monitored following the watch and wait approach, unless there is evidence of disease progression or disease-related symptoms (Hallek *et al*., 2018). Blood cell counts and clinical examinations should be carried out every 3-12 months after the first year, when 3-monthly intervals should be applied for all patients (Eichhorst *et al*., 2021).

Front-line treatment decision includes an assessment of IGHV and *TP53* status (IGHV UM and *TP53* mutated present aggressive features of CLL), as well as patient-related factors such as comedication, comorbidities, preferences, drug availability and potential of treatment adherence (Eichhorst *et al*., 2021). Different treatment regimens are available for front-line therapy (Eichhorst *et al*., 2021). In patients with unmutated IGHV genes, *TP53* wild type and less than 65 years of age, the disease is sensitive to chemoimmunotherapy and could therefore be treated with the FCR regimen, consisting of fludarabine, cyclophosphamide and the monoclonal anti-CD20 rituximab. If the patient is older than 65 years of age, the BR regimen is administered (rituximab and bendamustine) (Eichhorst *et al*., 2021; Fischer *et al*., 2016). Another option, currently more recommended, is treatment with first generation Bruton-tyrosine kinase inhibitor (BTKi) ibrutinib (Gribben et al., 2018).

Chemoimmunotherapy is associated with a spectrum of systemic adverse effects, including the potential induction of secondary malignancies. Conversely, BTKi, which are administered indefinitely, exhibit a quantifiable cardiotoxic profile. Consequently, the optimization of therapeutic regimens necessitates a comprehensive risk assessment tailored to each patient (Eichhorst *et al*., 2021). This includes a thorough evaluation of cardiac comorbidities, as well as pulmonary function, coagulation status, and others. In instances where ibrutinib is not well-tolerated, the therapeutic strategy may shift to the second-generation BTKi, acalabrutinib (Eichhorst *et al*., 2021; Gribben *et al*., 2018). This alternative is associated with a reduced incidence of adverse reactions while maintaining comparable efficacy, attributable to its enhanced selectivity for BTK over other kinases. For patients contraindicated for the aforementioned treatments, the CLBO protocol, comprising the chemotherapeutic agent chlorambucil and the monoclonal anti-CD20 antibody obinutuzumab, or the BCL2 inhibitor venetoclax in conjunction with obinutuzumab, may be considered (Eichhorst *et al*., 2021). The latter combination has demonstrated superior effectiveness to chemoimmunotherapy with a more favorable side effect profile. In contrast to BTKi, the venetoclax and obinutuzumab regimen offers a fixed

treatment course and improved tolerability, despite the requirement for intravenous delivery of the monoclonal antibody. In comparison, both BTKis and venetoclax are orally administered (Fischer Kirsten *et al*., 2019).

If the patient has a genetic abnormality of *TP53* (with unmutated IGHV), chemoimmunotherapy is never recommended. Instead, first-line treatment of these aggressive forms of CLL should preferentially be based on ibrutinib or acalabrutinib, as venetoclax based regimens have been shown to be less effective. Emerging therapies for CLL, including BTKi and venetoclax, have been associated with the emergence of drug resistance (Eichhorst *et al*., 2021). This resistance often manifests through the clonal selection of mutations within the *BTK* and *BCL2* genes, or via the upregulation of proteins downstream in the BTK and BCL2 pathways impeding the drugs' ability to bind covalently to their targets (Blombery *et al*., 2020; Quinquenel *et al*., 2019). Consequently, second-line treatments are tailored to employ alternative therapies that the patient has not yet resisted (Eichhorst *et al*., 2021). To address this resistance, ongoing clinical trials are exploring the efficacy of noncovalent BTKi like pirtobrutinib and various immunotherapeutic approaches (Frustaci *et al*., 2023; Perutelli *et al*., 2022).

Treatment for DLBCL-type RS primarily utilizes the R-CHOP chemoimmunotherapy regimen, comprising rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone. However, its efficacy is notably limited in patients with this disease (Mouhssine and Gaidano, 2022). This limitation has catalyzed the exploration of novel therapeutic avenues, currently under clinical investigation. Among these, bispecific antibodies, immune checkpoint inhibitors, and CAR-T cell therapies represent the most promising strategies for enhancing treatment outcomes (Mahmoud *et al*., 2023).

