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Department of Health Sciences

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Association of clonal hematopoiesis of indeterminate potential and Richter transformation in chronic lymphocytic leukemia

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Summary

Chronic lymphocytic leukemia (CLL) represents the most frequent leukemia in adults, characterized by considerable clinical and biological heterogeneity. Some patients exhibit an indolent form that does not require treatment for several years, while others have an aggressive form necessitating early intervention. CLL evolves from an indolent phase to an aggressive lymphoma in 5–10% of the cases. This clinicopathologic transformation is known as Richter transformation (RT). Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related condition characterized by the presence of somatic genomic alterations in hematopoietic cells of individuals without a hematologic malignancy. Notably, CHIP has garnered significant attention in research due to its association with an increased risk of developing myeloid malignancies. However, the role of CHIP in lymphoid neoplasms, particularly in CLL, remains less understood. CHIP has been recognized as a precursor to hematologic malignancies, including CLL. The association between CHIP and RT in CLL remains incompletely understood.

In this thesis, we conducted a cohort of 367 newly diagnosed CLL patients to investigate the prevalence and clinical significance of CHIP mutations. Genomic DNA was extracted from peripheral blood, and targeted next generation sequencing was performed to detect mutations in 28 genes associated with CHIP. Of the CLL patients analyzed, 45.5% harbored at least one CHIP mutation, the most frequently mutated genes were *DNMT3A*, *TET2*, and *ASXL1*. *ASXL1* mutations were identified as an independent predictor of a shorter time to RT, highlighting their role in disease progression. However, overall, the presence of CHIP mutations did not predispose CLL patients to RT. Monitoring CHIP mutations could become a crucial aspect of long-term patient care. Our findings underscore the prevalence of CHIP mutations in CLL and their association with *ASXL1* mutations as a predictor of accelerated RT. These results provide novel insights into the genetic landscape of CLL and suggest *ASXL1* mutation status as a potential biomarker for risk stratification in CLL patients. Further studies are warranted to validate these findings and explore their implications for clinical management. This research highlights the intricate relationship between CHIP mutations and RT, suggesting routine CHIP screening and a comprehensive understanding of how CHIP mutations influence RT to advance CLL management and enhance patient outcomes.

1 Introduction

1.1 Chronic lymphocytic leukemia (CLL)

Chronic lymphocytic leukemia (CLL), the most common leukemia in adults in western countries comprising approximately 40% of all adult leukemia cases (Fabbri & Dalla-Favera, 2016). In the United States, it exhibits an age-adjusted incidence rate of 4.5 per 100,000 individuals, At diagnosis, the median age of CLL patients is 70 years old the male to female ratio is 1.7 (Ou *et al*., 2022).CLL may manifest sporadically, a familial predisposition is observed in 5-10% of cases, often associated with a history of CLL and other non-Hodgkin lymphomas (NHLs) (Goldin *et al*., 2004). CLL is characterized by the accumulation of clonal B lymphocytes in peripheral blood, bone marrow, and lymphoid tissues. The World Health Organization classification system classifies CLL and its predominantly nodal variant, small lymphocytic lymphoma (SLL), as mature B cell neoplasms (Campo *et al*., 2011). CLL diagnosis requires the presence of≥5,000 clonal B lymphocytes per microliter of peripheral blood persisting for over 3 months (Hallek *et al*., 2008a).

Despite their uniform morphological and immunological characteristics, CLL exhibits diverse clinical outcomes, attributable in part to two distinct disease subtypes delineated by the mutational status of immunoglobulin heavy chain variable region (IGHV) genes: IGHV-mutated (IGHV-M) and IGHV-unmutated (IGHV-UM) CLLs. Traditionally, IGHV-M CLL associates with a good prognosis, while IGHV-UM CLL correlates with a poorer prognosis(Pérez-Carretero *et al*., 2021) , likely due to disparities in genetic lesions, clonal evolution, epigenetic modifications, signaling pathway activation, and microenvironment interactions within lymph nodes or bone marrow.

The progression of CLL often begins with an asymptomatic precursor phase termed monoclonal B cell lymphocytosis (MBL), wherein CLL-like cells are detected in the peripheral blood of otherwise healthy individuals (Strati & Shanafelt, 2015). MBL transform to CLL at a rate of approximately 1% per year, driven by cumulative genetic alterations, epigenetic modifications, and environmental influences (Strati & Shanafelt, 2015). Historically, the two most aggressive CLL clinical phenotypes encompass those resistant to fludarabine (FR-CLL) and those transforming into aggressive diffuse large B cell lymphoma (DLBCL) types, termed Richter transformation (RT) (Rossi & Gaidano, 2013; Stilgenbauer & Zenz, 2010; Tsimberidou *et al*., 2006).

1.2 CLL cell of origin

CLL is defined as a lymphoproliferative disorder, characterized by the clonal expansion of CD5+ mature B cells. The immunophenotype of CLL, marked by the expression of B cell markers such as CD23, CD19, and weakly CD20, along with the CD5 antigen and weak surface membrane immunoglobulin (sIg) expression, distinguishes it from other B cell-derived tumors(Mukkamalla *et al*., 2023). While the expression of CD5 has historically led to the belief that CLL originates from the innate-like B1 lineage of B cells rather than the B2 lineage involved in adaptive immunity (Baumgarth, 2010; "B-Chronic Lymphocytic Leukemia: A Malignancy of Anti-Self B Cells," 1996; Caligaris-Cappio *et al*., 1982; Stall *et al*., 1988)

The exact phenotype of the B cell responsible for clonally expanding to generate CLL is still under investigation, Kikushige *et al* suggests that the earliest genetic and epigenetic alterations leading to CLL may originate in pluripotent hematopoietic stem cells (HSCs). Immunogenetic studies and gene expression profiling (GEP) analyses have provided important information regarding the putative CLL progenitor (Fabbri G *et al*., 2016). Naïve B cell encounters the antigen and may proceed either through a T cell-dependent reaction occurring in the germinal center (GC) and leading to the generation of memory B cells that have undergone somatic hypermutation (SHM) of IGHV genes, or in T cell-independent immune responses that may lead to the formation of antigen-experienced B cells harboring unmutated IGHV genes. CLL, and the preceding MBL phase, may originate from both subsets of antigen-experienced B cells. CLLs originating from B cells that have experienced SHM carry mutated IGHV genes and are defined as mutated CLL (M-CLL). Conversely, CLLs originating from B cells that have been involved in T cell-independent

immune reactions harbor germline IGHV genes, without SHM, and are defined as unmutated CLL (U-CLL) (Playoust *et al*., 2023) (Figure 1).

