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Department of Health Sciences

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Evolution of clonal hematopoiesis of indeterminate potential gene mutations after therapy in chronic lymphocytic leukemia patients

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Index

Contents

Summary

Chronic lymphocytic leukemia (CLL) is one of the most common B-cell malignancies, characterized by significant 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. Historically, chemoimmunotherapy (CIT) was the primary treatment for CLL, but its use has significantly declined with the advent of pathway inhibitors like venetoclax, a BCL2 inhibitor. Clonal hematopoiesis of indeterminate potential (CHIP) is an age-related phenomenon defined by the presence of specific, cancer-associated somatic mutations in hematopoietic cells in the absence of a hematological malignancy. Importantly, CHIP has been the focus of several studies because of its link to a higher risk of myeloid malignancies. However, less is known about the role of CHIP in lymphoid neoplasms, particularly in CLL. Further research is needed to investigate the correlation between CHIP and CLL to enhance our understanding of this phenomenon and its clinical and prognostic implications. This thesis explores the genetic landscape and clonal evolution of CHIP in newly diagnosed CLL patients, focusing on the impact of different treatment regimens. In our study, we performed a mutational analysis of 367 CLL patients, through next generation sequencing (NGS) approach, using a panel of 28 genes. Our findings reveal a CHIP prevalence in 45.5% of the cohort. Notably, mutations in *DNMT3A*, *TET2,* and *ASXL1* being the most common, consistent with observations from previous studies. Our longitudinal analysis showed that CIT often led to an increase in CHIP mutations and variant allele frequency (VAF), suggesting that CIT may exacerbate CHIP. In contrast, venetoclax-based therapy exhibited heterogeneous effects, with some patients developed new CHIP mutations while others cleared their pre-existing mutations, indicating varied impacts of venetoclax on clonal expansion. Monitoring CHIP mutations could become a crucial aspect of long-term patient care. This study advocates for routine CHIP screening and further research into the mechanisms of clonal expansion post-therapy, aiming to improve personalized treatment strategies and patient outcomes. In conclusion, our research highlights the intricate relationship between CHIP and CLL, underscoring the importance of comprehensive genomic monitoring, and understanding how different therapies impact clonal dynamics is essential for advancing CLL treatment and enhancing patient care. This thesis contributes to the broader understanding of CLL pathogenesis and the potential for targeted interventions to mitigate therapy-related complications.

1. Introduction

1.1. Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is one of the most frequent B-cell malignancies and the most common type of leukemia in Western countries, with an incidence of approximately 5.1/100000 new cases per year. At diagnosis, the median age of CLL patients is 70 years old, and only 9.1% of them are younger than 45 years old. The disease affects men more often than women (1.7:1), and this gender difference appears to exist across all ethnic groups (Yao *et al*., 2022).

CLL is defined as a lymphoproliferative disorder, characterized by the clonal expansion of CD5+ mature B cells in the peripheral blood (PB), bone marrow (BM) and lymphoid tissues (lymph nodes and spleen) (Hallek *et al*., 2018a). According to the World Health Organization (WHO) CLL is invariably a disease of neoplastic B cells, is only distinguished from small lymphocytic lymphoma (SLL) by its leukemic presentation (Hallek *et al*., 2018).

Transcriptome analyses of CLL and the primary normal B cell subsets from human blood and spleen showed that CLL cells co-express the B-cell surface antigens CD19 and CD20 together with CD5, CD23, CD43 and CD200 (Seifert *et al*., 2012).

Although the precise cell of origin of CLL is still under investigation, 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 of immunoglobulin heavy chain variable region (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 somatic hypermutation (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) (Figure 1).

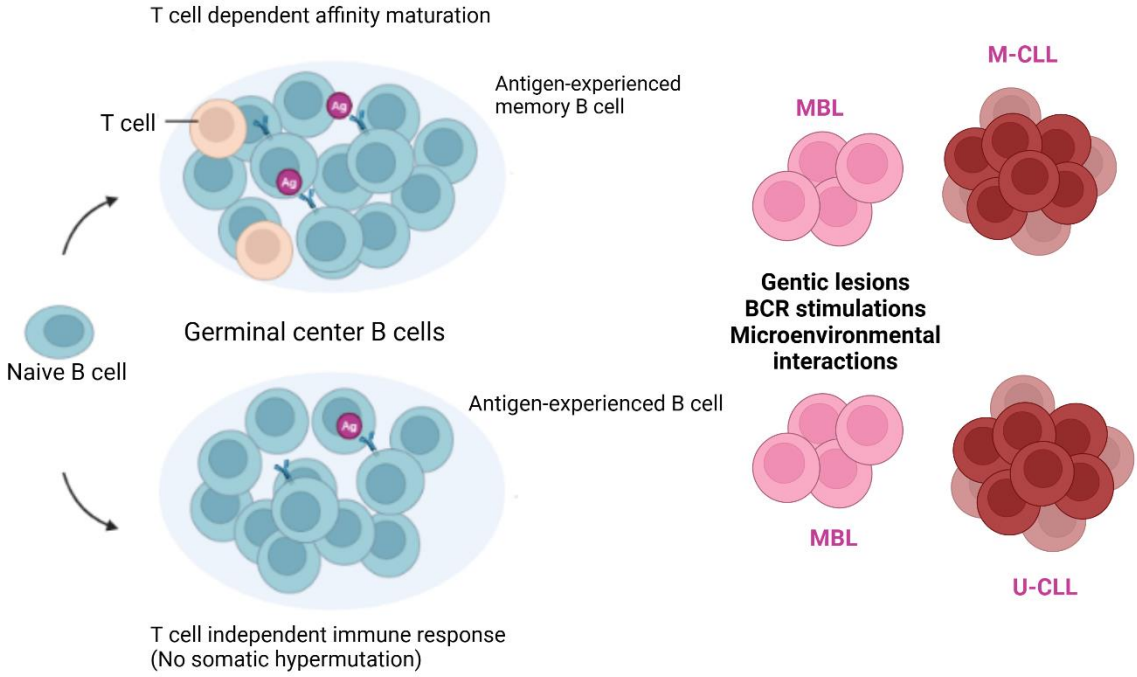


Figure 1. CLL subtypes may originate from two distinct cell types

CLL cells possessing unmutated IGHV are believed to originate from B cells that have not experienced somatic hypermutation in the IGV(D)J region, typically occurring in the germinal center (Stevenson *et al*., 2011). Conversely, CLL cells with mutated *IGV* seem to arise from post-germinal center B cells (Fais *et al*., 1998). Additionally, a small percentage of cases (6% to 10%) have undergone isotype switch recombination (Fais *et al*., 1998).

