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Ph.D. Thesis

Mechanisms of innate immunity – targets in lymphoid leukemias immunotherapy

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"Education is the most powerful weapon which you can use to change the world."
- Nelson Mandela

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TABLE OF CONTENTS

ABBREVIATIONS USED IN TEXT

CLL	Chronic Lymphocytic Leukemia
TME	Tumor microenvironment
SLL	Small Lymphocytic Lymphoma
MBL	Monoclonal B-cell lymphocytosis
BCR	B-cell receptor
Ig	Immunoglobulin
IgHV	Immunoglobulin Heavy Chain Variation
M-IgHV	Mutatetd- IgHV
U-IgHV	Unmutated-IgHV
IL	Interleukin
NF-κB	Nuclear factor kappa B
TTFT	Time to first treatment
IwCLL	International Workshop on CLL
FCR	Fludarabine-Cyclophosphamide-Rituximab
BTKi	Bruton tyrosine kinase inhibitor
ZAP-70	Zeta-chain-associated protein kinase 70
FISH	Fluorescence In Situ Hybridization
RS	Richter's Syndrome
NOTCH1	Neurogenic locus notch homolog protein 1
SF3B1	Splicing factor 3b, subunit 1
BIRC3	Baculoviral IAP repeat-containing protein 3
mRNA	Messenger RNA
miRNAs	MicroRNAs
RNA	Ribonucleic acid
TLR	Toll-like receptors
NETs	Neutrophil extracellular traps
VDR	Vitamin D receptors
NK	Natural Killer cells
Th	T helper cells
Treg	Regulatory T cells
TNFα	Tumour Necrosis Factor-alpha
INF	Interferon

TCL	Cytotoxic T cell
SIRPα	Signal-regulatory protein-alpha
IRAK1	Interleukin Receptor Associated Kinase 1
TARF	Tumour necrosis factor receptor-associated factors
R/R	Relapsed/Refractory
TN	Treatment naive
BR	Bendamustine-Rituximab
CR	Complete remission
CRi	Complete remission with incomplete count recovery
PR	Partial remission
SD	Stable disease
PD	Progressive disease
TTNT	Time to next treatment
PFS	Progression-free survival
OS	Overall survival
DCs	Dendritic cells
STAT	Signal transducer and activator of transcription
RISC	RNA-induced silencing complex
XPO5	Exportin 5

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a hematological disorder defined by the abnormal growth and accumulation of clonal B lymphocytes (CD5+, CD19+ and CD23+) in the hematopoietic marrow, lymphoid organs and peripheral blood, forming a tumor microenvironment (TM), which inhibits the immune response and promotes malignant proliferation and resistance to treatment. CLL accounts for about 30% of leukemias worldwide and is considered a disease of the elderly. The presence of comorbidities and a low performance index limits the indication for intensive treatment, so recently CLL treatment has evolved dramatically towards targeted therapy as a less aggressive therapeutic option. Indeed, for many years, chemo-immunotherapy was considered the standard of care for fit patients, but the paradigm was overturned with the advent of Bruton's tyrosine kinase inhibitors, which are suitable for elderly or low performance index patients as well as for fit patients.

At present there is a limited range of prognostic and predictive markers for this pathology, such as TP53 gene mutation, Immunoglobulin Heavy Chain Variation (IgHV) mutational status, presence of deletions 17p (del(17p)), 11q (del(11q)), 13q (del(13q)), 12 trisomies or a complex karyotype, which, however, in the setting of novel, targeted therapy have lost their relevance, suggesting that course of this disease is influenced by multiple factors and not just the patient's genetic and molecular landscape. Moreover, patients diagnosed with CLL have a highly variable time from diagnosis to the moment when treatment initiation is imposed, which can be in part predicted by the disease stage at diagnosis and genetic and molecular alterations, but not entirely.

Based on the idea that the heterogeneity of the disease resides partially in deregulated immune mechanisms, the present work aims to assess and study to a small extent their involvement in the course of the disease and response to treatment. In the first instance, we considered it necessary to start from a database including the status of patients diagnosed with CLL at the "Ion Chiricuță" Institute of Oncology in Cluj-Napoca, since data on the evolution of these patients outside clinical trials are limited, especially in Romania. Thus, we performed a retrospective analysis of these patients, focusing on the time elapsed from the time of diagnosis to the initiation of therapy, the type of treatment, the response and the evolution of the patients, and the turning point created by the emergence of the targeted therapy, in the era of chemo-immunotherapy as a gold standard. We then focused on patients receiving Bruton's tyrosine kinase inhibitors treatment, specifically ibrutinib, to assess whether there is a correlation between the biological, molecular and cytogenetic status, the stage of

disease at diagnosis and the course of treatment. Finally, a part of the initially included patients were randomized according to the time elapsed between diagnosis and treatment initiation. Vitamin D levels in serum were assessed, and miRNAs were extracted to investigate potential statistically significant correlations between them or other factors, such as time to first treatment and prognostic indicators. This analysis seeks to explore the relationship between miRNAs and vitamin D, disease progression and treatment response.

The results of this work will form a pillar for future research inquiring the causes of dysregulated immune mechanisms in CLL that favor disease progression and poor response to treatment.

CURRENT UNDERSTANDING OF THE SUBJECT

1. Chronic Lymphocytic Leukemia

1.1. Definition and Etiology

According to the International Workshop on Chronic Lymphocytic Leukemia (IwCLL), chronic lymphocytic leukemia (CLL) is defined by the presence of ≥ 5 × 10^9/L monoclonal B lymphocytes (CD5+, CD19+ and CD23+) in the peripheral blood, persisting at least 3 months, and their proliferation in the bone marrow and lymphoid organs (1). The proliferation of monoclonal lymphocytes in peripheral blood, bone marrow and lymphoid organs will support the formation of tumor microenvironment (TME), which will promote malignant proliferation (2). The localized form of CLL, in which tumor proliferation occurs mostly in lymphoid organs without the presence of lymphocytosis in the peripheral blood, is called lymphocytic lymphoma (SLL) (3).

Like most neoplastic diseases, the etiology of CLL has not been identified. Prolonged and heavy exposure to tobacco has been correlated with an increased frequency of leukemias, including CLL (4). However, there is no evident established link between exposure to chemicals, drugs or ionizing radiation and the development of this neoplasm (5). Some studies indicate a possible correlation between the risk of developing CLL and exposure to chemicals, such as benzene and aromatic hydrocarbons, among farmers and workers in the oil and wood industries (6). The issue of existing familial clustering has also been addressed, estimating that those who have a first-degree relative diagnosed with CLL are 2-7 times more likely to develop CLL than the general population (7). In addition, a synergy between genetic and environmental factors could generate an immune response leading to monoclonal B-cell lymphocytosis (MBL), considered by some authors as a CLL precursor(8, 9). It has been found that about 13%-18% of the offspring of patients diagnosed with CLL will develop MBL, but only a small percentage will develop the disease itself (10).

1.2. Epidemiology

CLL accounts for 25-30% of all leukemias in the Western world (11). Over 100,000 newly diagnosed CLL cases and over 40,000 deaths were reported Worldwide in 2019 (11). CLL is regarded as a disease predominantly affecting older adults, with the median age at diagnosis being around 70 years. Its incidence rises significantly with age and is notably twice as prevalent among men as it is among women (12, 13). Awareness of the importance of routine blood tests has led to more frequent incidental diagnoses of this pathology in the younger population, even under 55 years (14). The

age at diagnosis may also be influenced by heredity, as it has been found that the offspring of CLL patients will develop CLL at an age almost 10-20 years younger than their predecessors at the time of diagnosis (15). There was also a discrepancy between the frequency of CLL in Western countries and that in Asian countries, where the incidence of this neoplasia is lower. The same observation was also found among the Asian population immigrating to Western countries, suggesting that the reason behind the different incidence is an ethnic rather than a geographical factor (16).

1.3. Pathogenesis

The pathogenesis of CLL is not fully elucidated. Still, it results from disrupted immune responses, genetic aberrations and cell signalling pathways that ultimately will lead to immune evasion, resistance to apoptosis and chaotic proliferation of malignant cells. CLL is a proliferation of B lymphocytes. The response of B lymphocytes to antigenic stimulation is mediated by B cell receptor (BCR) modulation. Each B cell has a BCR that is the result of variable combinations of the V, D, J segments of the immunoglobulin (Ig) heavy chain gene and the V and J segments of the light chain encoding gene. In addition, BCR diversity is also ensured by the process of somatic hypermutation, which occurs at the germinal centre of the lymph node (17). The mutational status of Ig heavy chain variation (IgHV) is relevant in CLL, much more so than in other lymphoproliferative disorders. Depending on the degree of somatic IgHV mutations, patients can be classified as mutated or unmutated, this distinguishes between an indolent and an aggressive course (18, 19) (Figure 1).

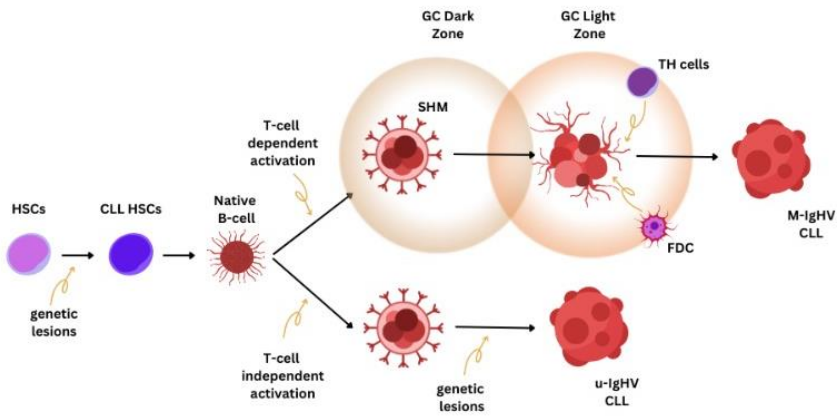

Figure 1: A possible explanation for the formation of CLL cells: Secondary to exposure to genetic lesions, CLL HSCs are born, and further evolve into naive B-cells, which will undergo a T-cell-dependent or independent activation. B cells that are T-cell-dependently activated, enter the dark zone of the germinal

center of the B-cell follicles, where they are exposed to clonal proliferation and SHM, which stimulates the mutation in the variable region of the immunoglobulin gene. Additionally, they move into the light zone of the germinal center, where they develop their B-cell receptor affinity through interactions with T helper cells and follicular dendritic cells, leading to M-IgHV cells formation. Naive B-cells that are T-cell-independently activated, do not undergo SHM, which will lead to the development U-IgHV cell cells. It is unclear in which step of the B-cell development and activation the genetic lesion that will lead to CLL cells formation intervenes (20, 21). Abbreviations: HSC-hematopoietic stem cell, MBL-monoclonal B-cell lymphpocytosis, GC-germinal center, SHM- somatic hypermutation, TH cells- T Helper cells, FDC-Follicular dentritic cell, M-IgHV -mutated IgHV, U-IgHV- unmutated IgHV.

The immunophenotypic profile of lymphocytes involved in the pathogenesis of CLL is different from the one of normal B lymphocytes, presenting the expression of CD5, CD19, and CD23 (20). These cells are functionally incompetent, with an impaired differentiation capacity, somewhere between the pre-B cell and mature B cell stage (22). Some researchers believe that the CD5+ cells involved in the pathogenesis of CLL are part of a separate cell lineage, with a gene expression profile that is different from that of CD5+ lymphocytes isolated from the umbilical cord, and rather similar to that of memory B lymphocytes (23). In terms of IgHV mutational status, cells with this M-IgHV derive from mature CD5+ CD27+ B lymphocytes and those with U-IgHV from CD5+, CD27- B lymphocytes (20).

For simplicity, the pathogenesis of CLL can be divided into two stages. The first represents the development of BML and the second is the progression to CLL.

1.3.1. **Monoclonal B-cell lymphocytosis**

MBL is a premalignant proliferative pathology, defined by the presence of monoclonal lymphocytes in peripheral blood < 5 x 10^9/L, in the absence of adenopathy or organomegaly (20). It has been observed in approximately 13-18% of people who have at least two relatives diagnosed with CLL, and in 3.5% of the general population aged over 40 years, with its prevalence increasing exponentially with age (10). As in CLL, a higher BML frequency has been found in males (10). The cells involved in the development of BML may show genetic abnormalities similar to those seen in CLL, suggesting that the former is a precursor to neoplasia itself. BML may have a low monoclonal lymphocyte count < 0.5 x 10^9/L, associated with a low rate of progression to CLL or other lymphoproliferative diseases, or an increased monoclonal cell count of 0.5-4.9 x 10^9/L, with a rate of progression to lymphoproliferative diseases of approximately 1-2% per year (20, 24, 25). There is little data on the genetic landscape involved in the BML development, but IgHV mutation was evidentiated in 70-80% of BML cases, with more frequent localization in the IgHV3-23 and IgHV4-59/61 regions, overlapping with indolent CLL (26). Comparative studies have shown increased

expression of CTLA4, LEF1, ROR1 and TCL1A oncogenes, but lower than in CLL patients (27). Identification of SF3B1, BIRC3, DDX3X, CHD2, NOTCH1 mutations, and the presence of CD38, CD49d and Zeta-chain-associated protein kinase 70 (ZAP-70) markers expression, were correlated with more rapid progression of BML to CLL (24, 28). The cytogenetic profile of BML is similar to that of early-stage CLL, with deletion 13q being the most common aberration, followed by trisomy 12. Cytogenetic abnormalities that are correlated with more aggressive disease progression, such as deletions 11q and 17p, were rarer (29).

1.3.2. **Chronic Lymphocytic Leukemia**

CLL is characterized by the sustained presence and advancement of monoclonal B-lymphocytes in the blood, bone marrow, and lymphoid tissues. This condition arises from resistance to apoptosis and uncontrolled cell growth, influenced by a combination of genetic mutations, B-cell receptor signaling pathways, immune system irregularities, and factors within the tumor microenvironment.

1.3.2.1. Tumor microenvironment

The tumor microenvironment plays a key role in the development of all neoplasms, including CLL, by providing contact between tumor cells and tumor tissue-associated T lymphocytes, stromal cells or macrophages, also known in CLL as nurse-like cells (30-33). Following these interactions, tumor B cells receive pro-survival signals (20). The proliferation of malignant lymphocytes in this pathology occurs in the lymph nodes, where malignant B lymphocytes express receptors for chemokines and adhesion molecules, which will activate the NF-κB signaling pathway and induce BCR signaling and activation (33).

1.3.2.2. Dysregulated immune mechanisms

Mechanisms used by tumor cells to inhibit CD8+ T cell-mediated immunity include disruption of antigen presentation, down-regulation of HLA molecules and induction of co-inhibitory molecules such as PD-L1 and PD-L2 (34). In addition, lymphocytes in CLL are able to secrete interleukin-10 (IL-10), which in turn will increase PD-L in malignant cells, underlining the degree of immunosuppression (16, 35). Another mechanism, this time involving natural killer (NK) cells, is the upregulation of NKG2D, MICA and B (major histocompatibility complex class I-related glycoproteins A and B) receptor ligands, which will lead to immune evasion of tumor cells (36).

1.3.2.3. B-cell receptor

Proper functioning of the BCR is required for the survival of B lymphocytes, whether malignant or healthy (37). BCR signaling will ultimately lead to activation of the AKT/mTOR (mammalian target of rapamycin), NF-κB or ERK (extracellular signal-regulated kinase) signaling pathways (20). As with most cancers, BCR signaling is heterogenic and can stimulate or inhibit B lymphocyte activation (16). Studies have correlated the activation of B lymphocytes by BCR signaling with U-IgHV and inhibition with M-IgHV (38). The importance of BCR has also been marked by the emergence and efficacy of tyrosine kinase inhibitors, which block BCR signaling (39).

1.3.2.4. Chromosomal aberrations

Approximately 80% of patients diagnosed with CLL will have at least one of the 4 chromosomal abnormalities typical for CLL. The most common is deletion 13q (del(13q)), in the region where microRNAs 15 and 16 (miR-15 and miR-16), which regulate the expression of proteins involved in apoptosis and cell cycle progression, are found (40). This abnormality is associated with a favorable prognosis. Del(11q) is the 2nd most common and is associated with alterations in the ataxia-telangiectasia mutated (ATM) gene which encodes proteins involved in DNA repair and is associated with a poor prognosis. Trisomy 12, the third most frequent abnormality, is associated with an intermediate prognosis. The last anomaly is del(17p), which is associated with the loss of the tumor suppressor gene TP53 and therefore a worse prognosis (41).

1.3.2.5. Somatic mutations

The heterogeneous manifestations of CLL reside in the diversity of mutations identified in this pathology. Recurrent somatic mutations have been identified with a role in DNA damage, chromatin modification, messenger RNA (mRNA) processing. One of the most common mutations is SF3B1, which is involved in the dysregulation of RNA processing, one of the main causes of CLL development (42). NOTCH1, a protein involved in cell differentiation, has been observed most frequently in patients with unmutated IgHV (U-IgHV) (43, 44). About 40% of patients with this mutation also have trisomy 12, suggesting their involvement in the pathogenesis of CLL (44). Mutations have also been identified in genes like MYD88, NFKBIE, BIRC3, and TRAF3, which modulate inflammatory responses, or in POT1, responsible for telomere protection (42, 45).

1.3.2.6. MicroARNs

CLL was the first pathology to be associated with miRNAs dysregulation. Carlo Croce and his colleagues first described miR-15 and miR-16 dysregulations in this pathology (46). MiR-15 and miR-16 regulate anti-apoptotic proteins, like the ones from the BCL-2 family, leading to an antiapoptotic effect in CLL (46). Of interest are also miR-29a/b, miR-29c, miR-34b, miR-181b, miR-155, which are involved in BCR signaling, respectively in proliferative and apoptotic processes, suggesting an important role in its pathogenesis (47-50).

1.4. Clinical Manifestations

1.4.1. Symptoms

Patients with CLL are often asymptomatic at initial presentation, and in many cases, the pathology is discovered fortuitously, following a routine blood count that reveals the presence of lymphocytosis. 5-10% of CLL patients will present with at least one of the "B symptoms" - fever >38°C for ≥ 2 weeks, outside of an infectious setting, unintentional weight loss ≥ 10% of body weight in the past 6 months, profuse night sweats outside of an infectious setting, or extreme fatigue with altered performance status (1).

Occasionally, they may present with recurrent infections and autoimmune complications such as hemolytic anemia, thrombocytopenia or pure red cell aplasia, manifested by extreme fatigability, dyspnea, palpitations, syncope, and hemorrhagic syndrome in thrombocytopenia. Exaggerated reactions to insect stings or bites have also been reported (51, 52) (53, 54)

1.4.2. Clinical signs

In addition to the secondary signs of thrombocytopenia and anemia manifested by the presence of ecchymoses, hematomas, purpuric lesions, skin and mucosa pallor, respectively tachycardia, on physical examination, 50-90% of CLL patients present with painless, firm, round and mobile adenopathies, localized or generalized. Painless splenomegaly is seen in 25-55% of cases. Painless hepatomegaly is seen in 15-25% of patients (55).

In addition to examination of the lymph nodes and organomegaly, rigorous cutaneous inspection is recommended, as this pathology may be associated with cutaneous neoplasms, manifested by papules, macules, plaques, ulcers, vesicles or

nodules. Non-specific secondary skin lesions may occur secondary to bleeding, infection and insect bites (56, 57). Also, in 4-27% of CLL cases, *leukemia cutis* has been described to be associated with a more aggressive disease course (57, 58).

1.5. Diagnostic and staging systems

1.5.1. Laboratory findings

In the mid-20th century, chronic lymphocytic leukemia (CLL) was initially characterized by the buildup of small, mature lymphocytes that appeared to have a smudged look after preparing peripheral blood smears, named "Gumprecht shadows". (1, 59). The most notable laboratory finding in CLL is peripheral lymphocytosis. While the definitive blood lymphocyte threshold for diagnosing CLL is set at 5 x 10^9/L of B lymphocytes, a notable number of patients exhibit counts reaching 100 x 10^9/L or more (1). In addition to lymphocytosis, cytopenias like anemia or thrombocytopenia can occur, having an autoimmune origin in 25% of instances (60). Rare cases of pure red cell aplasia or neutropenia have been described (51, 61). Biochemically, CLL may present hyperuricemia, changes resulting from hemolysis, hypogammaglobulinemia, and elevated levels of beta-2 microglobulin (62-64).

1.5.2. Diagnosis Confirmation

In addition to paraclinical changes, the presence of adenopathies and organomegaly were also found (59, 65). The accuracy of the diagnosis is important, all the more so as there are other lymphoproliferative diseases with similar clinical and paraclinical manifestations and immunophenotype, but with different therapeutic approaches and disease outcomes. To diagnose CLL, there must be a sustained presence of monoclonal B lymphocytes in the peripheral blood, specifically at least 5 × 10^9/L, characterized by CD5+, CD19+, and CD23+ markers, maintained for a minimum duration of three months. In 1994, Estella Matues proposed a system for the diagnosis of CLL, the "Matutes Score", which initially included the assessment of surface immunoglobulins, CD5, CD19, CD23, CD22 and FMC7 (66). CD22 was later replaced by CD79b (67). Depending on the presence or absence of the markers assessed, each is assigned a score between 1 and 0. A minimum score of 4 points is required for a definite diagnosis. Exceptions are cases of atypical CLL for which additional markers will be added (68). Other markers, such as the CD 200, have been proposed to be included in the Matutes Score, but for the time being it remains unchanged (68). The Matutes Score is detailed in Table I.

MARKERS	SCORE POINTS	SCORE POINTS
	1	0
CD 5	Positive	Negative
CD 23	Positive	Negative
SmIg	Weak	Strong
FMC7	Negative	Positive
CD22/CD79b	Weak	Strong

Table I: Matutes Score: Most CLL cases need at least a score of 4 points. SmIg= surface membrane immunoglobulin.

In contrast to CLL, SLL is characterized by the presence of peripheral adenopathy and organomegaly, in the absence of peripheral monoclonal lymphocytosis greater than 5×10^9/L; the diagnosis is confirmed by lymph node biopsy with histopathological examination including evaluation of cyclin D1, SOX11 and LEF1 expression - necessary for differential diagnosis with other lymphoproliferative lymphomas (25).

1.5.3. Staging and Scoring Systems

Even though Rai and Binet classification systems, based on clinical examination of the patient and standard laboratory findings, are over 40 years old, they are still in use currently (69, 70). Of course, with the emergence of new prognostic markers, the need for new staging modalities, such as the Integrated CLL Evaluation System (ICSS), the CLL International Prognostic Index (CLL-IPI), or the International Prognostic Score for Early Stage CLL (IPS-E), has emerged (71-73).