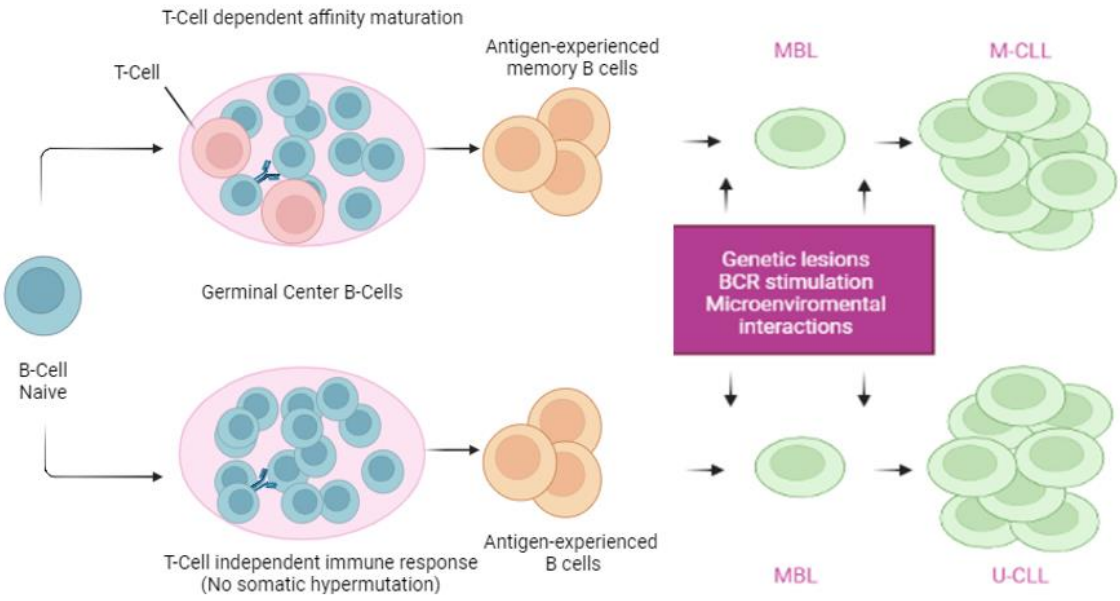


Figure 1: The cellular origin of CLL

1.3 Molecular pathogenesis of CLL

While only a few risk factors for CLL have been identified (Slager *et al*., 2014), it exhibits one of the strongest inherited predispositions among hematological malignancies, with approximately 10% of patients having a family history of the disease (Cerhan & Slager, 2015). Factors such as living on a farm, exposure to herbicides and pesticides, reduced sun exposure, history of atopic conditions, hepatitis C virus exposure, and common infections are associated with an increased risk (Landgren *et al*., 2007; Slager *et al*., 2014).

CLL is characterized by a marked degree of heterogeneity both at the clinical and at the biological level (Gaidano & Rossi, 2017). Comprehensive genomic analyses have identified a model of sequential genetic evolution in CLL, where the loss or gain of chromosomal material initiates leukemic transformation in most cases. Additional mutations or chromosome alterations acquired during the disease course contribute to increased aggressiveness and treatment resistance (Landau *et al*., 2015). Common initiating chromosomal aberrations include deletion of

chromosome 13q (del[13q]) in about 55% of cases, acquisition of chromosome 12 (trisomy 12) in 10-20% of cases. Deletion of chromosome 11q (del[11q]) is observed in around 10% of cases, and deletion of chromosome 17p (del[17p]) in about 5-8% of cases, typically occurring at later disease stages. Del(13q) leads to the loss of miRNAs (miR-15a and miR-16-1), initiating leukemogenesis (Calin *et al*., 2002; Klein *et al*., 2010). Del(11q) results in the loss of the *ATM* gene, encoding the DNA damage response kinase ATM (Calin *et al*., 2002; Klein *et al*., 2010), while del(17p) typically deletes the tumor suppressor gene *TP53*. Over 80% of cases with del(17p) also carry mutations in the remaining *TP53* allele, disrupting the TP53 pathway functionally (Shackelford *et al*., 2005). *TP53* mutations and del(17p) are collectively categorized as genetic *TP53* aberrations. Additional somatic gene mutations have been identified in *NOTCH1, XPO1, KLHL6, MYD88*, and *SF3B1* (Schaffner *et al*., 1999; Zenz *et al*., 2008). The survival of CLL cells also relies on a supportive microenvironment provided by cellular components such as macrophages, T cells, or stromal follicular dendritic cells, which stimulate essential survival and pro-proliferative signaling pathways by secreting chemokines, cytokines, and angiogenic factors or expressing distinct surface receptors or adhesion molecules (Burger & Gribben, 2014).

1.4 CLL diagnosis

The most frequent presentation of CLL typically involves the incidental detection of lymphocytosis during routine blood count analysis conducted for unrelated purposes. Lymphadenopathy represents the second most common clinical manifestation, while B symptoms (fever, night sweats, weight loss, fatigue) or cytopenias (anemia, thrombocytopenia, neutropenia) due to marrow infiltration occur less frequently. A diagnosis of CLL is typically established through immunophenotyping in the presence of an elevated B-cell count of at least 5000 B cells per µL peripheral blood (Hallek *et al*., 2008b).

Immunophenotyping of peripheral blood lymphocytes aids in distinguishing clonal from reactive causes of lymphocytosis or lymphadenopathy and differentiating CLL from other low-grade non-Hodgkin lymphoma subtypes (Hallek *et al*., 2008b). A lymph node biopsy should be done when immunophenotyping of peripheral blood lymphocytes fails to definitively establish the diagnosis (Morice *et al*., 2008).

Patients with a confirmed diagnosis of CLL should undergo risk stratification. Rai and Binet clinical staging systems (Table 1). These two staging techniques which rely on physical examination and standard laboratory tests without imaging studies, have been pivotal in prognostication over the past four decades (Hallek & Al-Sawaf, 2021). Both approaches define three major prognostic groupings with distinct clinical outcomes.

The Rai staging system classifies low-risk disease in patients with lymphocytosis, indicated by leukemia cells in the blood and/or bone marrow, where lymphoid cells exceed 30% (formerly Rai stage 0). Patients with lymphocytosis, enlarged lymph nodes anywhere in the body, and splenomegaly or hepatomegaly (regardless of palpable lymph nodes) are classified as having intermediate-risk disease (formerly Rai stages I or II). High-risk diseases include conditions such as disease-related anemia, with a hemoglobin (Hb) level below 11 g/dL (formerly stage III), or thrombocytopenia, defined by a platelet count lower than 100 x 10^9/L (formerly stage IV) (*The Rai Staging System for CLL (Rai et al. 1975)*.

The Binet staging system classifies disease based on the number of affected areas, indicated by enlarged lymph nodes larger than 1 cm in diameter or organomegaly, as well as the presence of anemia or thrombocytopenia. Stage A is defined by a hemoglobin level of ≥10 g/dL and a platelet count of ≥100 x 10^9/L, with no more than two areas of lymphoid tissue involved. Stage B is characterized by a hemoglobin level of ≥10 g/dL, a platelet counts of ≥100 x 10^9/L, and organomegaly involving three or more areas. Stage C is marked by a hemoglobin level of less than 10 g/dL and/or a platelet count of less than 100 x 10^9/L (Binet *et al*., 1981).

Risk Stratification	Rai Stage	Binet Stage
Low risk	0: Lymphocytosis only	A: <3 Lymphadenopathies
Intermediate risk	I: Lymphadenopathy II: Organomegaly (splenomegaly/ hepatomegaly)	B: >3 Lymphadenopathies
High risk	III: Anemia (hemoglobin <11 g/dL) IV: Thrombocytopenia (platelets $<100 \times 10^9$/L)	C: Hemoglobin <10 g/dL and/or platelets $<100 \times 10^9$/L

Table 1: Rai and Binet staging system (Binet *et al*1981; Rai *et al*. 1975)

1.5 Prognostic markers in CLL

In the past two decades, the advances in understanding the genetic and molecular biology of chronic lymphocytic leukemia has led to the identification of markers associated with risk of progression and survival, providing prognostic information that is complementary to the classical staging systems.(Landau *et al*., 2015.; Puente *et al*., 2011) In particular, *TP53* aberrations are currently the most significant prognostic factor in CLL (Gonzalez *et al*., 2011; Malcikova *et al*., 2018; Stilgenbauer *et al*., 2014),predict an aggressive disease course and refractoriness to chemoimmunotherapy. (Artmut *et al*., 2000; Zenz *et al*., 2010) The mutational status of the IGHV genes has been shown to be an independent prognostic factor in different prognostic models for overall survival, patients with unmutated IGHV genes have a more aggressive disease course than patients with mutated IGHV genes.(Hamblin *et al*., 1999; Thunberg *et al*., 2001) Other relevant prognostic parameters include expression of ZAP-70, CD38, and CD49d and serum concentrations of thymidine kinase37 and β2-microglobulin. (Bulian *et al*., 2014; Crespo *et al*., 2003; Gentile *et al*., 2009; Hallek *et al*., 1996) Finally, mutations or deletions in genes such as *NOTCH1* and *SF3B1,*

are associated with reduced survival. An international group of investigators did a comprehensive analysis to develop a prognostic index for chronic lymphocytic leukemia(Puente *et al*., 2011; Quesada *et al*., 2011).