1.2 Molecular Pathogenesis of CLL

CLL is characterized by a marked degree of heterogeneity both at the clinical and at the biological level (Gaidano & Rossi, 2017). The genetic landscape of CLL is significantly varied, including severe chromosomal anomalies such as del13q14, trisomy 12, del17p13, and del11q23, as well as mutations in numerous cancer-related genes (Fabbri & Dalla-Favera, 2016; Julio Delgado *et al*., 2020a; Putowski & Giannopoulos, 2021). The genes most commonly affected by molecular changes in CLL cluster into particular

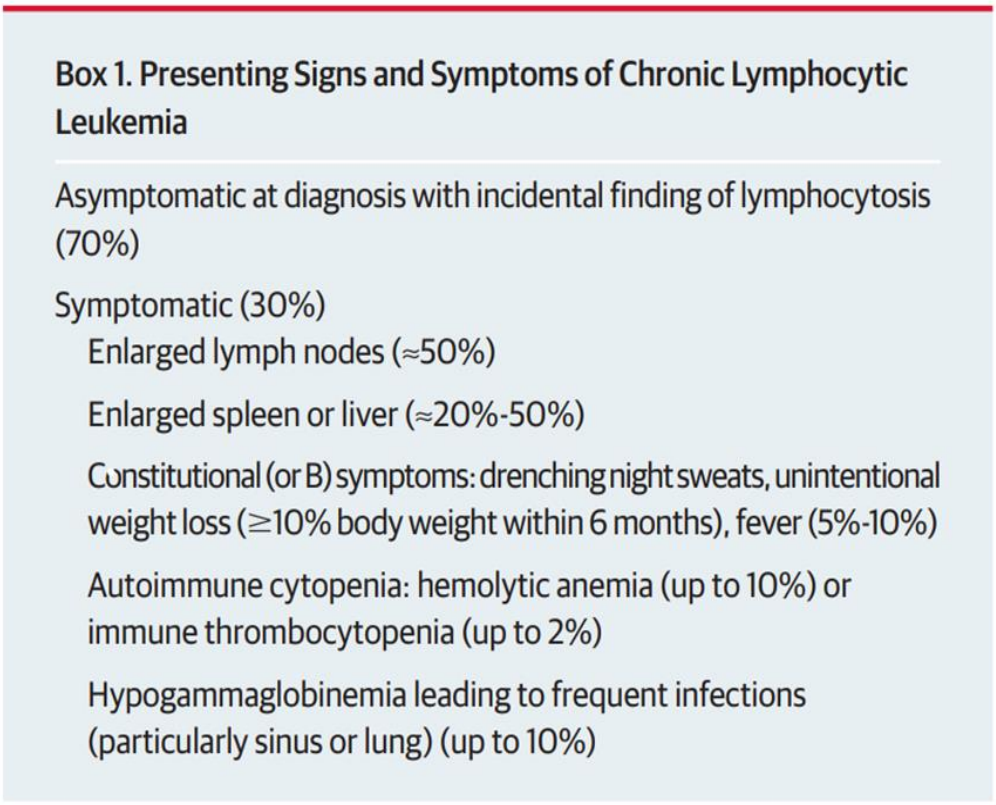
biological pathways , including NOTCH1 signaling (*NOTCH1* and *FBXW7*), DNA damage response (*ATM*, *TP53*, *POT1*), apoptosis (*miR15/16* and *BCL2*), BCR and toll-like receptor (TLR) signaling (*EGR2*, *BCOR*, *MYD88*, *TLR2*, *IKZF3*), NF-κB signaling (*BIRC3*, *NFKBIE, TRAF2, TRAF3*), and RNA splicing and metabolism (*SF3B1, U1, XPO1, DDX3X, RPS15*) (Fabbri & Dalla-Favera, 2016; Julio Delgado *et al*., 2020a; Putowski & Giannopoulos, 2021).

1.3 CLL Diagnosis

The clinical diagnosis of CLL requires the presence of ≥5x103/µl of monoclonal B lymphocytes in the peripheral blood (Fazi *et al*., 2011; Hallek *et al*., 2018b; Parikh *et al*., 2021). Patients with a lymphocyte count less than 5x103/µl may be diagnosed with monoclonal B-cell lymphocytosis (MBL), a condition where there is no evidence of enlarged lymph nodes. Albeit they do not meet the diagnostic criteria for CLL (Shadman, 2023) the rate of transformation to CLL in MBL patients is 1% to 2% annually *(*Fazi *et al.,* 2011*;* Hallek *et al*., 2018b; Parikh *et al*., 2021).

Approximately70% of individuals with CLL are diagnosed without symptoms at the time of diagnosis due to an inexplicable lymphocytosis that was accidentally found (Abrisqueta *et al*., 2009) (Box 1). Of the patients who exhibit symptoms, approximately half have lymphadenopathy symptoms, around 20% to 50% have hepatosplenomegaly symptoms, and approximately 5% to 10% have unintended weight loss of 10% or more within a 6-month period, fever, intense sweats at night, or extreme fatigue (B symptoms) (Nabhan & Rosen, 2014; Rai *et al*., 1975).

Additionally, patients may present with cytopenia due to immune-mediated complications such as immune thrombocytopenia (<2%) or autoimmune hemolytic anemia (<10%), or as a result of CLL cells involving the bone marrow (Nabhan & Rosen, 2014; Rai *et al*., 1975). To diagnose CLL, a peripheral blood flow cytometry test is required and is often suffice (Hallek *et al*., 2018b). Additional imaging and a bone marrow sample are not required in asymptomatic patients, and further evaluations may be postponed until therapy is administered (Hallek *et al*., 2018b; Wierda *et al*., 2022a).



Box 1. Presenting signs and symptoms in CLL

1.4 Clinical staging system

Two clinical staging systems applied to CLL patients to define disease burden and treatment indication: Rai and Binet staging systems (Binet *et al*., 1981; Rai *et al*., 1975). Both approaches define three major prognostic groupings with distinct clinical outcomes (Hallek & Al-Sawaf, 2021). These two staging techniques are straight forward and inexpensive, relying on a physical examination and conventional laboratory tests (Hallek & Al-Sawaf, 2021).

The Rai staging system classifies low-risk disease in patients exhibiting lymphocytosis, characterized by the presence of leukemia cells in the blood and/or bone marrow, with lymphoid cells exceeding 30% (former Rai stage 0). Patients presenting with lymphocytosis, enlargement of nodes at any location, and splenomegaly or hepatomegaly (regardless of whether the lymph nodes are palpable) are classified as having intermediate-risk disease (formerly considered Rai stage I or stage II). High-risk diseases encompass conditions such as disease-related anemia, characterized by a hemoglobin (Hb) level below 11 g/dL (previously

known as stage III), or thrombocytopenia, defined by a platelet count lower than 100 x 10^9/L (formerly stage IV) (Rai *et al*., 1975) (Table 1).

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, and the presence of anemia or thrombocytopenia. The Binet staging system classifies stage A by a hemoglobin level of ≥10 g/dL and platelet count of ≥100 x 10^9/L, with no more than two areas of lymphoid tissue involved.; Stage B is characterized by hemoglobin levels of ≥10 g/dL, platelet counts of ≥100 x 10^9/L, and organomegaly that exceeds the criteria for stage A, which includes enlargement in three or more areas of nodal or organ involvement. Stage C is characterized 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) (Table 2).

Table 1. Clinical Staging Systems in Chronic Lymphocytic Leukemia

Rai staging			Binet staging	
Stage	Risk category	Findings	Stage	Findings
0	Low	Lymphocytosis[a]	A	No cytopenia and ≤2 lymphoid area involvement
1	Intermediate	Lymphadenopathy[b]	B	No cytopenia and >3 lymphoid area involvement
2	Intermediate	Hepatosplenomegaly[b]	C	Presence of anemia or thrombocytopenia
3	High	Anemia[c]		
4	High	Thrombocytopenia[d]		

[a] Lymphocyte count greater than 5×10^9/L.
[b] On physical examination.
[c] Hemoglobin level less than 11 g/dL.
[d] Platelet count less than 100 000/µL

Table 1. Clinical Staging Systems in CLL

1.5 Prognostic markers in CLL

Molecular prognostic markers provide useful information about the biology and the natural history of CLL in the absence and/or independent of the treatment received (Lee & Wang, 2020). They include:

TP53 disruption

TP53 aberrations (including del(17p) and *TP53* mutations) are currently the most significant prognostic factor in CLL (Gonzalez *et al*., 2011; Malcikova *et al*., 2018;

Stilgenbauer *et al*., 2014a). *TP53* aberrations can be found in 10% of CLL patients on frontline therapy, 30% at relapse, and 50% at refractoriness (Gonzalez *et al*., 2011; Malcikova *et al*., 2018; Stilgenbauer *et al*., 2014a).

