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# **Association of single nucleotide polymorphisms with therapy-related toxicities in Chronic lymphocytic leukemia**

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## Abstract

Bruton's tyrosine kinase inhibitors (BTKi) have revolutionized the therapeutic paradigm of Chronic Lymphocytic Leukemia (CLL); however, their use is associated with a marked interindividual heterogeneity in terms of tolerability and efficacy. This study investigates the hypothesis that germline genetic variability in the host, through single nucleotide polymorphisms (SNPs), modulates pharmacokinetics and peripheral drug susceptibility, thereby explaining the phenotypic differences observed in clinical practice. A retrospective cohort of 107 CLL patients treated with BTKi was analyzed. Extracted genomic DNA was analyzed by NGS sequencing using a targeted panel of 74 genes involved in drug metabolism and organ toxicity pathways. Efficacy in terms of progression-free survival (PFS) was evaluated using the log-rank test, while the risk of adverse events was studied using logistic regression models. With a median follow-up of 3.8 years, CYP2B6 cytochrome variants (rs3745274-G/T and rs2279343-A/G) were associated with a significant prolongation of PFS compared to wild-type subjects (5-year probability: 66.4% vs 42.1%,  $p=0.024$ ; 78.6% vs 45.6%,  $p=0.003$ ). Regarding adverse events, ABCB1 efflux pump polymorphisms significantly increased the risk of hemorrhagic manifestations (occurring in 16.8% of the sample). The incidence of atrial fibrillation (12.1%) was increased in carriers of variants in the MGMT, BRCA1, and TGFB1 genes, while the development of arterial hypertension (13.1%) was strongly associated with the ABCG2 transporter variant rs2622604. The data demonstrated that the germline pharmacogenetic profile represents a critical determinant in modulating the therapeutic index of BTKi in CLL. The clinical integration of a pre-treatment screening for cytochrome and membrane transporter loci could prove fundamental for optimizing long-term efficacy and preventing adverse events through personalized pharmacological therapy.

# 1. Introduction

## 1.1 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is a type of cancer that affects the blood and bone marrow. It develops when the body produces too many abnormal lymphocytes, a type of white blood cell that helps fight infections. These abnormal cells accumulate over time and can interfere with the normal function of healthy blood cells<sup>1</sup>. CLL is the most common leukemia in adults and usually progresses slowly. Many people are asymptomatic when first diagnosed. When symptoms manifest, they may include fatigue, swollen lymph nodes, frequent infections, weight loss, fever, or night sweats. The exact cause of CLL is unknown, but factors such as age, family history, and certain genetic changes may increase the risk<sup>2</sup>. Diagnosis typically involves blood tests, bone marrow examination, and imaging studies.

Treatment depends on the stage of the disease and the patient's overall health. Some individuals with early-stage CLL may not need immediate treatment and are monitored through a strategy called “watchful waiting”<sup>3</sup>. When treatment is necessary, options may include targeted therapies, immunotherapy, chemotherapy, or a combination of these approaches. Although CLL is generally not curable, modern treatments can effectively control the disease and help many patients maintain a good quality of life for years.<sup>4</sup>

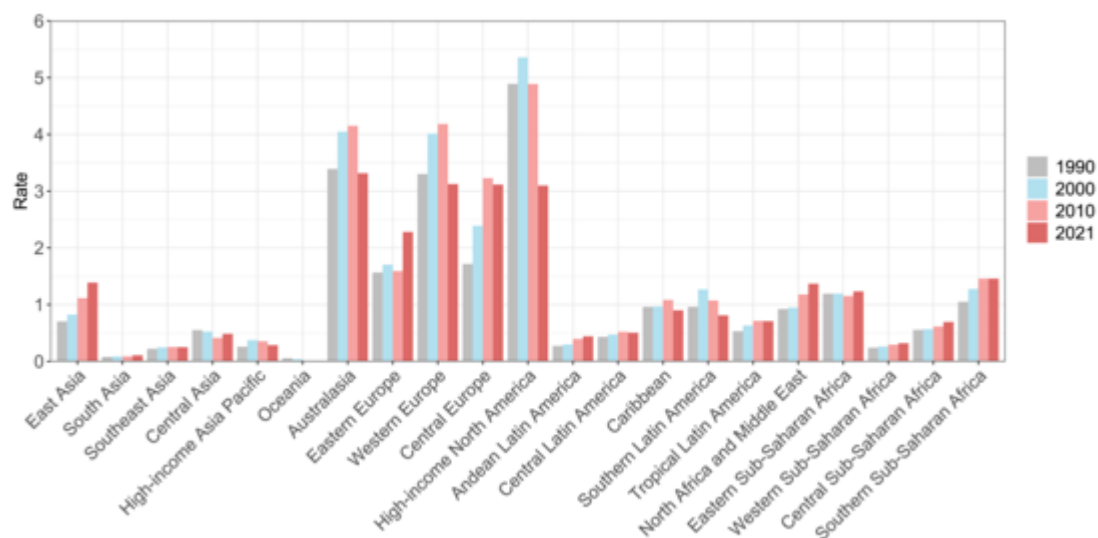


**Figure1. geographical distribution of CLL updated till 2021**

### 1.1.1 Epidemiology and pathogenesis

CLL is the most common leukemia in adults in Western countries, accounting for approximately 25–30% of all leukemia cases<sup>5</sup>. The disease primarily affects older individuals, with a median age at diagnosis of around 70 years. CLL is rare in people under 40 years of age and occurs more frequently in men than in women, with a male-to-female ratio of approximately 1.5–2:1.

The incidence varies geographically, being highest in Europe and North America and considerably lower in Asian populations. Family history is an important risk factor, as first-degree relatives of patients with CLL have a significantly increased risk of developing the disease, suggesting a genetic predisposition. The pathogenesis of CLL involves the accumulation of mature but functionally incompetent B lymphocytes in the blood, bone marrow, lymph nodes, and spleen. Unlike many cancers that result primarily from rapid cell proliferation, CLL develops largely because malignant B cells evade normal mechanisms of programmed cell death (apoptosis), allowing them to survive and accumulate over time.

Genetic abnormalities play a central role in disease development<sup>6</sup>. Common chromosomal alterations include deletion of chromosome 13q, deletion of 11q, trisomy 12, and deletion of 17p, which affects the TP53 tumor suppressor gene. Mutations in genes such as TP53, NOTCH1, SF3B1, and ATM contribute to disease progression and influence prognosis. Patients with TP53 abnormalities generally have a more aggressive disease course and reduced response to conventional chemotherapy. A key feature of CLL pathogenesis is abnormal signaling through the B-cell receptor (BCR). Continuous or excessive BCR activation promotes the survival, proliferation, and migration of leukemic cells. This process is supported by interactions between CLL cells and the tumor microenvironment, including stromal cells, T lymphocytes, and cytokines within lymphoid tissues. These interactions provide growth and survival signals that protect leukemic cells from apoptosis.