1.5.3.1. *Rai Staging*

The Rai classification originally included 5 prognostic groups, graded from 0 to 4, but was later modified to include only 3 (69). The Rai staging system categorizes low-risk CLL as the presence of monoclonal lymphocytosis exceeding 5 x 10^9/L, without any clinically evident lymphadenopathy or organ enlargement, corresponding to the former Rai stage 0. Patients exhibiting lymphocytosis along with lymphadenopathy and either splenomegaly or hepatomegaly are classified as having intermediate-risk disease, which aligns with the previous Rai stages 1 or 2. High-risk disease is characterized by the presence of anemia related to the disease, indicated by a hemoglobin level below 11 g/dL (previously Rai stage 3), or thrombocytopenia, identified by a platelet count of less than 100 x 10^9/L (previously Rai stage 4) (2).

1.5.3.2. *Binet Staging*

The Binet classification system categorizes patients based on the size of lymphadenopathy, as well as organomegaly and the presence or absence of anemia or thrombocytopenia. To facilitate assessment, five specific regions of interest are identified: the first encompasses the head and neck, including Waldeyer's ring; the second pertains to the axillary region; the third involves the groin; the fourth includes the spleen, and the fifth focuses on the liver. According to the Binet staging system, Stage A is characterized by hemoglobin levels of ≥10 g/dL and a platelet count of ≥100 x 10^9/L, with involvement limited to a maximum of two of the specified areas. Stage B is identified when hemoglobin remains ≥10 g/dL and platelet count is ≥100 x 10^9/L, but three or more areas are affected. Stage C is defined by hemoglobin levels falling below 10 g/dL and/or a platelet count of less than 100 x 10^9/L (2).

1.5.3.3. ICSS Score

The ICSS score is based on cytogenetic abnormalities, IGHV mutational status and CD38 expression. ICSS stratifies patients into 3 risk groups - low, intermediate and high - with different time to first treatment (TTFT) and survival in order to predict early disease progression (73).

1.5.3.4. CLL-IPI Score

The CLL-IPI is regarded as the most significant scoring system for the era of targeted therapies; however, it necessitates access to molecular and cytogenetic testing. This score has been validated in a separate cohort of patients who were newly diagnosed with CLL and is beneficial for predicting time to first treatment (TTFT) and the risk of disease progression in those undergoing first-line chemotherapy. The CLL-IPI incorporates five independent prognostic factors: the presence of a del(17p) or a TP53 mutation, the mutational status of the IgHV, serum β2-microglobulin levels, the Rai/Binet stage, and the patient's age. This model categorizes patients into four distinct risk groups: low, intermediate, high, and very high, each associated with varying 5-year survival rates (74).

1.5.3.5. *IPS-E Score*

The IPS-E score is the most recent and has been proposed as a predictor of TTFT for CLL patients with early disease (71). This score was developed based on the presence of U-IgHV, lymphocytosis greater than $15x10^9$ /L and the presence of palpable adenopathies to predict the 5-year cumulative risk for starting treatment, categorizing patients into three risk groups: low, intermediate and high (71).

1.6. Prognostic and Predictive Factors

The clinical progression of newly diagnosed CLL varies greatly, as some patients remain asymptomatic and maintain an active lifestyle for many years, while others may quickly experience symptoms or develop a high-risk form of the disease that necessitates prompt treatment initiation following diagnosis, potentially leading to death from complications related to the therapy or the disease itself. Nevertheless, the majority of patients fall somewhere between these two extremes in terms of their clinical course.

In the past twenty years, new clinical and genetic prognostic factors have been discovered in patients with CLL (75). These factors encompass age ≥65, gender, the Eastern Cooperative Oncology Group (ECOG) score, along with molecular and cytogenetic aberrations, abnormal expression of CD38 and ZAP-70 and β2-microglobulin levels (76-79).

1.6.1. Laboratory and flow cytometry prognostic markers

Patients with beta-2-microglobulin (B2M) >3.5mg/dl, thymidine kinase> 48.5 U/L and lymphocyte doubling time under 6 months tend to have a reduced overall survival (80, 81). In cases of CLL where B2M levels exceed 3.5 mg/dl, patients receiving chemo-immunotherapy exhibited reduced overall survival (OS) and lower rates of complete remission (CR) compared to those with lower B2M levels (82).

The positivity of CD38, CD49d, and Zap-70 markers on flow cytometry are correlated with lower OS (83-85). CD49d is considered the most significant among them due to its independent influence on prognosis, regardless of FISH tests or IGHV mutation status (86).Many studies indicate that CD38 and Zap-70 have a strong correlation with unmutated IGHV and could serve as useful surrogates (78). However, the variability and lack of standardization of these markers across different laboratories nationwide have limited their effectiveness.

1.6.2. Genetic aberrations as prognostic markers

The four most frequently identified chromosomal abnormalities detected through FISH studies at the time of diagnosis are the 13 (del(13q14)), observed in 55% of cases, 11 (del(11q23)), present in 18% of patients, trisomy 12, found in 16% of cases and 17 (del(17p)), detected in 7% at diagnosis (40, 41).

The del(13q) abnormality is one of the most prevalent chromosomal changes in CLL and identified as single cytogenetic aberration is associated with a good prognosis and a long median survival (87).

Conversely, del(11q) is associated with high-risk features and its prevalence increases over time (88). This deletion is predominantly observed in younger male patients and is typically linked with significant lymphadenopathy (89). It correlates with shorter TTFT and relapse after treatment (88). While the introduction of chemoimmunotherapy, particularly fludarabine in association with cyclophosphamide and rituximab (FCR), has considerably enhanced the outcomes for patients with del(11q), their prognosis remains poorer compared to patients without this aberration (88). Notably, recent follow-up data involving new therapies, such as ibrutinib, have demonstrated sustained responses in both treatment-naïve and relapsed-refractory patients with del(11q) (90). As the use of chemoimmunotherapy becomes more limited and the application of Bruton tyrosine kinase inhibitor (BTK) therapy rises, the prognostic significance of del (11q) is diminishing (91).

As a sole cytogenetic aberration, trisomy 12 is classified as an intermediate-risk abnormality (92, 93). It can be associated with unmutated IGHV, ZAP-70 positivity, and highly homologous stereotyped B-cell receptors. While TP53 mutations are uncommon in this group, NOTCH1 mutations are prevalent, occurring in 40% of individuals, associating a rapidly progressive clinical course. (92-94).

Del(17p) is the most critical prognostic and predictive marker in CLL, linked with poor outcomes and rapid disease advancement, as it has historically shown resistance to conventional fludarabine-based therapy (95, 96). While del(17p) is infrequently detected at diagnosis it tends to occur more frequently in previously treated patients, highlighting the importance of re-evaluating for this aberration during clinical progression due to its therapeutic implications (97). Approximately 80% of patients with del(17p) have mutations in the remaining TP53 allele, yet 4-5% of CLL patients can have TP53 mutations without del(17p) (98). Given the chemo-immunotherapy resistance observed in CLL patients with these abnormalities, TP53 mutations and del(17p) were initially utilized as predictive markers. Additionally, BTK inhibitors were recommended, leading to improved overall survival and progression-free survival (PFS) with this treatment (99, 100).

Similar to numerous other neoplastic conditions, a complex karyotype in CLL is linked to a shorter TTFT and a worse prognosis when treated with chemoimmunotherapy (101). However, in the context of targeted therapies and BCL-2 inhibitors, the prognostic and predictive significance of a complex (≥3 chromosomal aberrations) or highly complex (≥5 chromosomal aberrations) karyotype is yet to be decided (101).

The IgHV mutational status stays consistent throughout the progression of the disease and influences prognosis, dividing patients into two categories: those with unmutated IgHV and those with mutated IgHV (18). Unmutated IGHV is associated with poorer survival rates and shorter treatment-free intervals (18). Among patients

with mutated IgHV, those exhibiting somatically mutated VH3-21 have a worse prognosis compared to those without it (102). Testing for IGHV mutations is crucial, as it affects both prognosis and treatment decisions. In fit patients with CLL who have mutated IGHV, first-line chemoimmunotherapy with FCR has led to long-term remissions and improved overall survival (103). However, in U-IgHV BTK, proved to be superior, when compared to chemo-immunotherapy (104, 105).

Mutations like NOTHC1, SF3B1, BIRC3 and ATM have been linked to CLL pathogenesis and could also be used as prognostic factors (106). NOTCH1 is an important regulator of hematopoiesis, and it often corresponds to high ZAP-70 and CD38 expression, U-IgHV and the presence of trisomy 12, being associated with a shorter TTFT, resistance to fludarabine and increased likelihood of developing Richter's Syndrome (RS) (107) (108).

Mutations in SF3B1 result in mRNA splicing defaults that will alter key pathways involved in cycle-cell regulation, angiogenesis and apoptosis (109). Patients with SF3B1 mutations can develop resistance to fludarabine based therapies and shorter TTFT, but not lower OS (110).

BIRC3 and ATM are both encoded in the long arm of the 11th chromosome. Among those with del11q CLL, BIRC3 mutation occurs in 83% of cases (111).

Low BIRC3 expression is associated with more aggressive disease, but also with increased sensitivity to BCL-2 inhibitors, which suggests that low BIRC3 expression or loss-of-function mutations might stand to gain from venetoclax-based treatment (112).

ATM mutations are found in 25% of patients diagnosed with CLL and are frequently linked to unmutated IGHV, ZAP-70 expression, and the presence of del(11q) (108).

Since nearly all patients with del11q CLL also have deletions of ATM, it remains uncertain whether the negative prognostic impact of del11q CLL is due to deletions of ATM, BIRC3, or both (111).

2. Treatment of Chronic lymphocytic leukemia

2.1. Treatment initiation criteria

Current guidelines advise that the treatment should only be started for patients having symptomatic CLL, while for the rest, the optimal strategy is to postpone it until the disease progresses, as no survival benefit was achieved by treating early-stage CLL (1, 25, 113, 114).

Patients diagnosed with CLL can be divided into 3 categories: those who have minimal involvement and do not require initiation of treatment, those who have an indolent initial course followed by rapid worsening of the disease, and those whose disease has an aggressive course from diagnosis (115).

Defining criteria for initiating treatment for CLL has been challenging, particularly as risk stratification becomes more refined.

For patients with early-stage disease – Rai 0, Binet A "watchful waiting" is generally recommended, until progression. Those with intermediate and high-risk, meaning advanced Rai/Binet stages, benefit from treatment, but if the clinical condition allows it, they can be monitored in the absence of treatment, until there are signs of active disease.

2.1.1. Signs of active disease

At least one sign of active disease should be met, for the treatment initiation, according to IwCLL guidelines. Table II describes the treatment initiation criteria. However, these criteria should not be assessed independently of the performance status or the duration of their evolution. Additionally, leukocytosis alone should not serve as a criterion for initiating treatment, as leukostasis has been scarcely reported in CLL (116).

NUMBER	CRITERIA
1	Hb<10g/dl and/or plt< 100 x10^9/L secondary to marrow failure and not autoimmune causes
2	Massive/progressive/ symptomatic splenomegaly
3	Massive/progressive/ symptomatic lymph nodes
4	Lymphocyte doubling time< 6 months or increase ≥50% in 2 months
5	Autoimmune complications that are refractory to corticotherapy
6	Symptomatic extranodal involvement
7	B symptoms that alter the quality of life

Hb=hemoglobin, Plt=platelets.

Table II. CLL treatment initiation criteria (1)

2.2. Current Therapeutic Options

The management of patients with CLL may involve chemotherapy, a combination of chemotherapy and immunotherapy, or medications that target the signaling pathways responsible for the growth and/or survival of CLL cells.

One of the first steps in the treatment of CLL is associated with a series of unfortunate events: World War (WW) I and WW II. During that time a lot of compounds and chemicals were tested on the battlefield. One of the compounds used was mustard gas which was first synthesized in 1860 by Frederick Guthrie (117). A few years later Viktor Meyer 'improved' this toxic gas making it more toxic (118). Mustard gas is a vesicant, which smells like garlic and was frequently used in WWI, until it was forbidden by the Geneva Gas Protocol, in 1925. However, not all countries agreed to sign this Protocol (117). During WWII another compound related to mustard gas was used as a biological weapon: nitrogen mustard. Compared to mustard gas, nitrogen mustard had no smell, and apart from being a vesicant it also targeted the bone marrow, leading to severe aplasia (119). Later, two pharmacologists, Alfred Gilman and Louis Goodman, published an article where they showed they cured mice with lymphosarcoma with nitrogen mustard (120). The first clinical trial with nitrogen mustard was conducted at the Yale School of Medicine on a 47 year old patient with lymphosarcoma. At first, the patient responded very well but he relapsed very quickly and succumbed (119). Later, cyclophosphamide, an alkylating agent was synthetized from nitrogen mustard (121). Nowadays, cyclophosphamide is used in the treatment of Hodgkin's Lymphoma, non-Hodgkin's lymphoma, CLL, and multiple myeloma, (122) but also in the setting of allogeneic stem cell transplantation (123).

One of the first lines of treatment in CLL was chlorambucil, an alkylating agent, synthetized by Alexander Haddow in the 1950s. Later, in 1955, the first report on chlorambucil in treating lymphoma was published (124). In 1998 a randomized clinical trial showed that when comparing chlorambucil, in different schedules with no treatment, in patients with stage A CLL, there was no benefit on survival. Thus, it was concluded that doctors should postpone treatment and defer it until patients progress to more advanced stages (125). There were several studies that evaluated chlorambucil in the first line setting in patients with CLL. CLL1 study reported a 57%, and a CR rate of 13%, in CLL2 study, 21% of the patients' achieved CR and 75% had an ORR. In CLL3, the ORR was 74% and the CR rate of only 17% (124, 126). CLL4 compared fludarabine -an alkylating agent- with chlorambucil and fludarabine +

cyclophosphamide. There was no significant difference in survival rates among the three treatment groups; however, the combination of fludarabine and cyclophosphamide showed benefits in terms of complete response CR and PFS.

The introduction of rituximab, a monoclonal antibody targeting CD20, revolutionized the management of various hematologic cancers and beyond. Approved by the Food and Drug Administration (FDA) in 1997 for the treatment of follicular lymphoma, rituximab is now utilized in the therapy of CLL, non-Hodgkin's lymphoma, and several rheumatological disorders (127-129) or neurologic diseases (130, 131), and pemphigus vulgaris (132). Off-label, it is also used in refractory autoimmune hemolytic anemia (133) and immune thrombocytopenia (134) and thrombotic thrombocytopenic purpura (135). Rituximab approval was followed by the emergence of second generation anti CD20-monoclonal antibodies, such as ofatumumab and Obinutuzumab, used as well in CLL's treatment (136).

For many years chemoimmunotherapy (CIT) was the mainstay for CLL patients with indication for treatment initiation - the combination of fludarabine with cyclophosphamide and rituximab (FCR) for young and fit CLL patients, bendamustine and rituximab (BR) for patients who were not fit enough to receive FCR and chlorambucil associated or not with an anti-CD20 monoclonal antibody, for frail patients (137). The discovery of prognostic risk factors proved that patients should be stratified according to the presence/absence of del(17p), del(11q), del(13q), trisomy 12, TP53 mutations, IgHV mutational status, as those harbouring these genetic abnormalities had worse outcomes with CIT (138). In 2014, Bruton tyrosine kinase inhibitor, ibrutinib, was FDA-approved for R/R CLL or with the presence of del(17p)(139). Later, the RESONATE-2 phase 3 trial, showed that ibrutinib had better results than chlorambucil in frail patients, even in the absence of high-risk factors (140). A041202 and E1912 phase 3 trials proved once again ibrutinib's superiority when compared to BR, respectively FCR, regardless of the genetic and molecular landscapes of the patients (105, 141). A041202 study, also showed that rituximab addition to ibrutinib does not bring any benefit (105). Given the context, ibrutinib was approved as frontline therapy for all CLL patients. Second generation BTKi, acalabrutinib and zanubrutinib, were created to to inhibit BTK more selectively, with a better safety profile than ibrutinib (142, 143). Furthermore, ALPINE study proved that zanubrutinib has better OS and PFS in R/R CLL patients when compared to ibrutinib, holding lower rate of discontinuation and a better safety profile (143).

The approval of BCL-2 inhibitor, venetoclax, in R/R CLL, brought a new turn of events in CLL treatment, along with the hope of a fixed-duration treatment. Venetoclax in combination with rituximab or obinutuzumab, was compared to CIT in R/R and TN CLL patients, showing increased PFS, which led to the endorsement of venetoclax as

frontline therapy, and not just for R/R, regardless of the cytogenetic and molecular profile of the patient (144, 145). Ibrutinib and veneotclax combination proved good OS and PFS, with undetectable minimal residual disease (uMRD), an endpoint that was not validated in BTKi single-agent therapy, in 66% of patients treated with 24 cycles, however, at the expense of increased toxicity, when compared to single agent therapy (146). Needless to say, in comparison to CIT, namely chlorambucil-obinutuzumab, ibrutinib-venetoclax, showed greater PFS (147). Combinations of BTKi, monoclonal antibodies and venetoclax in AVO and BOVen pahase 2 clinical trials, showed the achievemnt of deeper responses and higher and durable uMRD rates, but at the hand of increased treatment related toxicity (139). It remains to be seen whether the associated adverse events of novel combinations are justified by the achievement and persistence of uMRD.

3. Immune mechanisms involved in CLL progression

Innate and adaptive immune responses are significantly dysregulated in CLL and support the formation of a tolerant environment that promotes disease progression. Downregulation of innate immunity, through evasion of the function of NK cells, T cells, dendritic cells (DCs) and macrophages, leads to reduced apoptosis and tumor growth. In addition to changes in cellular components, notable modifications in the humoral response also play a significant role in the immune evasion of tumors in CLL (148).

Besides the cytokines released by CLL cells that help tweak the immune response to their advantage, vitamin D deficiency can oversee the whole tumor formation process, from the initial stages to metastasis, by modulating the activity of immune cells and the inflammatory and anti-inflammatory cytokine production (149, 150). Moreover, research has indicated that vitamin D can influence miRNAs linked to inflammation and tumor progression, such as miR-155 (151, 152).

MicroRNAs are small, non-coding proteins that regulate gene expression (153). Additional research has shown that miRNAs play a crucial role in the physiological development of all living organisms, including humans (154). They help regulate important processes such as embryogenesis, the cell cycle, cell differentiation, proliferation, and apoptosis (155). Dysregulation of miRNAs can lead to the development of malignant diseases, including CLL (156).

3.1. Vitamin D3

Vitamin D3 or the “sunshine vitamin", significantly influences the immune system, cellular growth and differentiation, and calcium and phosphate balance (157, 158). It is a fat-soluble compound with several isoforms. The most pertinent forms are ergocalciferol, also known as D2, which is present in plant-based foods, and cholecalciferol, or vitamin D3, which is produced in the skin secondary to sun exposure or can be acquired from dietary sources (159, 160). The production of vitamin D includes both enzymatic and photosynthetic processes. Both isoforms need to be activated by hydroxylation. Vitamin D will be hydroxylated at the 25 position to form 25 hydroxyvitamin D (25(OH)-D) in the liver, and then, in the kidneys, skin or immune cells, it undergoes a second hydroxylation at the 1st position, to form 1,25 dihydroxycholecalciferol (1,25(OH)2-D) (157).

Vitamin D regulates both the innate and the adaptative immune system (161, 162).

3.1.1. Vitamin D and innate immunity

As a result of exposure to inflammatory cytokines, the levels of Cytochrome P450 Family 27 Subfamily B Member 1 (CYP27B1) and vitamin D receptors (VDR) on the surface of monocytes and macrophages increase. This boost helps convert 25(OH)D into its active form, 1,25(OH)2-D. The double hydroxylated form of vitamin D then triggers macrophages to produce cathelicidin and defensin, which have antiviral, antifungal, and antimicrobial properties (163, 164). Additionally, calcitriol lowers the expression of TLR2 and TLR4 on monocytes and suppresses the production of IL-2, IL-6, and IL-17, thus suppressing inflammation, and shielding the body from self-damage (165, 166). It also helps regulate monocytes and macrophages during immune challenges, affecting their differentiation, phagocytosis, and chemotaxis (163, 167, 168).

Like macrophages and monocytes, neutrophils and NK cells also express VDR, enabling them to secrete antimicrobial peptides, like cathelicidin and defensin (165, 169). Vitamin D deficiency lowers neutrophils' migration at the site of infection and impairs the formation of neutrophil extracellular traps (NETs) (170). The effect of vitamin D on NK cells is still a controversial topic, with some studies claiming that it has an inhibitory effect on NK cells, while others describe a stimulatory effect (171-173).

Additionally, vitamin D affects dendritic cells (DCs) by suppressing their proliferation, maturation, and differentiation. It also diminishes the synthesis of inflammation cytokines such as IL-12, IL-23, tumor necrosis factor-alpha (TNF-α), and gamma interferon (IFN-γ), which are involved in the regulation of Th1 and Th17 differentiation (174). At the same time, vitamin D promotes the production of the anti-inflammatory cytokine IL-10 (174).

3.1.2. Vitamin D and adaptative immunity

B cells acquire their VDR only when they become activated (165, 175). Calcitriol is known to block cytokine-mediated activation of B cells by targeting T-helper cells and encouraging the production of IL-10 from B cells (176). Furthermore, it inhibits the differentiation of mature B cells into plasma cells, inhibits immunoglobulin production, and it triggers apoptosis in both activated B cells and plasma cells (176, 177).

The immune response relies on the balance between Th1 and Th2 cells (178). 1,25(OH)2-D reduces the production of pro-inflammatory cytokines and lowers the proliferation and differentiation of T helper (Th) 1 cells while also decreasing the activity of Th17 cells. Conversely, it boosts the activity of Th2 and T regulatory (Treg) cells by promoting the production of anti-inflammatory cytokines (179-182).

3.1.3. Vitamin D in CLL

In CLL, vitamin D deficiency was linked to a shorter TTFT and poorer OS (183). Reports indicate that vitamin D administration *in vitro* activates caspase 3 and 9 dependent pathways and modulates apoptosis of B cells (184, 185). Furthermore, low vitamin D levels increase the activity of myeloid-derived suppressor cells and promote leukemia growth, possibly because CLL B cells display an elevated expression of the VRD compared to normal B cells, which may result in tumor cell proliferation (186, 187). In a study that included 3,474 patients, 931 individuals were supplemented with vitamin D for six months, resulting in a reduced TTFT and extended treatment-free survival (TFS) (188).