TP53 encodes a key regulator of the DNA damage response (Landau *et al*., 2013; Malcikova *et al*., 2015; Rossi *et al*., 2014). Chemotherapy is rarely effective in eliminating *TP53* mutant or deleted CLL cells due to their poor ability to undergo apoptotic induction (Landau *et al*., 2013; Malcikova *et a*l., 2015; Rossi *et al*., 2014). Furthermore, CLL cells with *TP53* abnormalities usually exhibit expansion upon recurrence after treatment (Landau *et al*., 2013; Malcikova *et al*., 2015; Rossi *et al*., 2014). CLL with del(17p) and/or *TP53* mutations, alone or in conjunction with other unfavorable prognostic markers, is considered very-high risk (Dreger *et al*., 2013). Prior to the advent of new medications, allogeneic stem cell transplantation was the only method for treating very-high-risk CLL (Dreger *et al*., 2013). B-cell receptor (BCR) pathway drugs appear to overcome *TP53*-induced treatment resistance and improve the subgroup's poor prognosis (Burger, 2020; Byrd *et al*., 2021; Farooqui *et al*., 2015).

IGHV mutational status

IGHV status has been shown to be an independent prognostic factor in different prognostic models for overall survival (Eichhorst & Hallek, 2016). The unmutated-IGHV status is present in about half of CLL cases (<2% deviation from the germline sequence) (Bosch & Dalla-Favera, 2019; Crombie & Davids, 2017; Julio Delgado et al., 2020b). These patients usually experience more unfavorable clinical outcomes and a shorter time between disease diagnosis and development. (Bosch & Dalla-Favera, 2019; Crombie & Davids, 2017; Julio Delgado *et al*., 2020b).

On the other hand, the clinical course of CLL patients with a mutated IGHV status - (≥2% deviation) is more slowly progressing (Bosch & Dalla-Favera, 2019; Crombie & Davids, 2017; Julio Delgado *et al*., 2020b). Atypical humoral and cellular immunological reactions to infections or vaccinations are observed in patients with CLL (Shadman *et al*., 2023).

***NOTCH1* mutations**

The *NOTCH1* gene coding for transmembrane proteins is mutated in 4% to 11% of CLL patients and is frequently associated with trisomy 12 (Rossi *et al*., 2012; Stilgenbauer *et al*.,

2014b; L. Wang *et al*., 2011). Multivariate analysis has revealed that NOTCH1 is an independent prognostic factor of poor prognosis—at least in the era of chemoimmunotherapy (CIT) (Cortese *et al*., 2014). Patients carrying *NOTCH1* mutation appear to have a higher risk of Richter transformation (Stilgenbauer *et al*., 2014b).

Comprehensive prognostic scores have been developed to distil prognostic information into a few clinically relevant, essential 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 Leukemia (CLL-IPI)', 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 with varying five-year survival rates (see Table 2). 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).

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) (Table 2).

Table 2. Prognostic Scoring Systems in Chronic Lymphocytic Leukemia

Variable	Points	Risk group (total points)	5-y cumulative risk of treatment start, %	
International Prognostic Score for Asymptomatic Early-stage Disease (IPS-E)				
Unmutated *IGHV*	1	Low (0)	8.4	
ALC ≥15 × 10^9/L	1	Intermediate (1)	28.4	
Palpable lymphadenopathy	1	High (2-3)	61.2	
CLL International Prognostic Index (CLL-IPI)			5-y overall survival, %	10-y overall survival, %
Del(17p12) or *TP53* variant	4	Low (0-1)	93	79
β_2 microglobulin >3.5 mg/L	2	Intermediate (2-3)	79	39
Unaltered *IGVH*	2	High (4-6)	63	22
Rai stage 1-4	1	Very high (7-10)	23	4
Age >65 y	1			

Abbreviations: ALC, absolute lymphocyte count; CLL, chronic lymphocytic leukemia; *IGHV*, immunoglobulin heavy chain gene.

Table 2. Prognostic Scoring systems in CLL

1.6 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 (Gaidano & Rossi, 2017).

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 (Herling *et al*., 2020; Langerbeins *et al*., 2022). Treating individuals with asymptomatic CLL does not appear to increase survival (Herling *et al*., 2020; Langerbeins *et al*., 2022). 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; Langerbeins *et al.*, 2022). 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*., 2018b; Wierda *et al*., 2022b).

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 secondary myeloid malignancy (Kutsch *et al*., 2020). The use of traditional CIT has significant decreased since the introduction of pathway inhibitors (Shadman, 2023). The ongoing advancements in comprehending the pathogenesis of CLL have broadened treatment options beyond conventional CIT (Shadman, 2023). This includes the emergence of innovative small molecule inhibitors that target key signaling pathways critical to the survival and proliferation of CLL cells (Shadman, 2023).

BCR Inhibitors

Since the BCR signaling pathway contributes to the pathophysiology of CLL, 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) (Byrd *et al*., 2014).

BTK inhibitors, are small molecules that occupy of the ATP binding domain inhibits the subsequent phosphorylation of BTK and blocks the downstream signaling pathways, which regulate cell survival and proliferation (Zain & Vihinen, 2021). They are administered as monotherapy indefinitely, as continuous inhibition of the target enzyme is essential for their antiproliferative effect (Barr *et al*., 2022a). 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*., 2022b). Conversely BTK is reversibly inactivated by the noncovalent inhibitor pirtobrutinib (E. Wang *et al*., 2022). 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 (Bonfiglio *et al*., 2023; Woyach *et al*., 2017).

As the first class covalent BTK inhibitor discovered, ibrutinib is linked to a 60% PFS at 7 years when used as first-line therapy, and a 40% PFS at 5 years when used in patients who experienced a relapse (Barr et al., 2022c; Mato et al., 2018; Munir et al., 2019) . However, side effects, including rash (16.7%), atrial fibrillation (25%), and arthralgia (42%), are frequent and

may require discontinuing the treatment (Barr *et al*., 2022c; Mato *et al*., 2018; Munir *et al*., 2019).

Acalabrutinib and zanubrutinib are examples of second-generation BTK inhibitors that specifically target the BTK enzyme (Brown *et al*., 2023; Byrd *et al*., 2021). Clinical trials directly comparing these drugs to ibrutinib have observed lower incidences of grade 3 or higher adverse events with acalabrutinib (68.8% vs. 74.9%) and zanubrutinib (67.3% vs. 70.4%) than with ibrutinib (Brown *et al*., 2023; Byrd *et al*., 2021).