The mutation status of the immunoglobulin heavy-chain variable region (IGHV) gene is another important biological factor. Patients with mutated IGHV genes generally experience a more indolent disease course, whereas unmutated IGHV is associated with more aggressive disease and poorer outcomes. CLL often develops from a precursor condition known as monoclonal B-cell lymphocytosis (MBL), in which small populations of clonal B cells are

present in the blood without clinical symptoms. Although only a minority of individuals with MBL progress to CLL, this condition is considered an early stage in the evolution of the disease. Overall, CLL results from a complex interplay of genetic alterations, disrupted apoptosis, abnormal immune signaling, and supportive microenvironmental interactions. These mechanisms drive the gradual accumulation of malignant B lymphocytes and underlie the clinical behavior of the disease. Modern targeted therapies have been developed based on these pathogenic pathways, significantly improving patient outcomes<sup>7,8</sup>.

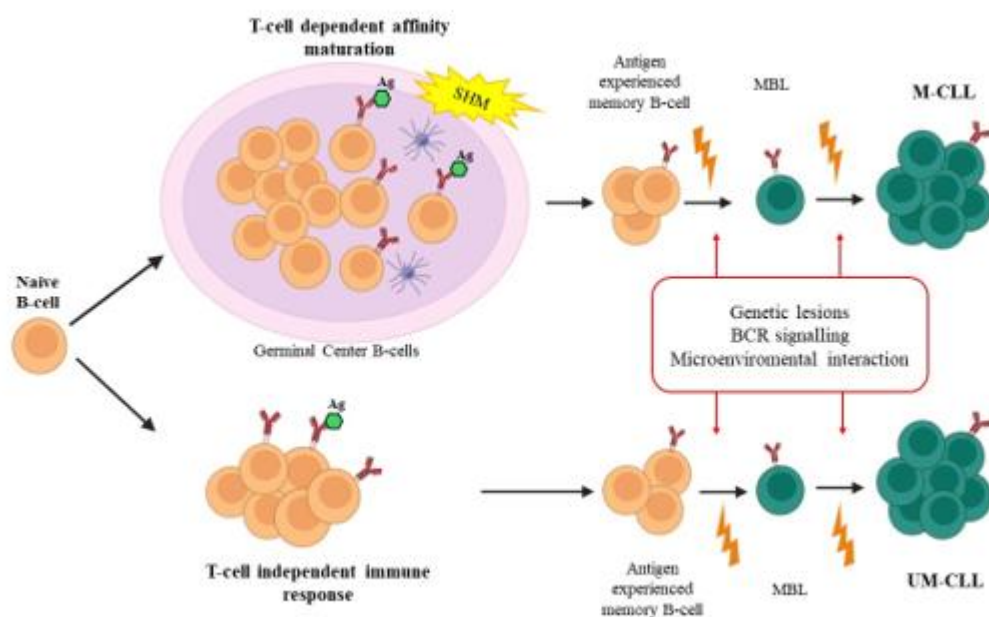


**Figure 2 CLL pathogenesis.** B cell can experience the germinal center and go through somatic hypermutation or can be activated independently from T-cell. Cells can be affected by mutations during the process and at the end it is possible to distinguish mutated IGHV CLL or unmutated IGHV CLL. SHM, somatic hypermutation. BCR, B-cell Receptor. MBL, Monoclonal B-Cell Lymphocytosis. M-CLL, mutated IGHV. UM-CLL, unmutated IGHV.

### 1.1.2 Diagnosis

CLL diagnosis is often made incidentally during routine blood tests showing persistent lymphocytosis. The initial evaluation includes a complete blood count (CBC), which typically reveals an increased absolute lymphocyte count of at least  $5 \times 10^9/L$  persisting for more than three months. A peripheral blood smear may demonstrate small, mature-appearing lymphocytes and characteristic “smudge cells.” The definitive diagnosis is established through flow cytometry of peripheral blood. CLL cells express a characteristic immunophenotype, including CD19, CD20 (dim), CD23, and the aberrant co-expression of CD5, a marker usually associated with T cells. Demonstration of this immunophenotypic profile confirms the clonal B-cell population required for diagnosis.

Additional investigations help assess prognosis and guide management. Cytogenetic and molecular studies, such as fluorescence in situ hybridization (FISH), identify chromosomal abnormalities including deletion 13q, trisomy 12, deletion 11q, and deletion 17p<sup>9</sup>. Testing for TP53 mutations and IGHV mutation status provides important prognostic information and influences treatment decisions. Bone marrow biopsy is not routinely required for diagnosis but may be useful in selected cases to evaluate cytopenias or treatment response<sup>8</sup>.

Imaging studies are generally reserved for patients with symptoms or suspected organ involvement. Following diagnosis, disease staging is commonly performed using the Rai or Binet staging systems, which classify patients according to lymphadenopathy, organ enlargement, anemia, and thrombocytopenia. Accurate diagnosis and risk stratification are essential for determining prognosis and selecting the most appropriate management strategy, ranging from active surveillance to targeted therapeutic interventions<sup>10</sup>.

### 1.1.3 Prognostic and Predictive Biomarkers

Prognostic and predictive biomarkers play a crucial role in the management of CLL helping to estimate disease courses and guide treatment selection. Prognostic biomarkers provide information about the likely outcome of the disease regardless of therapy, whereas predictive biomarkers indicate the likelihood of response to a specific treatment<sup>11</sup>. Among the most important prognostic biomarkers in CLL is the mutation status of the IGHV gene.

Patients with mutated IGHV generally experience a more indolent disease course and longer survival, while unmutated IGHV is associated with more aggressive disease and earlier need for treatment. Genetic abnormalities detected by fluorescence in situ hybridization (FISH) also have significant prognostic value. Deletion of chromosome 13q as a sole abnormality is associated with a favorable prognosis, whereas deletion 11q and trisomy 12 confer intermediate risk. Deletion 17p, involving the TP53 gene, is linked to poor outcomes and resistance to conventional chemoimmunotherapy. TP53 mutations represent one of the most important predictive biomarkers in CLL<sup>8</sup>.

Patients harboring TP53 disruption, including either a TP53 mutation or 17p deletion, respond poorly to chemotherapy but benefit from targeted therapies such as Bruton tyrosine kinase (BTK) inhibitors and BCL-2 inhibitors<sup>12</sup>. Consequently, TP53 testing is recommended before initiating treatment. Other biomarkers, including elevated expression of CD38, ZAP-70, and  $\beta$ 2-microglobulin, have been associated with more aggressive disease, although their clinical use is less central than IGHV and TP53 testing.