3.2. MicroRNAs

MicroRNAs are tiny, non-coding molecules typically made up of about 22 nucleotides, and they play a key role in regulating gene expression (153). In 1993, lin-4 became the first miRNA ever discovered, and it was found to lower lin-14 mRNA levels, subsequently decreasing protein synthesis (189). Seven years later, the first human miRNA, let-7, which regulates lin-41 mRNA, was identified (190). Additional research concluded that miRNAs play a crucial role in the physiological development of humans, through regulating key processes, such as cell differentiation, proliferation and apoptosis (191). A new milestone was reached in 2002 when Croce and his collaborators were the first to describe that miR-15 and miR-16 were deregulated in CLL cells (192).

MiRNAs' role in cancers was extensively investigated, however, no guideline includes their use as diagnostic or prognostic markers and even less as therapeutical targets. However, ongoing research and clinical trials look promising.

3.2.1. MicroRNAs synthesis

MicroRNAs can be synthesized via either the canonical or non-canonical pathway. The non-canonical pathway is not as well understood. It usually skips at least one step from the canonical process, and the role of the resulting miRNAs is still somewhat unclear (193).

In the canonical pathway, most miRNAs are generated from introns by RNA polymerase II or III, resulting in a hairpin-shaped primary miRNA. In the nucleus, the Microprocessor complex, featuring the RNase III protein Drosha, starts the maturation of primary miRNA by cutting it at the base of the stem loop, producing the precursor miRNA, which is exported into the cytoplasm by a complex that includes exportin 5 (XPO5). In the cytoplasm, the Dicer RNase III-endonuclease removes the hairpin loop, resulting in a short RNA duplex. One of the strands will pair up with Argonaute family

members to form the RNA-induced silencing complex (RISC), which will specifically target an mRNA, leading to translation inhibition and gene silencing(194) . The canonical pathway is described in Figure 2.

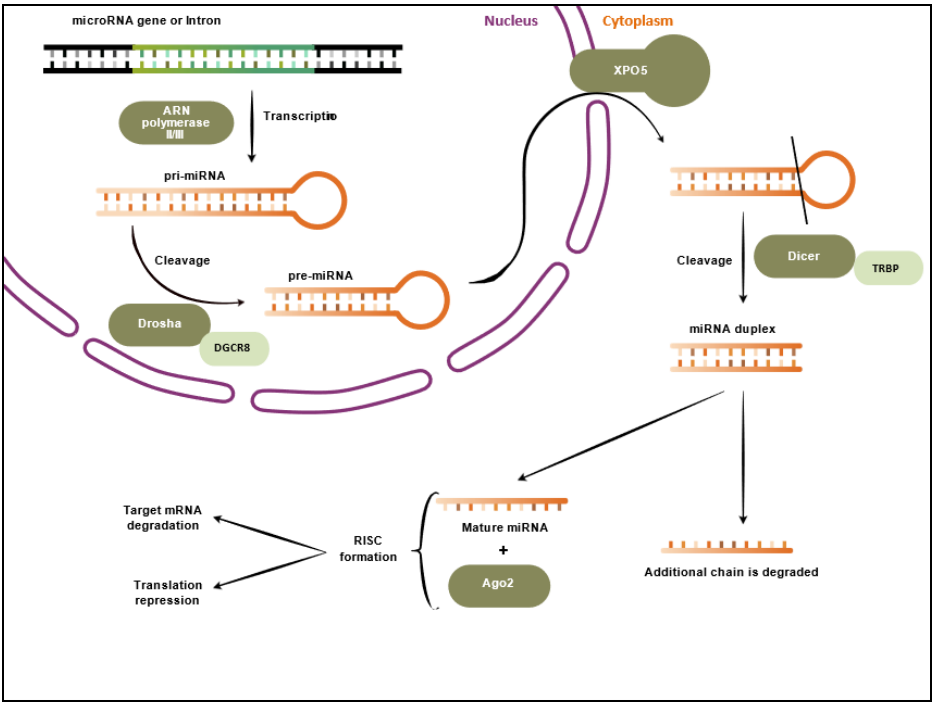

Figure 2: The canonical pathway of miRNAs biogenesis: Inside the nucleus the primary miRNA is transcribed by RNA polymerase II/III. The Microprocessor Complex (Drosha+DGCR8 proteins) cleaves the primary miRNA and the precursor miRNA that results, is exported by XPO5 into the cytoplasm, where the Dicer protein, along with TRBP, cuts it into a duplex. One of the resulting strands is used to form the RISC. The other one is discarded and degraded (194). Abbreviations: XPO5= exportin 5, TRBP= transactivation response element RNA-binding protein.

3.2.2. MicroRNAs and immune modulation

MicroRNAs regulate innate and adaptative immunity responses, but they also play a dual role in cancer biology, acting as tumor suppressors or oncogenes (195, 196). It has been suggested that miRNAs act as immune modulators in tumors, by diminishing the effectiveness of immune cells, interfering with the immune response, and altering apoptosis (30, 197).

MiR-15a and miR-16-1 were among the earliest microRNAs identified as playing a role in cancer development. In approximately 2/3 of CLL cases, these microRNAs are

found to be downregulated. The deletion of chromosome 13 (del(13q)) is linked to reduced expression of miR-15a and miR-16-1 since these microRNAs are located on the 13th chromosome (192, 198).

MiR-15a/16-1 modulate the response of CD8+ T cells (CTLs) against neoplasia. In a study on mice with GL261-derived glioma, miR-15a/16-1 levels were elevated. These CTLs secreted high levels of pro-inflammatory cytokines, such as IFN-γ and TNF-α, and reduced PD-1, Tim-3, and LAG-3 expressions, suggesting a higher sensitivity to immune checkpoint inhibitors therapy (30, 199).

MiR-155 is crucial for the maturation and functionality of B-cells and hematopoiesis (30, 200-202). Furthermore, miR-155 contributes to tumor progression in hematological cancers, including CLL (49).

Elevated levels of miR-155 have been detected in individuals with MBL, and even higher in CLL patients (203). Its overexpression is linked to ZAP-70 expression, U-IgHV, del (17q) and del(11q), which are all associated with a more aggressive form of the disease (203-205).

MiR 155 is prevalent in both innate and adaptive immune cells and has a pro-inflammatory impact (206). Its deficiency was linked to impaired functioning of Th1 and Th2 cells (201). Research on T cell immune responses indicates that heightened levels of miR-155 boost T cell sensitivity to homologous cytokines, such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (207) (208). This results in improved T-cell survival and greater antitumor effectiveness (207). Furthermore, miR-155 regulates PI3K/AKT and STAT pathways and promotes cytokine production, contributing to inflammatory response (207). In lymphoproliferative syndromes, miR-155 reduces cell survival and inhibits cell-cycle progression (209).

Research shows that miR-29 is downregulated in CLL and might have a role in disease development and progression (210). Its overexpression has been associated with a good prognosis (30, 156). MiR-29 inhibits inflammation by specifically targeting and disrupting TNF ligands, which are responsible for activating the NF-kB signaling pathway (211). Furthermore, it modulates Th cell activity, by lowering INF-γ production and causing immune dysfunction (212). Studies have shown that blocking miR-29 expression will result in enhanced activity of Th1, secondary to INF-γ production (213). Moreover, MiR-29 mitigates CD8+ T cells development and function and activates NK-cell immune responses (214, 215).

MiR-181 regulates apoptosis, cell cycle, and modulates TME (30, 216). In CLL, it acts as a regulator of B-cell differentiation by modulating the NF-κB pathway; lower levels of miR-181 were associated with treatment resistance (217). Furthermore, it regulates TCL and NK cells, by interfering with PI3K/AKT pathway (217, 218). If overexpressed miR-181 can induce anti-inflammatory effects, by promoting Treg differentiation, reducing macrophage polarization towards their inflammatory subtype, M1 and reducing DCs activity (219, 220).

MiR-223 was initially identified as a regulatory molecule involved in stem cell differentiation and immune response processes (221). In cancer development, miR-223 plays a significant role in modulating various signaling pathways, notably the NF-κB and PI3K/AKT pathways (222).

Patients with unmutated immunoglobulin heavy chain variable region (IgHV) showed reduced levels of miR-223, as did those with elevated ZAP70 expression. Additionally, increased expression of miR-223 was associated with the presence of del13q (223, 224).

Mir-223 inhibition stimulates the production of Il-6, IL1β and TNF-α, well-known inflammatory citokynes, through activation of the sigaling pathways transducer and activator of transcription 3 (STAT3) and NF-κB (225). MiR-223 reduces macrophage polarization towards the M1, inflammatory subtype (226). Furthermore, miR-223 inhibits neutrophil and DCs activation and chemotaxis (225).

The miR-17/92 cluster consists of six microRNAs: miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a-1, often referred to as oncomiR-1 (30, 227). In aggressive CLL with U-IgHV, this miRNA cluster downregulates genes that promote cell death and inhibit growth (228).

MiR-17/92 plays a role in B-cell maturation, with its expression decreasing as the cells advance in development. Additionally, it contributes to the synthesis of IgM antibodies (229). It enhances inflammatory responses by encouraging the proliferation and function of Th1 cells while suppressing Tregs. Additionally, the miR-17-92 cluster influences T cell movement into the B cell follicle and germinal center via the PI3K signaling pathway (230, 231).

Specific elements of the miR-17-92 cluster, particularly miR-17 and miR-20a, facilitate the differentiation and maturation of monocytes. Additionally, they influence macrophage activation by downregulating Signal Regulatory Protein α (SIRPα), which in turn enhances the levels of pro-inflammatory cytokines like TNF-α, IL-6, and nitric oxide (NO). On the other hand, miR-92 mitigates inflammatory responses by targeting the Mitogen-activated protein kinase kinase 4 (MKK4), resulting in lowered production of inflammatory cytokines (232, 233).

Along with miR-155, mir-146 is expressed in immune cells and modulates their function (234). In CLL low levels of miR-146b-5p were positively correlated with a shorter TTFT (235).

MiR-146 downregulation promotes inflammation by stimulating the production of high IL-6 and TNFα levels (236). Furthermore, it was associated with STAT1 pathway activation and decreased INF-γ (237).

The levels of miR-146 are elevated in monocytes while remaining unchanged in lymphocytes. Research revealed that miR-146 suppresses IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) function, leading to endotoxin tolerance and inhibition of monocytes' functions (231, 238, 239).

Furthermore, miR-146 modulates DCs' production of pro-inflammatory cytokines, without impacting their differentiation (240).

The expression of MiR-146 is elevated in Tregs. MiR-146 inhibits STAT1, which hinders Th1 differentiation and subsequently affects the functionality of Tregs (237).

PERSONAL CONTRIBUTION

1. Hypothesis

The present thesis started from the idea of CLL's robust mechanisms of immune evasion that contribute to disease progression and treatment resistance, which raises significant challenges in predicting disease progression and tailoring treatment strategies. In recent years, targeted therapies that act on specific proteins involved in CLL progression, such as BTKi and BCL-2 inhibitors have been developed. These therapies have brought a change of paradigm in CLL treatment, as they are proven to be well tolerated and have demonstrated at least similar efficacy in comparison to conventional chemo-immunotherapy, which is known to be of significant toxicity.

I hypothesised that, by investigating the outcome of CLL patients in recent years, in watch and wait, or treated with conventional and with the newer, non-chemotherapy regimens, we can understand both pluses and shortcomings of our current therapeutic paradigms, identify predictive and/or prognostic associations, and, finally, investigate new potential markers associated with particular disease evolution and treatment response.

In pursuit of this goal, we conducted a retrospective analysis of patients diagnosed and treated at the Hematology Department of IOCN over six years. The objectives were to characterize the population with CLL, assess the impact of targeted therapies on treatment strategies, and evaluate treatment responses and OS.

Further, we questioned whether BTKi therapy could have a different effect, in terms of response to treatment, OS and the number of adverse events, on a real-world population compared to the carefully selected participants in clinical trials. Consequently, we utilized the database we established to select and to examine disease outcome, O, and the tolerance of BTKis within our patient cohort.

Finally, the evolution and progression of CLL are influenced by the interplay between the patient's genetic mutation landscape and dysregulated immune mechanisms. Specifically, we propose that microRNAs and vitamin D deficiency are significant factors that interfere with innate immunity in CLL patients and may serve as potential modulators of disease progression.

2. General Methodology

The research discussed in my thesis was conducted with the approval of the Ethics Council of the "Iuliu Haţieganu" University of Medicine and Pharmacy in Cluj-Napoca, as of the Ethics Council of the "Prof. Dr. Ion Chiricuta" Institute of Oncology, in Cluj-Napoca (IOCN), for data collection.

A retrospective study was performed on clinical and paraclinical data of CLL patients diagnosed and treated in Hematology Department at "Prof. Dr. Ion Chiricuta" Institute of Oncology, in Cluj-Napoca. The relevant data concerning diagnosis, treatment, and disease progression was gathered from the patients' observation sheets and the IOCN database. This information was then compiled into an Excel spreadsheet, and statistical analyses were conducted using Excel spreadsheet and GraphPad 10.3 (GraphPad Software, Inc., La Jolla, CA). We analyzed the correlations between clinical and paraclinical data with treatment response, complete remission (CR), and overall survival rate.

At the time of diagnosis or prior to the initiation of treatment, a 5 ml vacutainer containing a coagulation activator, and a separating gel was collected from each patient. This sample was centrifuged for 25 minutes at a temperature of 20°C and at a speed of 3000g. A unique laboratory ID was assigned to each patient. The resulting serum was then aliquoted into Eppendorf tubes, with each tube containing 500 µL, and stored at -80°C.

One 500 µL Eppendorf has been outsourced to an accredited laboratory for testing the vitamin D level.

For miRNA profiling, the sera from patients were thawed and processed for miRNA extraction according to the manufacturer's protocol included in the Small RNA and DNA from Plasma NucleoSpin™ miRNA Plasma kit by Macherey-Nagel. cDNA was synthesized from reverse-transcribed RNA using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Thermo Fisher Scientific). The PCR reaction mixture was prepared following the manufacturer's instructions with TaqMan™ Small RNA Assays from Applied Biosystems (Thermo Fisher Scientific). The miRNAs' expression levels were correlated with clinical and paraclinical data and with vitamin D level.

3. An Overview of CLL in the Experience of Hematology Department of "Prof. Dr. Ion Chiricuta" Institute of Oncology, in Cluj-Napoca. A Retrospective Study (1st Study)

3.1. Introduction

CLL is a lymphoproliferative neoplasm characterized by the presence of clonal B lymphocytes in the peripheral blood and lymphoid organs (1).

Although it is regarded as an indolent disease, there were 40,000 reported deaths attributed to it in 2019 (11). Accounting for 30% of all leukemias, CLL is a disease of the elderly, with a median age at diagnosis around 70 years (2, 241). The advanced age, which correlates with increased frailty and higher comorbidities rates, could be a reason for the number of deaths reported.

The standard recommendation for treating fit patients with CLL typically involved chemo-immunotherapy (CIT), which combines rituximab—an anti-CD20 monoclonal antibody—with fludarabine and cyclophosphamide (FCR), a regimen which was mostly well tolerated and obtained good ORR, but with the possible risk of developing secondary malignancies to fludarabine (242-244). However, for older, unfit CLL patients, this regimen was not indicated, and milder types of treatment, involving chemotherapy alone, such as chlorambucil or cyclophosphamide-vincristine-prednisone (COP), anti-CD20 monoclonal antibodies, or a combination between the two of them was the preferred therapeutic option.

The introduction of prognostic markers like del(17p), TP53 mutations, and IgHV mutational status has revealed that CIT is not an appropriate treatment option for all TN CLL patients, and even less for those with R/R disease (245).

The introduction of targeted therapy with the approval of BTKi, in 2014, transformed the treatment landscape, particularly for patients with unfavorable prognostic factors such as TP53 mutations, del(17p), or U-IGHV status. This provided a valid, well-tolerated, and chemotherapy-free treatment alternative. Over time, BTKi therapy was extended to encompass all patient categories, regardless of their risk stratifications, demonstrating a superior safety profile compared to conventional CIT and achieving a favorable ORR. However, clinicians faced challenges in clinical practice related to the indefinite duration of treatment and concerns over the novelty of the therapy, which might lead to unforeseen long-term adverse effects in the settings of real-world patients. The emergence of the BCL2-inhibitor venetoclax has come to

clinicians' rescue, offering the option of a fixed treatment duration by validating the combination of venetoclax with the monoclonal antibodies anti-CD20, rituximab and obinutuzumab. These treatments offer spectacular results in terms of ORR and PFS and are well tolerated by patients with multiple comorbidities (145, 246).

3.2. Hypothesis

The approval of targeted therapy brought CLL into a new era. Furthermore, the emergence of prognostic markers has ruled out the idea of CIT as the standard of care for CLL patients. This study aims to assess the extent to which the emergence of targeted therapies and prognostic markers have changed the approach and survival in CLL, outside clinical trials, the results of this switch and what can be improved in clinical practice.

3.3. Materials and Methods

We conducted a retrospective study examining the clinical and paraclinical data of CLL patients who were diagnosed and treated between January 1st, 2017, and December 31st, 2022, at the Hematology Department of the "Prof. Dr. Ion Chiricuta" Institute of Oncology in Cluj-Napoca. The relevant data concerning diagnosis, treatment, and disease progression was gathered from the patients' observation sheets and the IOCN database. Patients were censored as of the 31st of December 2023, and any patients lost to follow-up were censored at their last clinic visit.

The inclusion criteria for the retrospective study consisted of the following:

- Individuals aged 18 years and older, diagnosed with MBL/CLL/SLL at the Hematology Department of the IOCN.
- Individuals who received treatment within the aforementioned department.

The following criteria were established for excluding participants from the retrospective study:

- Individuals under the age of 18
- Individuals diagnosed with other chronic lymphoproliferative disorders, including CLL-like conditions
- Individuals who were diagnosed and/or treated at different medical institutions
- Individiuls for which we couldn’t gather sustainable data from their medcal records
- Individuals who did not provide their consent by signing the consent form

The collected data was entered into an Excel spreadsheet and cross-verified with each patient's medical record for statistical analysis. The numerical (quantitative) variables were summarized by their mean, standard deviation and mean confidence

interval (CI) of 95%, median, minimum, and maximum values. We also studied the distribution pattern of values and differences from the normal distribution. Categorical variables were summarized by relative frequency, expressed as numbers and percentages. We assessed the existence of correlations between the studied variables by using appropriate statistical tests. Thus, for categorical variables, we used the chi2 test. For quantitative variables, we used the t-test for independent samples as well as the Pearson correlation coefficient obtained from the linear regression. We considered that a value of this coefficient between 0.35 and 0.7 indicates a moderate correlation between the two variables, and a value above 0.7 a strong correlation. For values below 0.35 we considered the 2 variables as independent.

For the survival analysis we considered the date of diagnosis as the reference date, for studied intervals. For OS the event of interest was death. For patients who at the study closure date were alive the survival interval was considered the censored date.

Univariate survival analysis was performed with the Kaplan- Meier estimation method, and log-rank test to assess the differences between them. For multivariate analysis, we used Cox proportional hazards regression model to assess the simultaneous influence of several factors on survival.

We considered the statistical significance limit for all tests to be $p<0,05$.

All statistical analyses were performed using the ExcelSpreadsheet and GraphPad 10.3 (GraphPad Software, Inc., La Jolla, CA).

We analyzed the correlations between clinical and paraclinical data with treatment response, complete remission, and overall survival rate.

3.4. Results

We included 308 patients, out of which 11 were diagnosed with MBL, 265 with CLL and 32 with SLL (Figure 3).

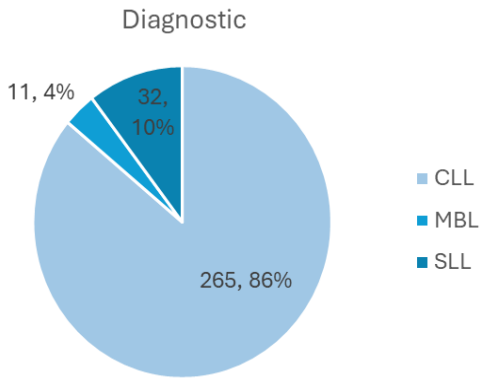

Figure 3: Patients according to their diagnosis.

Table III presents the biological characteristics of our many patients.

Biological parameter	Mean (min-max)
ALC x 10^9/L	23 (0.6 -520)
AMC x 10^9/L	0.8 (0-216)
Hb g/dl	13 (4.1 – 18.5)
Plt x 10^9/L	177 (1-423)
LDH UI/L	344 (94-1555)
IgG mg/dl	859 (103-3566)
Creatinine mg/dl	1 (0.27-9)
Uric acid mg/dl	6 (1.5- 14.8)
ESR mm/1h	9 (1-112)

***Table III*:** Patient's biological characteristics Abbreviations: ALC-absolute lymphocyte count; AMC-absolute monocyte count; MCV-mean corpuscular volume, Hb- hemoglobin, Plt-platelets, LDH- Lactate Dehydrogenase, IgG-immunoglobulin G, ESR-erythrocyte sedimentation rate

The distribution of patients by year of diagnosis was relatively consistent from 2017 to 2019. However, in 2020, we noted a decrease in the number of patients diagnosed with CLL, followed by a significant rise in the subsequent years (Figure 4).

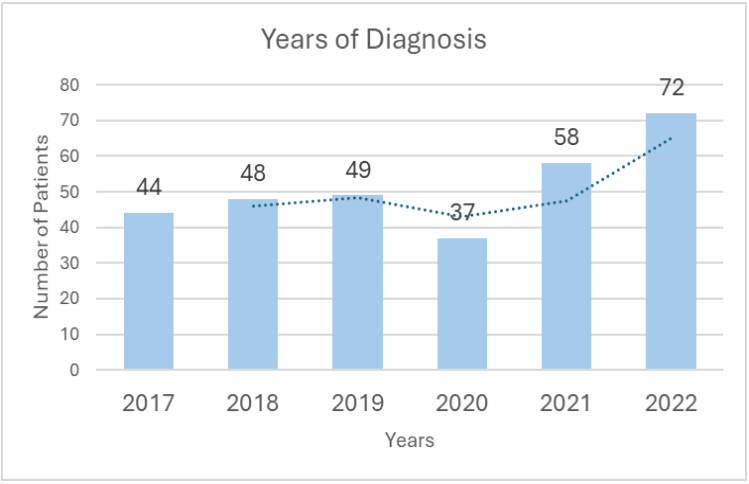

Figure 4: Distribution of patients based on their year of diagnosis

In our patient population, we observed a higher prevalence of individuals from urban backgrounds. Specifically, 54% of our patients were from urban areas, compared to 46% from rural areas (Figure 5).