In patients with indolent B-cell lymphomas, PI3K inhibitors effectively suppress the δ isoform of PI3K (Shouse *et al*., 2022). For the treatment of CLL, idelalisib (an inhibitor of the δ isoform) and duvelisib (an inhibitor of the δ and γ isoforms) are approved (Shouse *et al*., 2022). Higher prevalence of all infections and a number of immune-mediated side effects are linked to PI3K inhibitors (Sharman *et al*., 2019). Every month, patients taking idelalisib should be evaluated for elevated liver enzyme levels (39%), diarrhea (29%), pneumonitis (cough, dyspnea, and hypoxemia) (5.5%) and colitis (4.5%) (Sharman *et al*., 2019).

BCL2 Inhibitors

The apoptotic mitochondrial pathway is inhibited by BCL2 proteins (Chong & Davids, 2020). The pathophysiology of CLL heavily relies on the overexpression of BCL2 family proteins (Chong & Davids, 2020). For about 70% of CLL patients, limited duration treatment with venetoclax, the only clinically available BCL2 inhibitor, results in complete responses without measurable disease in the blood or marrow; this effect lasts longer than six months for more than 60% of patients even after the drug is stopped (Shadman, 2023).

The extracellular region of the CD20 antigen is bound by monoclonal anti-CD20 antibodies, which can cause cell death directly, through complement-dependent cell toxicity, or through antibody-dependent cell toxicity (Maloney, 2012). These antibodies include chimeric (rituximab) and humanized (obinutuzumab and ofatumumab) (Maloney, 2012). Novel agents, including venetoclax, are coupled with monoclonal antibodies (Maloney, 2012).

1.7 Clonal Hematopoiesis of Indeterminate Potential (CHIP)

Recent genome-wide studies have revealed that aging or chronic inflammation can cause clonal expansion of cells in normal tissues Clonal Hematopoiesis (CH) is the type of

clonal proliferation that has been studied the most among the different forms of clonal expansion (Genovese *et al*., 2014a; Jaiswal *et al*., 2014a; Xie *et al*., 2014).

The term "CH of indeterminate potential (CHIP)" is defined by to the presence of at least one somatic mutation in hematopoietic stem cells of peripheral blood in the healthy individuals with no history of hematological malignancy individuals, with a variant allele frequency (VAF) of at least 2% (Steensma *et al*., 2015). It is considered an aging phenomenon because the prevalence increases with age reaching 10–30% in individuals over 70. 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 mutation found in CHIP is a C to T single nucleotide substitution in the coding region, which results from an age-dependent increase in the rate of spontaneous deamination of 5-methyl-cytosines at CpG loci (Australian Pancreatic Cancer Genome Initiative et al., 2013). The most frequently mutated genes in CHIP are those coding for the epigenetic regulators DNMT3A, TET2, and ASXL1, and these mutations provide a fitness advantage to HSCs, potentially leading to their expansion *(*Asada *et al., 2018, 2019;* Izzo *et al*., 2020). Mutations in *DNMT3A* and *TET2* influence DNA methylation whereas those in ASXL1 alter histone modifications, and thereby influence hematopoiesis (Asada *et al*., 2018, 2019; Izzo *et al*., 2020).

CHIP is a significant risk factor for myeloid malignancies including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasms (MPN) suggesting that CHIP is a precursor state for hematological malignancy that occurs less frequently (Jaiswal *et al*., 2014a, 2017a; Steensma *et al*., 2015; Welch *et al*., 2012; Xie *et al*., 2014). Furthermore, it appears that certain lymphoid cancers emerge from CHIP (Asada & Kitamura, 2021). CHIP is also common in patients with chemotherapy-treated solid tumors and has also been linked to poor prognosis (Coombs *et al*., 2017).

Significantly, CHIP is associated with increased risk of cardiovascular disease and all-cause mortality (Jaiswal *et al*., 2014a, 2017a; Steensma *et al*., 2015; Welch *et al*., 2012; Xie *et al*., 2014). Moreover, CHIP has been found in non-malignant diseases such as acquired aplastic anemia (AA) which is an autoimmune disease (Coombs *et al*., 2017), Erdheim-Chester disease, and hereditary bone marrow failure syndromes (Cohen Aubart *et al*., 2021; Tsai & Lindsley,

2020; Yoshizato *et al*., 2015). These findings indicate that there is a need for future studies to focus on the implications of CHIP (Figure 2).

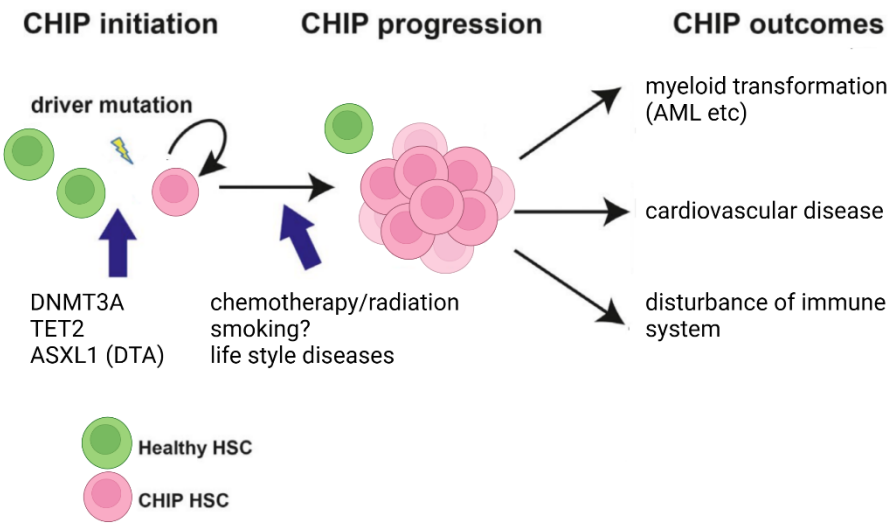


Figure 2. Overview of CHIP and its implication

1.8 CHIP and CLL

Numerous hematologic malignancies, such as CLL, multiple myeloma (MM), and AML, are known to have well-established precursor conditions that come prior to the manifestation of overt cancer (Condoluci & Rossi, 2018). CLL is invariably preceded by a high MBL count (Landgren, Albitar, *et al*., 2009). MM is almost always preceded by Monoclonal Gammopathy of Undetermined Significance (MGUS) (Landgren, Kyle, *et al*., 2009), and at least one-fourth of patients with MDS progress to AML (Pfeilstöcker *et al*., 2016). Deep genomic sequencing in healthy individuals has shown that as humans age, the expansion of one or more hematopoietic stem and progenitor cells (HSPCs) leads to clones that disproportionately contribute to the production of mature blood cells (Condoluci & Rossi, 2018). As such, CHIP is characterized by the proliferation of HSPC clones that carry specific,

disruptive, and recurrent genetic variants in individuals who do not have a definitive diagnosis of hematologic malignancies (Jaiswal *et al*., 2014b). Numerous studies have shown that MDS are often preceded by CHIP (Malcovati *et al*., 2017). Furthermore, certain mutations associated with CHIP may elevate the risk of leukemia, while others may heighten the risk for heart disease and diabetes. Several studies demonstrated that, MDS are frequently preceded by CHIP, moreover, some CHIP related mutations can increase the risk for leukemia (Genovese *et al*., 2014b), while other mutations possibly increase the risk for heart disease and diabetes (Jaiswal *et al*., 2017b). Prior research has established a proof of principle that CHIP may be associated with the expansion of B-cell clones exhibiting a CLL phenotype, thereby linking CHIP with MBL and CLL in a continuum of evolution from HSCP clones to mature B-cell clones (Condoluci & Rossi, 2018).