1.2 Cytokines in CLL

1.2.1 CLL Microenvironment

The development and progression of CLL are intricately linked to the tumor microenvironment (TME), which provides vital support to leukemic cells (Svanberg *et al*.,

2021). Accumulation of leukemic cells in specialized niches such as lymph nodes, spleen, and bone marrow allows for multi-directional signal exchange between CLL cells and the surrounding myeloid cells, mesenchymal stromal cells, T- and NK-cells, collectively creating a leukemia-supportive niche (Nguyen *et al*., 2019). This communication facilitates the creation of a leukemia-supportive niche that fosters the growth and survival of CLL cells (Vitale *et al*., 2021). These dynamic interactions promote a self-sustaining multicellular network where CLL and microenvironmental cells continuously interact, leading to altered phenotypes and subsequent acquisition of divergent functions. (Nguyen *et al*., 2019). The altered phenotypes and functions of these cells contribute to the complex pathology of CLL, including enhanced survival of leukemic cells, increased cellular proliferation, evasion of immune surveillance, and development of resistance to therapeutic interventions (Skånland and Mato, 2021).

1.2.1 Imbalanced cytokines expression in CLL

Cytokines and chemokines play a significant role in mediating this crosstalk. Cytokines, typically less than 40kDa, are proteins secreted by almost all cells and they play a crucial role in regulating and modulating the immune system's response (Takeuchi and Akira, 2010). Chemokines are cytokines that are potent activators, guiders, and chemoattractants for white blood cell subpopulations and some nonhemopoietic cells (Murdoch and Finn, 2000). The dysregulation of cytokines and chemokines result in a pro-inflammatory state, which promote cancer predisposition and progression (Allegra *et al*., 2020; Haseeb *et al*., 2018).

Tumor necrosis factor α (TNFα) is a cytokine involved in cancer predisposition and progression. It is produced by activated macrophages and its release is further enhanced by interleukin-2 in activated B type CLL cells (Foa *et al.,* 1990; Larsson *et al*., 1993). Indeed, CLL patients present elevated levels of TNFα compared to healthy individuals (Foa *et al*., 1990). Furthermore, nurse-like cells produce a variety of chemokines and cytokines, such as B-cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), which lead to the increased expression of anti-apoptotic genes (Filip *et al*., 2015).

CLL results protected from apoptosis also through the increased release of interleukin-4 (IL-4), potentially conferring resistance to chemotherapeutic agents (M. Dancescu *et al*, 1992).

In addition, the tumor growth in CLL is influenced by angiogenesis, which is driven by the dynamic interplay of cytokines functioning through paracrine and autocrine mechanisms (Yoon *et al*., 2012). The increased release of inflammatory cytokines in CLL patients, including IL-6, IL-8 and IL-10 influence the microenvironment of CLL, thereby promoting angiogenesis and so the progression of the disease (Yoon *et al*., 2012). Higher concentrations of these cytokines are associated with shorter survival (Fayad *et al*., 2001; Letilovic *et al*., 2006).

CLL is characterized also by altered levels of vascular endothelial growth factor (VEGF) (Aguirre Palma *et al*., 2016; Piechnik *et al*., 2013). VEGF promotes survival, movement, and interaction between CLL cells and their microenvironment, contributing to disease progression. Studies have shown that CLL patients with elevated VEGF levels face a higher risk of disease progression (Lozano-Santos *et al*., 2014).

Moreover, the irregular release of various chemokines foster a microenvironment supportive of disease advancement (Burger and Gribben, 2014); in fact the expression of C-X-C motif ligand 9 is associated with the worsening of CLL conditions (Haseeb *et al*., 2018). Furthermore, overexpression of C-X-C motif chemokine receptor 4 (CXCR4) in CLL cells is associated with enlarged lymph nodes, splenomegaly, and bone marrow infiltration (Burger *et al*., 1999). In addition, the secretion of chemokine (C-C motif) ligand 3 (CCL3) by malignant B cells enhances the interaction between CLL cells and their microenvironment, correlating with advanced-stage disease (Choi *et al*., 2016; Molica *et al*., 2002; Sivina *et al*., 2011).

The cytokine network in CLL is complex and dynamic, with various cytokines playing multifaceted roles in the disease's pathology. The interaction between CLL cells and the microenvironment, particularly through cytokine networks, may influence the homing and migration patterns of the malignant cells, affecting disease progression and dissemination (Burger JA *et al*., 2009).

2. Objectives

This study aimed to *(i)* determine the impact of cytokine levels on overall survival in patients, providing insights into the prognostic value of these biomarkers; *(ii) t*o identify key signaling pathways associated with cytokine dysregulation in order to elucidate the underlying mechanisms contributing to disease progression and patient outcomes; *(iii)* to examine correlations between cytokine levels and clinical parameters, enhancing the understanding of how these factors interact and affect disease prognosis.

3. Materials and Methods

3.1 Patients

The study was conducted on a real-life cohort composed of 100 CLL patients, referred at AOU Maggiore della Carità of Novara. For each patient, plasma separated from peripheral blood was obtained. Clinical and biological data were available for each patient, such as age, sex, blood count, biochemical profile, immunophenotype via flow cytometry, Fluorescent In situ Hybridization (FISH) analysis, mutational status of IGHV genes, mutational analysis of the TP53 gene via DNA sequencing, Rai and Binet staging, TTFT and OS.