Advanced prognostic scores have been formulated to condense prognostic information into a set of clinically pertinent parameters by combining clinical, biological, and genetic data (Cortese *et al*., 2014; Pflug *et al*., 2014; Shanafelt *et al*., 2009; Wierda *et al*., 2007). The most pertinent prognostic score at present is the CLL International Prognostic Index (CLL-IPI) ("An International Prognostic Index for Patients with Chronic Lymphocytic Leukaemia (CLL-IPI): A Meta-Analysis of Individual Patient Data," 2016)

It employs a weighted grading system based on five independent prognostic factors: *TP53* deletion and/or mutation (together referred to as *TP53* dysfunction), the mutational status of the immunoglobulin heavy chain variable (IGHV), serum β2-microglobulin levels, clinical stage, and age. The CLL-IPI distinguishes four groups (low-risk, intermediate-risk, high-risk and very high-risk) with varying five-year survival rates. The prognostic significance of the CLL-IPI is being re-evaluated for the application of targeted therapies ('An International Prognostic Index for Patients with Chronic Lymphocytic Leukemia (CLL-IPI)', 2016) (Table 2).

A recently proposed system, the International Prognostic Score for Early-stage CLL (IPS-E), predicts the time to first treatment in patients with early, asymptomatic CLL (Condoluci *et al*., 2020a). Three covariates: an unmutated IGHV gene, an absolute lymphocyte count higher than 15 x 10^9/L, and the presence of palpable lymph nodes, were combined to predict a 5-year cumulative risk for the initiation of treatment at 8.4%, 28.4%, and 61.2% for low-risk, intermediate-risk, and high-risk patients, respectively. The IPS-E is beneficial for counselling patients with early-stage CLL (Condoluci *et al*., 2020b).

Prognostic factor	Points
Del17p on FISH or *TP53* mutation	4
Unmutated *IGHV* genes	2
Serum β2 microglobulin >3.5 mg/L	2
Rai stage I–IV	1
Age >65 years	1

Cumulative CLL-IPI score	Risk category	5-year TFS[a]
0–1	Low risk	78%
2–3	Intermediate risk	54%
4–6	High risk	32%
7–10	Very high risk	0%

FISH fluorescence in situ hybridization, *IGHV* immunoglobulin heavy chain gene, *TFS* treatment-free survival
[a]For the Mayo validation cohort

Table 2: international prognostic index for patients with chronic lymphocytic leukemia (*The CLL-International Prognostic Index 30*)

1.6 CLL treatment

Clinically, CLL is characterized by a marked degree of heterogeneity. Some patients have a condition that is indolent and will not require treatment for several years (Gaidano & Rossi, 2017). On the other hand, some patients have an aggressive form of the disease that requires prompt treatment after diagnosis, or may subsequently transform into an aggressive lymphoma, known as Richter's syndrome (Douglas, 2022).

Asymptomatic patients without anemia, neutropenia, or thrombocytopenia are typically managed with a watch-and-wait strategy, meaning that treatment is not required at the time of diagnosis for most patients (Owen *et al*., 2023). Treating individuals with asymptomatic CLL

does not appear to increase survival. For instance, fludarabine, cyclophosphamide, and rituximab treatment did not improve survival over observation without treatment at five years (82.9% vs. 79.9%; P =.86) in a clinical trial involving 201 patients with asymptomatic CLL (Herling *et al*., 2020). In the event that a patient develops significant anemia or cytopenia (hemoglobin level <10 g/dL or platelet count <100 000/μL), hepatosplenomegaly (≥10 cm for lymph nodes and ≥6 cm below the left costal margin for spleen), recurrent infections, or refractory autoimmune thrombocytopenia/anemia related to CLL, treatment is required (Hallek *et al*., 2018).

Chemoimmunotherapy (CIT) was the main therapy of the treatment of CLL. Chemotherapy agents such as alkylating agents (such as bendamustine and chlorambucil), and purine analogues (such as fludarabine and pentostatin) in combination with the anti-CD20 monoclonal antibodies (Kutsch *et al*., 2020). It should be mentioned that over a median follow-up of 58 months, the fludarabine-cyclophosphamide-rituximab (FCR) combination is linked to a roughly 7% risk of a secondary myeloid malignancy (Kutsch *et al*., 2020).

The use of traditional CIT chemotherapy agents such as alkylating agents (such as bendamustine and chlorambucil), and purine analogues (such as fludarabine and pentostatin) in combination with the anti-CD20 monoclonal antibodies was previously the mainstay of the treatment of CLL but has significant decreased since the introduction of pathway inhibitors (Odetola & Ma, 1234). The continuous improvement in the understanding of CLL pathogenesis has expanded the treatment choices beyond traditional chemoimmunotherapy (CIT) with the introduction of novel small molecule kinase/pathway inhibitors such as the development of novel therapies that specifically target pivotal signaling pathways of CLL cells (Burger & O'Brien, 2018).

B-Cell Signaling Pathway Agents

Since the BCR signaling pathway is essential for CLL pathogenesis and proliferation, typical treatment for the disease involves the use of medications that block the BCR pathway's enzymes, particularly Bruton tyrosine kinase (BTK) and phosphoinositide 3′-kinase (PI3K) (Burger & Chiorazzi, 2013).

BTK inhibitor:

BTK is a crucial intracellular protein downstream of the BCR, whose expression is upregulated in CLL cells (Montresor *et al*., 2018). BTK inhibitors are small molecules that occupy the ATP binding domain inhibits the subsequent phosphorylation of BTK and blocks the downstream signaling pathways, which regulate cell survival and proliferation. They are administered as monotherapy indefinitely, as continuous inhibition of the target enzyme is essential for their antiproliferative effect(Barr *et al*., 2022). BTKi can be grouped into covalent BTKi (ibrutinib, acalabrutinib, and zanubrutinib) and non-covalent BTKi (pirtobrutinib). Covalent BTK inhibitors like ibrutinib, acalabrutinib, and zanubrutinib irreversibly inhibit the BTK enzyme, and over 90% of patients respond to these treatments (Barr *et al*., 2022). Conversly BTK is reversibly inactivated by the noncovalent inhibitor pirtobrutinib. CLL patients may develop resistance against BTKi by different mechanisms Including mutations of the BTK binding site and of the gene encoding phospholipase C Gamma 2 (PLCG2), which acts downstream of BTK in the BCR signaling cascade (Wang *et al*., 2022).

PI3K inhibitors:

In patients with indolent B-cell lymphomas, PI3K inhibitors medicines effectively suppress the δ isoform of PI3K. Idelalisib results in inhibition of the δ isoform of PI3K (PI3Kδ), which is highly expressed in malignant B-cells. This inhibition results in the apoptosis of malignant tumor cells. In addition, idelalisib inhibits several signaling pathways, including B-cell receptor, C-X-C chemokine receptor type 4 (CXCR4), and CXCR5 signaling(Visentin *et al*., 2020). Idelalisib is approved by the FDA and is used in relapsed/refractory settings in combination with rituximab(Shah & Mangaonkar, 2015).