However, less is known on the role of CHIP in lymphoid neoplasms, particularly in CLL. Further research on the correlation between CHIP and CLL is necessary to better understand this phenomenon and its clinical and prognostic implications.

2. Aim of the study

The aims of the present study are:

i) Investigate the prevalence and incidence of CHIP in a prospective cohort of 367 newly diagnosed CLL patients referred to our institution.

ii) Evaluate the changes in CHIP status at baseline and following treatment.

3.Materials and methods

3.1 Patients

The study was conducted on a real-life cohort composed of 367 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 Diagnostics™ 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). 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 because 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-iT™ 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

The mutational analysis in 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 gDNA. Libraries were generated using the KAPA HyperPrep kit (Roche Diagnostics, Pleasanton, CA, USA) and enrichment of regions of interest was achieved using a KAPA Hyper Choice 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 flow cell, to which they are linked by special adapters. On the flow cell 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 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 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.

3.6 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 Richter syndrome measured from the date of diagnosis to the time of transformation to Richter syndrome. The analyses were carried out with SPSS 24 software and R-Studio 2021.09.1.

4.Results

4.1 Patients characteristics

In our study, 367 newly diagnosed CLL patients have been analyzed. 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 and 236 (65.6%) had mutated IGHV genes. Out of 367 patients, 36 (9.9%) had *TP53* disruptions. Concerning additional chromosomal anomalies, 61 individuals (16.7%) exhibited Trisomy 12, 20 individuals (5.5%) had a deletion in 11q, and 175 individuals (47.9%) showed a deletion in 13q. Out of the study group, 23 patients (6.3%) exhibited Richter transformation. 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.

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

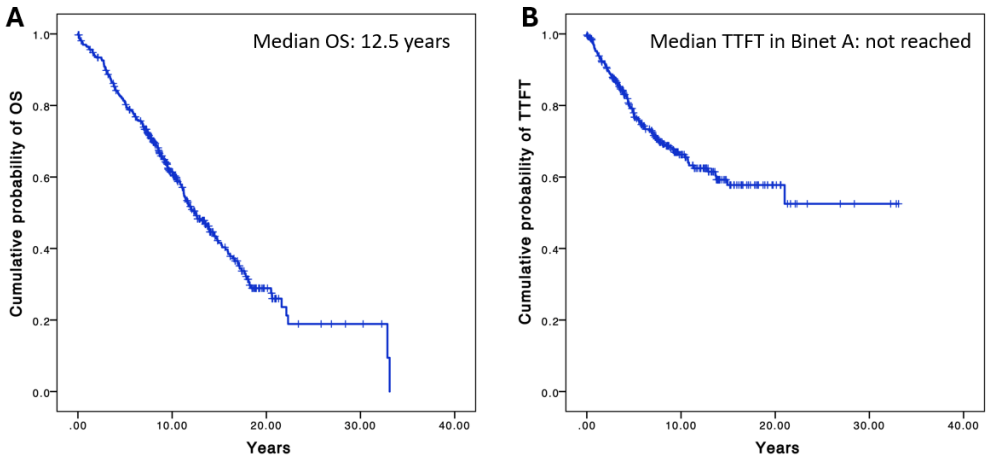


Figure 3. Survival analysis with Kaplan-Meier curves. A: Global PFS of the cohort. B: Global TTFT of the cohort.

4.2 Chip prevalence and the mutational analysis

Out of 367 patients analyzed for CHIP, 167 (45.5%) exhibited at least one CHIP mutation. As expected, the most frequently mutated gene was *DNMT3A in* 89 (24.3%) patients followed by *TET2* in 52 (14.2%), *ASXL1* in 10 (2.7%) and*TP53* in 9 (2.5%) (Figure 4).

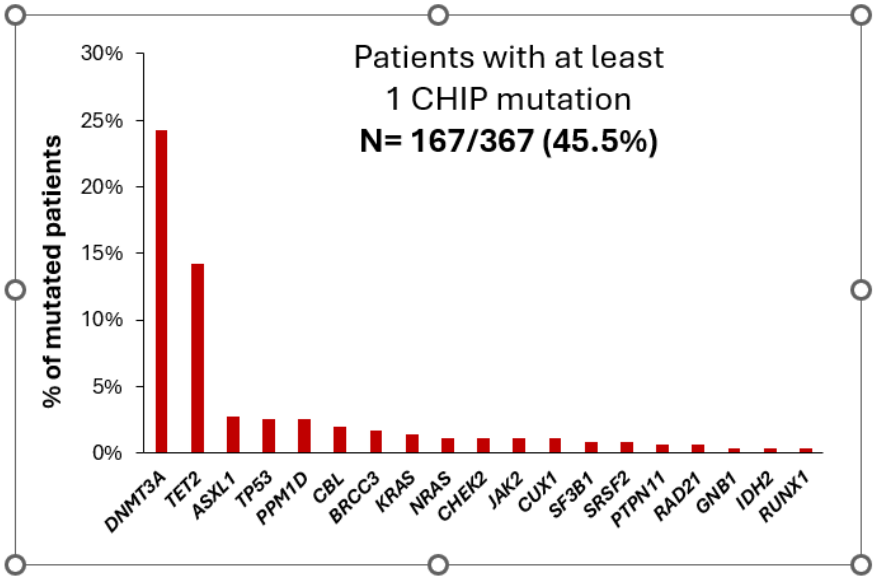


Figure 4. CHIP mutations. The histogram presents the 19 genes found mutated in the patients on the x-axis, and the absolute number of mutations on the y-axis.

Regarding *DNMT3A* mutations, the largest number of mutations were non-synonymous SNVs (N=63), followed by frameshift deletions (N=16), and stopgain mutations (N=11). The most frequent TET2 mutations were non-synonymous SNVs and stopgain mutations (N=20 for each. *ASXL1* was most frequently affected by frameshift deletions and frameshift insertions (N=4 for each) (Figure 5).

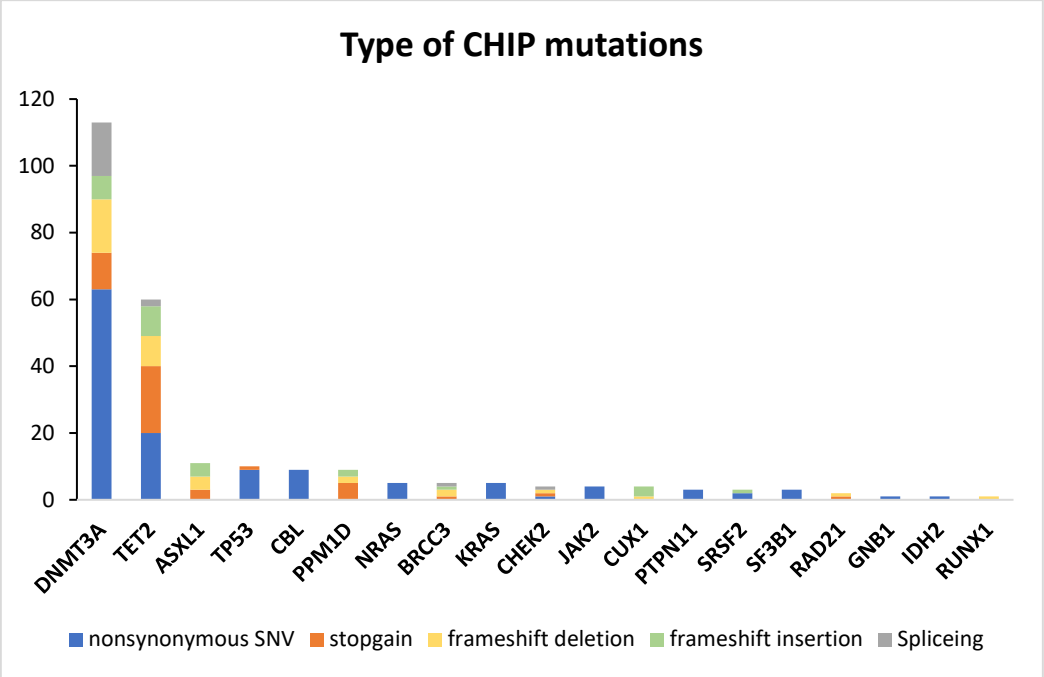


Figure 5. Type of CHIP mutations. The histogram presents the 19 genes found mutated in the patients on the x-axis, and the number of mutations on the y-axis. The legend allows you to distinguish the type of mutation for each gene, based on a color scale. SNV, single-nucleotide variant.