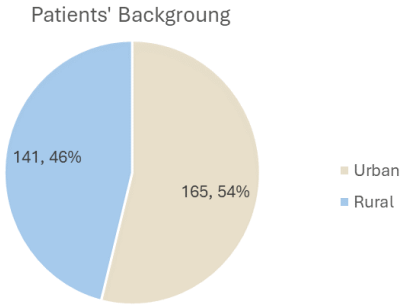

Figure 5: Distribution of patients based on their background

Our patient group showed a higher number of males, accounting for 62% (N=191) of the total, while females' percentage was only 38% (N=117) (Figure 6).

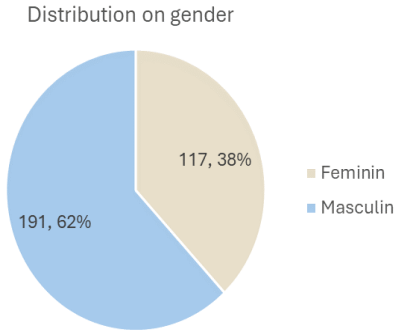

Figure 6: Distribution of patients based on their gender

Among the 265 patients diagnosed with CLL, 30.2% (N=80) were classified as Rai stage 0, 26.8% (N=71) as Rai stage 1, 18.9% (N=50) as Rai stage 2, 9.4% (N=25) as Rai stage 3, and 14.7% (N=39) as Rai stage 4. In terms of the Binet staging system, 41.9% (N=111) of the patients were in stage A, 34% (N=90) in stage B, and 24.2% in stage C (Figure 7).

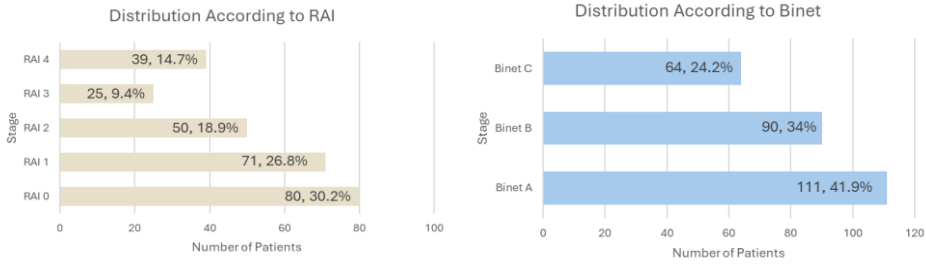

Figure 7: Distribution according to Rai/Binet staging systems

The distribution of patients by age revealed a peak incidence in the sixth decade of life, followed by the seventh. Only two patients were aged 90 or older. The median age at diagnosis was 66 years (range: 34-92) (Figure 8).

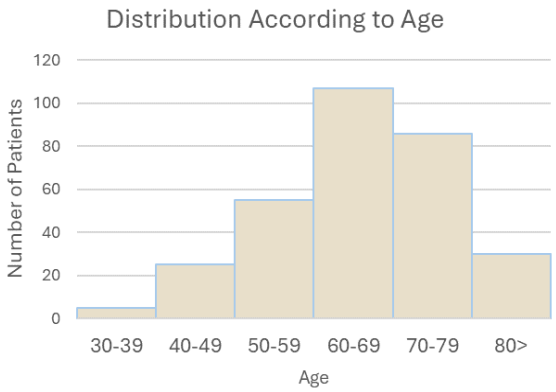

Figure 8: Distribution according to age at diagnosis

IgHV mutational analysis was conducted on 173 patients, revealing that 35.5% (N=61) had M-IgHV. Del(17p) was examined in 176 patients, with a confirmed presence in 7.4% (N=13) of cases. TP53 mutation analysis was performed on 177 patients, where 6.7% (N=12) exhibited the TP53 mutation (Figure 9). Among these, 8 patients had both TP53 mutation and del(17p), while 4 had only the TP53 mutation, and 5 displayed isolated del(17p).

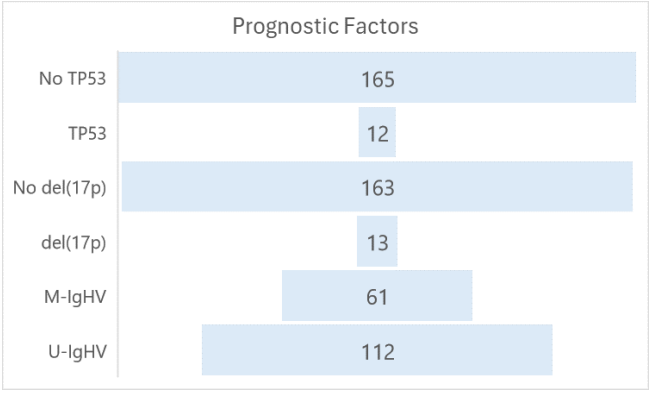

Figure 9: Distribution according to prognostic factors

Of the 308 patients, 58% (N=180) required the initiation of treatment (Figure 10).

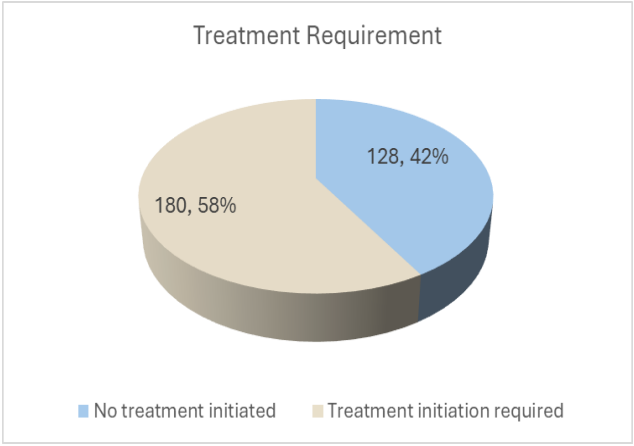

Figure 10: Distribution according to treatment initiation

For our group median time to first treatment was only two months, ranging from zero to 61 months. Figure 11 describes the variation of TTFT. 113 patients required treatment initiation in less than 6 months from the moment of diagnosis.

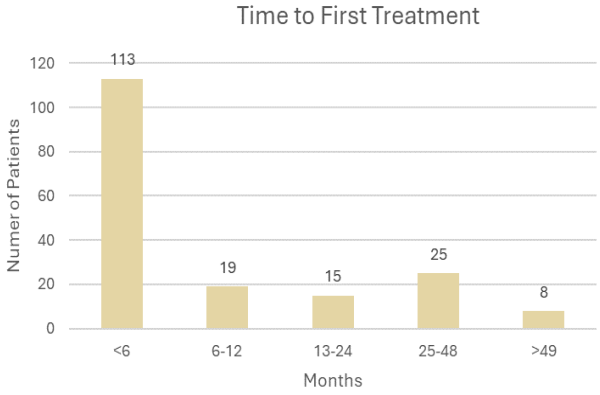

Figure 11: Time to first treatment

The median age at the beginning of treatment was 66 years (range from 34 to 92 years) (Figure 12).

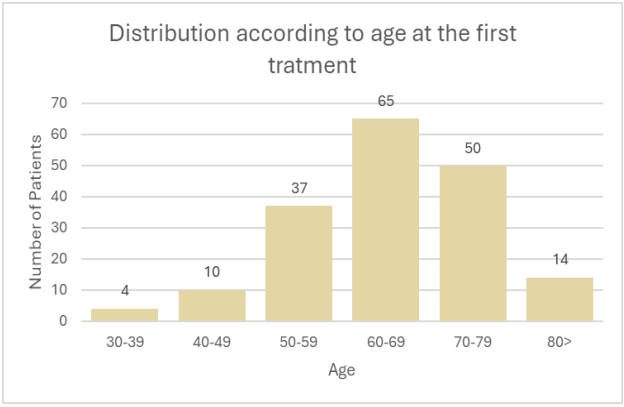

Figure 12: Distribution according to age at treatment initiation

As the initial line of treatment, 44.7% (N=67) received targeted therapy, mostly represented by venetoclax-obinutuzumab (ven-obi) and ibrutinib. 55.3% (N=83) received chemo-immunotherapy (CIT), represented by either R-COP, FCR, R-leukeran, or O-leukeran (figure 13). Table II presents details about the treatment regimens that are used less frequently.

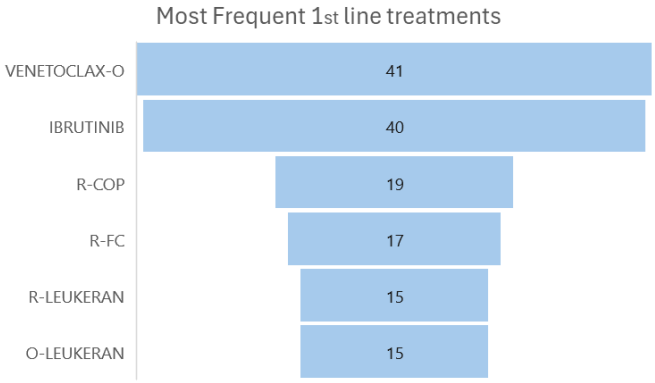

Figure 13: Distribution according to treatment initiation. **Abbreviations:** O=Obinutuzumab; R=Rituximab; R-COP=Rituximab-Cyclophosphamide-Vincristine-Prednisone; R-FC=Rituximab-Fludarabine-Cyclophosphamide ;

Regimen	Number of patients
LEUKERAN	6
VEN-R	5
IBRUTINIB-O	5
O	5
ACALABRUTINIB-O	2
OFA-LEUKERAN	2
BENDAMUSTINE-R	2
COP	2
VEN	1
PIRTOBRUTINIB	1
R-CHOP	1
IBRUTINIB-VEN	1

***Table IV*:** Less used 1st line treatments. Abbreviations: Ven=venetoclax; R=rituximab; O=obinutuzumab; OFA=ofatumumab;R-CHOP=Rituximab-Cyclophosphamide-Doxorubicin-Vincristine-Prednisone; COP= Cyclophosphamide-Vincristine-Prednisone

The effectiveness of the first-line treatment was assessed in 155 out of 180 patients. Among the others, 25 were lost to follow-up or had medical records with unavailable data. According to the IwCLL response criteria, the overall response rate (ORR), which includes complete responses (CR), complete responses with incomplete marrow recovery (CRi), and partial responses (PR), was 89.7% (N=139) (see Figure 14). Additionally, 3.2% (N=5) of patients experienced intolerance to the first-line treatment, necessitating a change in therapy.

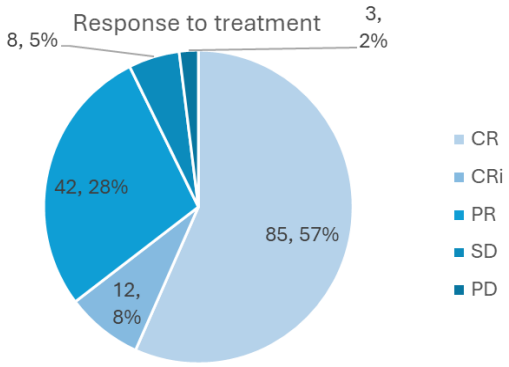

Figure 14: Response to treatment. **Abbreviations:** CR= complete responses, CRi= complete responses with incomplete marrow recovery, PR= partial responses, SD=stable disease, PD=progressive disease

Among the 308 patients, 16.2% (N=50) required a second line of treatment, with a preference for ibrutinib and venetoclax-rituximab (Figure 15). Responses to treatment were unable to be assessed for 28% of the patients, while 2% exhibited

treatment intolerance. Of the patients evaluated, 38% achieved CR and 32% experienced PR. No cases of SD or PD were reported.

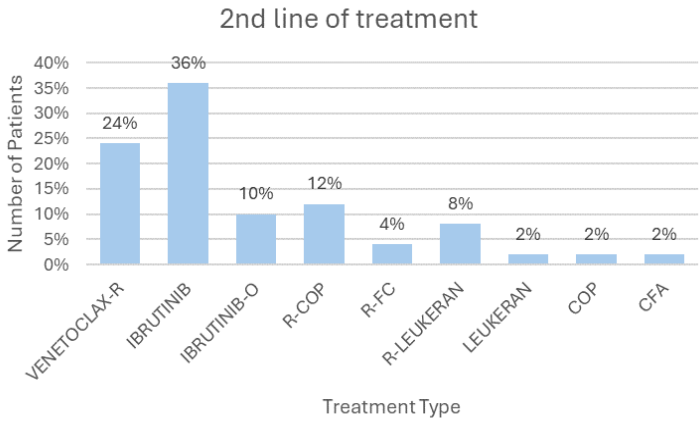

Figure 15: Second line treatment. **Abbreviations:** O=Obinutuzumab; R=Rituximab; R-COP=Rituximab-Cyclophosphamide-Vincristine-Prednisone; R-FC=Rituximab-Fludarabine-Cyclophosphamide; CFA= Cyclophosphamide

Eight patients required a third line of treatment, and three of them developed RS. None of the patients diagnosed with MBL required treatment during the follow-up period. At the time of censoring, 62 patients (20.1%) had died, while 246 patients (79.9%) remained alive. The median follow-up duration was 25.3 months, with a range of 1 to 93.6 months. The median OS was not reached during the median follow-up period. As anticipated, patients with monoclonal MBL have a superior OS rate compared to those with CLL/SLL. In our cohort, CLL patients exhibited a 3-year OS rate of 79.5% [95% confidence interval (CI): 74.1-85.2], whereas SLL patients demonstrated a 3-year OS rate of 81.4% [95% CI: 65-100] (Figure 16).

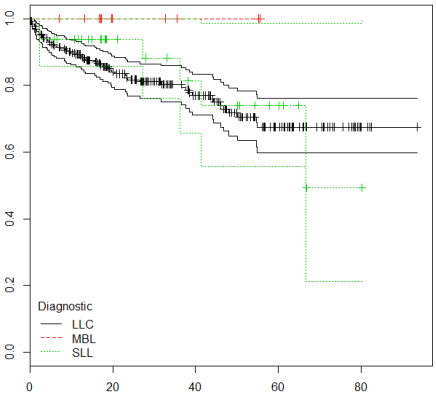

Figure 16: Survival depending on the diagnosis: MBL SLL, CLL. The median overall survival (OS) was not reached at the median follow-up period of 25.3 months.

We analyzed the relationship between the assessed prognostic factors and OS. When examining the association between the presence of del(17p) and OS, we found no statistically significant result (p = 0.914, which is greater than 0.05). Similarly, there was no significant correlation between TP53 mutations and OS (p = 0.529, also greater than 0.05). However, individuals without del(17p) or TP53 mutations seem to have improved survival, as indicated by a higher survival curve in the graphs (Figure 17).

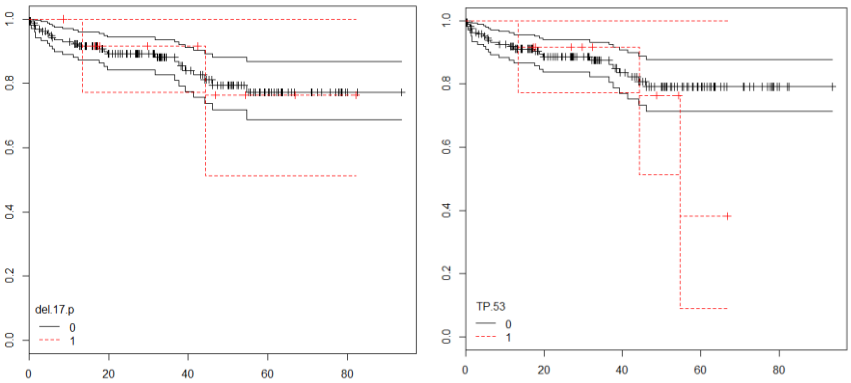

Figure 17: Survival depending on del(17p) and TP 53 mutation

IgHV mutational status was correlated with OS. No statistical significance was observed in our group of patients (p= 0.978>0.05) (Figure 18).

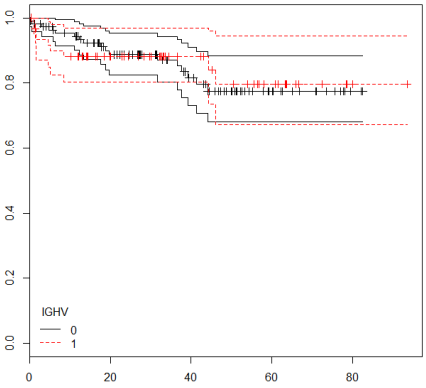

Figure 18: Survival depending on IgHV mutational status

We assessed OS based on whether patients received targeted therapy or conventional chemotherapy. The results indicated a statistically significant improvement in OS for the group that received targeted therapy compared to the conventional chemotherapy group (p= 0.0006< 0.05) (Figure 19).

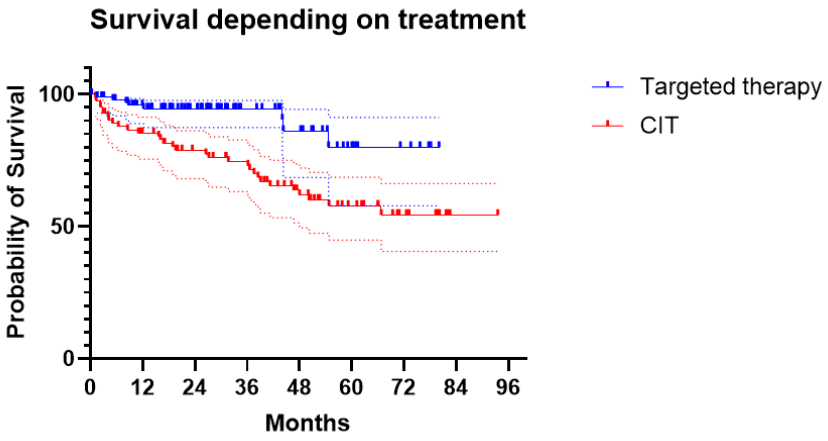

Figure 19: Survival depending on the choice of treatment.
Abbreviation: CIT=chemo-immunotherapy

3.5. Discussions

We evaluated 308 MBL/CLL/SLL diagnosed and treated at the IOCN, over 6 years. The patients were followed until the 31st of December 2023.

Regarding the year of diagnosis, a lower incidence can be observed between 2017 and 2019, which is probably due to a lower number of patients presenting for routine check-ups. The lowest incidence of CLL was recorded in 2020, which does not mean that the incidence of CLL is decreasing, but it is explainable in the context of the SARS-COV2 pandemic and the limitation of access to standard health care. The years 2021 and 2022 describe the highest incidence of CLL, in IOCN, explained by an increase in people's concern about their own health and an increase in the number of routine check-ups.

Compared to Worldwide records, our cohort of patients has a preference as well for the male sex, but it is slightly younger, with a median age at diagnosis of 66, ranging from 34 to 92 (2).

As described in most clinical trials, in our cohort most patients had Rai 0/Binet A at diagnosis (247, 248). Unfortunately, data that could help evaluate any comorbidity index or performance status, was inconsistent.

Prognostic factors were evaluated in over 50% of the population studied and in 66.7% of the patients who began treatment (data not shown). This is consistent with findings from other real-world clinical trials (247-250). Del(11q), del(13q) and trisomy 12, were assessed in a small number of patients, less than 10%, as access to these evaluations was financially challenging. According to the IwCLL recommendations, the CLL-IPI should be regarded as a prognostic score. However, we were unable to calculate it for our patients because B2M was measured in only a limited number, which would diminish the score's statistical significance.

Median TTFT was only two months, ranging from 0 to 61 months. Such a small median TTFT is explained by the fact that 113 of our patients had treatment indication in less than 6 months from the moment of diagnosis.

Out of 308 patients, 180 required treatment initiation. Among them, 84 were administered CIT, while 96 underwent targeted therapies, indicating a slight preference for the latter. 25 of the patients were lost to follow-up and the response to treatment could not be evaluated, and 5 patients were intolerant to initial therapy, and needed treatment change. For the remaining patients ORR, which included CR, CRi and PR, was 89.7%, which follows real-world clinical data (249-252). However, 16.2% of patients needed a 2nd line of therapy, due to intolerance, SD, PD or relapse. In the 2nd line, preference for the targeted therapy is obvious, accounting for 60% of the regimens used. None of the patients diagnosed with MBL needed treatment initiation during the follow-up period, and as anticipated, and confirmed by the literature, patients with monoclonal MBL have a superior OS rate compared to those with CLL/SLL (253).

At the end of the follow-up period, 20.1% of patients had died, while 79.9% were alive. At the median follow-up of 25.3 months, the median OS was not reached, an aspect supported by the existing literature (145, 242, 243, 246, 254).

We correlated OS with the presence/absence of risk prognostic markers, such as del(17p), TP53 mutation and IgHV mutational status. No statistically significant correlations were found, which is explicable in the context of almost two equal groups of patients (CIT group had 83 patients *versus* the targeted treatment group, which had 67). This has also been observed in other real-life studies. However, even if the correlation is not statistically significant, the graph suggests a slightly worse survival for patients with del17p and/or TP53 mutation. This was not observed in patients with mutated vs unmutated IgHV. Our findings line up with the literature (141).

Further, we correlated OS with the type of treatment - CIT or targeted therapy. It was observed that similar to clinical trials and real-life studies, the group of patients treated with targeted therapy had a better OS than the group with CIT (145, 246, 255-257).

3.6. Conclusions

The objective of this study was to assess the outcomes achieved in a real-world clinical environment and to examine how clinicians adapted to the introduction of targeted therapies. The SARS-COV2 pandemic has had disastrous effects on Romania's healthcare system, but it seems to have stimulated the population to have more frequent routine check-ups, which led to a higher incidence of CLL's diagnostic in its initial, asymptomatic stages. Although initially, targeted therapy was viewed with circumspection by hematologists, there is now a preference for targeted therapy over the classic CIT in patients requiring treatment for R/R disease, and beyond. This effect may occur with the emergence of limited-duration targeted therapies such as venetoclax-obinutuzumab or venentoclax-rituximab. The value of known prognostic markers is decreasing in the light of these novel therapies.

4. The impact of ibrutinib in the management of CLL (2nd Study)

4.1. Introduction

CLL is a lymphoproliferative neoplasm characterized by the presence of clonal B lymphocytes in the peripheral blood, bone marrow, lymph nodes, and spleen, specifically those that are CD5+, CD23+, and CD19+, with a count of at least 5 x 10^9/L (1).