The study was approved by the intercompany ethics committee of the AOU Maggiore della Carità of Novara (CE 120/19).

3.2 Plasma Separation

PB samples were collected and centrifuged at 800 relative centrifugal force (RCF) for 10 minutes, at 4°C to separate plasma from cells. Plasma was then further centrifuged at 13.000 rpm for 10 minutes at 4°C to pellet and remove any remaining cells. The plasma was stored at -80°C.

3.3 Bio-Plex Pro 27 Human Cytokine Assay

3.3.1 Running the Assay

The Bio-Plex Pro 27 Plex Human Cytokine Assay (Bio-Rad Laboratories Ltd., Hercules, CA, USA) was employed to simultaneously measure 27 secreted cytokines in the plasma of newly diagnosed CLL patients. Initially, serially diluted standards and diluted plasma samples (1:4 dilution) were prepared. These prepared solutions were then added to a microfilter plate pre-coated with antibody-coupled beads (50 µL) specific for each of the 27 cytokines. The plate was incubated at room temperature (RT) for 30 minutes with

continuous shaking (850 ± 50 rpm) to facilitate the binding of cytokines to their respective beads. Following the initial incubation, the microfilter plate underwent three washing steps to remove unbound substances. Biotinylated detection antibodies (25 µL) were subsequently added to the plate and incubated with continuous shaking (850 ± 50 rpm for 30 minutes at RT), enhancing the detection specificity for each cytokine. After the detection antibody incubation, the plate was subjected to another set of three washing steps to eliminate excess detection antibodies. Streptavidin-Phycoerythrin (SA-PE) (50 µL) was then added to each well, and the plate was incubated at room temperature with shaking (850 ± 50 rpm for 10 minutes). This step is crucial as SA-PE binds to the biotinylated antibodies, allowing the detection of cytokines via fluorescent signals. Assay buffer (125 µL) was added to each well of the microfilter plate before being read on a Bio-Plex 200 machine.

3.3.2 Data Acquisition

Data acquisition was performed using the Bio-Plex Manager Software, with specific settings for bead count, sample size, and gating. Post-acquisition, the data were analyzed to remove outliers and ensure quality control. The obtained concentrations were compared against expected ranges to validate the assay performance.

3.4 Statistical Analysis

Patient characteristics at diagnosis were compared with cytokine concentration levels using Pearson's chi-squared correlation test. The cytokine concentration cut-off that better predicts shorter overall survival (OS) was determined through MaxStat statistical test, with adjustments made by the Bonferroni correction. Survival analysis was performed using the Kaplan-Meier method and differences between groups were compared using the Log-rank test. This part of the analysis was performed with the Statistical Package for the Social Sciences (SPSS) software v.24.0 (Chicago, IL, USA) and R-studio v.4.2.2. Statistical significance was defined as p-value <0.05. Cellular pathway involvement analysis was performed using the EnrichR website (https://maayanlab.cloud/Enrichr/).

4. Results

4.1 Patient Characteristics

A total of 100 patients were enrolled in the study, with a median age of 67.92 years. Of these patients, 53 (53.00%) were male and 47 (47.00%) were female. The median hemoglobin (Hb) level was 13.85 g/dL, the median lymphocyte count was 90.55 x10^3/µl, the median platelet count was 20.90 x10^3/µl, and the median LDH level of the population was 359. Regarding genetic characteristics, 34.00% of patients had unmutated IGHV status and 63.00% had mutated IGHV status. The presence of key deletions and chromosomal abnormalities included del 17p (8.00%), del 13q (45.00%), del 11q (7.00%), and trisomy 12 (20.00%). Furthermore, while 64 (64.00%) patients experienced their first infection, 36 (36.00%) remained infection free, to the contrary, 36 (36.00%) developed a secondary cancer while 64 (64.00%) did not exhibit such malignancies (Table 4.1).

Characteristics	(N, %)
Gender	
Male	53 (53.00%)
Female	47 (47.00%)
Age (median)	67.92
Hemoglobin g/dL (median)	13.85%
Lymphocyte x10^3/microliter (median)	90.55%
Platelet x10^3/microliter (median)	20.90%
IGHV status	
Unmutated	34.00%
Mutated	63.00%
Del 17p	
Present	8.00%
Absent	92.00%
Del 13q	
Present	45.00%
Absent	55.00%
Del 11q	
Present	7.00%
Absent	93.00%
Trisomy 12	
Present	20.00%
Absent	80.00%
First Infection	
Yes	64.00%
No	36.00%
Secondary Cancer	
Yes	36.00%
No	64.00%

Table 4.1. **Patient characteristics**

The Kaplan-Meier survival curve depicted in Figure 4.1 illustrates the cumulative probability of OS for the cohort over a period of up to 30 years. The median overall survival for the entire patient cohort was determined to be 13.7 years. This median value indicates that half of the patients lived longer than 13.7 years, while the other half had shorter survival times. The survival probability steadily declines over time, with notable drops at various intervals, reflecting the impact of disease progression and other clinical factors on patient survival. The curve provides a comprehensive overview of the long-term survival outcomes for the cohort, highlighting the importance of monitoring and managing factors that could potentially influence survival rates in patients with elevated cytokine levels.