Duvelisib results in the dual inhibition PI3Kδ and PI3Kγ. Inhibition of the δ isoform results in apoptosis of malignant tumor cells, whereas inhibition of the γ isoform reduces differentiation and migration of support cells in the tumor microenvironment. It also inhibits B-cell receptor signaling pathways and CXCR12-mediated chemotaxis of malignant B-cells. Duvelisib is approved by the FDA and is used in patients with relapsed/refractory CLL who have received at least 2 prior therapies (Faehling *et al*., 2023).

B-cell lymphoma 2 inhibitor:

The B-cell lymphoma 2 (BCL-2) protein inhibits the apoptotic mitochondrial pathway. BCL2 is overexpressed in CLL and mediates leukemic B-cell survival. Venetoclax directly inhibits BCL-2 protein, activating the proapoptotic proteins and restoring the apoptotic process (Qian *et al*., 2022). Venetoclax is approved by the FDA for use in the relapsed/refractory setting either alone or in combination with rituximab. Because of the high incidence of tumor lysis syndrome during clinical trials, venetoclax has a weekly dose ramp-up schedule, starting at a very low dose of 20 mg and this has been shown to greatly reduce tumor lysis syndrome in high-risk patients.(Sharma & Rai, 2019)

1.7 Richter transformation (RT)

RT was defined for the first time in 1928 by Maurice N. Richter as "reticular cell sarcoma". Nowadays, according to the World Health Organization (WHO) classification of Tumors of Hematopoietic and Lymphoid Tissues, RT is defined as the occurrence of an aggressive lymphoma in patients previously diagnosed with CLL or small lymphocytic lymphoma (SLL) (Krewski D *et al.,2019)*. RT is currently categorized into two distinct pathological variants: the diffuse large B-cell lymphoma (DLBCL) variant, characterized by sheets of large neoplastic post-germinal center B lymphocytes, and the Hodgkin lymphoma (HL) variant (Mao *et al*., 2007).

RT incidence ranges from 1 to 7% (Rossi *et al*., 2012).The transformation from CLL to DLBCL-type RT has been shown to associate with somatic genetic events acquired in the CLL phase, including *TP53*, *CDKN2A*, *NOTCH1* or *MYC*, while the biology of the HL-type RT is largely unknown (Rossi *et al*., 2014).

From the phenotypic standpoint, the neoplastic cells of DLBCL-type RT express CD20, and less commonly CD5 and CD23. MYC expression is observed in 30% to 40% of cases and its expression accompanies gene translocation. Moreover, the DLBCL-type RT frequently expresses BCL2 similar to CLL (Cabeçadas *et al*., 2021). However, the *BCL2* gene is rarely affected by genetic lesions in DLBCL-type RT compared with the prevalence of BCL2 translocations and

somatic mutations which are commonly found in *de novo* DLBCL (Testoni *et al*., 2015). Another difference between DLBCL-type RT and de novo DLBC, is the PD-1 expression which is documented in up to 80% of DLBCL-type RT, whereas this marker is poorly expressed in *de novo* DLBCL. The majority of DLBCL-type RT are Epstein-Barr virus negative (EBV^-)(Rossi *et al*., 2018).

The Analysis of the IGHV rearrangement allows the assessment of the clonal relationship between the RT and the CLL phase of the same patient and identifies two distinct RT types, i) clonally related RT, has a rearrangement of immunoglobulin gene related to the CLL phase (~80% of DLBCL-type RT cases), thus documenting that this histologic shift is a true transformation event from the previous indolent phase. ii) clonally unrelated RT that are characterized by a rearrangement of immunoglobulin gene different from the CLL phase, these cases are less frequent (~20% of DLBCL-type RT cases), documenting a clonally unrelated origin of RT, considered as a de novo lymphoma and favored by the intrinsic higher incidence of secondary tumors in CLL patientsa (Mao *et al*., 2007)

HL-type RT resembles its de novo counterpart, featuring Hodgkin and Reed–Sternberg cells with a CD30+/CD15+/CD20− immunophenotype, and the majority of cases of the HL-type RT are EBV+. (Mao *et al*., 2007; Xiao *et al*., 2016).

The prognosis of DLBCL-type RT is unfavorable, and the outcome of HL-type RT appears to be better. The most important prognostic factor is the clonal relationship between the CLL and DLBCL clones. The clonally unrelated RT cases display a better prognosis with a longer median survival (~5 years) compared with clonally related RT cases. As a consequence, investigating the clonal relationship in DLBCL-type RT patients is clinically relevant, especially considering that clonally unrelated DLBCL may be managed as a de novo DLBCL arising in the context of CLL, rather than a true transformation (Favini *et al*., 2022).

RT poses a significant challenge in clinical practice, with limited targeted treatment options and often ineffective therapies leading to patient mortality (Condoluci & Rossi, 1912). The R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) regimen, is widely used in de novo DLBCL, showed an overall response rate (ORR) of 67% in RT patients, with a

complete response (CR) of only 7%, a median overall survival (OS) of 21 months and a median progression-free survival (PFS) of 10 months. The most common severe adverse events were hematological toxicity (grade ≥3 anemia, neutropenia and thrombocytopenia) and infections (Langerbeins *et al*., 2014; Rogers *et al*., 2018; Rossi & Gaidano, 2009). On the other hand, The understanding of the molecular pathways involved in RT pathogenesis has led to

the identification of novel druggable targets, namely BTK, BCL2, PD-1 and PD-L1 (Mouhssine & Gaidano, 2022a). Ongoing clinical trials exploring pathway inhibitors and monoclonal antibodies offer promising avenues for RT treatment, suggesting potential advancements in precision medicine approaches for RT management in the future.(Mouhssine & Gaidano, 2022b)

1.8 Clonal hematopoiesis of indeterminate potential (CHIP)

Clonal Hematopoiesis of Indeterminate Potential (CHIP) is a recently recognized aging phenomenon with significant implications for health. It is characterized by the presence of at least one somatic mutation in HSCs of peripheral blood, occurring in the absence of hematological malignancy or other clonal disorders with a variant allele frequency (VAF) of at least 2%. (Jaiswal *et al*., 2014a; Jaiswal & Ebert, 2019; Natarajan *et al*., 2018; Steensma *et al*., 2015a). These mutations are believed to provide a selective advantage to HSCs, allowing them to proliferate clonally while maintaining normal hematopoietic function(Murphy *et al*., 2022). CHIP is considered an aging-related phenomenon, as its prevalence increases with age, affecting 10–30% of individuals over 70 (Bick, Weinstock, *et al*., 2020; Genovese *et al*., 2014a; Jaiswal *et al*., 2014a; McKerrell *et al*., 2015). The clinical data suggest that aging is the strongest risk factor for CHIP (Australian Pancreatic Cancer Genome Initiative *et al*., 2013). In addition to age, the second most major risk factor for CHIP is a history of chemotherapy or radiation (Coombs *et al*., 2017).

The most common mutations associated with CHIP involve loss-of-function or truncating alleles in epigenetic regulator genes such as *DNMT3A*, *TET2*, and *ASXL1*, frequently observed in myeloid cancers. Importantly, the presence of these mutations elevates the risk of developing hematological cancer by approximately tenfold, indicating CHIP as a precursor state for haematological malignancy (Genovese *et al*., 2014a; Jaiswal *et al*., 2014a).