Due to its prevalence, accounting for 30% of all leukemias, CLL is a condition of significant interest (241). Despite being classified as an indolent disease, CLL can exhibit highly heterogeneous progression, resulting in varying durations from diagnosis to the start of treatment.

The conventional approach for treating fit patients with CLL used to involve chemo-immunotherapy, which combines rituximab—an anti-CD20 monoclonal antibody—with fludarabine and cyclophosphamide (244).

With the emergence of prognostic markers, such as del(11q), de(13q), del(17p), trisomy12, TP53 mutations, and IgHV mutational status it became clear that the FCR regimen is not a suitable treatment option for all fit patients, even more so, for patients with relapsed/refractory (R/R) disease (245). The Food and Drug (FDA) approval for Bruton tyrosine kinase inhibitor, ibrutinib, in 2014, has paved the way for more personalized treatment approaches, particularly for patients with refractory disease and those with del(17p) and/or TP53 mutation, which were the primary indications for this therapy. Nowadays, prognostic factors have become less significant, given that BTK inhibitors are generally better tolerated than chemo-immunotherapy, and consequently, their use has expanded beyond just patients with poor prognoses.

In Romania, ibrutinib received its first approval in 2017 as a single-agent treatment for R/R CLL or fost CLL with del(17p) and/or TP53 mutation, and subsequently for all CLL cases that met therapeutic indications, whether used alone or in combination with other drugs.

4.2. Hypothesis

CLL is a non-aggressive disease, with heterogenous manifestations. In 2017, BTK inhibitors were approved in Romania for the treatment of CLL, presenting the opportunity for enhanced patient outcomes. However, these advantages come with challenges, including the necessity for indefinite treatment duration and a variable

spectrum of side effects, particularly in patients who may differ significantly from those who participated in clinical trials. This study aims to assess the response to ibrutinib in patients with CLL through a retrospective analysis of real-world data, to contribute to the development of more effective strategies for selecting CLL therapies.

4.3. Materials and methods

We performed a retrospective study on clinical and paraclinical data of CLL patients diagnosed and treated with single agent ibrutinib, either as first line of therapy or for R/R disease, in Hematology Department at "Prof. Dr. Ion Chiricuta" Institute of Oncology, in Cluj-Napoca, between January 1st, 2017, to December 31st, 2022. The relevant data concerning diagnosis, treatment, and disease progression was gathered from the patients' observation sheets and the IOCN database. Patients were censored as of July 31, 2024, and any patients lost to follow-up were censored at their last clinic visit. Patients with unconfirmed diagnoses of CLL/SLL, and those who did not receive ibrutinib treatment during the follow-up period were excluded from the analysis, along with those who chose not to give their consent.

The collected data was compiled into an Excel spreadsheet, and statistical analyses were conducted using Excel spreadsheet and GraphPad 10.3 (GraphPad Software, Inc., La Jolla, CA). The numerical (quantitative) variables were summarized by their mean, standard deviation and mean confidence interval (CI) of 95%, median, minimum, and maximum values. We also studied the distribution pattern of values and differences from the normal distribution. Categorical variables were summarized by relative frequency, expressed as numbers and percentages. We assessed the existence of correlations between the studied variables by using appropriate statistical tests. Thus, for categorical variables, we used the chi2 test. For quantitative variables, we used the t-test for independent samples as well as the Pearson correlation coefficient obtained from the linear regression. We considered that a value of this coefficient between 0.35 and 0.7 indicates a moderate correlation between the two variables, and a value above 0.7 a strong correlation. For values below 0.35 we considered the 2 variables as independent.

For the survival analysis we considered the date of diagnosis as the reference date, for studied intervals. For OS the event of interest was death. For patients who at the study closure date were alive the survival interval was considered the censored date.

Univariate survival analysis was performed with the Kaplan- Meier estimation method, and log-rank test to assess the differences between them. For multivariate analysis, we used Cox proportional hazards regression model to assess the simultaneous influence of several factors on survival.

We considered the statistical significance limit for all tests to be $p<0,05$.

All statistical analyses were performed using the Excel spreadsheet and GraphPad 10.3 (GraphPad Software, Inc., La Jolla, CA).

4.4. Results

In total, we enrolled 61 patients with treatment-naïve or relapsed/refractory CLL/SLL who were treated with ibrutinib. Among these, 55 patients (90%) were diagnosed with CLL, while 5 patients (10%) were diagnosed with SLL (Figure 20). Of the total, 40 patients (65.6%) received ibrutinib as a frontline treatment, whereas 21 patients (34.4%) were treated for relapsed or refractory disease (Fig. 20).

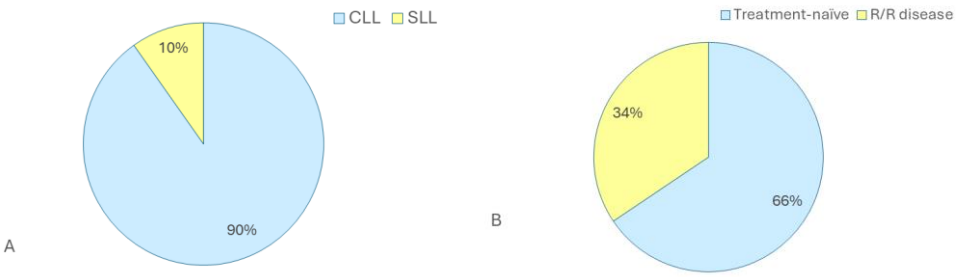

Figure 20: Chart A: 10% of patients diagnosed with SLL; 90% of patients diagnosed with CLL; **Chart B:** 66% of patients were treatment-naïve; 34% of patients had R/R disease.

The distribution of patients based on their year of diagnosis remained fairly uniform, except 2022, when only four patients met the inclusion criteria (Fig. 21). One possible explanation for the small number of patients for whom ibrutinib was initiated in 2022 is that more patients are choosing to be treated by their local hematologist.

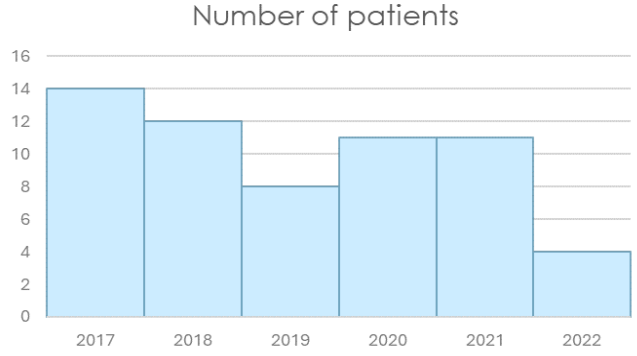

Figure 21: Distribution of the patients based on their year of diagnosis

Our patient group showed a higher number of males, accounting for 57.4% (N=35) of the total, while females were 42.6% (N=26) (Fig. 22).

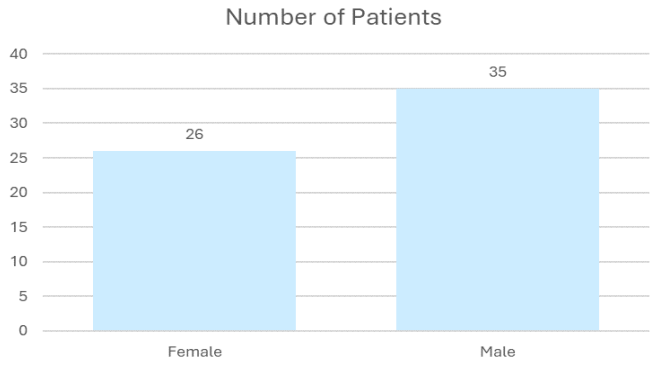

Figure 22: Distribution of the patients based on their gender

The median age at diagnosis was 63 years, with an age range of 34 to 83 years, while the median age at the start of treatment was 65 years, spanning from 34 to 83 (Fig. 23).

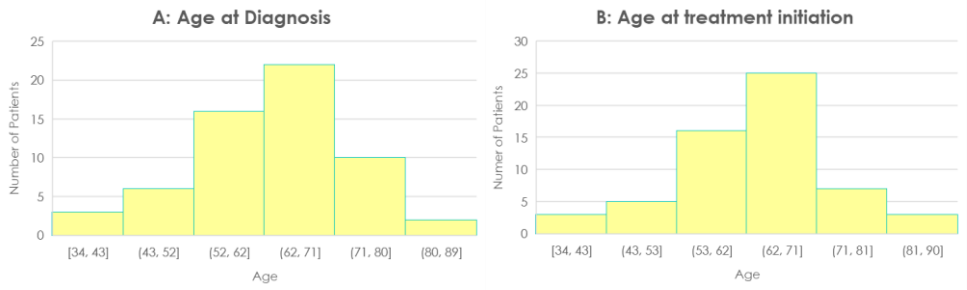

Figure 23:**A**: Distribution of the patients based on their age at diagnosis; **B:** Distribution of the patients based on their age at treatment initiation

Regarding CLL staging, 42.6% of participants fell under Binet stage A, 26.2% under Binet stage B, and 21.3% under Binet stage C. In terms of Rai staging, 69% were classified as Rai stages 0-2, while 23% were classified as Rai stages 3-4. It's important to mention that the Rai/Binet stages were not evaluated for the five patients diagnosed with SLL; instead, Ann Arbor (AA) staging was utilized (Figure 24).

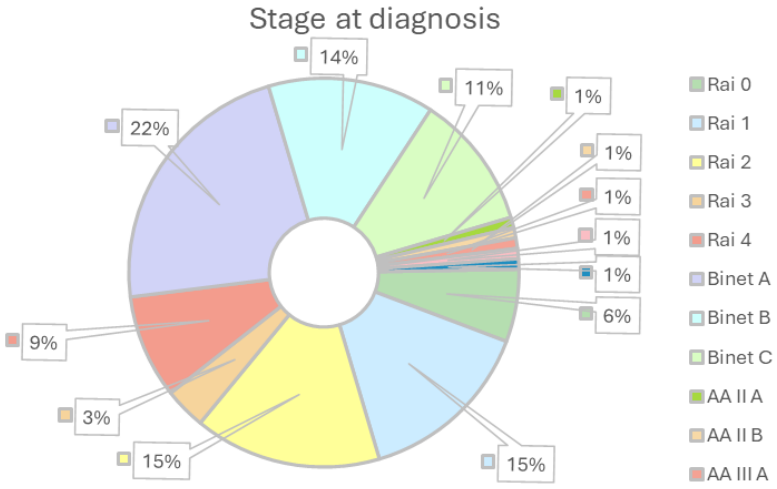

Figure 24: Distribution of the patients based on their stage at diagnosis. AA=Ann Arbor

IgHV mutational status, TP53 mutation, del(17p) presence, and CD38+ marker were performed to assess the risk profile. IgHV mutational status was determined for 78.7% of the patients, TP53 and del(17p) were evaluated for 82%, and CD38 for 70.5%. Among those included, 21.3% displayed mutated IgHV, 8.2% showed TP53 mutations, 11.5% had del(17p), and 11.5% were CD38+ (Fig.25).

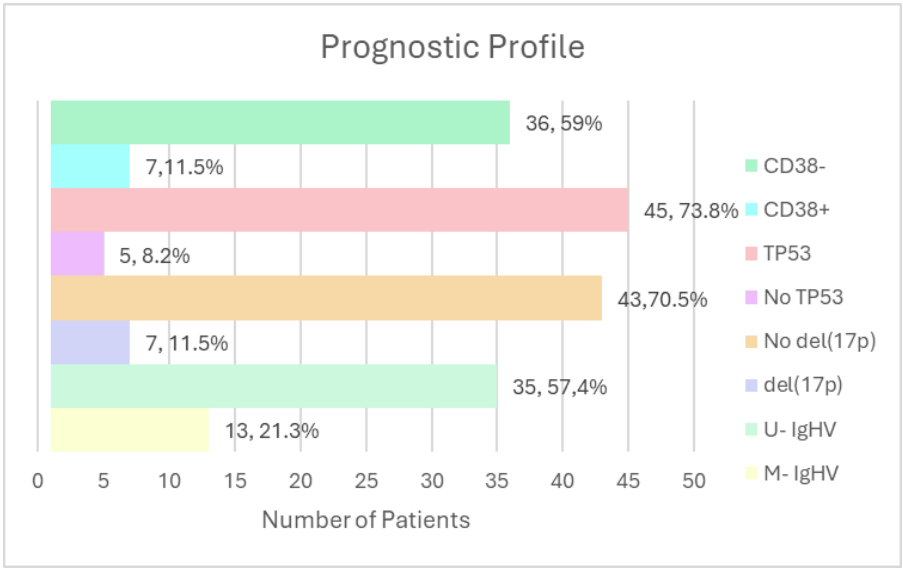

Figure 25: Distribution of the patients based on their prognostic profile

Our cohort of patients had a median TTFT of 7 months (range 0-58 months). Their biological characteristics and Cumulative Illness Rating Scale-Geriatric (CIRS) index at diagnosis are presented in Table V.

Parameter		**Ibrutinib in 1st line, N=40 (65.6%)**	**Ibrutinib in 2nd line, N=16 (26.2%)**	**Ibrutinib in 3rd line, N=5 (8.2%)**	**Overall, N=61 (100%)**
CIRS index	Median	4(4-11)	6 (4-14)	5 (4-7)	5 (4-14)
	NA	13	3	0	16
Biological parameter	ALC x 10^9/L	42 (2 -326)	26.3 (2.8-394)	102 (0.6-176)	34 (0.6-394)
	AMC x 10^9/L	1 (0.37-216)	0.8 (0.16-10)	1.79 (0.84-25)	1 (0.16-216)
	Hb g/dl	13.7 (4.8 – 16)	11.9 (6-16.7)	10.7 (6.7-14.7)	13 (4.8-16.7)
	MCV fl	87.8 (80.5-107.1)	90 (80.7-112.3)	96.6 (85.6-100)	88 (80.5-112.3)
	Plt x 10^9/L	164 (54 -331)	155 (35-326)	90 (17-253)	161 (17-331)
	LDH UI/L	413 (210-912)	454 (306-1555)	292 (248-430)	419 (210-1555)
	IgG mg/dl	856 (315-3242)	755 (504-1640)	696 (616-875)	794 (315-3242)
	Creatinine mg/dl	1.08 (0.56-1.45)	1.04 (0.3-1.68)	1.08 (0.83-1.19)	1.08(0.3-1.68)
	Uric acid mg/dl	5.6 (3.26-10.6)	5.4 (2.9-10)	5.7 (4.59-8.51)	5.55(2.98-10.6)

Table V: Patient's biological characteristics and CIRS index at diagnosis. Abbreviations: ALC-absolute lymphocyte count; AMC-absolute monocyte count; MCV-mean corpuscular volume, Hb-hemoglobin, Plt-platelets, LDH- Lactate Dehydrogenase, IgG-immunoglobulin G.

Among the patients studied, 65.6% (N=40) received ibrutinib as their initial treatment, while 34.4% (N=21) had previously undergone at least one treatment regimen prior to ibrutinib (Figure 26).

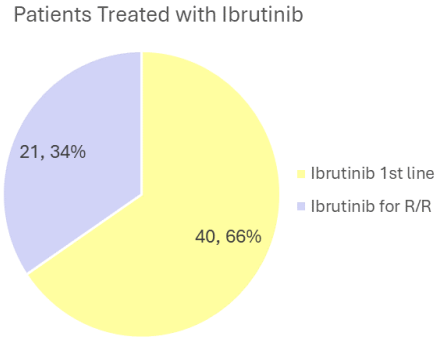

Figure 26: Patients' distribution according to the timing of their ibrutinib administration.

In the treatment-naïve group, 15.2% (N=9) of patients required another line of therapy following ibrutinib, compared to 9.1% (N=6) of patients with relapsed/refractory disease. Table VI outlines the therapies provided both before and after the initiation of ibrutinib.

Regimen	1st line	2nd line	3rd line
Ibrutinib	40 (65.6%)	16 (26.2%)	5 (8.2%)
R-FC	2 (3.3%)	1 (1.6%)	-
R-CVP	7 (11.5%)	1 (1.6%)	-
CVP	1 (1.6%)	1 (1.6%)	-
R-Clb	4 (6.6%)	2 (3.3%)	-
O-Clb	3 (4.9%)	-	-
O	3 (4.9%)	-	-
Clb	1 (1.6%)	-	-
Acala	-	5 (8.2%)	1 (1.6%)
R-Ven	-	4 (6.6%)	1 (1.6%)
O-Ven	-	-	1 (1.6%)
Ven	-	-	1 (1.6%)

Table VI: Therapies given prior to and following ibrutinib. Abbreviations: R-FC- rituximab, fludarabine and cyclophosphamide; R-CVP, rituximab, cyclophosphamide, vincristine, and prednisone; CVP cyclophosphamide, vincristine, and prednisone; R-Clb, rituximab and chlorambucil; O-Clb, obinutuzumab and chlorambucil; O-Obinutuzumab single agent; Clb-chlorambucil single agent; Acala-acalabrutinib; R-Ven – rituximab and venetoclax,; O-Ven - obinutuzumab and venetoclax; Ven-venetoclax single agent.

The median follow-up period from the start of treatment was 35.5 months, ranging from 2 to 74 months. The median time to the next treatment (TTNT) was 33 months, with a range of 5 to 74 months.

The assessment of the treatment response was performed in accordance with the protocols set forth by the International Working Group on Chronic Lymphocytic Leukemia (IwCLL) (1).

Two patients were excluded, as they were lost to follow-up. According to the IwCLL response criteria, the overall response rate (ORR) for both patient groups - encompassing complete responses (CR), complete responses with incomplete marrow recovery (CRi), and partial responses (PR) - was 90.2% (Figure 27).

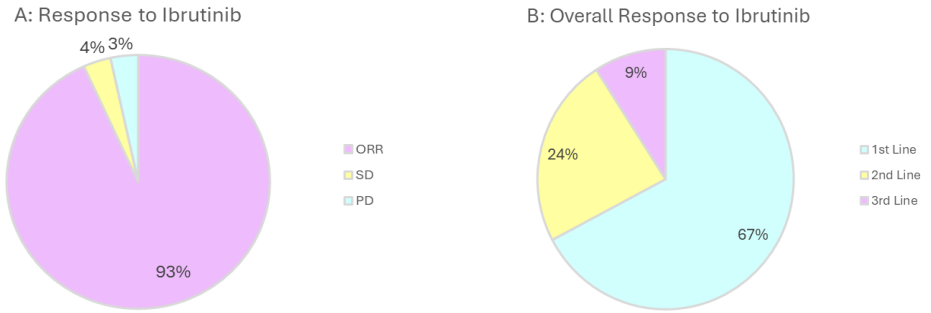

Figure 27. A: Response to ibrutinib; **B:** ORR depending on which line ibrutinib was administered

Ibrutinib therapy was mostly well-tolerated; however, in our cohort, 27.1% (N=16) had discontinued ibrutinib by the censoring point. Among those, 13,5% (N=8) ceased treatment because of disease progression, which included 1 patient who developed RS. Additionally, 13.5% (N=8) patients had stopped ibrutinib treatment: 6 for AEs and 3 by physician's choice (Figure 28).

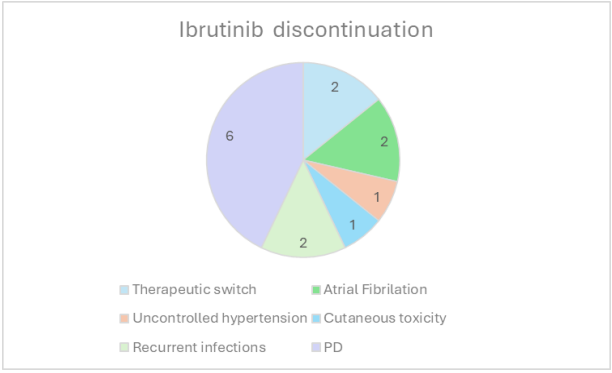

Figure 28. Adverse events during ibrutinib treatment. For the ones with therapeutic switches, the reason was not mentioned in their medical records.

At the time of data censoring, out of the 61 patients included in the study, 2 were lost to follow-up, 9 (14.8%) were deceased, and 50 (83.6%) were still living. The causes of death for six patients were not recorded in their medical files. One patient died secondary to a pulmonary infection, while two others died as a result of RS. The median overall survival (OS) had not been reached at the median follow-up duration of 55 months (Figure 29).

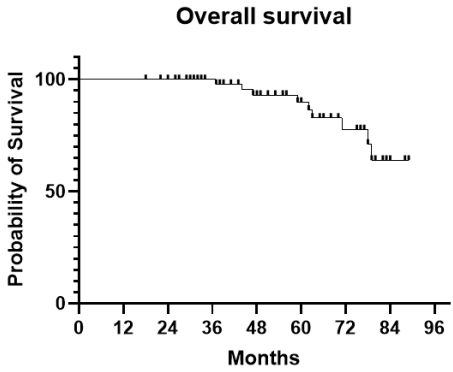

Figure 29. The median overall survival (OS) was not reached at the median follow-up period of 55 months.

The subgroup analysis revealed no significant correlation between CIRS index ≥ 6 points at diagnosis or age ≥65 years at diagnosis and progression-free survival (PFS) (Figure 10). On the other hand, a link was observed between advanced Binet/Rai stages at diagnosis and PFS (Figure 30).

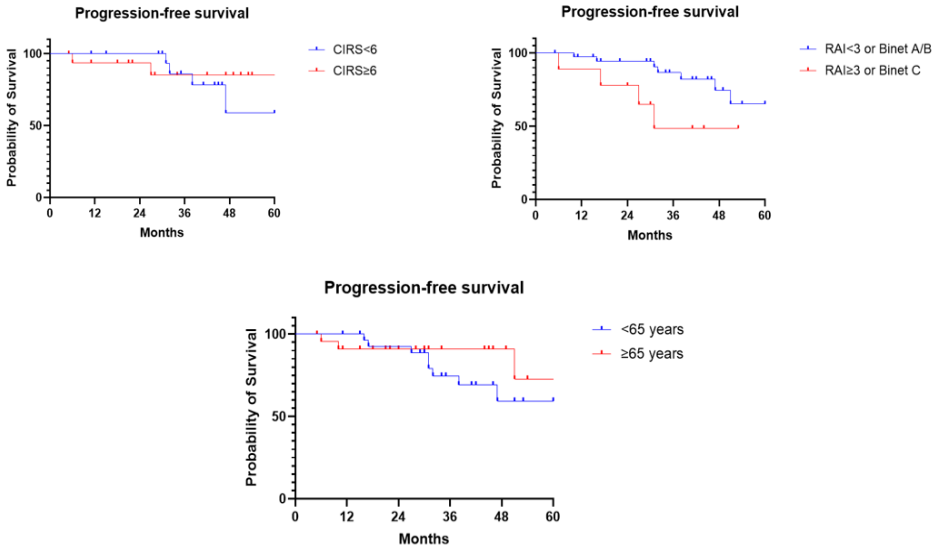

Figure 30. Progression-free survival (PFS) depending on CIRS index, RAI/Binet stage and age at diagnosis

We correlated the evaluated prognostic factors, namely IgHV mutational status, TP53 mutation, del(17p) and CD38 with PFS and OS (Figure 31). We did not obtain any statistically significant correlations, except for the association of M-IgHV with a shorter PFS.