Minimal residual disease (MRD) assessment has also emerged as an important predictive marker, as achieving undetectable MRD after therapy is associated with longer progression-free survival. Overall, the integration of prognostic and predictive biomarkers into clinical practice enables risk-adapted management and supports the selection of the most effective therapeutic strategies for individual patients with CLL<sup>13</sup>.

| Parameters                            | Score |
|---------------------------------------|-------|
| Age>65 years                          | 1     |
| Rai stage I-IV or Binet B-C           | 1     |
| Unmutated IGHV genes                  | 2     |
| Serum $\beta$ 2-microglobulin>3,5mg/L | 2     |
| Del(17p) and/or TP53 mutation         | 4     |

**Table1. CLL international prognostic index**

### 1.1.4 Clinical Staging System

Clinical staging is an essential component of CLL management, providing information about disease extent, prognosis, and treatment planning. The two most widely used staging systems are the Rai system, developed in the United States, and the Binet system, commonly used in Europe. The Rai staging system classifies patients into five stages (0–IV) based on physical examination findings and blood counts. Stage 0 is characterized by isolated lymphocytosis and is considered low risk. Stages I and II involve lymphadenopathy, splenomegaly, or hepatomegaly and are categorized as intermediate risk. Stages III and IV are defined by anemia (hemoglobin <11 g/dL) and thrombocytopenia (platelet count <100×10<sup>9</sup>/L), respectively, and are considered high risk. Survival generally decreases with advancing stage<sup>14</sup>.

| Stage             | Characteristics                                                                              |
|-------------------|----------------------------------------------------------------------------------------------|
| Low risk          | only lymphocytosis in the peripheral blood and bone marrow infiltration (same as stage 0)    |
| Intermediate risk | presence of lymphadenopathies (same as stage I) and/or hepatosplenomegaly (same as stage II) |
| High risk         | presence of anemia (same as stage III) and or thrombocytopenia (same as stage IV)            |

**Table2. Rai staging system for CLL.**

The Binet staging system is based on the number of involved lymphoid areas and the presence of anemia or thrombocytopenia. Five lymphoid regions are evaluated: cervical, axillary, and inguinal lymph nodes, spleen, and liver. Stage A includes fewer than three involved areas without anemia or thrombocytopenia and is associated with a favorable prognosis. Stage B includes three or more involved areas without anemia or thrombocytopenia and represents intermediate-risk disease. Stage C is defined by the presence of anemia (hemoglobin <10 g/dL) and/or thrombocytopenia (platelet count <100 × 10<sup>9</sup>/L), regardless of the number of involved areas, and indicates advanced disease. Although both staging systems remain valuable for estimating prognosis, modern CLL management increasingly incorporates molecular and genetic biomarkers, such as TP53 abnormalities and IGHV mutation status, to refine risk assessment. Nevertheless, Rai and Binet staging continue to provide a simple and effective framework for the initial clinical evaluation and follow-up of patients with CLL<sup>15</sup>.

| Stage | Characteristics                                                                                                                             |
|-------|---------------------------------------------------------------------------------------------------------------------------------------------|
| A     | no anemia (Hb > 10 g/dl) or thrombocytopenia (Plt > 100 000/mm <sup>3</sup> ) and up to 2 lymphoid sites involved                           |
| B     | no anemia (Hb > 10 g/dl) or thrombocytopenia (Plt > 100 000/mm <sup>3</sup> ) and more than 2 lymphoid sites involved                       |
| C     | presence of anemia (Hb < 10 g/dl) and/or thrombocytopenia (Plt < 100,000/mm <sup>3</sup> ) regardless the number of lymphoid sites involved |

**Table 3. Binet staging system**

### 1.1.5 Treatment

The treatment of CLL depends on the stage of disease, symptoms, patient age, comorbidities, and genetic risk factors. Not all patients require immediate therapy. Individuals with early-stage, asymptomatic CLL are often managed with a “watch-and-wait” approach, involving regular clinical assessments and blood tests, as early treatment has not been shown to improve survival<sup>16</sup>. Treatment is initiated when patients develop symptomatic disease, progressive bone marrow failure, significant lymphadenopathy or splenomegaly, rapidly increasing lymphocyte counts, or constitutional symptoms such as fever, night sweats, and weight loss. In recent years, targeted therapies have largely replaced traditional chemotherapy as the standard of care<sup>17</sup>. Overall, modern targeted therapies have significantly improved outcomes in CLL, leading to prolonged progression-free survival, better quality of life, and reduced treatment-related toxicity compared with traditional chemotherapy<sup>18</sup>.

#### 1.1.5.1 Chemoimmunotherapy Era

The rationale behind immunochemotherapy lies in the synergistic interaction between chemotherapy and immunotherapy<sup>19</sup>. Chemotherapeutic agents reduce tumor burden by inducing apoptosis and inhibiting cell proliferation, while monoclonal antibodies target surface antigens such as CD20, promoting immune-mediated destruction of malignant cells through complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and direct induction of apoptosis to achieve durable remissions, reduce disease burden, improve

progression-free survival (PFS) and overall survival (OS) and also enhancing quality of life while minimizing treatment-related toxicity<sup>19</sup>.

The most used combinations of drugs are Fludarabine Cyclophosphamide Rituximab (FCR), Bendamustine-Rituximab (BR) and chlorambucil based strategies. The FCR regimen has historically been considered the gold standard for fit, younger patients with previously untreated CLL. Fludarabine, a purine analog, and cyclophosphamide, an alkylating agent, are combined with rituximab, an anti-CD20 monoclonal antibody. Clinical studies demonstrated that FCR significantly improves response rates, compared with chemotherapy alone. Long-term follow-up has shown that patients with mutated IGHV genes may experience prolonged remissions and, in some cases, functional cure-like outcomes. However, FCR is associated with substantial toxicities, including myelosuppression, increased risk of infections, secondary malignancies, and prolonged immunosuppression. BR emerged as an alternative for older patients or those unable to tolerate FCR<sup>20</sup>. Bendamustine possesses both alkylating and purine analog-like properties, providing effective antileukemic activity. Compared with FCR, BR generally exhibits lower toxicity, reduced rates of severe neutropenia and better tolerability in elderly patients<sup>21</sup>.

Rituximab was the first monoclonal antibody widely incorporated into CLL treatment. By targeting CD20 on B cells, it enhances the efficacy of chemotherapy and contributes significantly to improved clinical outcomes. Obinutuzumab is a glycoengineered type II anti-CD20 antibody with enhanced antibody-dependent cellular cytotoxicity. Clinical trials have demonstrated superior efficacy compared with rituximab in certain patient populations, particularly when combined with chlorambucil. Ofatumumab is a fully human anti-CD20 antibody that binds a distinct epitope of CD20. It has shown activity in relapsed or refractory CLL and has been incorporated into several immunochemotherapy regimens. So, the choice of immunochemotherapy depends on several factors such as the overall health status of the patient and the genetic mutation that cause leukemia. In fact, Assessment of performance status, renal function, and comorbidities is essential. Fit patients may tolerate intensive regimens such as FCR, whereas elderly or frail individuals may benefit from less intensive approaches<sup>22</sup>.