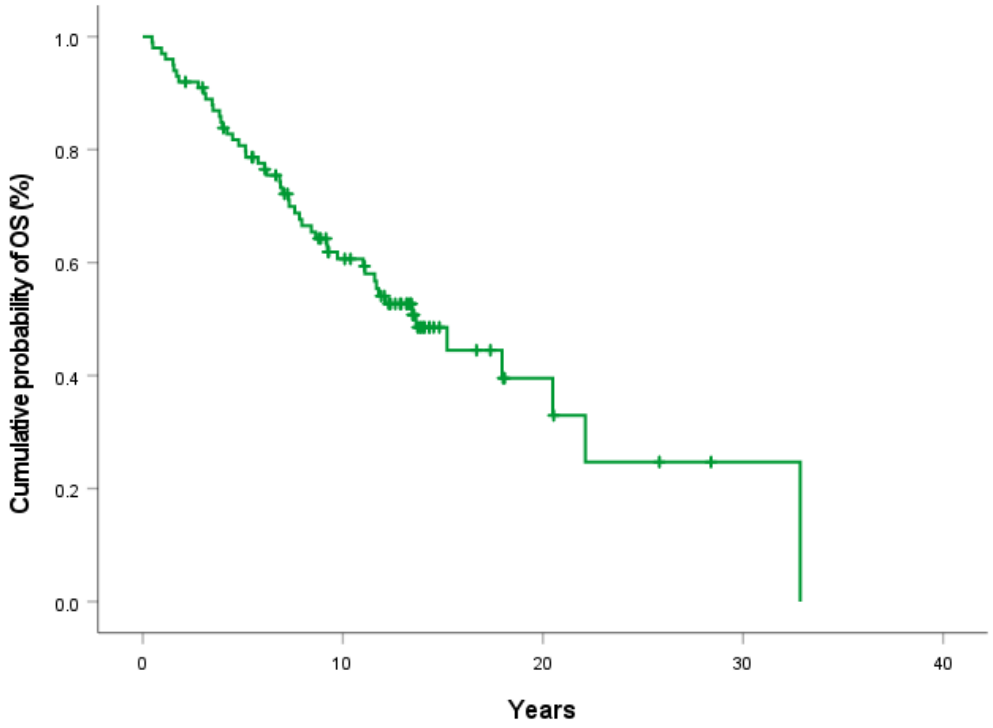

Figure 4.1. **Kaplan-Meier analysis of OS (Overall Survival) of the 100 patients.**

4.2 Cytokine cut-off levels for predicting OS

The cytokine concentration cut-off that best predicts shorter OS was determined using the MaxStat statistical test, with adjustments made by the Bonferroni correction. Statistically significant impacts were observed for eight out of the twenty-six cytokines studied (Table 4.2). VEGF was excluded from the analysis due to the very low number of patients with available data. To address the issue of type I α error, the Bonferroni correction was applied, reducing the likelihood of false positives and ensuring the reliability of the findings.

	Cut off OS	p-value OS	p-Bonferroni OS
Eotaxin	78.06	0.0019	0.04940000
FGFbasic	46.70	0.013	0.33800000
GCSF	34.12	0.0033	0.08580000
GMCSF	0.86	0.026	0.67600000
INFg	14.74	0.021	0.54600000
IL1b	5.14	0.013	0.33800000
IL1ra	57.83	0.06	1.56000000
IL2	0.25	0.0019	0.04940000
IL4	5.86	0.013	0.33800000
IL5	1.46	0.000003	0.00007800
IL6	0.18	0.00014	0.00364000
IL7	27.49	0.086	2.23600000
IL8	5.20	0.072	1.87200000
IL9	441.29	0.0029	0.07540000
IL10	0.97	0.09	2.34000000
IL12p70	11.30	0.01	0.26000000
IL13	2.78	0.047	1.22200000
IL15	0.00	0.11	2.86000000
IL17	15.07	0.062	1.61200000
IP10	257.40	0.00026	0.00676000
MCP1MCAF	32.83	3.12E-08	0.00000081
MIP1a	0.93	0.00029	0.00754000
MIP1b	167.71	0.0054	0.14040000
PDGFb	51.71	0.000204	0.00530400
RANTES	1150.42	0.017	0.44200000
TNFa	71.79	0.0038	0.09880000