Although CHIP itself does not manifest as hematological malignancy, it substantially heightens the likelihood of such cancers. Moreover, while the association between CHIP and

hematological malignancies was anticipated, several large-scale studies have unexpectedly linked CHIP with cardiovascular diseases (CVD) such as coronary artery disease (CAD) and stroke. CHIP carriers exhibit a 1.5–2-fold increased risk of developing CAD compared to non-carriers, along with nearly four times the risk of early-onset myocardial infarction (Bhattacharya *et al*., 2022; Bick, Pirruccello, *et al*., 2020; Jaiswal *et al*., 2014a, 2017a). Recent advancements have shed light on the underlying causes and consequences of CHIP over the past few years.(Ahmad *et al*., 2022)

1.9 CHIP in hematological malignancies

Several hematologic malignancies, including CLL, Multiple Myeloma (MM), and Acute myeloid leukemia (AML), are characterized by well-defined precursor states that precede the onset of overt cancer. CLL is consistently preceded by a high count of MBL, while MM typically arises following monoclonal gammopathy of undetermined significance (MGUS). Additionally, at least a quarter of patients with Myelodysplastic Syndromes (MDS) progress to AML (Landgren, Albitar, *et al*., 2009; Landgren, Kyle, *et al*., 2009; Pfeilstöcker *et al*., 2016). Through deep genomic sequencing of normal individuals, it has been revealed that during human aging, the expansion of one or more Hematopoietic Stem and Progenitor Cell (HSPC) clones leads to sustained contributions to the production of mature blood cells. Since CHIP is defined as the expansion of HSPC clones, harboring specific, disruptive, and recurrent genetic variants, in individuals without clear diagnosis of hematologic malignancies (Jaiswal *et al*., 2014b). Several studies demonstrated that, MDS are frequently preceded by CHIP, (Malcovati *et al*., 2017), moreover, some CHIP related mutations can increase the risk for leukemia, while others potentially elevate the risk of heart disease and diabetes (Genovese *et al*., 2014b; Jaiswal *et al*., 2017b). From a pathogenetic perspective, the study conducted by Agathangelidis *et al*. provided the proof of principle that CHIP may also associate with expansion of B-cell clones with CLL phenotype and connects CHIP with MBL and CLL in a continuum of evolution from HSCP clones to mature B-cell clones. (Kikushige *et al*., 2011).

However, the role of CHIP in lymphoid neoplasms, especially in CLL, is not well understood. More research is needed to explore the correlation between CHIP and CLL to gain a clearer understanding of this phenomenon and its clinical and prognostic implications.

2 Aim of study

The aims of the present study are:

i) To examine the occurrence and prevalence of CHIP in a prospective cohort of 367 newly diagnosed CLL patients at our institution.

ii) To assess the relationship between CHIP and RT.

3 Materials and methods

3.1 Patients

The study was conducted on a real-life cohort composed of xxx CLL patients. For each patient, samples of genomic DNA (gDNA) extracted from granulocytes were obtained.

Clinical and biological data were available for each patient, such as age, sex, blood count, biochemical profile, immunophenotype on flow cytometry, FISH analysis, mutational status of IGHV genes, mutational analysis of the *TP53* gene via DNA sequencing, Rai and Binet staging, TTFT and OS.

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

3.2 Separation of granulocytes from peripheral blood (PB)

PB from patients with CLL was collected in special tubes. Granulocytes were isolated from the same peripheral blood samples and used as a source of gDNA.

PB granulocytes were separated by Ficoll gradient density centrifugation, PB was diluted in 1:2 ratio with physiological solution (NaCl 0.9%) and then centrifuged at 1800 revolutions per minute (rpm) for 25 minutes in a gradient differentiation Sigma DiagnosticTM Histopaque®-1077 Cell Separation Medium (Sigma-Aldrich, St. Louis, MO, USA) solution to obtain granulocytes and mononuclear cells (monocytes and lymphocytes).

3.3 DNA extraction

Granulocytes gDNA were extracted either by using the “salting out” protocol (Miller *et al*., 1988)(Miller *et al*., 1988)(Miller *et al*., 1988). Cells were lysed with Lysis Buffer (Tris-HCl 1M, pH 8.2, NaCl 5M, 20 EDTA 0.5M), sodium dodecyl sulphate (SDS) 20% and digested with 20 mg/ml of proteinase enzyme (pronase E). Samples were incubated at 37°C overnight in a shaking

incubator. Proteins were precipitated with 6M NaCl, and subsequently discarded after centrifugation at 3200 rpm for 20 minutes. DNA was isolated by precipitation with pure ethanol and the lactescent "jellyfish" of DNA, formed as a result of the addition of ethanol, was recovered with glass loops and washed three times in 75% ethanol. The excess of ethanol was evaporated, and the DNA was dissolved with TE Buffer (Tris-HCl 1M, pH 8.2, EDTA 0.5M).

3.4 DNA quantification and fragmentation

gDNA were quantified using the Quant-iTTM PicoGreen dsDNA Assay kit (ThermoFisher Scientific, Eugene, OR, USA). PicoGreen is a molecule that binds selectively to double helix DNA and allows to obtain a precise estimate of the amount of DNA. The fluorimetric reading was performed using the Infinite F200 fluorometer (TECAN, Männedorf, Switzerland) using the Magellan software. The fluorimetric readings were obtained at a wavelength of 485 nm in absorption and 530 nm in emission. For quantification a standard curve was prepared using a DNA of known concentration and performing serial 1:2 scalar dilutions. Quant-iTTM PicoGreen dsDNA Assay kit was used at the 1:200 dilutions.

The gDNA was fragmented by sonication with the M220 focused ultrasonicator (Covaris® Woburn, MA, USA) before the library preparation to obtain 250/300 base pair fragments, the optimal length for analysis using the MiSeq and NextSeq 550 platforms (Illumina, San Diego, CA, USA).

The size of the fragments was checked by using the 2100 Bioanalyzer Instrument with the High Sensitivity DNA kit (Agilent Technologies, St. Clara, CA, USA)

3.5 Library design for hybrid selection

A targeted resequencing gene panel, including coding exons and splice sites of 28 genes (target region: 29710 bp) that are recurrently mutated in CLL and CHIP, has been specifically designed for this project.

3.6 Next generation sequencing (NGS)

The mutational analysis in next generation sequencing (NGS) was performed using the MiSeq and NextSeq 550, which allows for massive high-throughput sequencing of the genomic regions of interest. The sequencing workflow involves the following phases: i) generation of libraries containing the regions of interest; ii) sequencing; and iii) data analysis.

i) Generation of libraries

Library preparation is a process that consists of generating a collection of DNA fragments suitable for analysis by NGS sequencing. In the current study, libraries were prepared from gDNAes

Libraries were generated using the KAPA HyperPrep kit (Roche Diagnostics, Pleasanton, CA, USA) and enrichment of regions of interest was achieved using a KAPA HyperChoice probe system (Roche Diagnostics, Pleasanton, CA, USA).

ii) Sequencing

The MiSeq and NextSeq 550 sequencer are based on sequencing by synthesis technology, in which DNA libraries are transferred onto a solid support, called flowcell, to which they are linked by special adapters. On the flowcell the libraries are amplified by a method called bridge amplification, which generates clusters of identical DNA molecules, each derived from the amplification of a single molecule. Sequencing is based on the reversible cyclic termination method, with a by-synthesis approach, which includes three steps: the incorporation of the nucleotide, the detection of the fluorescence image and the cut.

In the first phase of the cycle, the DNA polymerase elongates a specific primer by adding a nucleotide covalently bound to a fluorophore. This presents a block on the 3'- OH of ribose which does not allow polymerization with other nucleotides. Each nucleotide base is bound to a fluorophore of a specific colour. It follows the detection step of the image that recognizes the specific emission wavelength of the fluorophore. Next, the cut removes both the fluorophore and the inhibitory group present at the 3'-OH end, allowing the beginning of a new cycle. Libraries were sequenced by pair-end sequencing. The library pool was denatured using 0.2N NaOH.