### 1.1.5.2 Target Therapy Era

The introduction of targeted agents such as the Bruton's tyrosine kinase (BTK) inhibitors and BCL-2 inhibitors has transformed CLL management. Agents such as ibrutinib, acalabrutinib, zanubrutinib, and venetoclax-based combinations have demonstrated superior efficacy and improved safety profiles in many patient populations especially in patient with chemo refractory mutations such as TP53 mutation or del 17p and del 11p<sup>23</sup>.

#### 1.1.5.2.1 BTK Inhibitors

Bruton's tyrosine kinase inhibitors (BTKi) have significantly transformed the management of CLL<sup>18</sup>. BTK is a key component of the B-cell receptor signaling pathway, which promotes the survival, proliferation, and migration of malignant B lymphocytes. By inhibiting BTK activity, these agents disrupt signaling pathways essential for CLL cell growth and survival. The first-generation BTK inhibitor, ibrutinib, demonstrated substantial improvements in progression-free survival and overall survival compared with traditional chemoimmunotherapy<sup>24,25</sup>. Its success established BTK inhibition as a standard treatment option for both treatment-naïve and relapsed/refractory CLL patients<sup>25</sup>. However, off-target effects have been associated with adverse events such as atrial fibrillation, hypertension, bleeding, and musculoskeletal symptoms<sup>26</sup>.

To improve tolerability while maintaining efficacy, second-generation BTK inhibitors such as Acalabrutinib and Zanubrutinib were developed. These agents exhibit greater selectivity for BTK and are associated with lower rates of certain cardiovascular toxicities. Clinical trials have shown that both drugs provide excellent disease control and are effective treatment options across different CLL patient populations. BTKi therapy is generally administered continuously until disease progression or unacceptable toxicity. These agents are particularly valuable for patients with high-risk genomic features, including deletion 17p and TP53 mutations, who historically had poor outcomes with chemoimmunotherapy. Long-term follow-up studies have demonstrated durable responses and prolonged disease control in these high-risk groups. Despite their effectiveness, resistance to BTKi therapy can occur, often through mutations affecting BTK or downstream signaling molecules<sup>27</sup>.

New therapeutic strategies, including non-covalent BTK inhibitors and combination approaches with BCL-2 inhibitors or anti-CD20 monoclonal antibodies, are being investigated

to overcome resistance and further improve patient outcomes. In conclusion, BTK inhibitors have become a cornerstone of modern CLL treatment, offering highly effective, chemotherapy-free options that improve survival and quality of life for many patients. Continued research is expected to further refine their use and expand treatment possibilities for patients with CLL.

#### 1.1.5.2.2 Bcl2 Inhibitors

B-cell lymphoma 2 (BCL-2) inhibitors have become an important component of modern treatment strategies for CLL. The BCL-2 protein is an anti-apoptotic molecule that is overexpressed in CLL cells, allowing them to evade programmed cell death and survive for prolonged periods. Targeting BCL-2 restores the apoptotic process, leading to the elimination of malignant lymphocytes. The most widely used BCL-2 inhibitor in CLL is Venetoclax (Tausch et al., 2020). Venetoclax selectively binds to the BCL-2 protein, displacing pro-apoptotic factors and triggering rapid apoptosis of CLL cells. Clinical studies have demonstrated high response rates and deep remissions in both treatment-naïve and relapsed/refractory patients, including those with high-risk genetic abnormalities such as TP53 mutations and deletion 17p.

One of the major advantages of venetoclax-based therapy is the possibility of fixed-duration treatment. Unlike Bruton's tyrosine kinase inhibitor (BTKi) therapy, which is typically administered continuously, venetoclax is commonly given for a predefined period in combination with other agents. In the frontline setting, venetoclax combined with the anti-CD20 monoclonal antibody Obinutuzumab has shown excellent efficacy and durable remissions. In relapsed or refractory CLL, venetoclax combined with Rituximab has also demonstrated significant improvements in progression-free survival and rates of undetectable minimal residual disease (uMRD). A unique consideration with venetoclax treatment is the risk of tumor lysis syndrome (TLS)<sup>28</sup>, particularly in patients with a high disease burden. To minimize this risk, therapy is initiated using a gradual dose-escalation schedule accompanied by careful laboratory monitoring, adequate hydration, and, when appropriate, prophylactic medications. The achievement of undetectable minimal residual disease has emerged as an important therapeutic goal in CLL management.

Venetoclax-based regimens frequently induce deep molecular responses, and uMRD status is associated with prolonged progression-free survival and improved long-term

outcomes. Consequently, BCL-2 inhibitor therapy has become a preferred option for many patients seeking a time-limited treatment approach.

## 1.2 Single nucleotide polymorphisms

Despite their effectiveness, chronic usage of BTKi is associated with a toxic effect due to off target Kinase's inhibition (different from BTK), that has in common a cysteine residue fundamental for the ATP binding<sup>29</sup>. Atrial fibrillation and hypertension are tightly correlated to the inhibition of TEC kinase and PI3K-AKT pathway; those are detrimental for cardiomyocyte protection from oxidative stress and apoptosis<sup>30</sup>. The deregulation of cardiac ionic channel mediated by those events increases the sensitivity of the atrium to electrical stimuli, generating an arrhythmic beating<sup>25</sup>. Another common side effect is diarrhea is caused by the inhibition of EGFR (Epidermal Grow Factor Receptor) express by the intestinal epithelium. Signal interference of EGFR can modify the mucosal structure and adsorption process, generating diarrhea symptoms<sup>24</sup>.

Although CLL does not itself cause bone marrow failure, BTKi can induce neutropenia through transient inhibition of myeloid precursor. The mechanism appears to involve interference with SRC and TEC family kinases, which are necessary for the correct maturation and release of granulocytes from the bone marrow into the bloodstream. Regarding infection risk, the causes are multifactorial. In addition to CLL intrinsic hypogammaglobulinemia, BTKi inhibits BTK function also in macrophages (Kupffer cells and alveolar macrophages) and neutrophils, compromising phagocytosis and the production of reactive oxygen species (ROS) mediated by Toll-like receptors (TLR). This expose patients to an increased risk of opportunistic infections particularly of the upper and lower respiratory tract, in the early phase of the treatment. Another commonly encountered symptom in patients receiving active treatment is fatigue<sup>31</sup>.

Chronic fatigue represents a complex symptom, often linked to low grade systemic toxicity and modulation of pro-inflammatory cytokines. Although the exact molecular mechanism remains partly elusive, it is believed that chronic inhibition of ubiquitous kinases pathway may alter cellular energy metabolism and contribute to the sensation of exhaustion reported by patients. The durations and intensity of an adverse event are closely linked to systemic drug level. The pharmacokinetics of BTKi is largely dependent on hepatic metabolism,

mediated primarily by cytochrome P450 system (particularly CYP3A4/5) and cellular efflux transporters (such as the p Glycoprotein encoded by the ABCB1 gene).