Table 4.2. **Statistically significant cytokines and their impact on overall survival with Bonferroni correction.**

The impact of cytokine levels on OS in patients was evaluated, dividing the cohort into two groups based on predefined cut-off values for each cytokine. The findings demonstrated a significant difference in survival outcomes between patients with cytokine levels above the cut-off and those with levels below the cut-off. An analysis of overall survival was conducted for each statistically significant cytokine, utilizing specific cut-off values corresponding to each cytokine. Patients with cytokine levels above the cut-off values had shorter overall survival compared to those with cytokine levels below the cut-off. Patients with high eotaxin levels (above the cut-off) had a median overall survival of 5.7 years, whereas patients with low eotaxin levels (below the cut-off) had a median overall

survival of 17.9 years, which is three times longer (Figure 4.2 A). Regarding IL-2, patients with high IL-2 levels had a median OS of 7.9, compared to the 17.9 years for those below the threshold (Figure 4.2 B). Similarly, patients with high IL-5 levels had a median OS of 7.8 years, while those with low IL-5 levels presented a survival rate of 22.1 years, more than twice as long (Figure 4.2 C). High IL-6 levels were associated with a median OS of 9.7 years, in contrast to a median OS of 22.1 years for patients below the cut-off (Figure 4.2 D).

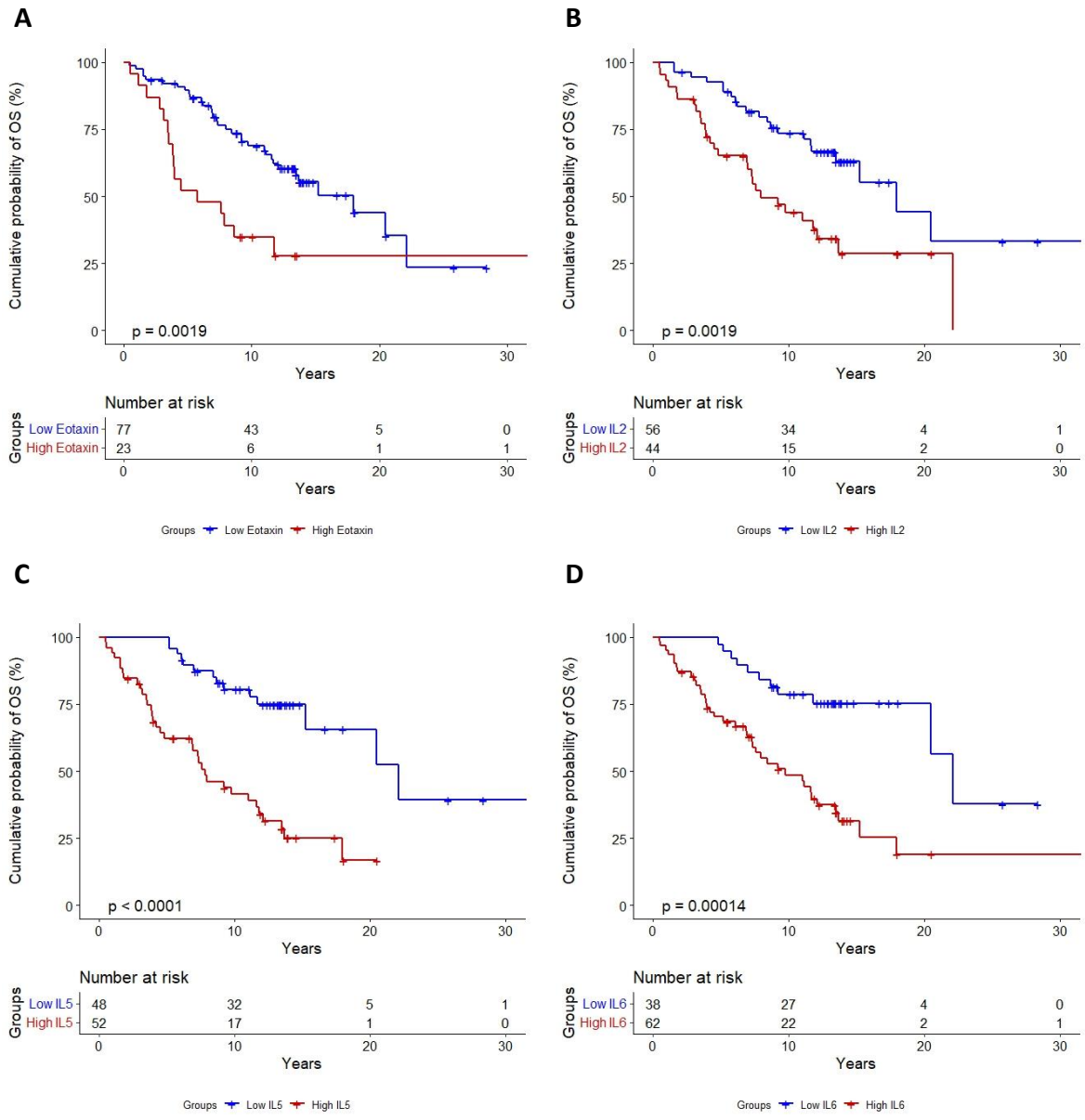

Figure 4.2. **OS curves of the significant cytokines.** Patients with cytokine levels lower than the specified cut-off (in blue) were associated with longer OS compared to patients with cytokine levels above the specified cut-off (in red), which displayed a shorter OS.

Similarly, patients with high IP-10 displayed a median OS 7.6 years compared to 15.2 years of the patients below the cut-off (Figure 4.3 A). Regarding MCP-1/MCAF, patients above the cut-off had a median survival rate of 1.8 years, contrasting with 15.2 years for those below the cut-off, more than eight times as long (Figure 4.3 B). The same tendency is observed for MIP1-α and PDGF-β, with patients above the cut-off exhibiting median OS of 9.2 years and 6.8 years, respectively. For MIP1-α, patients with lower cytokine levels had not yet reached the median OS, while for PDGF-β, patients below the cut-off had a median survival rate of 17.9 years (Figure 4.3 C-D).