An amount of of 6 to 9.5 pM of denatured DNA (for the MiSeq platform) and 1.3 pM (for the NextSeq 550 platform) was loaded into the cartridge, which also contained all the reagents necessary for the sequencing reaction.

iii) Data analysis

During the sequencing run, the integrated software for real-time primary analysis (RTA, Real Time Analysis, Illumina) performs image analysis and identification of the bases and assigns a qualitative score (Phred score) to each base for each cycle. Once the primary analysis is completed, A second software integrated into the sequencer, MiSeq Reporter Software (MSR) for the MiSeq platform and Local Run Manager for the NextSeq 550 platformthe perform a secondary analysis on the data generated by the RTA through a series of procedures that include: i) de-multiplexing, which separate and identify data from different samples sequenced and pulled together based on the specific sample index sequences; ii) FASTQ generation, which are files containing all the reads obtained from sequencing.

FASTQ sequencing reads were subjected to deduplication by using the FastUniq v1.1. Then, the deduplicated FASTQ sequencing reads were locally aligned to the GRCh37/hg19 version of the human genome assembly using the BWA v.0.6.1 software with the default setting, and sorted, indexed and assembled into a mpileup file, using SAMtools v.1.

The calling of single-nucleotide variants (SNVs), deletions and insertions (indel) at good quality nucleotide positions (Phred score >20), the process of identifying gDNA differences compared to the reference sequence GRCh37/hg19, was carried out using the somatic function of the VarScan2 program. The use of this method allowed us to exclude any polymorphisms and to consider only the somatic variants (SNV, indels).

The variants called by VarScan 2 were annotated using the wANNOVAR (https://wannovar.wglab.org/). Variants annotated as (single-nucleotide polymorphisms) SNPs according to according to the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/snp/), with the exception of *TP53* variants that were manually solved and scored as SNPs according to the International Agency for Research on Cancer *TP53* database (http://p53.iarc.fr). Intronic variants, mapping >2 bp before the start or after the end of coding exons, and synonymous variants were then filtered out.

Among the remaining variants, only protein truncating variants (i.e., indels, stop codons and splice site mutations), as well as missense variants, not included in the dbSNP and annotated as somatic in the COSMIC v96 database (https://cancer.sanger.ac.uk/cosmic), were retained.

All the variants were visualized using IGV (Integrative Genomics Viewer) software.

3.7 Statistical analysis

Patient characteristics at diagnosis were compared with respect to the presence of CHIP using the Mann-Whitney test for continuous variables and the Chi-square test for categorical variables. The clinical end points were: i) OS measured from the date of diagnosis until the date of death from any cause; ii) the TTFT measured from the date of diagnosis until the start of the first line of therapy; iii) the time to transformation to RT measured from the date of diagnosis to the time of transformation to RT. The analyzes were carried out with SPSS 24 software and R-Studio 2021.09.1.

4 Results

4.1 Patients characteristics

In our cohort, 367 newly diagnosed CLL patients have been sequenced. The median age at diagnosis was 70.3 years old, 206 (56.1%) were male and 161 (43.9%) were female. The median lymphocyte count was 9.1 x 10^3 /µL, the median Hb level was 13.8 g/dL, and the median PLT count was 205.5 x 10^3 /µL. As for CLL biological features, 124 (34.4%) patients harbored unmuted IGHV genes, 236 (65.6%) patients harbored mutated IGHV genes, 36 (9.9%) patients had *TP53* disrupted, 61 (16.7%) Trisomy 12, 20 (5.5%) Del 11q, 175 (47.9%) Del 13q, and 23 (6.3%) had RT. Among the 155 patients (42.2%) who received treatment, chemo-immunotherapy was the initial treatment for 141 (91.0%) of them. The main characteristics of the patients are shown in table 3.

Characteristics	N (%)
Gender (n. %)	
Male	206 (56.1%)
Female	161 (43.9%)
Age (median)	70.3
Lymphocyte x10^3/µL (median)	9.1
Hb g/dL (median)	13.8
Platelet x10^3/µL (median)	205.5
IGHV status	
Unmutated	124 (34.4%)
Mutated	236 (65.6%)
***TP53* disrupted**	
Yes	36 (9.9%)
No	328 (90.1%)
Trisomy 12	
Present	61 (16.7%)
Absent	304 (83.3%)
Del11q	
Present	20 (5.5%)
Absent	345 (94.5%)
Del13q	
Present	175 (47.9%)
Absent	190 (52.1%)
Richter transformation	
Yes	23 (6.3%)
No	344 (93.7%)
Treated	
Yes	155 (42.2%)
No	212 (57.8%)
Type of 1st line treatment	
Chemo-immunotherapy	141 (91.0%)
BTKi or BCL2i	14 (9.0%)

Table 3: Patients Characteristics

After a median follow-up of 13.9 years, the median OS was 12.5 years and the median TTFT in Binet A was not reached (Figure 2).

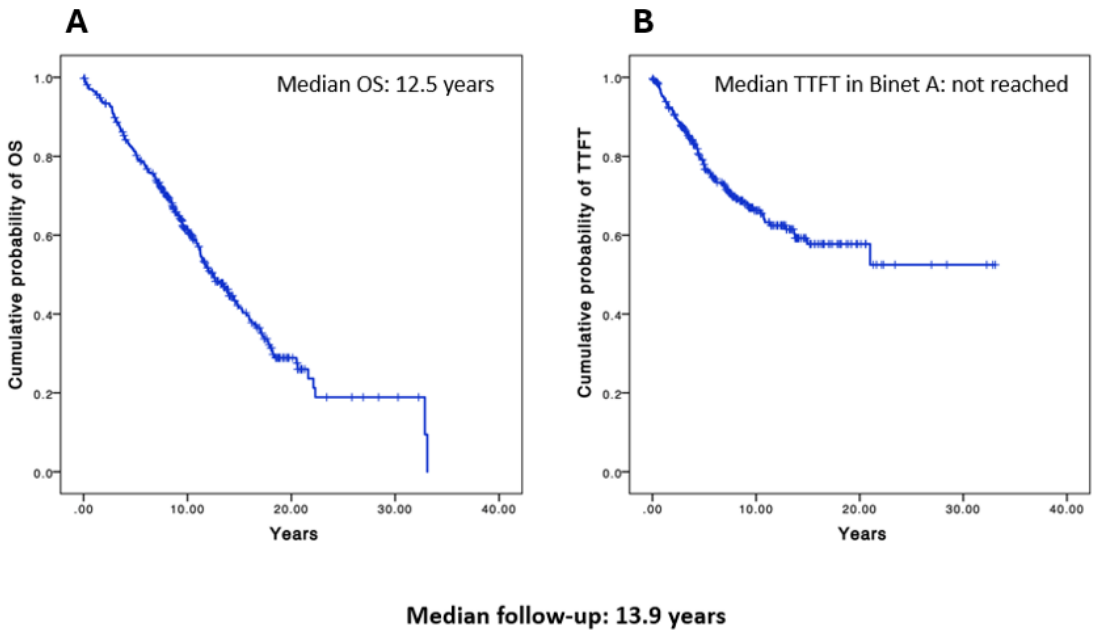


Figure 2. Survival analysis with Kaplan-Meier curves. A: Global PFS of the cohort. B: Global TTFT of the cohort.

4.2 Chip prevalence and the mutational analysis

Out of 367 patients analyzed for CHIP, a total of 167 (45.5%) patients showed at least 1 CHIP mutation with different types of mutations. As expected, *DNMT3A* had the highest frequency of mutations observed in 89 (24.3%) patients, followed by *TET2* in 52 (14.2%) patients and *ASXL1* in 10 (2.7%) patients (Figure 3).