Polymorphism in genes encoding these enzymes and transporters profoundly alter exposure to active and inactive metabolites.<sup>29</sup> Subject carrying genotypes that confer a poor metabolizer phenotype may experience a marked increase in the drug's half-life. This reduced clearance exposes healthy tissue to persistent plasma concentrations, exacerbating severity and prolonging the duration of adverse events, delaying clinical recovery even after dose reduction or temporary discontinuation (drug holiday)<sup>32</sup>.

Despite the biological rationale linking the patient genomic profile to the incidence, severity, and distribution of BTKi toxicities, very little literature currently exists on the topic. This knowledge gap currently makes priori clinical risk profiling impossible. Only by filling this gap can the translation be made from empirical administration to true precision medicine, capable of proactively guiding the clinician toward the safest molecule or dosage for the individual patient<sup>33</sup>.

## 2. Aim of the study

The present study arises from the clinical need to understand the marked individual heterogeneity in BTKi tolerability. Although these agents have revolutionized the CLL therapeutic algorithm, the management of toxicities remains a complex challenge. Based on the hypothesis that host polymorphism plays a cardinal role in the pharmacokinetics and pharmacodynamics of these molecules, a cohort of CLL patients who receive BTKi was analyzed. The study objectives are therefore to evaluate the correlation between specific single nucleotide polymorphism (SNP) and: (i) BTKi efficacy and (ii) the risk of developing clinically relevant adverse events during BTKi therapy.

## 3. Materials and methods

### 3.1. Patients

This retrospective analysis is based on a cohort of 107 patients, enrolled by those regularly followed up at the Università del Piemonte Orientale hematology unit and the Ospedale Maggiore della Carità of Novara. The study protocol was reserved for patients with a confirmed diagnosis of CLL who had received a first line or subsequent line pharmacological treatment based on BTK inhibitor (Novara inter-institutional Ethics Committee, EC 120/19). To ensure maximum accuracy, the research database was compiled by associating with each individual patient a comprehensive set of clinical, biological, and molecular variables.

For each patient, hematological parameters were first recorded, combined with fundamental demographic data (age and sex), along with a basic's biochemical assessment. Simultaneously, biological characterization of the leukemic clone was performed, collecting reports relating to the immunophenotypic profile assessed by flow cytometry, cytogenetic investigation by FISH, the mutational status of the variable portion of the immunoglobulin heavy chains (IGHV), analysis of the molecular lesions in the TP53 gene (the latter conducted by direct DNA sequencing), and other common CLL molecular alterations.

### 3.2. Separation Of Granulocytes from Peripheral Blood

To obtain the genomic DNA (gDNA) necessary for the subsequent polymorphic analyses, the granulocyte fraction was isolated from peripheral blood samples collected from enrolled patients. Operationally, whole blood samples were first subjected to a 1:2 dilution using normal saline solution (0.9% NaCl). The resulting mixture was layered onto a density gradient separation medium using Ficoll Histopaque®-1077 reagent. The preparation was then centrifuged at 1800 rpm for a total of 25 minutes. This specific analytical step ensured optimal cellular stratification: the density gradient difference allowed granulocyte to precipitate and be recovered, cleanly isolated from both plasma supernatant and the intermediate buffy coat consisting of mononuclear cells, such as tumor lymphocytes and monocytes.

### 3.3. DNA Extraction

For the isolation of genomic DNA (gDNA) from the granulocyte fraction, the salt precipitation extraction protocol, known as the “salting out” technique, was used<sup>34</sup>. Cells were initially dissolved in a lysis buffer (composed of Tris-HCl 1 M at pH 8.2, NaCl 5 M, EDTA 0.5 M, and 20% SDS) in conjunction with enzymatic digestion by Proteinase K at a concentration of 20 mg/ml. The mixture was then incubated at 37°C overnight. The following day, the addition of 6M NaCl and subsequent centrifugation (3,200 rpm for 20 minutes) allowed the protein component to be precipitated and removed. The actual recovery of gDNA was archived by precipitation with absolute ethanol. The genetic material was mechanically collected with a glass loop, subjected to three purification steps with 75% ethanol, and finally resuspended and stored in T.E. buffer (Tris-HCl 1 M, pH 8.2; EDTA 0.5 M).

### 3.4. DNA Quantification and Fragmentation

Extraction yield was evaluated fluorometrically using the Quanti-iT™ PicoGreen® dsDNA assay (ThermoFisher scientific, Eugene, OR, USA). This method provides highly specific and sensitive quantification, exploiting a fluorochrome capable of selectively intercalating exclusively with double strand DNA. This reagent, prepared at a 1:200 dilution, was incubated with the extracted samples. Concentrations were calculated by interpolation on a calibration curve, established in parallel using serial dilution (1:2) of a known concentration DNA standard. Fluorescent signal acquisition was performed using the Infinite® F200 microplate reader (Tecan, Männedorf, Switzerland), setting the optical parameters at 485 nm for excitation and 530 nm for emission. In preparation for genomic library construction, the quantified DNA was subjected to physical fragmentation. The M220 focused ultrasonicator (Covaris®, Woburn, MA, USA) was used to uniformly shear the gDNA, obtaining fragments between 250 and 300 base pairs, a size considered optimal for subsequent sequencing procedures on Illumina platform (MiSeq and NextSeq550 systems). To validate the fragmentation accuracy, the size distribution and exact concentration (express in pg/μL) of amplicons were carefully verified using microfluidic technology of the Bioanalyzer 2100 system, equipped with dedicated chips from the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA).

### 3.5. Library Design for Hybrid Selection

Genomic library preparation represents a crucial step in generating a pool of nucleotide fragments suitable for subsequent analysis by massively parallel sequencing (NGS) platforms. Within this experimental protocol, the entire preparation procedure was conducted starting from the previously extracted genomic DNA (gDNA) samples. Technically library preparation was performed using the KAPA HyperPrep Kit commercial system (Roche Diagnostic, Pleasanton, CA, USA). In order to specifically isolate and amplify only the genomic regions relevant to the study, a targeted enrichment (target enrichment) method was used, implemented through the KAPA HyperChoice capture probe panel (Roche Diagnostic, Pleasanton, CA, USA), which enabled analysis of 74 genes involved in polymorphism related to BTKi metabolism.

### 3.6. Next Generation Sequencing

The MiSeq and NextSeq550 platforms exploit sequencing by synthesis (SBS) technology, which allows massively parallel high-throughput sequencing of genomic region of interest. DNA libraries are transferred to a solid support, called flow cell, to which they bind via special adapter inserted into the fragment during the library preparation phase. On the flow cell, libraries are amplified in a process called bridge amplification, which generates cluster of identical molecules, each derived from the amplification of a single DNA molecule. Sequencing is based on reversible cyclic termination method with a by-synthesis approach, comprising three steps: (i) nucleotide incorporation; (ii) image detection; and (iii) cleavage. In the first phase of the cycle, the DNA polymerase bound to the template extends a specific primer by adding a nucleotide covalently linked to a fluorophore. This has a block on the 3'-OH of the deoxyribose that prevents polymerization with other nucleotides. Each nitrogen base is linked to a fluorophore of a specific color. Incorporation of the labeled nucleotide only occurs when the complementary base is present on the template sequence. This is followed by the image detection phase that recognizes the specific emission wavelength of the fluorophore. Cleavage then removes both the fluorophore and the inhibitor group present on the 3'-OH, allowing the start of a new cycle. Libraries were sequenced using pair end sequencing. The library pool was denatured using 0.2N NaOH. An amount of 9 pM of denatured DNA (for the MiSeq platform)

and 1.3 pM (for the NextSeq550 platform) was loaded into the cartridge also containing all reagents necessary for the sequencing reaction to occur.