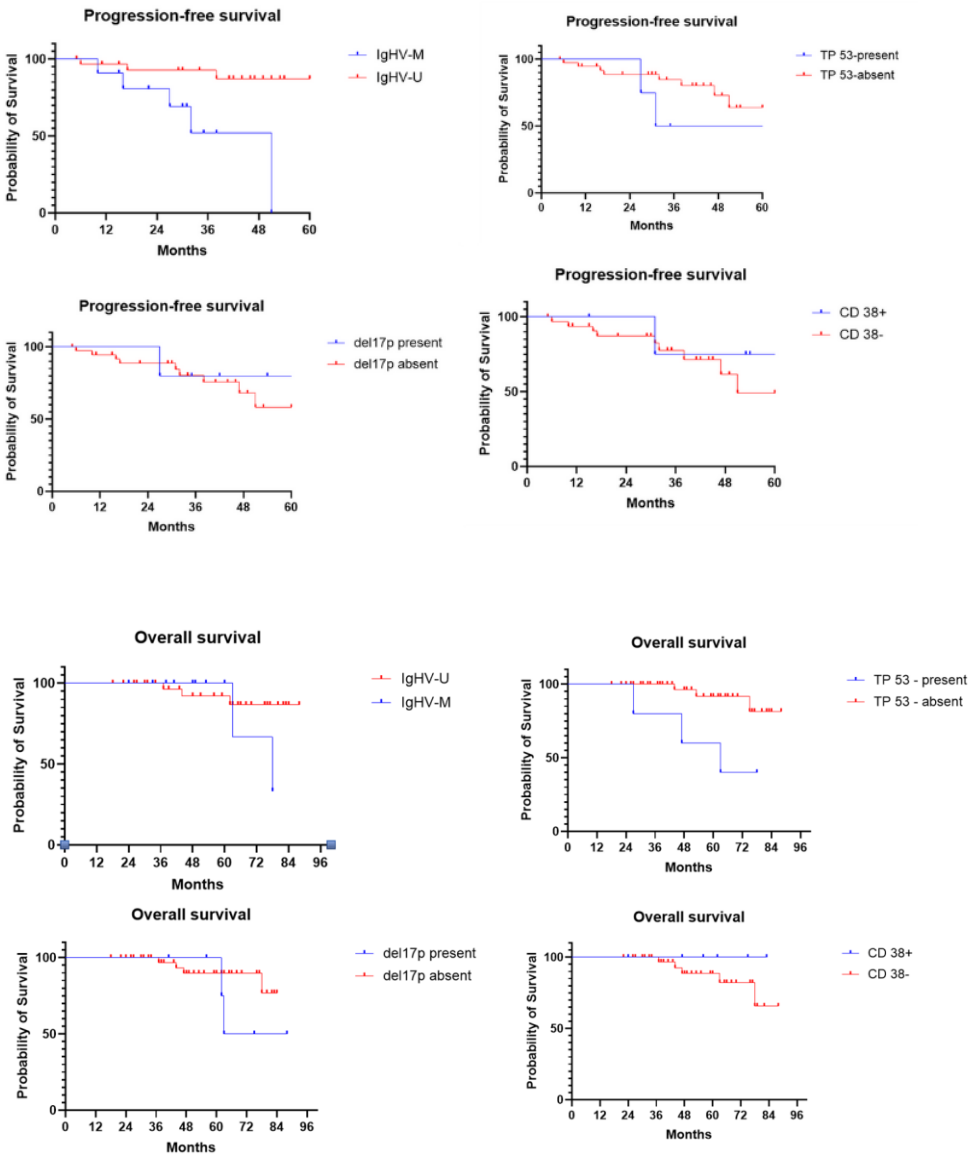

Figure 31. Progression-free survival (PFS) and overall survival (OS) correlations with prognostic factors

4.5. Discussions

Compared to the median age at diagnosis described in the World Wide records, which is 70 years old, our cohort of patients is young, as the median age at diagnosis is 63 and at the start of treatment is 65, which could explain the good tolerance of ibrutinib and lower CIRS index (2).

Ibrutinib has improved OS and PFS in clinical trials and real-world settings when compared to traditional chemo-immunotherapy, even in the presence of adverse prognostic factors, such as del(17p), del(11q), 12 trisomy, U-IgHV and TP53 mutations (141).

Although our study involved a limited patient cohort, we seek to assess real-world data on the effectiveness of ibrutinib as a monotherapy for patients with TN or R/R CLL. Patient follow-up commenced at the start of treatment and lasted for a median of 38 months. The median PFS for those receiving ibrutinib was 31 months, while median OS was not reached. The ORR, which included CR, CRi and PR was recorded at 90.2%, with an OS rate of 83.6%. Our results align with findings from clinical trials and other retrospective studies conducted in real-world settings (251, 252).

We evaluated the statistical significance of the correlation between age and PFS. Our patients were categorized into two groups: those aged≥ 65 and those < 65. No significant correlation was found. Consistent with previous research, age alone did not have an independent impact on PFS (258). However, real-world data revealed that age-related conditions, such as comorbidities or poor performance status were associated with shorter OS and PFS while on ibrutinib (259).

A study involving 145 R/R CLL patients, treated with ibrutinib found that a CIRS score ≥7 points was associated with poorer median EFS and lower 2-year OS (260).

We were unable to assess ECOG due to the limited information available in the patient's medical records. However, we were able to evaluate the CIRS index for 73.8% of our patients, despite its complexity. We investigated whether a CIRS score≥ 6 points in our patient cohort is associated with a shorter PFS. However, no statistically significant correlations were applied to our group. Our findings were confirmed by a study on 123 CLL patients diagnosed and treated with ibrutinib in Fundeni Clinical Institute, Bucharest, Romania (250).

With the rise of targeted therapies, traditional prognostic indices like Rai/Binet appear to be losing their relevance. In response, IwCLL recommends the evaluation of CLL-IPI as a novel prognostic scoring system (261) (2). However, due to the lack of a standardized protocol, B2M is not regularly assessed in our clinic, thus we were unable to calculate the CLL-IPI for a substantial number of patients. As a result, we concentrated solely on the Rai and Binet staging systems.

The Rai and Binet staging systems were analyzed at the time of diagnosis for all patients, with the exception of those diagnosed with SLL, for whom the Ann Arbor staging was utilized. For our group of patients, Rai stages 3-4 and Binet C. at diagnosis, were associated with reduced PFS. Real-world data on this topic is inconsistent. In most cases, PFS with ibrutinib treatment appears to be independent of the Rai/Binet stages at the time of diagnosis (262). However, we found real-world studies that corroborated our results (259).

In the study, the mutational status of IgHV was assessed in 78.7% of the participants, TP53 mutation and del(17p) in 82%, and the CD38 marker in 70.5%.

This suggests a satisfactory alignment of the Hemaology Ward at IOCN to IwCLL standards.

Nonetheless, our results were rather unexpected, given that it is generally accepted that M-IgHV is associated with a positive prognosis and a milder course of the disease. In the group of patients we studied, M-IgHV was associated with a shorter PFS, a result that does not align with findings from real-world studies or clinical trials (252, 255, 256). It is worth mentioning that 11 of the 12 patients who had M-IgHV were from the TN group, and none had IgHV:3-21 serotype, which is usually associated with worse disease outcomes (263). One possible reason for this correlation may be attributed to the limited size of the population studied, along with the lack of a laboratory utilizing a standardized protocol, which could lead to inaccurate IgHV testing results.

Upon investigating the association between TP53 mutations, del (17p), and CD38+ and PFS, we did not identify any significant correlation. This finding aligns with previous observations from both real-world settings and clinical trials (250, 252, 255, 262).

Regarding safety, our group of patients usually handled ibrutinib effectively, aligning with results observed in other real-world clinical studies (250, 255, 264, 265).

At the time of the censoring, 27.1% of the patients had discontinued ibrutinib therapy permanently, comprising 16.9% (N=10) from the TN group and 10.2% (N=6) from the R/R group. Among those who stopped treatment, 13.5% did so because of PD, which included 1.6% (N=1) who developed RS. An additional 13.5% (N=8) ceased treatment for other reasons, including two patients with atrial fibrillations, one with uncontrolled hypertension, two with recurrent infections, and one experiencing cutaneous toxicity. In the case of two patients, their physician opted to switch them to Acalabrutinib, although the rationale for this change was not documented in the medical records.

At the time of censoring, 9 patients (15.3%) had passed away. One individual died of a pulmonary infection and two others due to RS. However, the causes of death for the other six patients were not noted in the medical record. Among the deceased, 8.5% (N=5) were from the TN group, reflecting a higher death rate than that observed in the R/R group, which contradicts existing literature (254). It is important to

consider that ibrutinib was administered as a first-line treatment for 40 of the patients, while only 21 received it for R/R disease. Therefore, if we assess the death rate based on the individual groups rather than the entire cohort, it becomes evident that the mortality rate in the TN group is lower compared to those with R/R disease, consistent with previous findings in the literature.

4.6. Conclusions

In contrast to the median diagnosis age of 70 years reported globally, our patient cohort is younger, with a median age of 63 at diagnosis and 65 at the initiation of treatment. This younger demographic may contribute to the lower CIRS index observed at diagnosis and the favorable tolerance to ibrutinib (2). In 2022, only four patients were diagnosed and treated with ibrutinib in the Hematology Ward of IOCN. This could suggest that with the arrival of new targeted therapies, like the combination of monoclonal antibodies and BCL-2 inhibitors that offer improved PFS and limited treatment durations, ibrutinib could be losing its relevance.

While the evaluation of prognostic factors at IOCN is consistent with the IwCLL recommendations, there is still a need for improved standardization in diagnostic methods. Additionally, a method accredited by the European Research Initiative on CLL (ERIC) for assessing TP53 mutations, IgHV mutational status, del(17p), del(13q), del(11q), and trisomy 12 is also necessary.

5. MicroRNA Dysregulation and Vitamin D Deficiency in CLL (3rd Study)

5.1. Introduction

The immune system functioning is not entirely deconstructed, but it is a well-known fact that dysregulated immunity can lead to the development of autoimmune diseases and even malignancies. Immune dysregulation arises from the interplay of various factors, including the microenvironment, and different types of proteins (266). MicroRNAs, small, non-coding molecules, that regulate gene expression, seem to influence the development, differentiation, and function of immune cells, as much as they influence any other processes that takes place in the human body (266). Their ability to modulate the expression of multiple target genes allows miRNAs to orchestrate intricate signaling pathways essential for a robust and adaptive immune response. Recently, there has been growing interest in understanding the complex interactions between miRNAs and various environmental factors, such as vitamin D, in the regulation of immune responses.

Vitamin D, a fat-soluble vitamin primarily obtained through sunlight exposure and dietary sources, is recognized for its immunomodulatory effects. It plays an essential role in controlling the cells involved in innate and adaptative immunity (267). Deficiency in vitamin D has been associated with increased susceptibility to autoimmune diseases, infections and even cancers, highlighting its significance in maintaining immune system homeostasis (182, 268). In CLL, vitamin D deficiency was positively correlated with TTFT and OS (188). Research suggests that vitamin D may influence the expression of specific miRNAs, thereby impacting immune cell function and inflammatory processes (151).

The association between miRNAs expression and cancer evolution was first described in CLL, when Calin et. al proved that MiR-15 and miR-16 regulate CLL cells (192). The discovery of miR-15 and miR-16 in CLL has paved the way for the identification of additional microRNAs that significantly influence the disease's progression and pathology.

Vitamin D level regulates the expression of miRNAs involved in immune modulation, such as miR-146, miR-155 and miR-150 and cancer development, including CLL (151, 269).

5.2. Hypothesis

CLL is a generally non-aggressive disease, with a heterogenous evolution. Patients diagnosed with this pathology experience variable TTFT. Hypothesizing that the heterogenous evolution of CLL doesn't rely solely on the patient's genetic profile, but also, among many factors, on the dysregulated immune mechanisms, we aimed to assess the vitamin D status of patients and investigated the expression of several miRNAs known to be associated with deregulated immune responses.

5.2.1. Objectives:

- To assess the association vitamin D status of patients at diagnosis and the time from diagnosis to the first treatment (the principles of CLL management include a period of non-intervention, called "watch and wait", as explained above, and the active treatment is being started only when disease progresses and affects either the quality of life or the life expectancy of patients). To this end, we used calcidiol serum levels dosage, as this is regarded as the best parameter reflecting vitamin D status.
- Assessment of the expression levels of miRNAs involved in regulating immune mechanisms and comparing their expression in patients with short versus those with long time to first treatment, in order to identify potential new targets for modulating immunity in CLL.
- Find if there is a statistically significant correlation between vitamin D levels and the studied miRNAs' expressions.
- To evaluate the prognostic/predictive value of vitamin D status and miRNA expression, by investigating the association between their expression and known prognostic/predictive markers like absolute leukocyte count (ALC), absolute monocyte count (AMC) and Binet stage.

5.3. Materials and Methods

From the database that was initially created for the 1st study of this thesis, we selected the treatment-naïve patients. Ideally, at the moment of diagnosis or at a time point before treatment initiation, a 5 ml vacutainer with or without a coagulation activator, and a separating gel was collected from each patient. This sample was centrifuged for 25 minutes at a temperature of 20°C and at a speed of 3000g. A unique laboratory ID was assigned to each patient. The resulting serum was then aliquoted into Eppendorf tubes, with each tube containing 500 µL, and stored at -80°C. The aim was to obtain at least 3 Eppendorf tube samples.

The included patients were further divided into two groups based on the dates of sample collection and their time to first treatment. The initial group comprised 22

patients whose TTFT<12 months from the sampling date, while the second group consisted of 16 patients with TTFT>12 months from the sampling moment.

From the obtained serum, one 500 μL Eppendorf tube was outsourced to an accredited laboratory for testing the vitamin D level.

We revised the literature and selected three miRNAs that are known immune modulators.

One of them was miR-29a, which regulates immunity by activating NF-kB signaling pathway and modulating Th cell, CTLs and NK cells development and function (211-215). MiR-29a is upregulated in patients with early-stage CLL, when compared to those with advanced disease (210) (30, 156). In a study on prostate cancer patients, it was found that levels of miR-29 were upregulated after treatment with testosterone and vitamin D (270).

The second selected miRNA, was miR-339, shown to have a role in cancer immunity by inhibiting CTLs action on cancer cells (271). Furthermore, it was found to be overexpressed and to promote Stem cell leukemia/lymphoma cells (272). MiR-339 expression was found to be increased after vitamin D supplementation (273).

The third miRNA we included in our study, was miR-133a. This miRNA is recognized for its role in immune regulation by stimulating sirtuin-1 (SIRT1) and inducing oxidative stress and inflammation (274, 275). Additionally, it affects immune responses by regulating cytokine production and leukocyte migration via TLR7 (276). Furthermore, it influences macrophage development, by regulating Granulocyte-Macrophage Colony-stimulating Factor Expression (277).

MiR-133a was studied in relation to vitamin K; however, we did not find any research regarding vitamin D and miR-133a association (278).

To the best of our knowledge, miR-339 and miR-133a's expressions were studied and confirmed to be involved in acute leukemias, but not in CLL (279, 280).

For the miRNA profiling, the sera from patients were thawed and processed.

MiRNA separation was realised following the manufacturer's protocol provided in the Small RNA and DNA from Plasma NucleoSpin™ miRNA Plasma kit by Macherey-Nagel. We utilized 500 μL of serum from each patient. RNA was stored at -80 until processing.

The extracted RNA was subjected to a specific miRNA reverse transcription, using the specific miRNA kit TaqMan™ Small RNA Assays from Applied Biosystems and High-Capacity miRNA cDNA Reverse Transcription Kit from Applied Biosystems (ThermoFisher Scientific). Components of the reverse transcription kit were thawed on ice. Reverse transcription for a single short-strand RNA was performed according to the manufacturer's protocol and for each miRNA specifically, by taking 10 μL of the RNA sample and adding 10μL of 2x RT Master Mix. The samples were centrifuged and placed in a BioRad™ thermal cycler for the reverse transcription, at temperatures according to the manufacturer's recommendations. The Reverse transcription conditions were set for a 20 μL sample and 4 incubation steps: 30 min at 16°C, 30 min

at 42°C, 5 min at 85°C and storage at 4°C. The obtained cDNA was stored in microtubes at -20°C, before for subsequent processing.

The PCR reaction mix was prepared per the manufacturer's guidelines using TaqMan™ Small RNA Assays from Applied Biosystems (Thermo Fisher Scientific). The primers, provided in a 750 μL (20x) format, were purchased from TaqMan™. 9.34μL of the reaction mix was added to each well of the 96-well reaction plate together with 1 μL of the cDNA extracted from each patient. After adding the sample and reaction mix to each well, the reaction plate was briefly centrifuged. Further, using the StepOnePlus System real-time thermal cycler (StepOnePlus System from Applied Biosystems for TaqMan™ Assays), we set the reaction volume for 10 μL and the cycling steps: enzyme activation at 95°C for 20 seconds for one cycle, denaturation at 95°C for 1 second, and annealing/extension at 60°C for 20 seconds. The last two steps were repeated for 40 cycles. All reactions were conducted in duplicate.

The microRNAs' results were interpreted by normalizing each obtained expression value of the inquired miRNA to the one of miR-25, the selected housekeeping miR. The result was further normalized to the highest obtained value and used to calculate the fold expression (FE).

The collected data was entered into an Excel spreadsheet and cross-verified with each patient's medical record for statistical analysis. The numerical (quantitative) variables were summarized by their mean, standard deviation and mean confidence interval (CI) of 95%, median, minimum, and maximum values. We also studied the distribution pattern of values and differences from the normal distribution, using the Shapiro-Wilk test. Categorical variables were summarized by relative frequency, expressed as numbers and percentages. We assessed the existence of correlations between the studied variables by using appropriate statistical tests. Thus, for categorical variables, we used the chi2 test. For quantitative variables, we used the t-test for independent samples as well as the Pearson correlation coefficient obtained from the linear regression. We considered that a value of this coefficient between 0.35 and 0.7 indicates a moderate correlation between the two variables, and a value above 0.7 a strong correlation. For values below 0.35 we considered the 2 variables as independent.

For the survival analysis, we considered the date of diagnosis as the reference date, for studied intervals. For OS the event of interest was death. For patients who at the study closure date were alive the survival interval was considered the censored date.

Univariate survival analysis was performed with the Kaplan- Meier estimation method, and log-rank test to assess the differences between them. For multivariate analysis, we used Cox proportional hazards regression model to assess the simultaneous influence of several factors on survival. We considered the statistical significance limit for all tests to be $p<0,05$.

All statistical analyses were performed using the Excel Spreadsheet and GraphPad Prism 10.3 (GraphPad Software, Inc., La Jolla, CA).

5.4. Results

We have included 38 patients diagnosed with CLL, out of which 22 had a TTFT < 12 months, while 18 had a TTFT>12 months. The median TTFT was 63.5 months, ranging from 0-243 months. Figure 32 describes patients' distribution according to TTFT.

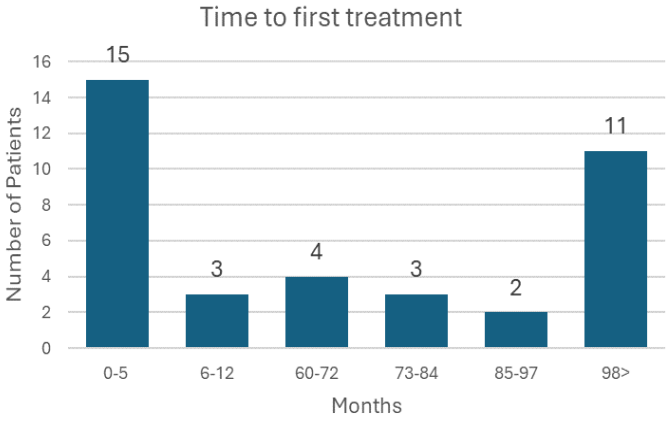

Figure 32: Distribution according to time to first treatment

The difference in survival between the two groups of patients was statistically significant. The median survival for the group of patients with TTFT>12 months was not reached, and for the group of patients with TTFT<12 months was 158 months (figure 33).

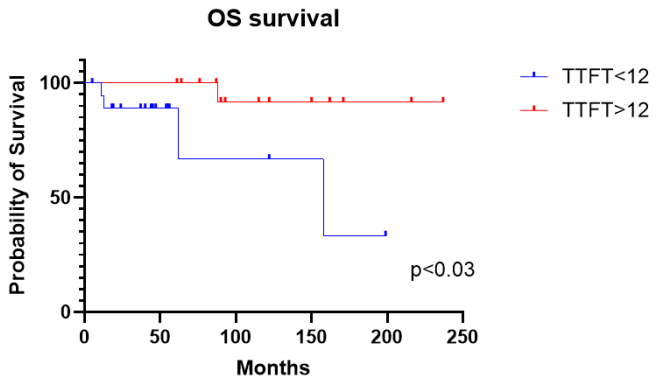

Figure 33: Overall survival in the two groups of patients

In the TTFT<12 months group, the median age at diagnosis was 62 years (range: 43-91), whereas in the TTFT>12 months group, the median age was 60.5 years (range: 41-85). Among our patients, there was a notable male predominance, with males accounting for 58% and females for 42%. Regarding their backgrounds, the distribution was fairly balanced, with 45% of patients originating from rural areas and 55% from urban settings (Figure 34).