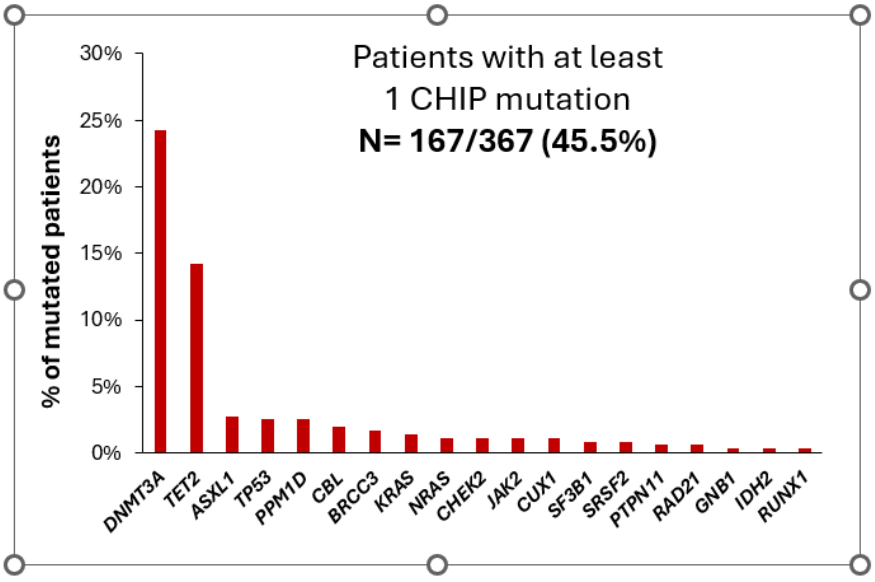


Figure 3. CHIP mutations. The histogram presents the 19 genes found mutated in the patients on the x-axis, and the absolute number of mutations on the y-axis.

In our analysis of CHIP mutations, we observed a diverse distribution of mutation types across the different genes. For *DNMT3A*, the most common mutations were non-synonymous SNVs (N=63), followed by frameshift deletions (N=16), and stopgain mutations (N=11). In the case of *TET2*, the most frequent mutations were equally divided between nonsynonymous SNVs and stopgain mutations (N=20 each). *ASXL1* predominantly exhibited frameshift deletions and frameshift insertions (N=4 each) (Figure 4).

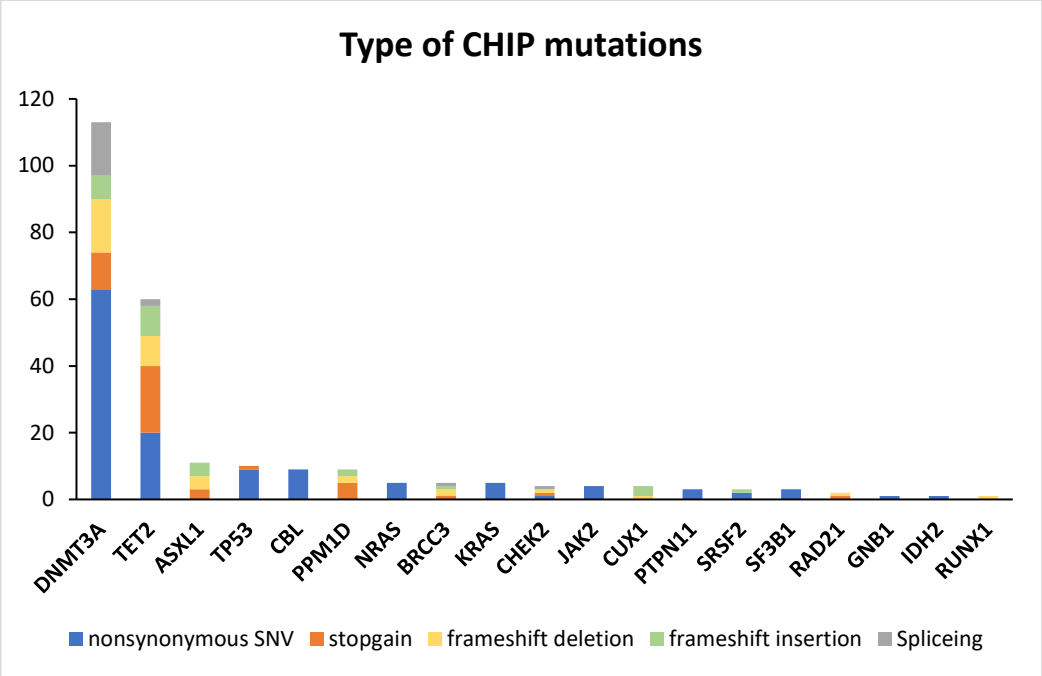


Figure 4. Type of CHIP mutations. The histogram presents the 19 genes found mutated in the patients on the x-axis, and the number of mutations on the y-axis. The legend allows you to distinguish the type of mutation for each gene, based on a color scale. SNV, single-nucleotide variant.

4.3 CHIP contribution in RT

Therefore, we have selected 22 patients who experienced RT to assess the potential contribution of CHIP in RT. Among these patients 8 (36.4%) harbored at least one CHIP mutation (CHIP-positive) and 14 patients didn't have CHIP mutation (CHIP-negative). Among the CHIP-positive group, *ASXL1* was the most frequently mutated gene followed by *TET2* (Figure 5).

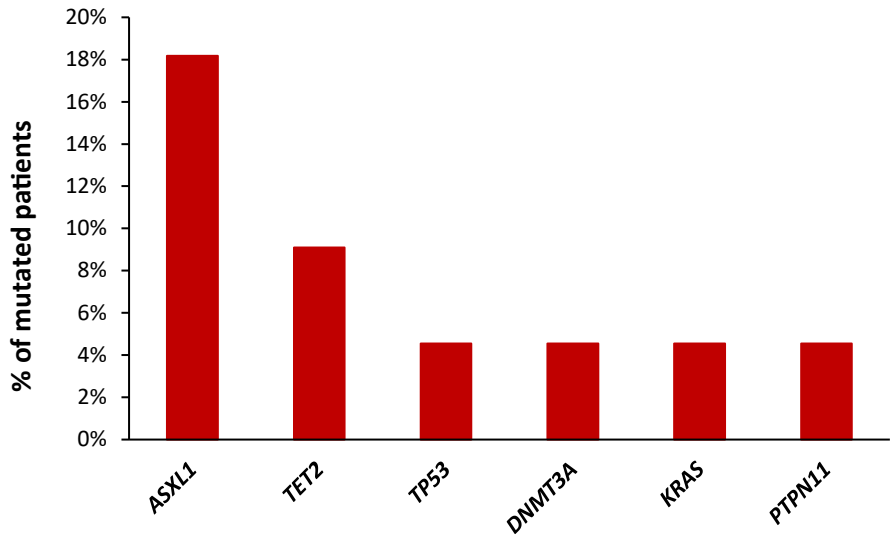
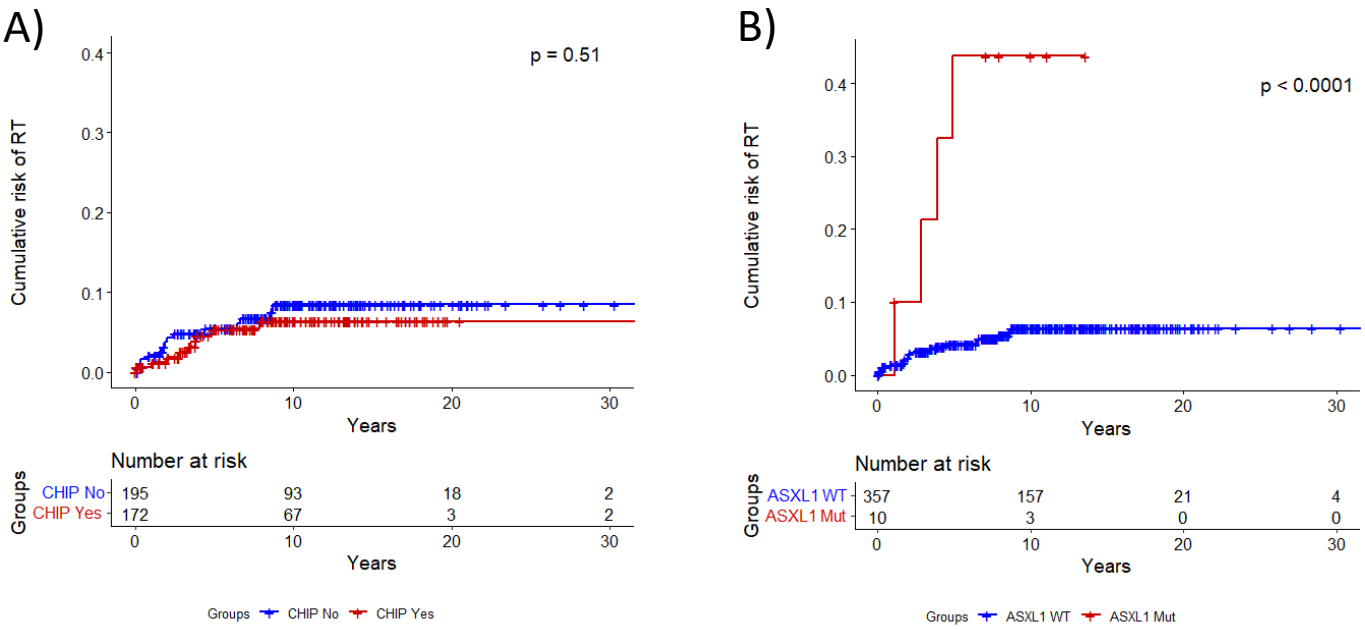