### 3.7. Data and Statistical Analysis

Primary data analysis was performed in real time using the Illumina Real Time Analysis (RTA) software. It provides a qualitative measurement of the reading of individual nucleotide based expressed as a Phred score, a quality assessment scale for DNA sequences. A second software integrated in the sequencer -Miseq Reporter Software (MSR) for the MiSeq platform and Local Run Manager for the NextSeq550 platform processes the data generated by primary analysis through a series of procedures: (i) the demultiplexing phase which allow data from pooled samples to be separated and identified based on short index sequences; (ii) the FASTQ file generation phase, which contains all reads obtained from sequencing. Sequences in the FASTQ file were duplicated using the FastUniq v1.1 software, subsequently aligned to the GRCh37/hg19 version of the human genome using SAMtool v1 software. Polymorphisms were defined as homozygous when the VAF was >99% or heterozygous when the VAF was between 33% and 63%<sup>35</sup>.

PFS and OS were used as study endpoints. PFS was calculated from the date of BTKi initiation until disease progression according to iwCLL 2018 criteria or patient death. OS was calculated from the date of BTKi initiation until patient death. Survival analysis was performed using the Kaplan-Meier method, and differences between groups were compared using the log-rank test. A logistic regression analysis was used to correlate SNPs with therapy related toxicities.

## 4. Results

### 4.1 Patient Characteristics

In this study, the examined cohort consisted of 107 patients with a confirmed diagnosis of CLL, characterized by a median age at baseline of 78.5 years (range: 45-96 years) and a prevalence of male patients as expected for CLL (71 cases, 66.4% vs 36 females 33.6%). The initial clinical assessment, evaluated according to the Binet system, showed 17 subjects (16.2%) at stage A, 43 (41.0%) at stage B, and 42 (40.0%) at stage C. In parallel, the Rai classification identified 2 patient (1.9%) at stage I, 36 (33.6%) at stage II, 21 (19.6%) at stage III, and 21 (21.6%) at stage IV. For three cases, Binet and Rai classification was unavailable as they had a diagnosis of small lymphocytic lymphoma, i.e., the CLL variant without lymphocytosis and with predominantly nodal involvement. Median patient hemoglobin was 120 g/L, median platelet count  $144 \times 10^3/\text{ml}$  and median lymphocyte count of 25200/ml

Biological characterization of the leukemic clone revealed an unmutated IGHV status in 79 patients (73.8%) and mutated in 24 (22.4%). Cytogenetic investigation by FISH documented, deletion of chromosome 13q (del13q) in 42.1% of cases (45 patients), deletion of chromosome 11q (del 11q) in 23.4% of cases (25 patients), trisomy 12 in 20.6% of cases (22 patients) and deletion of 17p (del 17p) in 15.0% of cases (16 patients). Completing the high-risk molecular profile, TP53 gene mutation was found in 24 subjects (22.4%).

Regarding the therapeutic management, the BTKi was used as first-line systemic treatment in 60.4% of cases (64 patients). In the remaining subject the drug was administered in subsequent lines: second line 23 patients (21.7%), third line 11 patients (10.4%), and fourth line or higher 8 patients (7.5%). Regarding the type of BTKi used within the cohort, ibrutinib was used in 67 cases (61.68%), acalabrutinib in 20 cases (18.69%), and Zanubrutinib in 16 cases (14.95%). Finally, the non-covalent inhibitor pirtobrutinib was reserved for a smaller subset of cases, equal to 4 patients (3.74%). The different prevalence of molecules reflects the different timing of approval of the various drugs in clinical practice. Against median observation time from the start of BTKi treatment of 3.8 years, the statistical survival analysis conducted, according to the Kaplan-Meier method, documented a median PFS of 5.3 years and a median OS of 8.0 years.

| Characteristic       | Value                            |
|----------------------|----------------------------------|
| Age at diagnosis     | Median 78.5 years (range 45–96)  |
| Male                 | 71 (66.4%)                       |
| Female               | 36 (33.6%)                       |
| Binet A              | 17 (16.2%)                       |
| Binet B              | 43 (41.0%)                       |
| Binet C              | 42 (40.0%)                       |
| Rai I                | 2 (1.9%)                         |
| Rai II               | 36 (33.6%)                       |
| Rai III              | 21 (19.6%)                       |
| Rai IV               | 21 (21.6%)                       |
| Hemoglobin           | Median 120 g/L                   |
| Platelet count       | Median 144 × 10 <sup>9</sup> /L  |
| Lymphocyte count     | Median 25.2 × 10 <sup>9</sup> /L |
| IGHV unmutated       | 79 (73.8%)                       |
| IGHV mutated         | 24 (22.4%)                       |
| del(13q)             | 45 (42.1%)                       |
| del(11q)             | 25 (23.4%)                       |
| Trisomy 12           | 22 (20.6%)                       |
| del(17p)             | 16 (15.0%)                       |
| TP53 mutation        | 24 (22.4%)                       |
| First line           | 64 (60.4%)                       |
| Second line          | 23 (21.7%)                       |
| Third line           | 11 (10.4%)                       |
| Fourth line or later | 8 (7.5%)                         |
| Ibrutinib            | 67 (61.68%)                      |
| Acalabrutinib        | 20 (18.69%)                      |
| Zanubrutinib         | 16 (14.95%)                      |
| Pirtobrutinib        | 4 (3.74%)                        |

**Table4. Patients characteristics in the cohort**

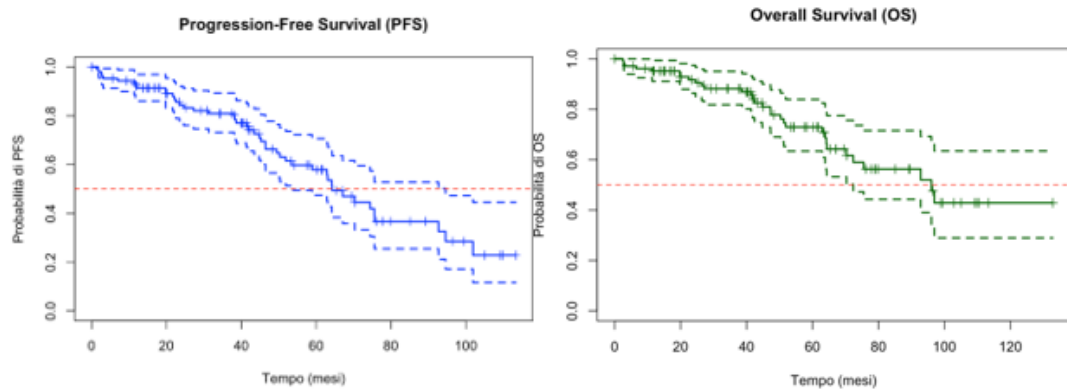


**Figure3.** Progression free survival & overall survival of patient cohort analyzed. On the x axis there is time express in month. On the y axis the probability of illness progression.