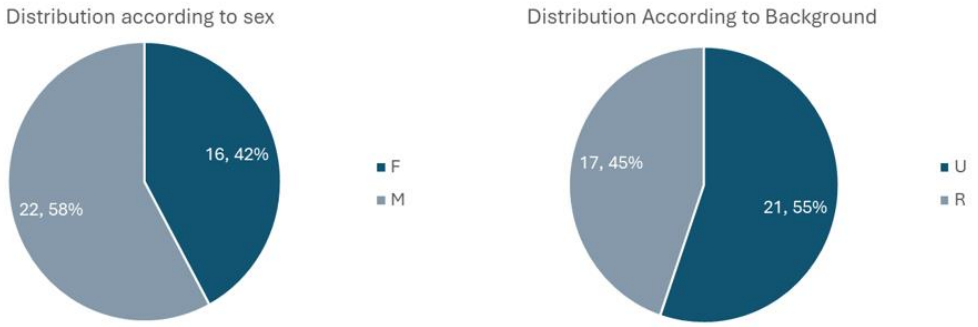

Figure 34: Distribution according to gender and background

At the moment of diagnosis, most of our patients were considered as stage Rai 0, Binet A (Figure 35).

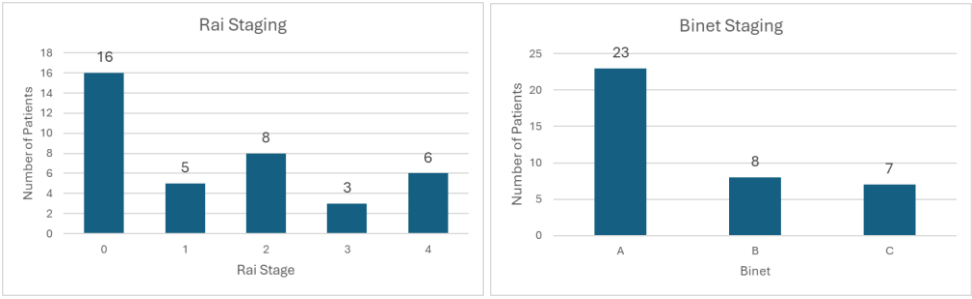

Figure 35: Distribution according to gender and background

Table VII describes the biological parameters at diagnosis.

Biological parameter	**Mean (min-max)**
Vitamin D ng/ml	18.5 (6 -94.3)
ALC x 10^9/L	40.6 (11.1 – 411.22)
ALM x 10^9/L	0.82 (0 - 9)
Hb g/dl	13.2 (4.2 - 16.4)
PLT x 10^9/L	171 (6 - 401)
LDH UI/L	457 (227 - 1555)
IgG mg/dl	868.5 (103 - 2084)

Table VII: Biological parameters of included patients. Abbreviations: ALC=absolute leucocyte count, AMC=absolute monocyte count, Hb= hemoglobin, plt= platelets, LDH= Lactate dehydrogenases, IgG= immunoglobulin G

An important aspect is the vitamin D level, which is known to be relevant to innate and adaptative immune systems. It is also suspected that vitamin deficiency could be, in part, responsible for CLL's evolution.

- *Vitamin D status in our group of patients. Correlations between vitamin D clinical and paraclinical parameters, disease progression and OS of the included CLL patients*

Figure number 36 illustrates the distribution of patients based on their vitamin D levels. Most patients exhibited vitamin D values below 20ng/mL, indicating a deficiency. Additionally, 8 patients were classified as severely deficient. Among the cohort, only 9 patients had vitamin D levels exceeding 30 ng/mL, which is considered optimal.

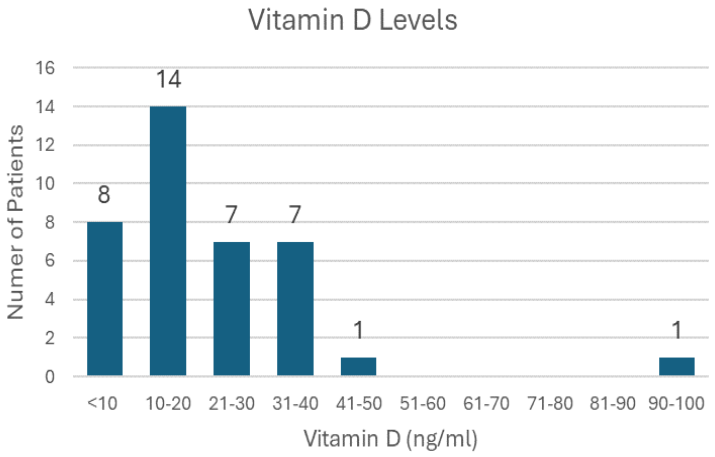

Figure 36: Distribution according to vitamin D level

We examined the relationship between vitamin D levels and factors such as age at diagnosis, absolute lymphocyte count, absolute leukocyte count, absolute monocyte count, platelet count, hemoglobin, and IgG levels. Our analysis revealed no statistically significant correlations, with the exception of a negative correlation between age and vitamin D levels (figure 37).

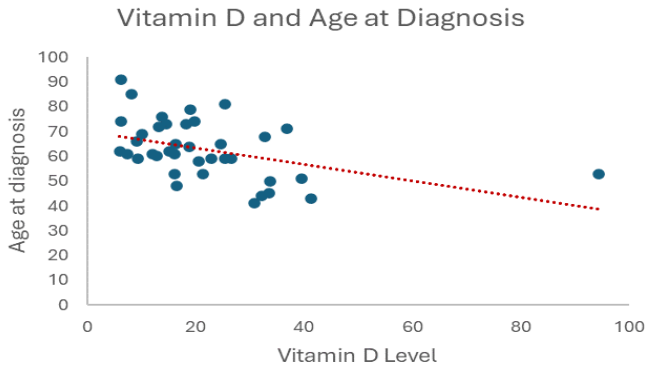

Figure 37: Vitamin D correlation with age at diagnosis

Given the fact that vitamin D deficit is associated with TTFT in the literature, a correlation between TTFT and vitamin D levels was assessed. Firstly we used the Shapiro–Wilk test to verify the distribution of our variables, then we used the unpaired T-test to assess the statistical significance. We obtained a p=0.22, which suggests that

there is no statistically significant correlation. The statistics were performed in Excel spreadsheet.

A survival analysis was conducted to investigate the relationship between the severity of vitamin D deficiency and OS, but no statistically significant association was found (figure 38).

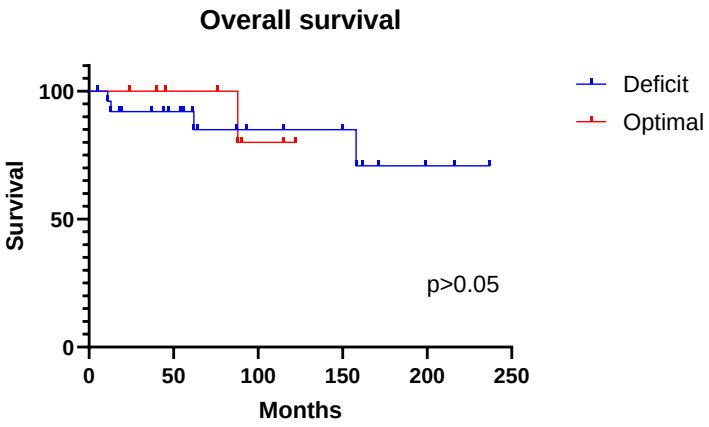

Figure 38: OS depending on vitamin D levels

- *Evaluation of MiR-339, miR-133a and miR-29a expressions in the sera of included CLL patients*

An important point of the present study was to assess whether miR-339, miR-133a and miR-29a are differentially expressed in the selected CLL patients. We extracted RNA from the sera of the selected patients, following the technique described in "Materials and Methods". Each extracted RNA sample was stored at -80ºC, until RT. Further, we performed the specific RT for each miRNA. We stored the obtained cDNA at -20ºC overnight and the following day we performed the PCR, according to the techniques described in "Materials and Methods". The microRNAs' results were interpreted by normalizing each obtained expression value of the inquired miRNA to the one of miR-25, the selected housekeeping miR. The result was further normalized to the highest obtained value and used to calculate the fold expression (FE). The results were obtained using Excel Spreadsheet. Our analysis showed that miRNAs are expressed at different levels in CLL.

To better visualize the miRNAs expressions in both groups of patients, we created a heatmap using GraphPad Prism 10.3 (figure 39). The red colour represents the highest miRNA expression.

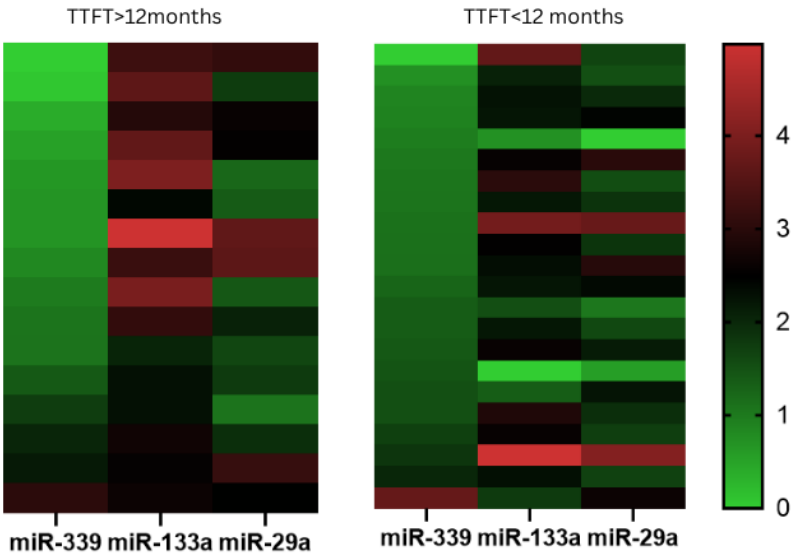

Figure 39: Heatmap that illustrates the expression levels of miR-339, miR-133a and miR-29a in CLL. The initial three columns display the expression of miR-339, miR-133a, and miR-29a in CLL patients with a time to TTFT>12 months. Conversely, the last three columns represent the expression of miR-339, miR-133a, and miR-29a in CLL patients with a TTFT < 12 months. High miRNA expression is indicated by red, while green indicates lower miRNA expression.

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and vitamin D levels.*

Further, we wanted to assess, whether vitamin D levels are correlated with the studied miRNAs expressions. First, we used the Shapiro–Wilk test to verify the distribution of our variables, then we used the Wilcoxon signed-rank test to analyze the correlation between vitamin D levels and each studied miRNA. We did not obtained p-values < 0.05, showing there is no statistically significant correlation between vitamin D levels and miR-133a, miR-339 and miR-29a. However, the correlation between vitamin D level and miR-29a expression level resulted in a p-value=0.065, which is at the limit of statistical significance. Statistics were realized using Excel Spreadsheet. To better illustrate the results, we logarithmized the values of the variables and created a heatmap using GraphPad Prism 10.3 (figure 40).

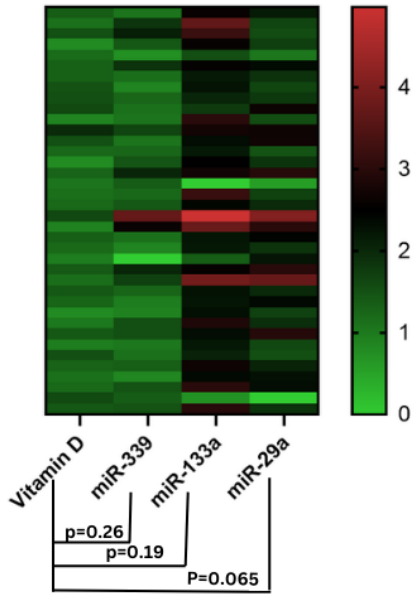

Figure 40: Heatmap depicting the correlation between vitamin D levels and the expression of miR-339, miR-133a, and miR-29a. The vitamin D levels range from 6 to 94.3 ng/mL, with lower values at the bottom and higher values at the top. To generate this aspect of the heatmap, all values were converted to their logarithmic form.

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and prognostic factors.*

Our next hypothesis was to determine if the examined miRNAs could serve as prognostic markers. We analyzed the expression of each miRNA in relation to various prognostic indicators, such as Binet staging, absolute leukocyte count, and monocyte count.

- miR-339, miR-133a, and miR-29 expressions and ALC-

To evaluate the expression values of miRNAs and ALC, we first checked for normality and then employed the Wilcoxon signed-rank test to analyze their correlation. When examining the relationship between MiR-339, miR-133a, and miR-29a with leukocyte count at diagnosis, we found p-values less than 0.05 for each miRNA, indicating statistically significant correlations among the measured values. Statistics were realized using Excel Spreadsheet.

We logarithmized the values of the variables and generated a heatmap using GraphPad Prism 10.3, to illustrate the expression of miRNAs alongside leukocyte count (Figure 41).

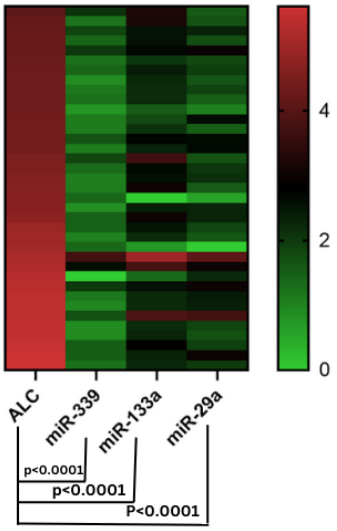

Figure 41: Heatmap illustrating the correlation between absolute leucocyte count at diagnosis and studied miRNAs expressions. The ALC levels range between 11.1 – 411.22 x10^9/L, with lower values at the bottom and higher values at the top. To generate this aspect of the heatmap, all values were converted to their logarithmic form.

- miR-339, miR-133a, and miR-29 expressions and AMC-

The next step was to correlate the studied miRNAs with the absolute monocyte count at diagnosis. We first checked the normal distribution of the variables by using the Shapiro–Wilk test. We used the Wilcoxon signed-rank test to assess the correlation between all three miRNAs and AMC. We obtained p values <0.05, when correlating miR-339 and miR-29a with AMC, which confirmed that there is a statistically significant relationship between the aforementioned variables. The correlation between miR-133a and AMC resulted in a p=0.55, which infirmed the existence of any statistically significant correlation. Statistics were realized using Excel Spreadsheet.

Figure 42 illustrates a heatmap created using GraphPad Prism 10.3, that describes the monocyte count and miRNAs expressions.

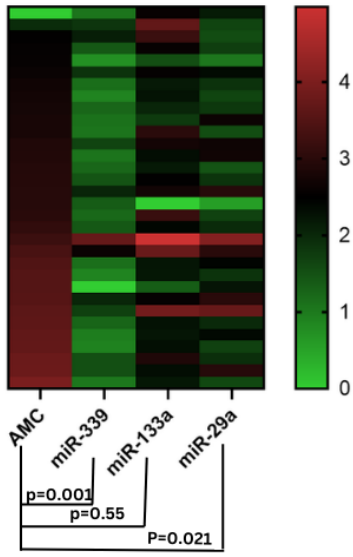

Figure 42: Heatmap illustrating the correlation between absolute monocyte count at diagnosis and studied miRNAs expressions. The AMC ranges from 0 to 9 x 10^9/L, with lower values at the bottom and higher values at the top. To generate this aspect of the heatmap, all values were converted to their logarithmic form.

- miR-339, miR-133a, and miR-29 expressions and Binet stage-

We also examined the relationship between the expressions of miR-339, miR-133a, and miR-29a and the Binet stages at diagnosis. After assessing the normality of our variables, we employed Welch's t-test to analyze any potential correlation between the examined miRNAs and the Binet stages at diagnosis. Although we did not find a statistically significant p-value for either correlation, the association between Binet stages A and C and miR-29a approached statistical significance (p=0.054). Statistics were realized using GraphPad Prism 10.3.

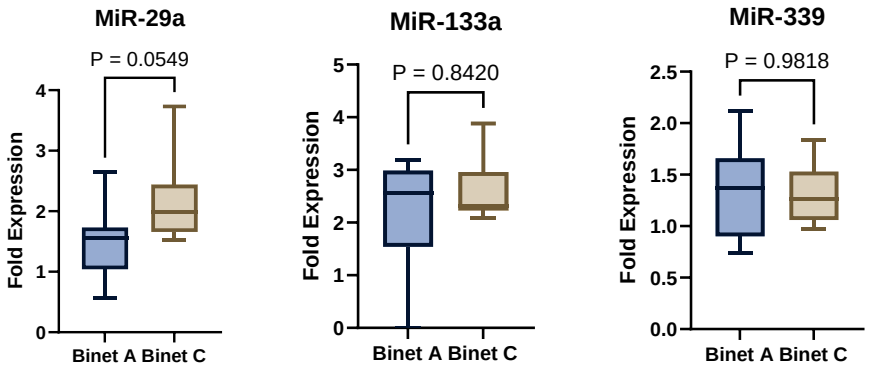

Figure 43: miR-29a's expression is increased in Binet A CLL when compared to Binet C at diagnosis

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and TTFT*

We aimed to evaluate whether there is a correlation between miRNA expressions in the TTFT<12 months group compared to the TTFT>12 months group. To do this, we examined the normality of the variables and applied Welch's t-test to analyze the relationship between miRNA expressions and TTFT. Our findings did not reveal any statistically significant correlations (Figure 44). Statistics were realized using GraphPad Prism 10.3.

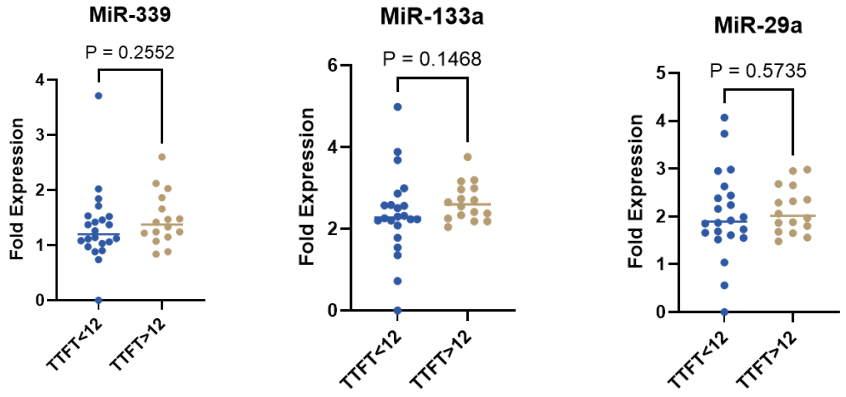

Figure 44: Correlations between studied miRNAs and TTFT

5.5. Discussions

We evaluated 38 CLL patients diagnosed and treated in the Hematology Ward of IOCN. The samples used were collected at diagnosis or at some point before the treatment initiation, the primary condition being that they were treatment naïve. Patients were further divided into 2 groups: those who had < 12 months (N=22) and those who had > 12 (N=16) months from the time of collection to the initiation of treatment.

In our cohort of patients, there was a slight preference for the male sex, as CLL is more frequent in the male population (1). We also observed that the majority of patients came from urban backgrounds. The groups analyzed were somewhat younger than the median age at diagnosis reported in the literature, with a median age of 62 years (range: 43-91) for the TTFT<12 months group, while the TTFT>12 months group had a median age of 60.5 years (range: 41-85) (1).

The TTFT exhibited a wide range of values, ranging from 0 to 243 months, with a median of 63.5 months. The survival analysis indicated a significant advantage in OS for the group with a longer TTFT ($p<0.05$), where the median survival had not yet been reached. In contrast, the group with a shorter TTFT achieved a median survival of 158 months.

Most patients, regardless of group were considered as Binet A/Rai 0 at the time of diagnosis, which aligns with current literature, that indicates that most CLL patients are diagnosed in the early stages (281). CIRS index or CLL-IPI could not be evaluated.

- *Vitamin D status in our group of patients. Correlations between vitamin D clinical and paraclinical parameters, disease progression and OS of the included CLL patients*

Among the biological parameters measured, we analyzed vitamin D levels, which exhibited a median of 18.5 ng/mL, ranging from 6 to 94.3 ng/mL. Most patients had vitamin D levels below 20 ng/mL, indicating a deficiency, and 8 patients had levels lower than 10 ng/mL, suggesting a severe deficit, as expected in a normal adult population before the coronavirus pandemic. For comparison, in the normal adult population in Europe, almost 40% of the included subjects had vitamin D deficiency, while in 13% of cases, there was a severe deficit (282). Statistical analysis was realized in Excel SpreadSheet.

Furthermore, we correlated vitamin D levels with the age at diagnosis absolute lymphocyte count, absolute leukocyte count, absolute monocyte count, platelet count, hemoglobin, and IgG levels. Our analysis revealed no statistically significant

correlations, with the exception of a negative correlation between age and vitamin D levels. Vitamin D value was negatively correlated with age. Findings that are confirmed by the literature, as vitamin D synthesis lowers with age (283).

To assess the association between vitamin D status and disease progression, serum calcitriol levels were evaluated, and compared between the short and long time to progression groups. Firstly we used the Shapiro–Wilk test to verify the distribution of our variables, then we used the unpaired T-test to assess the statistical significance. We obtained a p=0.22, which suggests that vitamin D status did not significantly differ between the two groups, thus not supporting the hypothesis that the lack of vitamin D might accelerate cancer progression in CLL. Our findings do not align with those described in the literature, where lower levels of vitamin D were associated with shorted TTFT (183, 188, 284). The statistics were performed in Excel SpredSheet.

Finally, we correlated vitamin D levels with OS and obtained a p>0.05, with suggested that there is no correlation between vitamin D levels and OS, findings that are not confirmed by those described in the literature, where lower levels of vitamin D were associated with inferior OS in CLL patients (183, 188, 284). This result might be attributed to the limited number of patients with optimal vitamin D levels, as only 7 patients had levels exceeding 30 ng/mL, the threshold for optimal. Nevertheless, we identified 2 patients – one from each group- with higher levels—one reaching 94.4 ng/mL and another surpassing 40 ng/mL—suggesting that some of the included individuals may have been taking vitamin D supplements at the time of blood sample collection, which could introduce bias into our analysis. The statistical analysis was conducted using GraphPad Prism 10.3.

- *Evaluation of MiR-339, miR-133a and miR-29a expressions in the sera of included CLL patients*

We revised the literature and selected three miRNAs that are known immune modulators. One of them was miR-29a, which regulates immunity by activating NF-kB signaling pathway and modulating Th cell, CTLs and NK cells development and function (211-215). MiR-29a is upregulated in patients with early-stage CLL, when compared to those with advanced disease (210) (30, 156). In a study on prostate cancer patients, it was found that levels of miR-29 were upregulated after treatment with testosterone and vitamin D (270).

The second selected miRNA, was miR-339, shown to have a role in cancer immunity by inhibiting CTLs action on cancer cells (271). Furthermore, it was found to be overexpressed and to promote Stem cell leukemia/lymphoma cells (272). MiR-339 expression was found to be increased after vitamin D supplementation (273).