Figure 5. Mutation frequency of CHIP genes in RT patinas. The bar graph displays the percentage of mutations observed for each gene.

From the clinical standpoint, the presence of any CHIP mutation did not correlate with a reduced time to RT (TTRT). Where the difference in the risk of RT between CHIP-positive and CHIP-negative patients was not statistically significant (P=0.51). Conversely, by analyzing the impact of each gene, *ASXL1*-mutated patients showed a shorter TTRT compared to those with wild-type *ASXL1* ($p<0.0001$) .

Moreover, in multivariate analysis *ASXL1* mutations maintained an independent association with shorter TTRT (HR 6.80, 95% CI 1.54-30.14, p=0.01) when adjusted for *TP53* disruption and *NOTCH1* mutations (Figure 6).



C)

Variable	N	Hazard ratio	p
NOTCH1 Mut	302	1.96 (0.56, 6.91)	0.29
TP53 Mut/Del	302	3.97 (1.27, 12.43)	0.02
ASXL1 Mut	302	6.80 (1.54, 30.14)	0.01

Figure 6. Survival analysis with Kaplan-Meier curves and forest plot for the impact of CHIP and *ASXL1* status on the cumulative risk of Richter transformation (RT). A) The Kaplan-Meier curve comparing CHIP-positive and CHIP-negative patients. B) The Kaplan-Meier curve comparing patients with wild-type (WT) versus mutated (Mut) *ASXL1* ($p < 0.0001$). C) Multivariate analysis of *ASXL1* mutations in relation to the main negative prognostic factors. The forest plot presents hazard ratios and p-value for RT based on mutations in *NOTCH1*, *TP53*, and *ASXL1*.

5 Discussion

CLL is one of the most prevalent B-cell malignancies, marked by significant clinical and biological heterogeneity (Meijers *et al*., 2020). Conversely, CHIP refers to the expansion of mutated hematopoietic cells in otherwise healthy individuals without a history of hematological malignancy (Gondek, 2021). CHIP is a well-established precursor for myeloid neoplasms, but its role in lymphoid neoplasms, particularly CLL, is less understood (Steensma, 2018). To deepen our understanding of the clinical and prognostic implications of CHIP in CLL, further research is required. This study aims to investigate the prevalence of CHIP in CLL patients and the relationship between CHIP and RT.

In this study, we investigated the prevalence and clinical implications of CHIP in a cohort of 367 newly diagnosed CLL patients. Our findings reveal several important insights into the role of CHIP mutations, particularly focusing on their association with RT and clinical outcomes.

The observed prevalence of CHIP in our CLL cohort was notable, with 45.5% of patients harboring at least one CHIP mutation. This finding aligns closely with previous studies suggesting a high prevalence of CHIP in hematologic malignancies, including CLL (Steensma *et al*., 2015b). *DNMT3A*, *TET2 and ASXL1* were the most frequently mutated genes, consistent with their known roles in hematopoiesis and leukemogenesis.

Contrary to our initial hypothesis, the presence of any CHIP mutation did not predispose CLL patients to an increased risk of RT. This finding suggests that while CHIP mutations are prevalent in CLL, they may not directly contribute to the transformation process to aggressive RT. This is consistent with recent literature indicating that the impact of CHIP on cancer progression can vary depending on the specific genetic context and the type of malignancy involved (Reed *et al*., 2023).

Interestingly, our study identified *ASXL1* mutations as an independent predictor of a shorter time to RT (TTRT) in CLL patients. Patients with *ASXL1* mutations had a significantly higher risk of progressing to Richter syndrome compared to those without these mutations. This finding underscores the specific role of *ASXL1* alterations in CLL pathogenesis, highlighting their potential utility as a prognostic biomarker for identifying patients at higher risk of aggressive disease progression.

The clinical implications of our findings are twofold. Firstly, while CHIP mutations are common in CLL (Von Beck *et al*., 2023), they do not appear to directly predispose patients to RT. This challenges the notion that CHIP universally accelerates CLL progression to more aggressive forms of the disease. Secondly, *ASXL1* mutations emerge as a critical genetic marker associated with adverse outcomes in CLL, particularly concerning the risk of RT. Clinically, monitoring *ASXL1* status could aid in risk stratification and treatment decision-making, potentially prompting closer surveillance or more aggressive therapeutic interventions for patients with *ASXL1* mutations.

However, this study is among the first to analyze CHIP in CLL, providing new insights into its implications in lymphoid diseases. Using samples from real-life patients, thereby offering a realistic clinical perspective that is more representative of actual patient populations compared to the idealized cohorts typically enrolled in clinical trials. Moreover, the application of NGS technology, noted for its high sensitivity and rapid execution, enhances the accuracy of the genetic analysis. NGS is poised to become the clinical standard for DNA sequencing soon, further underscoring the relevance of this study.

Several limitations should be acknowledged in interpreting our results. The study's retrospective nature and single-institutional cohort may limit the generalizability of findings to broader CLL populations. Additionally, the relatively small sample size of patients experiencing RT restricted our ability to comprehensively analyze the full spectrum of genetic and clinical factors influencing this transformation process.

Future studies incorporating larger, multicenter cohorts and longitudinal follow-up are warranted to validate our findings and elucidate additional genetic and environmental factors influencing RT development.

6 Conclusion

In conclusion, our study provides novel insights into the prevalence and clinical implications of CHIP mutations in CLL. While CHIP itself does not predispose CLL patients to RT, *ASXL1* mutations independently predict a shorter time to transformation, highlighting their potential as a prognostic marker in CLL management. These findings underscore the complex interplay between genetic mutations, disease progression, and clinical outcomes in CLL, warranting further investigation into personalized treatment strategies based on mutational profiling.

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