## 4.2 Clinical Impact of Single Nucleotide Polymorphism in terms of PFS

Analysis of the Kaplan-Meier curves reveals that certain SNPs are associated with a significant difference in PFS. Carriers of CYP2B6 cytochrome variants rs3745274-G/T and rs2279343-A/G show a significantly longer PFS compared to subject without this variant, with a five years PFS probability of 66.4% vs 42.2% ( $p=0.024$ ) and 78.6% vs 45.6% ( $p=0.003$ ) respectively. A favorable trend is also observed for BRACA1 rs1799950 in variant carriers (5 years PFS 87.5% vs 49.7%  $p=0.051$ ). These results suggest a possible predictive role of specific SNPs in the long term response to BTKi therapy.

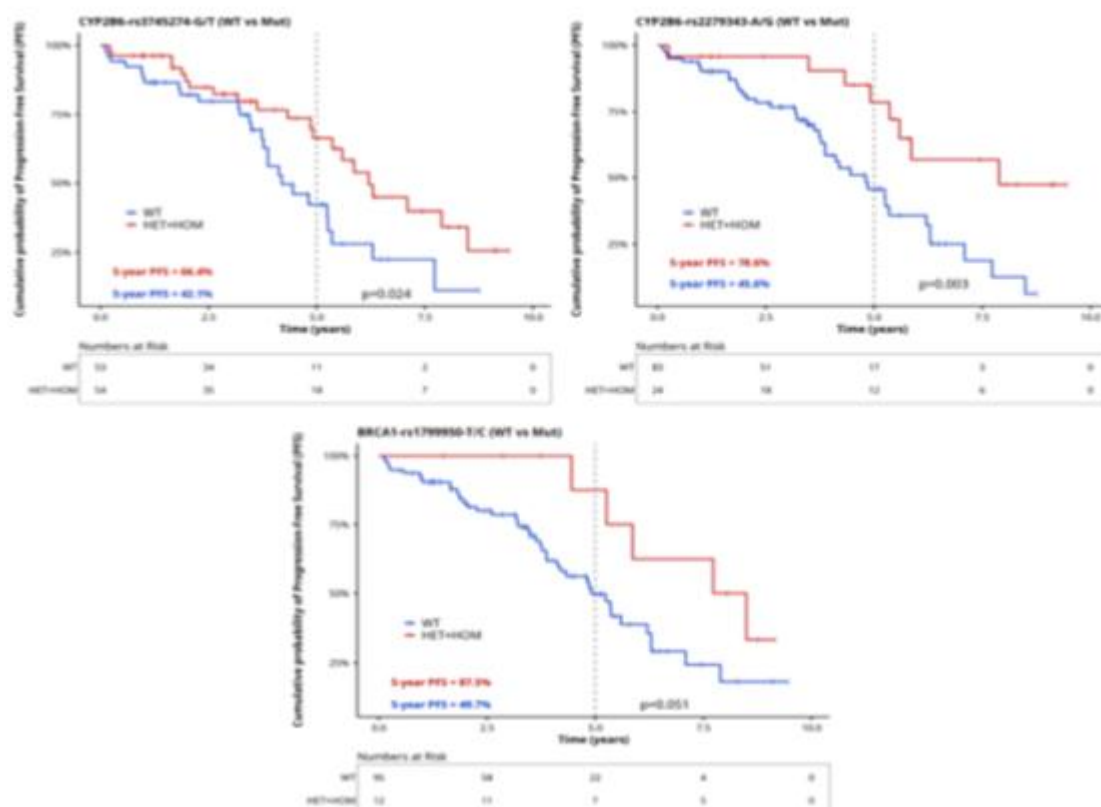


**Figure4.** progression free survival of treated patients differentiated by the presence of polymorphism. Patients with the SNP are represented in red; instead patient without the SNP are represented in blue. P-values are represented near the curves.

### 4.3 Distribution of BTKi Adverse Event in the Analyzed Cohort

On the incidence of adverse events, infectious complications represented by far the most frequent problem within the cohort, affecting more than half of the study subjects with a total of 56 recorded cases (52.34%). This finding closely correlates with the second most frequent hematological finding, namely neutropenia, which affected 20 patients (18.69%).

Regarding extra-medullary and organ toxicities, coagulation disorders and cardiovascular events positioned at an intermediate level of incidence. Specifically, hemorrhagic manifestations, classified predominantly as minor bleedings, involved 18 patients (16.82%), followed by vascular and cardiac problems. New-onset arterial hypertension or worsening of a pre-existing condition affected 14 subjects (13.08%), while atrial fibrillation (the historically sentinel adverse event of primary interest for the BTK inhibitor drug class) was

documented in 13 patients in the cohort (12.15%). Regarding other known BTKi adverse events, arthralgia affected 10 patients (9.35%), chronic fatigue 8 subjects (7.48%), and persistent diarrhea 6 patients (5.61%).