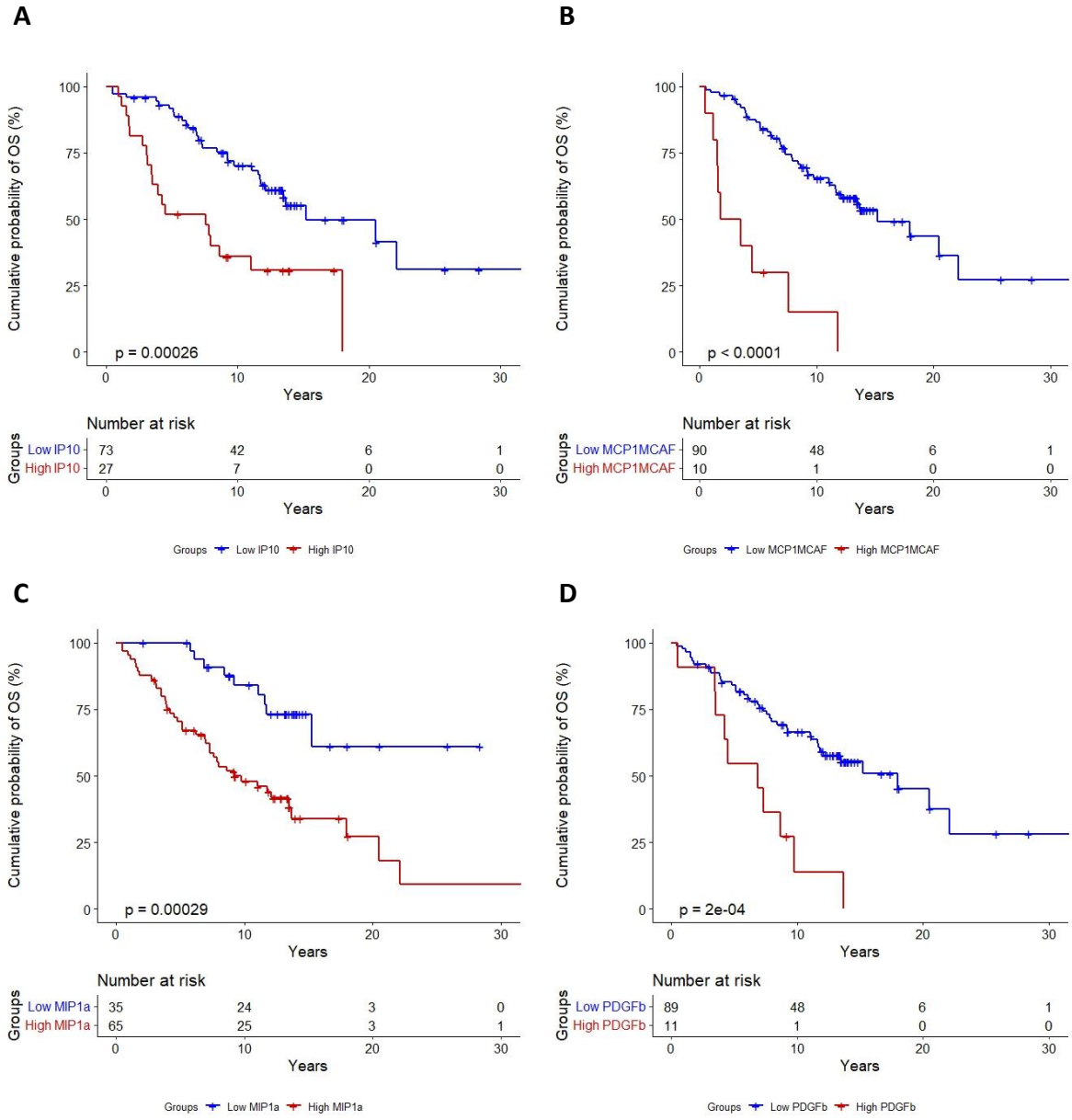

Figure 4.3. **OS curves of the significant cytokines.** Patients with cytokine levels lower than the specified cut-off (in blue) were associated with longer OS compared to patients with cytokine levels above the specified cut-off (in red), which displayed a shorter OS.

4.3 Pathway Analysis and Cytokine Involvement

The pathway analysis identified several key signaling pathways significantly involved in the disease process, as illustrated in Figure 4.4. The top pathways include cytokine-cytokine receptor interaction, IL-17 signaling pathway, JAK-STAT signaling pathway, chemokine signaling pathway, Toll-like receptor signaling pathway, TNF signaling pathway, graft-versus-host disease, PI3K-Akt signaling pathway, and Th1 and Th2 cell differentiation. These pathways are critical in mediating immune responses and inflammation.

The cytokines evaluated in this study—Eotaxin, IL-2, IL-5, IL-6, IP-10, MIP-1α, PDGF-β, and MCP-1/MCAF—play pivotal roles in these pathways. Eotaxin and IL-5 are involved in the chemokine signaling pathway, which recruits immune cells to sites of inflammation, potentially exacerbating tissue damage and disease progression.