The third miRNA we included in our study, was miR-133a. This miRNA is recognized for its role in immune regulation by stimulating sirtuin-1 (SIRT1) and

inducing oxidative stress and inflammation (274, 275). Additionally, it affects immune responses by regulating cytokine production and leukocyte migration via TLR7 (276). Furthermore, it influences macrophage development, by regulating Granulocyte-Macrophage Colony-stimulating Factor Expression (277).

MiR-133a was studied in relation to vitamin K; however, we did not find any research regarding vitamin D and miR-133a association (278).

To the best of our knowledge, miR-339 and miR-133a's expressions were studied and confirmed to be involved in acute leukemias, but not in CLL (279, 280).

An important point of the present study was to assess whether miR-339, miR-133a and miR-29a are differentially expressed in the selected CLL patients. We extracted RNA from the sera of the selected patients, following the technique described in "Materials and Methods". Each extracted RNA sample was stored at -80ºC, until RT. Further, we performed the specific RT for each miRNA. We stored the obtained cDNA at -20ºC overnight and the following day we performed the PCR, according to the techniques described in "Materials and Methods". The microRNAs' results were interpreted by normalizing each obtained expression value of the inquired miRNA to the one of miR-25, the selected housekeeping miR. The result was further normalized to the highest obtained value and used to calculate the fold expression (FE). The results were obtained using Excel Spreadsheet. Our analysis concludes that the studied miRNAs are differently expressed in CLL.

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and vitamin D levels.*

Further, we wanted to assess, whether vitamin D levels are correlated with the studied miRNAs expressions. First, we used the Shapiro–Wilk test to verify the distribution of our variables, then we used the Wilcoxon signed-rank test to analyze the correlation between vitamin D levels and each studied miRNA. We did not obtained p-values < 0.05, showing there is no statistically significant correlation between vitamin D levels and miR-133a, miR-339 and miR-29a. However, the correlation between vitamin D level and miR-29a expression level resulted in a p-value=0.065, which is at the limit of statistical significance. Statistics were realized using Excel Spreadsheet. The existing literature described that miR-29a and miR-339 are regulated by vitamin D levels, however, to our knowledge, there is no available literature assessing the relationship between miR-133a and vitamin D, which left us without a comparative basis for our findings (284). Furthermore, the correlation between miR-339, miR-133a and miR-29a and vitamin D in CLL was not evaluated in any research study, to our knowledge.

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and prognostic factors.*

Our next hypothesis was to determine if the examined miRNAs could serve as prognostic markers. We analyzed the expression of each miRNA in relation to various prognostic indicators, such as Binet staging, absolute leukocyte count, and monocyte count.

- miR-339, miR-133a, and miR-29 expressions and ALC-

To evaluate the expression values of miRNAs and ALC, we first checked for normality and then employed the Wilcoxon signed-rank test to analyze their correlation. When examining the relationship between MiR-339, miR-133a, and miR-29a with leukocyte count at diagnosis, we found p-values less than 0.05 for each miRNA, indicating statistically significant correlations among the measured values. Statistics were realized using Excel Spreadsheet. MiR-339 and miR-133a were not evaluated in CLL, but research has shown that miR-29a is overexpressed in the early stages CLL, which might suggest that there is an association with ALC and miR-29a (156, 285).

- miR-339, miR-133a, and miR-29 expressions and AMC-

The next step was to correlate the studied miRNAs with the absolute monocyte count at diagnosis. We first checked the normal distribution of the variables by using the Shapiro–Wilk test. We used the Wilcoxon signed-rank test to assess the correlation between all three miRNAs and AMC. We obtained p values <0.05, when correlating miR-339 and miR-29a with AMC, which confirmed that there is a statistically significant relationship between the aforementioned variables. The correlation between miR-133a and AMC resulted in a $p=0.55$, which did not confirm our hypothesis that there is any statistically significant correlation. Statistics were realized using Excel Spreadsheet.

To the best of our knowledge, neither of the studied miRNAs has been associated in other research with AMC in CLL, however, miR-133 is known to influence macrophage development, by regulating Granulocyte-Macrophage Colony-stimulating Factor Expression (277).

- miR-339, miR-133a, and miR-29 expressions and Binet stage-

We also examined the relationship between the expressions of miR-339, miR-133a, and miR-29a and the Binet stages at diagnosis. After assessing the normality of our variables, we employed Welch's t-test to analyze any potential correlation between

the examined miRNAs and the Binet stages at diagnosis. We did not find a statistically significant p-value for either correlation, however, the association between Binet stages A and C and miR-29a approached statistical significance (p=0.054). Statistics were realized using GraphPad Prism 10.3. As mentioned above, research has described higher miR-29a values in Binet A/Rai 0 stages, thus associating it with a good prognosis (285, 286). In our case, the borderline statistical significance of the correlation between miR-29a and Binet staging may be attributed to the small size of the patient cohort. Further research is warranted, as we did find research that associated miR-29 overexpression with advanced Rai/Binet stages at diagnosis(287).

MiR-339 and miR-133a, to the best of our knowledge, were not evaluated in CLL, so we do not have any means to compare our results. However, we did find that both miRNAs are expressed in our cohort of patients.

- *Relationship between the expression levels of miR-339, miR-133a, and miR-29a and TTFT*

We aimed to evaluate whether there is a correlation between miRNA expressions in the TTFT<12 months group compared to the TTFT>12 months group. To do this, we examined the normality of the variables and applied Welch's t-test to analyze the relationship between miRNA expressions and TTFT. Our findings did not reveal any statistically significant correlations. Statistics were realized using GraphPad Prism 10.3.

Other studies have linked the overexpression of miR-29a to an extended TTFT and a positive prognosis (285, 286). However, one publication indicated that increased expression of miR-29a was correlated with shorter TTFT, more advanced disease stages, and poorer outcomes (287). Additional research is necessary to better understand the significance of miR-29a in relation to this disease. However, miR-133a and miR-339 were not studied in CLL, to the best of our knowledge.

Limitations of the study include the fact that groups of patients are not equal and are relatively small. Another aspect would be the highly variable time from the moment of blood collection to the initiation of treatment, which makes the group of patients with TTFT>12 very heterogeneous. Moreover, we did not have control at the time of vitamin D dosing, this being done at a distance from the time of sampling and without taking into account the fact that some patients might have supplemented vitamin D, this probability being relatively high, considering the age at diagnosis and the frequent administration of vitamin D for the prevention and treatment of osteoporosis.

5.6. Conclusion

We were not able to obtain significant data on the hypothesis that vitamin D values influence TTFT, but we observed that its levels are negatively correlated with age. In our cohort of patients, miR-29a, miR-133a and miR-339 were not correlated with vitamin D levels. However, we did find that miR-133a, miR-29a and miR-339 are associated with ALC at diagnosis and that miR-29a and miR-339 are associated with AMC at diagnosis. We also obtained some correlations at the limit of statistical significance, like miR-133a's correlation with TTFT in CLL and miR-29a's correlation with early Binet stages. Further research is required to confirm this information.

6. General Discussions

CLL is a non-aggressive lymphoproliferative syndrome, in most cases, being diagnosed by chance, in the early stages, in asymptomatic patients. Data on the frequency of CLL in Romania are limited, hence in the first part of this work, we decided to create a database. with CLL patients diagnosed

We included 308 MBL/CLL/SLL diagnosed and treated at the Hematology Department of IOCN, over 6 years, from the 1st of January 2017 to the 31st of December 2022. The patients were followed until the 31st of December 2023.

Regarding the year of diagnosis, we observed a lower CLL incidence between 2017 and 2019. To determine if the incidence was indeed lower during those years, we would need access to National Registries, which, to the best of our knowledge, do not exist in Romania for this specific condition. One possible explanation for this incidence is that many individuals may not have undergone regular check-ups, leading to several undiagnosed cases until symptoms manifest. The lowest incidence of CLL was observed in 2020, likely due to the impact of the SARS-CoV-2 pandemic and the resulting restrictions on access to standard healthcare. The years 2021 and 2022 describe the highest incidence of CLL, in IOCN, explained by an increase in people's concern about their health and an increase in the number of routine check-ups.

62% of the participants were male, supporting the Worldwide observation of a higher prevalence of CLL among male patients. The median age at diagnosis was 66 years, which showed that our population is slightly younger than reported in the Western World (2).

About 50% of the patients in our study were diagnosed with early-stage disease, a finding that has also been observed in clinical trials conducted in other countries (247, 248). Unfortunately, data that could help evaluate any comorbidity index or performance status was inconsistent.

Prognostic factors were evaluated in over 50% of the population studied and in 66.7% of the patients who began treatment (data not shown). This is consistent with findings from other real-world clinical trials (247-250). Del(11q), del(13q) and trisomy 12, were assessed in a small number of patients, less than 10%, as access to these evaluations was financially challenging. According to the IwCLL recommendations, the CLL-IPI should be regarded as a prognostic score. However, we were unable to calculate it for our patients because B2M was measured in only a limited number, which would diminish the score's statistical significance.

Median TTFT was only two months, ranging from 0 to 61 months. This short median TTFT can be attributed to the fact that 113 of our patients required treatment within six months of their diagnosis.

Out of 308 patients, 180 required treatment initiation. Among them, 84 were administered CIT, while 96 underwent targeted therapies, indicating a slight preference for the latter. ORR, which included CR, CRi and PR, was 89.7%, which follows real-world clinical data (249-252). 16.2% of patients needed a 2nd line of therapy, due to intolerance, SD, PD or relapse. In the 2nd line, preference for the targeted therapy is obvious, accounting for 60% of the regimens used. None of the patients diagnosed with MBL needed treatment initiation during the follow-up period, and as anticipated, and confirmed by the literature, patients with monoclonal MBL have a superior OS rate compared to those with CLL/SLL (253).

At the end of the follow-up period, 20.1% of patients had died, while 79.9% were alive. At the median follow-up of 25.3 months, the median OS was not reached, an aspect supported by the existing literature (145, 242, 243, 246, 254).

No statistically significant correlations were found between OS and del(17p), TP53 mutation and IgHV mutational status, which is explicable in the context of almost two equal groups of patients (CIT group had 83 patients *versus* the targeted treatment group, which had 67). This has also been observed in other real-life studies (141). However, when correlating OS with the type of treatment - CIT or targeted therapy, the group of patients treated with targeted therapy had a better OS than the group with CIT, as it was observed in other real-world clinical trials (145, 246, 255-257).

In Romania, 2017 was a turning point for the therapeutic approach in CLL, as it was the year when the first targeted therapy – ibrutinib, a BTKi- was approved. It was initially approved for CLL with del(17p) or R/R, and subsequently endorsed as front-line therapy. Ibrutinib has improved OS and PFS in clinical trials and real-world settings when compared to traditional chemo-immunotherapy, even in the presence of adverse prognostic factors, such as del(17p), del(11q), 12 trisomy, U-IgHV and TP53 mutations (141). Thus, in the second study, from the initial cohort, we only selected the patients receiving ibrutinib, either as first-line therapy or for R/R CLL and followed them until the 31st of July 2024.

We managed to include 61 patients, and although our study involved a limited patient number, we aimed to provide real-world data on the effectiveness of ibrutinib as a monotherapy for patients with TN or R/R CLL. The median follow-up was 38 months, with a median PFS for those receiving ibrutinib of 31 months, while median OS was not reached. The ORR, which included CR, CRi and PR was recorded at 90.2%, with an OS rate of 83.6%. Our results align with findings from clinical trials and other retrospective studies conducted in real-world settings (251, 252).

Given that our data were not sufficient to assess other prognostic scores, we focused on correlating advanced Rai/Binet stages with PFS. Our findings indicated that, within the studied population, Rai stages 3-4 and Binet stage C at diagnosis were

linked to a decreased PFS. Real-world data on this topic is inconsistent. In most cases, PFS with ibrutinib treatment appears to be independent of the Rai/Binet stages at the time of diagnosis (262). However, we found real-world studies that corroborated our results (259).

In the study, the mutational status of IgHV was assessed in 78.7% of the participants, TP53 mutation and del(17p) in 82%, and the CD38 marker in 70.5%.

Nonetheless, our results were rather unexpected, given that it is generally accepted that M-IgHV is associated with a positive prognosis and a milder course of the disease. In the group of patients we studied, M-IgHV was associated with a shorter PFS, a result that does not align with findings from real-world studies or clinical trials (252, 255, 256). It is worth mentioning that 11 of the 12 patients who had M-IgHV were from the TN group, and none had IgHV:3-21 serotype, which is usually associated with worse disease outcomes (263). One possible reason for this correlation may be attributed to the limited size of the population studied, along with the lack of a laboratory utilizing a standardized protocol, which could lead to inaccurate IgHV testing results.

Ibrutinib was well tolerated in our cohort of patients. At the time of the censoring, 27.1% of the patients had discontinued ibrutinib therapy permanently, and 15.3% had died. Adverse events were encountered in 6 of the included patients. Among the deceased, 8.5% (N=5) were from the TN group, reflecting a higher death rate than that observed in the R/R group, which contradicts existing literature (254). It is important to consider that ibrutinib was administered as a first-line treatment for 40 of the patients, while only 21 received it for R/R disease. Therefore, if we assess the death rate based on the individual groups rather than the entire cohort, it becomes evident that the mortality rate in the TN group is lower compared to those with R/R disease, consistent with previous findings in the literature.

The third study focuses on dysregulated immune mechanisms, aiming to evaluate the vitamin D status of CLL patients and explore its correlations with the expression of various miRNAs that are known to be linked to altered immune responses. After reviewing the literature, we selected miR-29a, miR-133 and miR-339, known for their implications in mediating immunity. From the initial database, we selected 38 patients, from which we collected blood samples. We further divided them into 2 groups: those who had < 12 months (N=22) and those who had > 12 (N=16) months from the moment of collection to the initiation of treatment.

The median age at diagnosis was 62 years (range: 43-91) for the TTFT<12 months group, while the TTFT>12 months group had a median age of 60.5 years (range: 41-85) (1). The TTFT exhibited a wide range of values, ranging from 0 to 243 months, with a median of 63.5 months. The survival analysis indicated a significant advantage in OS for the group with a longer TTFT ($p<0.05$), where the median survival

had not yet been reached. In contrast, the group with a shorter TTFT achieved a median survival of 158 months.

Most of the included patients had vitamin D deficiency. In Europe, almost 40% of the population has a vitamin D deficiency; in 13% of cases, there is a severe deficit (282). Contrary to existing literature, vitamin D levels did not show a correlation with TTFT in the studied population, nor with the OS (183, 188, 284). The only statistically significant correlation we obtained with vitamin D was the one with the age of the patients. Vitamin D value was negatively correlated with age. Findings that are confirmed by the literature, as vitamin D synthesis lowers with age (283). The obtained results might be attributed to the limited number of patients with optimal vitamin D levels, as only 7 patients had levels exceeding the threshold for optimal vitamin D value. Nevertheless, we identified 2 patients – one from each group- with higher levels—one reaching 94.4 ng/mL and another surpassing 40 ng/mL—suggesting that some of the included individuals may have been taking vitamin D supplements at the time of blood sample collection, which could introduce bias into our analysis.

Further, we confirmed that miR-339, miR-133a and miR-29a are differently expressed in CLL. These miRNAs are involved in immune modulation. Our next step was to correlate vitamin D levels with the studied miRNA's expressions. We did not obtain p-values < 0.05, which showed that there is no statistically significant correlation between vitamin D levels and miR-133a, miR-339 and miR-29a. However, the correlation between vitamin D level and miR-29a expression level resulted in a p-value=0.065, which is at the limit of statistical significance. The existing literature described that miR-29a and miR-339 are regulated by vitamin D levels, however, to our knowledge, there is no available literature assessing the relationship between miR-133a and vitamin D, which left us without a comparative basis for our findings (284). Furthermore, the correlation between miR-339, miR-133a and miR-29a and vitamin D in CLL was not evaluated in any research study, to our knowledge.

Another hypothesis of ours was that these miRNAs can be used as prognostic markers and are correlated with disease evolution. Thus, we evaluate the relationship between the expression levels of miR-339, miR-133a, and miR-29a and prognostic factors. Correlating the studied miRNAs with ALC at diagnosis, we obtained p-values < 0.05 for each miRNA, indicating statistically significant correlations. MiR-339 and miR-133a were not evaluated in CLL, but research has shown that miR-29a is overexpressed in the early stages CLL, which might suggest that there is an association with ALC and miR-29a (156, 285).

Further, we assessed whether miR-339, miR-29a and miR-133a are correlated with AMC at diagnosis. P values <0.05, for AMC with miR-339 and miR-29a correlations, which confirmed that there is a statistically significant relationship between the aforementioned variables. The correlation between miR-133a and AMC resulted in a p=0.55, which did not confirm our hypothesis that there is any statistically significant correlation. To the best of our knowledge, neither of the studied miRNAs has been associated in other research with AMC in CLL, however, miR-133 is known to influence macrophage development, by regulating Granulocyte-Macrophage Colony-stimulating Factor Expression (277).

Finally, we correlated the studied miRNAs with Binet stage at diagnosis. We did not find a statistically significant p-value for either correlation, however, the association between Binet stages A and C and miR-29a approached statistical significance (p=0.054). As mentioned above, research has described higher miR-29a values in Binet A/Rai 0 stages, thus associating it with a good prognosis (285, 286). In our case, the borderline statistical significance of the correlation between miR-29a and Binet staging may be attributed to the small size of the patient cohort. Further research is warranted, as we did find research that associated miR-29 overexpression with advanced Rai/Binet stages at diagnosis(287). MiR-339 and miR-133a, to the best of our knowledge, were not evaluated in CLL, so we do not have any means to compare our results. However, we did find that both miRNAs are expressed in our cohort of patients.

Eventually, we aimed to evaluate whether there is a correlation between miRNA expressions in the TTFT<12 months group compared to the TTFT>12 months group. To do this, we examined the normality of the variables and applied Welch's t-test to analyze the relationship between miRNA expressions and TTFT. Our findings did not reveal any statistically significant correlations. However, when we analyzed the expression of miR-133a in the two groups (TTFT < 12 months and TTFT > 12 months), we observed a p-value of 0.082, which is the closest to suggesting statistical significance. Other studies have linked the overexpression of miR-29a to an extended TTFT and a positive prognosis (285, 286). However, one publication indicated that increased expression of miR-29a was correlated with shorter TTFT, more advanced disease stages, and poorer outcomes (287). Additional research is necessary to better understand the significance of miR-29a in relation to this disease. However, miR-133a and miR-339 were not studied in CLL, to the best of our knowledge.

All of the above-mentioned studies should be considered in light of the fact that the data collected are not sourced from medical records designated for clinical trials, but rather reflect the actual medical practice. Additionally, although the patient

cohorts are small in size, they provide valuable starting points for further research that could uncover new prognostic markers or therapeutic targets.

7. General Conclusions

1. Despite the severe impact of the SARS-CoV-2 pandemic on Romania's healthcare system, it appears to have encouraged the population to engage in more frequent routine check-ups, resulting in a higher diagnosis rate of CLL at initial, asymptomatic stages.

2. Initially met with scepticism, targeted therapies are now preferred by hematologists over classic chemotherapy for patients with R/R disease. This shift may be partly driven by the advent of limited-duration therapies such as venetoclax combined with obinutuzumab or rituximab.

3. The significance of established prognostic markers is diminishing in light of these novel therapeutic options.

4. In contrast to the global median diagnosis age of 70 years, our patient cohort is younger, with a median age of 63 at diagnosis and 65 at treatment initiation. This younger demographic may contribute to the lower CIRS index observed at diagnosis and the favourable tolerance displayed toward ibrutinib.

5. While the assessment of prognostic factors at IOCN aligns with the IwCLL recommendations, there remains a need for enhanced standardization in diagnostic methods. Accreditation by the ERIC for evaluating TP53 mutations, IgHV mutational status, deletions (del) of 17p, 13q, and 11q, and trisomy 12 is also warranted.

6. In our study group, vitamin D levels showed a negative correlation with age. Since CLL primarily affects older individuals, this relationship, while not validated in the current work, was confirmed by other studies that have linked higher vitamin D levels to prolonged TTFT. Therefore, vitamin D supplementation and dosing could be considered as part of the CLL treatment regimen. However, further research is necessary to explore this potential connection.

7. We did not find any statistically significant correlation between vitamin D levels and miR-339, miR-133a and miR-29a. However, previous studies suggest that vitamin D can affect the expression of various miRNAs, including miR-339 and miR-133a. Therefore, this concept should not be researched further.

8. We achieved a correlation at the threshold of statistical significance between miR-29a and Binet staging, which would be worth exploring in larger patient cohorts, especially as it has been described in other studies.

9. MiR-339, miR-133a, and miR-29a demonstrated statistically significant relationships with leukocyte and monocyte counts, indicating that they may serve as predictive markers and potential therapeutic targets.

8. Originality and Scientific Contribution of the Thesis

We conducted a retrospective analysis of CLL patients diagnosed and treated at the Hematology Department of the "Prof. Dr. Ion Chiricuta" Institute of Oncology in Cluj Napoca from January 1, 2017, to December 31, 2022. In a country where disease registers are sporadical and unsystematic, and where epidemiological data is lacking, our study comes to contribute with real-world data obtained over a long period in a rather large centre of regional relevance.

Furthermore, our study resulted in the establishment of a biobank with 105 samples of CLL patients, including cells and sera. This is an invaluable resource which served for the current studies but has an enormous potential to be used in future research projects.

It is well known that the heterogeneity of CLL cannot be attributed solely to genetic and molecular abnormalities; immune dysregulation significantly contributes to the disease's progression. Vitamin D level is known to impact the CLL's evolution, likely due to its role in modulating immune responses. Therefore, we aimed to investigate the correlation between vitamin D and the expression of microRNAs known to play a role in immune modulation. Notably, two of the selected miRNAs have not been previously studied in CLL. Our findings indicate that miR-29a, miR-133a, and miR-339 are expressed at different levels in CLL. Additionally, we sought to correlate the expression of these miRNAs with vitamin D levels, a connection that, to our knowledge, has not been explored in the literature regarding these specific miRNAs and CLL. Albeit small, we came up with a contribution towards the feasibility of miRNA dosage in plasma samples in CLL and their potential relevance.

If all of the above will prove to have been the first steps towards the establishment of a regular practice that will evolve in magnitude over time, resulting in a dedicated systematic disease registry, a functional biobank, and further studies on miRNAs in CLL both as predictors and as therapeutic targets, then this research would be confirmed as fruitful indeed.

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