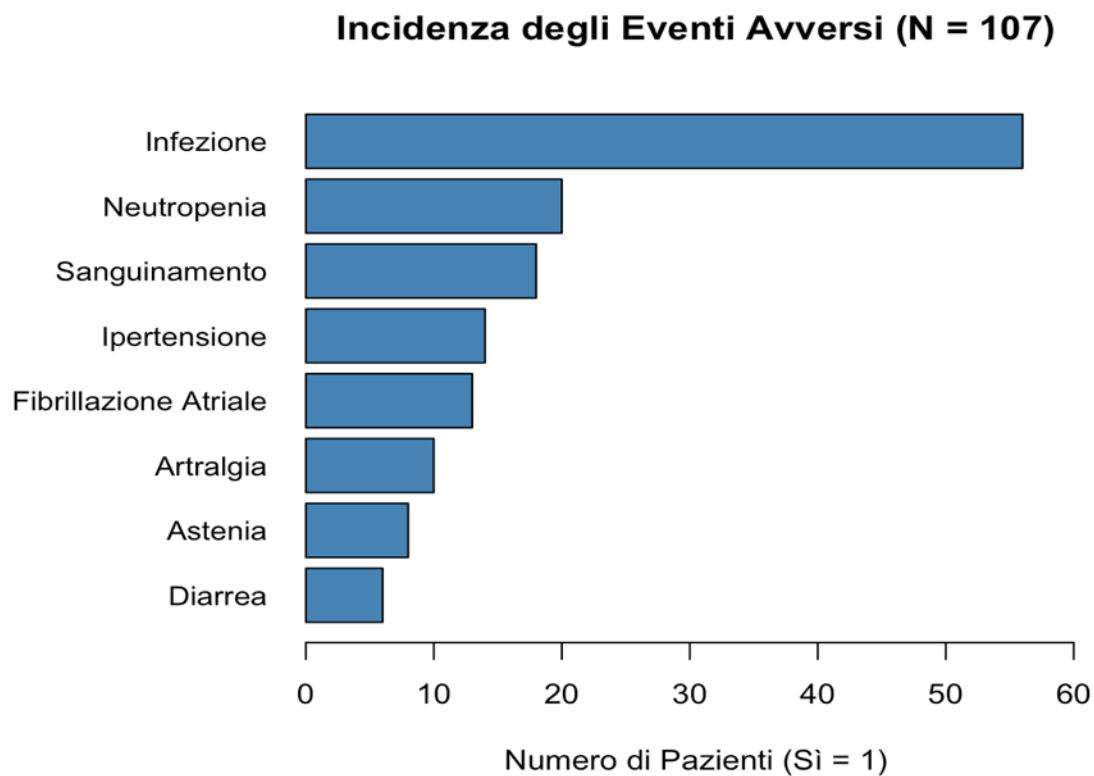


**Figure5.** adverse events incidence. On the x axis there is the number of the patients. On the y axis the most common adverse events of BTKi therapy.

#### 4.4. Association of SNPs and Risk of Adverse Events During BTKi Treatment

Logistic regression identified several polymorphisms associated with the occurrence of adverse events in BTKi-treated patients. Regarding atrial fibrillation, the most significant associations were observed for MGMT rs16906252, BRCA1 rs4986850, and TGFB1 rs1800471, associated with an increased event risk, while variants of ROCK2 rs10178332, ROCK2 rs965665, and XRCC6 rs132788 were associated with a reduction in risk. In the development of hypertension, the most relevant signal was provided by ABCG2 rs2622604, associated with increased risk, along with variants of NCF4 rs1883112, XRCC3 rs861539, GSTM3 rs36120609, and GSTM1 rs111436983.



## 5. Discussion

The advent of BTKi has radically redefined the therapeutic algorithm and long-term prognosis of CLL patients; however, the management of these therapies remains burdened by significant interindividual heterogeneity in terms of toxicity and clinical efficacy. The impact of the host's germline genetic variability on pharmacokinetic and pharmacodynamic processes remains a partially unexplored field. The rationale of this study is founded on the hypothesis that single nucleotide polymorphisms (SNPs) may modulate peripheral tissue susceptibility to BTKi, thereby explaining the differences in side effects observed in clinical practice among patients undergoing the same therapeutic regimen.

The study aimed to systematically evaluate the correlation between an SNP panel and the main clinical endpoints — represented by long-term therapeutic efficacy, expressed as PFS and OS, and by the safety profile, defined through the probability of developing clinically relevant adverse events. The methodology involved NGS sequencing of a targeted panel of 74 genes involved in the metabolic and functional pathways of the drugs under examination, applied to genomic DNA extracted from a cohort of 107 CLL patients. Statistically, survival analysis was conducted using Kaplan-Meier curves, while the association with organ toxicities was determined by association analysis.

The study population, characterized by a median age of 78.5 years and a male prevalence of 66.4%, accurately reflects the expected epidemiological characteristics of the disease. With a median follow-up of 3.8 years, the cohort showed a median PFS of 5.3 years and a median OS of 8.0 years, confirming the high overall efficacy of BTKi, represented predominantly by ibrutinib, followed by acalabrutinib and zanubrutinib. From the analysis of PFS correlations with patient genotype, a statistically significant association was identified between the presence of CYP2B6 cytochrome variants (rs3745274-G/T and rs2279343-A/G) and PFS prolongation, with a 5-year survival probability of 66.4% and 78.6%, respectively, compared to 42.1% and 45.6% in subjects without these variants, while a similar trend, albeit at the limits of statistical significance, was observed for the BRCA1 rs1799950 variant.

Regarding cardiac and vascular toxicity, against an atrial fibrillation incidence of 12.15%, logistic regression revealed a significant increase in risk in subjects carrying SNPs MGMT rs16906252, BRCA1 rs4986850, and TGFB1 rs1800471, while a protective effect against

arrhythmogenesis was documented for variants of ROCK2 rs10178332 and rs965665 and XRCC6 rs132788. In the development of arterial hypertension, found in 13.08% of cases, the strongest predictive signal was provided by the ABCG2 transporter variant rs2622604, in association with polymorphisms in the NCF4, XRCC3, GSTM3, and GSTM1 genes. Finally, regarding hemorrhagic diathesis, hemorrhagic manifestations — which affected 16.82% of the cohort — were strongly correlated with the genetic profile of membrane transporters, showing an increased risk in carriers of SNPs in the ABCB1 gene (rs1128503, rs2032582, rs1045642) and SULT1C4 rs1402467, balanced by significant protective effects for variants of GATA4 rs804280, TGFB1 rs1800469, and XPA rs1800975.

The obtained results are coherently integrated with literature data on BTKi pharmacokinetics, expanding their clinical value, given that BTK inhibitor drugs are known substrates of the hepatic cytochrome enzyme system and cellular efflux pumps such as P-glycoprotein, encoded by the ABCB1 gene. The finding of an increased hemorrhagic risk in patients carrying ABCB1 variants suggests that such polymorphisms may determine loss of function or reduced expression of the transporter, reducing drug efflux and leading to an increase in free plasma and tissue BTKi concentrations; this accumulation consequently amplifies the inhibitory effect on platelet signaling downstream of GPVI and CLEC-2 receptors, exacerbating hemorrhagic diathesis. Conversely, the protective effect on PFS observed for CYP2B6 variants finds a biological basis in the hypothesis of a slow or intermediate metabolizer phenotype, in which reduced metabolic clearance allows the maintenance of stable and therapeutically effective blood drug concentrations, optimizing selective pressure on the leukemic clone and delaying the emergence of resistant clones.

## 6. Conclusion

In conclusion, this work demonstrates that the host's profile represents a significant determinant in conditioning BTKi therapy in CLL patients, finding a precise molecular correlation between SNPs, therapeutic responses, and adverse effects. The future perspectives arising from this study reside in the need to validate these pharmacogenetic biomarkers within larger prospective multicentric cohorts, evaluating whether these SNPs maintain the same predictive value.

The clinical application of these findings aims at the implementation, within the diagnostic pathway of the CLL patient, of a pre-treatment pharmacogenetic screening, since the preventive identification of a genetic profile at high risk for severe toxicities could allow the clinician to personalize therapeutic choice with preventive dose modulations or alternative therapeutic strategies such as BCL2 inhibitors, concretely translating the paradigms of precision medicine into everyday hematological practice.

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