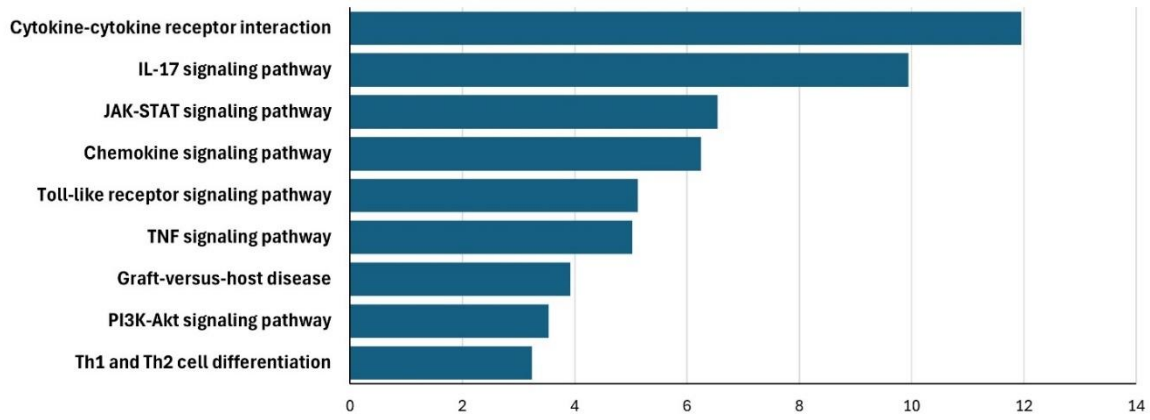

Figure 4.4. **Major pathways in which the eight cytokines are involved.**

4.4 Pearson's correlation analysis

Pearson correlation test was conducted to examine the relationships between cytokine levels and various clinical parameters. The results, summarized in Table 4.3, reveal several significant correlations. Eotaxin levels exhibited negative correlation with platelet count, IgG, and a positive correlation with age. IL-5 levels showed an indirect correlation with del 11q and a positive correlation with age. IL-6 levels correlated positively with del 17p and age. In addition, IP-10 levels had a positive correlation with lymphocyte count, LDH levels, and significant positive correlation with age. MCP-1/MCAF levels correlated with del 17p and indirectly with platelet count. MIP-1α levels exhibited a strong positive correlation with IGHV unmutated, del17p, and LDH levels, whereas it strongly negatively correlated with IgA levels. Lastly, PDGF-β levels were positively correlated with neutrophil count.