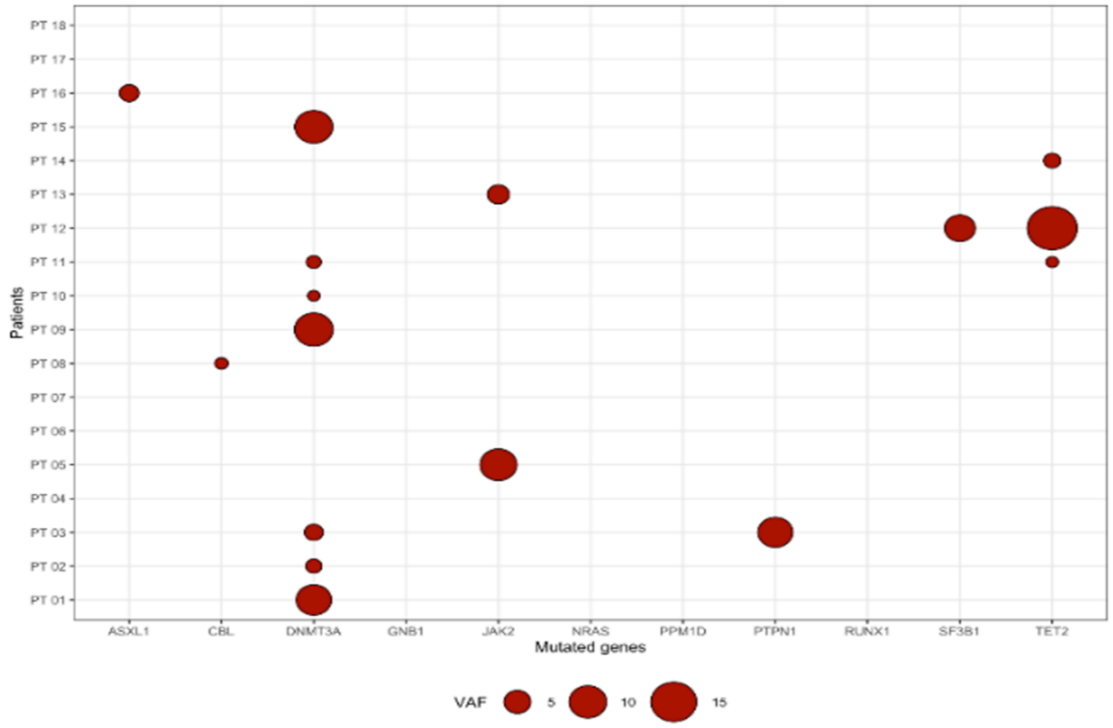
4.3 The clonal evolution of CHIP following the therapy

In this study, we collected and analyzed sequential patient samples at multiple critical stages specifically before CIT (pre-CIT) and after CIT therapy (post-CIT), as well as before and after venetoclax based therapy. This longitudinal approach aims to provide a comprehensive understanding of how therapy influences CHIP over time.

A) Chemoimmunotherapy (CIT)

In a cohort of 25 patients selected to evaluate the CIT impact on CHIP, 13 displayed at least one detectable mutation associated with CHIP in their pre-CIT samples. On the other hand, post-treatment analyses showed that 18 of the 25 patients had at least one detectable CHIP mutation in their samples. This represents an increase in the number of patients with CHIP following CIT (Figure 6).

A) Pre-CIT



B) Post-CIT

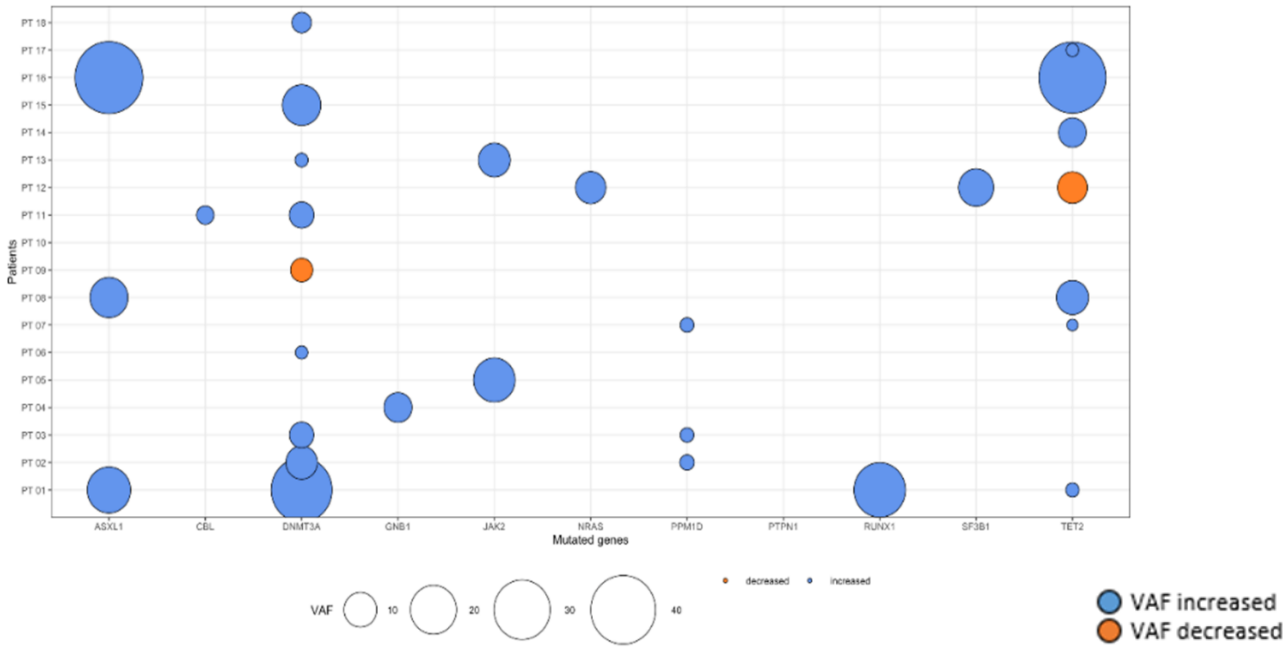


Figure 6. Comparative Analysis of Gene Mutation Frequencies Pre and Post-CIT. The x-axis represents mutated genes. The y-axis represents individual patients. The size of the plot represents the variant allele frequency (VAF), with larger blots indicating a higher VAF. A) Pre–CIT, the blots are red signifying the mutations present before CIT. B) Post-CIT, represents the mutations present after CIT, in blue with VAF increased and in orange with VAF decreases.