	Eotaxin	IL2	IL5	IL6	IP-10	MCP-1	MIP1α	PDGFβ
Gender F								
IGHV unmutated								
Trisomy 12								
Del 17p								
Del 11q								
Del 13q								
Lymphocyte count								
Hemoglobin levels								
Platelet count								
LDH levels								
Neutrophil count								
IgG level								
IgA level								
IgM level								
Binet stage								
First infection								
Second cancer								
Age								

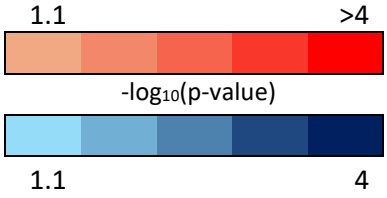

Table 4.3. **Pearson Correlation Between Cytokine Levels and Clinical Parameters**. LDH, lactate dehydrogenase.

5. Discussion

In this study, analysis was carried out on a cohort of 100 patients with a median age of 67.92 years, including 53 males and 47 females. Key clinical parameters such as hemoglobin (Hb) levels, lymphocyte counts, and platelet counts were assessed, revealing median values of 13.85 g/dL, 90.55 x10^3/µL, and 20.90 x10^3/µL, respectively. Genetic analysis showed that 34.00% of patients had unmutated IGHV status, while 63.00% had mutated IGHV status. Chromosomal abnormalities, including del 17p (8.00%), del 13q (45.00%), del 11q (7.00%), and trisomy 12 (20.00%), were also identified. These characteristics are consistent with previous findings in similar patient populations and provide a robust foundation for examining the impact of cytokine levels on disease outcomes.

The analysis of cytokine levels and the determination of the best cut-off that predicts OS, revealed that elevated levels of specific cytokines were associated with significantly shorter overall survival. Evidently, patients with high eotaxin levels had a median overall survival of 5.7 years, compared to 17.9 years for those with low eotaxin levels. Similar trends were observed for the other cytokines, underscoring the potential of these biomarkers in predicting patient prognosis. The Kaplan-Meier survival curves and corresponding statistical analyses consistently demonstrated that higher cytokine levels correlate with poorer survival outcomes, highlighting the need for further investigation into targeted therapies that can modulate cytokine activity.

Pathway analysis identified several critical signaling pathways involved in the disease process, including cytokine-cytokine receptor interaction, IL-17 signaling pathway, JAK-STAT signaling pathway, chemokine signaling pathway, Toll-like receptor signaling pathway, TNF signaling pathway, graft-versus-host disease, PI3K-Akt signaling pathway, and Th1 and Th2 cell differentiation. These pathways are known to play essential roles in immune response regulation and inflammation. The involvement of cytokines such as Eotaxin, IL-2, IL-5, IL-6, IP-10, MIP-1α, PDGF-β, and MCP-1 (MCAF) in these pathways underscores their significance in mediating disease progression and patient outcomes.

Pearson's correlation test was conducted to explore the relationships between cytokine levels and various clinical parameters. It was found that age directly correlated with several cytokines, including eotaxin, IL-5, IL-6, IP-10. This suggests that aging is closely linked to an upregulation of these inflammatory mediators, potentially reflecting broader age-related

changes in immune function. Advanced Binet stage positively correlated with MCP-1/MCAF and MIP-1α, indicating their potential role in disease progression. In addition, del 17q was associated with increased levels of IL-6, MCP1/MCAF and MIP-1α, suggesting genetic interactions influencing cytokine expression. IGHV unmutated status was associated with higher expression of MIP-1α, indicating its possible involvement in U-CLL pathogenesis. Furthermore, LDH levels directly correlated with increased IP-10 and MIP-1α levels, highlighting that these cytokines could contribute to the worse outcomes observed in patients with elevated LDH.

The significant correlations between elevated cytokine levels and poorer survival outcomes underscore the potential of cytokines as prognostic biomarkers. These findings suggest that cytokine profiling could be integrated into clinical practice to improve risk stratification and guide therapeutic decision-making. Targeting specific cytokines and their associated signaling pathways may offer novel therapeutic approaches to modulate immune responses and improve patient outcomes. Further research is needed to validate these findings in larger cohorts and explore the underlying mechanisms driving cytokine dysregulation and its impact on disease progression.

6. Conclusion

This study provides significant insights into the role of cytokine levels in predicting overall survival and their relationship with baseline clinical characteristics. Elevated levels of specific cytokines, including Eotaxin, IL-2, IL-5, IL-6, IP-10, MIP-1α, MCP-1 (MCAF), and PDGF-β, were associated with shorter overall survival rates. These findings underscore the importance of cytokine profiling in the prognosis and management of patients. The correlation between cytokine levels and various clinical and genetic parameters highlights the complex interplay between the immune system and disease pathophysiology. Key signaling pathways such as cytokine-cytokine receptor interaction, IL-17 signaling, and JAK-STAT signaling were identified as critical mediators of inflammation and immune response, offering potential targets for therapeutic intervention. Overall, this study emphasizes the need for further research to validate these biomarkers and develop targeted therapies to improve patient outcomes.

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