The mean variant allele frequency (mVAF) before CIT was 1.13%, which increased to 2.85% after CIT. A p-value of 0.0042 indicates that the increase in mutation frequency following CIT is statistically significant (Figure 7).

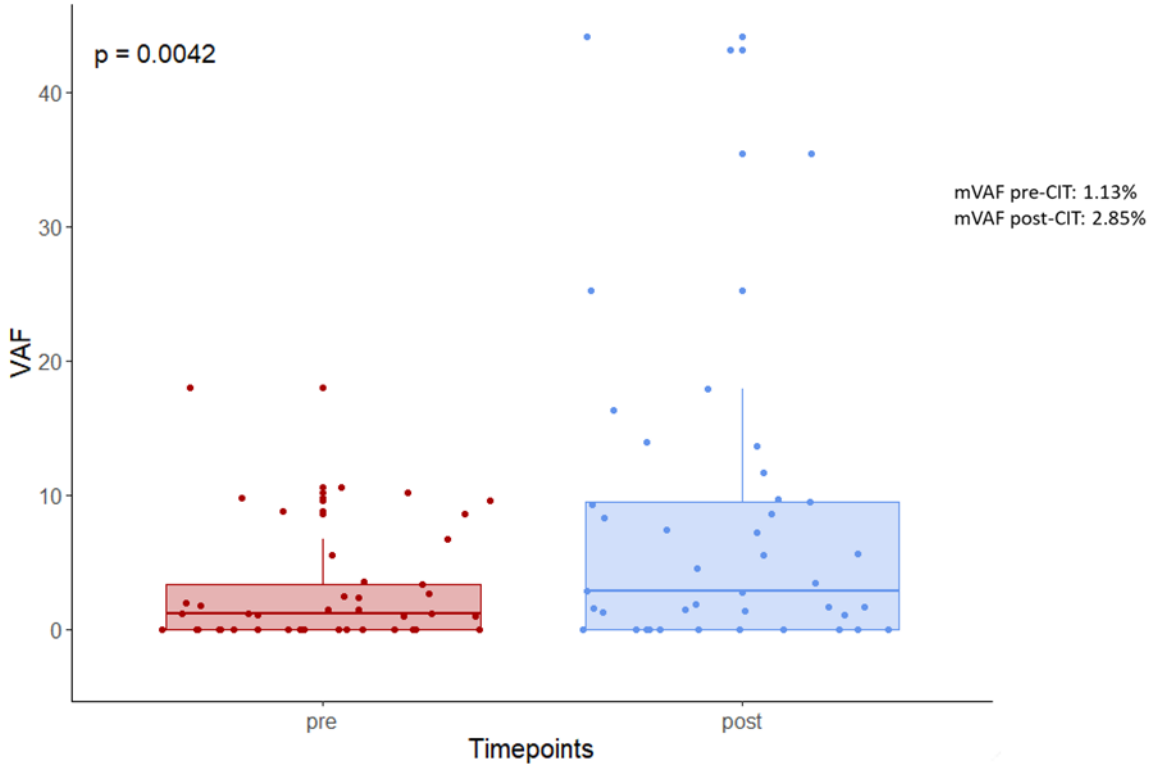


Figure 7. mVAF in CHIP Mutations Pre- and Post-CIT. X-Axis has two timepoints are shown: "pre" (pre-CIT) and "post" (post-CIT). Y-Axis indicates the mean VAF.

B) Venetoclax

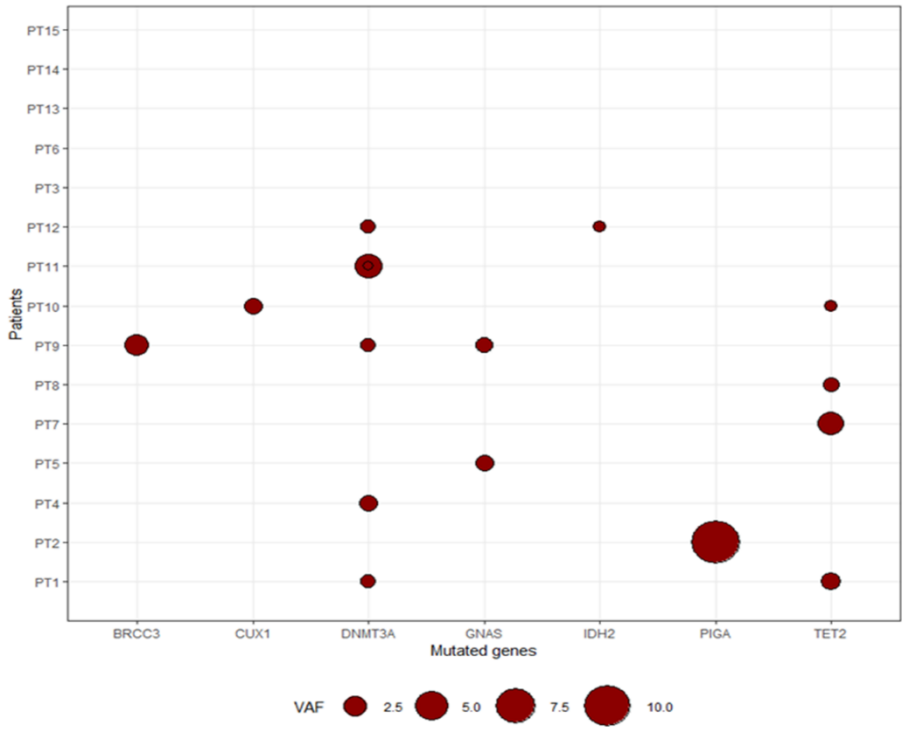
We selected 15 CLL patients treated with Obinutuzumab-venetoclax or R-venetoclax to study the clonal evolution of CHIP after venetoclax based therapy.

The VAF of seven different mutated genes patients was compared before and after the treatment. The studied mutated genes were *BRCC3*, *CUX1*, *DNMT3A*, *GNAS*, *IDH2*, *PIGA*, and *TET2*. In this cohort of patients, before the venetoclax therapy ten patients were CHIP-positive and five were CHIP-negative.

In the group of five CHIP-negative patients at baseline, two developed CHIP mutations (*DNMT3A* and *TET2*) following venetoclax treatment. Among the ten CHIP-positive patients, four cleared their CHIP mutations after venetoclax therapy and six continued to exhibit CHIP mutations post-venetoclax.

No significant differences were observed in the VAF of the mutated genes between pre-therapy and post-therapy measurements. The mVAF was 1.6% before treatment and 2.3% following therapy (p-value = 0.704). (Figure 8).

A) Pre–venetoclax



B) Post-venetoclax

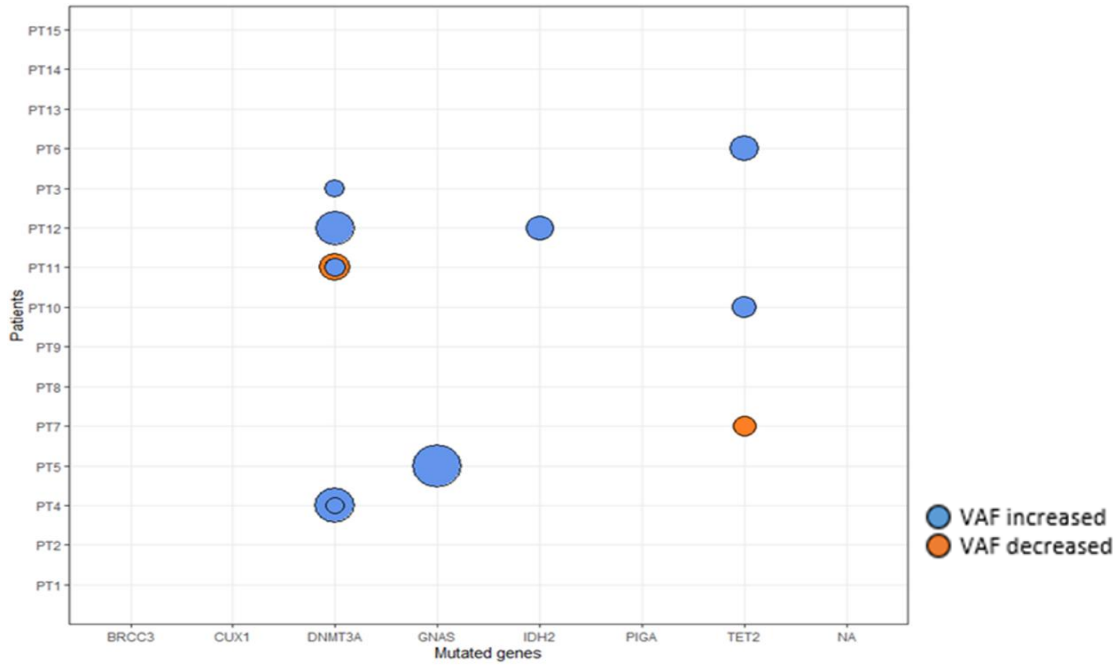


Figure 8. **Comparative Analysis of Gene Mutation Impact on Variant Allele Frequency in venetoclax based therapy.** The x-axis represents mutated genes. The y-axis represents individual patients. The size of the blots represents the variant allele frequency (VAF), with larger blots indicating a higher VAF. A) Pre–venetoclax, the blots are red signifying the mutations present before CIT. B) Post-venetoclax, represents the mutations present after venetoclax, in blue with VAF increased and in orange with VAF decreases.

5. Discussion

CLL is one of the most frequent B-cell malignancies. It is characterized by a high degree of heterogeneity both at the clinical and at the biological level (Scarfò & Ghia, 2019). On the other hand, the term CHIP is the clonal expansions of mutated hematopoietic cells in otherwise healthy individuals without history of hematological malignancy (Reed et al., 2023). CHIP is a well-characterized precursor lesion for the development of myeloid neoplasms (Arber et al., 2022). However, less is known on the role of CHIP in lymphoid neoplasms, particularly in CLL. Further research is needed to explore the correlation between CHIP and CLL to gain a deeper understanding of this phenomenon and its clinical and prognostic implications. Consequently, this study aims to investigate the prevalence of CHIP in CLL patients and its evolution following different treatment regimens.

In this study, CHIP was analyzed in a cohort of 367 CLL patients, with a median age of 70.3 years, predominantly consisted of males (56.1%). Baseline hematological parameters indicated a typical profile for CLL patients, with median lymphocyte, hemoglobin, and platelet counts aligning with expectations for this patient group. The IGHV mutation status, a well-established prognostic marker, revealed that a majority (65.6%) of the patients had mutated IGHV genes, which is generally associated with a more favorable prognosis. This finding is consistent with studies by Damle *et al* and Hamblin *et al*, who reported that unmutated IGHV genes are associated with more aggressive forms of CLL (Damle et al., 1999; Hamblin et al., 1999).

The chromosomal anomalies observed in our study, including trisomy 12, deletions in 11q and 13q, and *TP53* disruptions, are consistent with known genetic alterations in CLL. Notably, 9.9% of patients exhibited *TP53* disruptions, which are linked to poorer outcomes and resistance to conventional therapies, as supported by (Zenz *et al*., 2010).

The survival analysis showed a median overall survival (OS) of 12.5 years, with the time to first treatment (TTFT) not reached for patients in Binet stage A, suggesting a relatively indolent course for a significant subset of patients. This aligns with historical data, such as the findings of (Hallek *et al*., 2010), which suggest that many CLL patients, especially those with favorable prognostic markers, can have prolonged survival even without immediate treatment.

NGS analysis highlighted the presence of CHIP in 45.5% among our CLL cohort. This prevalence aligning closely with the prevalence observed in older age groups within the general population, where CHIP is studied in healthy individuals and observed in 12.5% of individuals regardless of age, and up to 50% in individuals over 85 years old (Zink et al., 2017).

DNMT3A, *TET2*, and *ASXL1* were the most frequently mutated genes, which mirrors findings in other hematologic malignancies and aging populations. Jaiswal and Ebert also reported a similar prevalence of these mutations in their study (Jaiswal & Ebert, 2019), indicating a commonality in CHIP across different conditions. The predominance of nonsynonymous SNVs in these genes further underscores the potential functional impact of these mutations on hematopoietic clones.

Our longitudinal analysis revealed distinct patterns of clonal evolution in response to CIT and venetoclax-based therapies. The increase in the number of patients with detectable CHIP mutations following CIT, along with the significant rise in mVAF, suggests that CIT may contribute to the expansion of pre-existing CHIP. This finding is significant as it implicates conventional therapy in the potential exacerbation of CHIP, which could have long-term implications for patient management. (Genovese et al., 2014b) also observed that certain therapies might influence the prevalence of clonal mutations, supporting our findings.

In contrast, venetoclax-based therapy demonstrated a more complex relationship with CHIP. While some patients developed new CHIP mutations post-therapy, others cleared their pre-existing mutations. The lack of a significant change in mVAF suggests that venetoclax, unlike CIT, does not universally drive clonal expansion. This differential impact on clonal dynamics may influence treatment decisions, especially in patients with existing CHIP mutations. Pollyea *et al* reported that venetoclax-based regimens could disrupt clonal dynamics differently, which aligns with our observations (Pollyea et al., 2018).

The presence and dynamics of CHIP mutations in CLL patients have important clinical implications. As CHIP is associated with increased risks of cardiovascular disease and secondary malignancies, monitoring CHIP in CLL patients could become a crucial aspect of long-term patient care. Fuster and Walsh in there study, highlighted the broader health implications of CHIP (Fuster & Walsh, 2018), underscoring the importance of our findings. Our study advocates for further research into the mechanisms driving clonal expansion post-therapy and the development of strategies to mitigate these effects.

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 in the near future, further underscoring the relevance of this study.

Despite this, our study also has a limitation, represented by the use of purified granulocyte DNA obtained through ultracentrifugation of blood samples, which therefore may also contain residues of lymphocyte DNA, potentially contaminating the samples. To definitively overcome this limitation, it would be appropriate to use a flow cytometric-based cell sorting technique, in order to separate the cell lines and sequence them individually.

Future studies should aim to elucidate the biological underpinnings of CHIP in CLL and explore the potential for targeted interventions. Additionally, integrating CHIP screening into routine clinical practice could help identify patients at higher risk for adverse outcomes, thereby informing personalized treatment approaches.

In conclusion, our study underscores the significance of CHIP in CLL and highlights the need for comprehensive genomic monitoring to optimize patient outcomes. The interplay between treatment regimens and clonal dynamics warrants further investigation to enhance our understanding of CLL pathogenesis and therapy-related complications.

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