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IMMUNOLOGICAL AND CLINICAL EFFECTS OF BRUTON TYROSINE KINASE INHIBITORS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Immunological and Clinical Effects of Bruton Tyrosine Kinase Inhibitors in Chronic Lymphocytic Leukemia

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By

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Blod, svett och några tårar

Popular science summary of the thesis

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Western countries. It develops when abnormal B lymphocytes gradually accumulate in the blood, bone marrow, and lymph nodes. Many patients live with the disease for years, but CLL can gradually affect both general health and the immune system.

In recent years, treatment of CLL has improved greatly through drugs called Bruton tyrosine kinase inhibitors (BTKi), including ibrutinib, acalabrutinib, and zanubrutinib. These medicines can control the disease effectively, often for a long time. However, they are usually taken continuously, which means that side effects including those on the immune system become important.

This thesis examined how BTKi treatment affects patients with CLL, both clinically and biologically. The first study found that long-term treatment with ibrutinib in routine clinical practice was often associated with side effects such as heart rhythm disturbances, bleeding, dose reductions, and treatment discontinuation. The second study showed that repeated SARS-CoV-2 vaccination during zanubrutinib treatment led to limited antibody responses, while T-cell responses appeared to be better preserved. These findings support continued vaccination in this patient group. The third study found that treatment with acalabrutinib and zanubrutinib changed the T-cell compartment over time, although the immune system did not return fully to normal. The fourth study examined whether selected patients could stop and later restart ibrutinib treatment. It suggested that treatment interruption could be feasible in some patients and gave insight into which biological effects of treatment were reversible and which were not.

Taken together, the results show that BTKi treatment affects more than the leukemia itself. It is also associated with toxicity, changes in immune function, and broader biological effects over time. Better understanding of these effects may help improve future treatment strategies for patients with CLL.

Abstract

Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous B-cell malignancy in which B-cell receptor signaling and tumor–microenvironment interactions are central to disease pathogenesis. Covalent Bruton tyrosine kinase inhibitors (BTKis) have transformed CLL treatment and provide durable disease control, but are commonly given continuously, raising questions regarding toxicity, immune effects, and the biological consequences of long-term treatment.

The overall aim of this thesis was to investigate clinical and immunological effects of BTKi therapy in CLL. Four studies were performed. **Paper I** was a retrospective real-world study of 134 patients treated with ibrutinib. **Paper II** examined local and systemic immune responses to repeated SARS-CoV-2 vaccination in 9 zanubrutinib-treated patients with CLL, with comparator cohorts. **Paper III** was a prospective longitudinal immune-monitoring study of 34 patients treated with acalabrutinib or zanubrutinib. **Paper IV** was a prospective multicenter phase 1b/2 study of repeated response-guided interruption and re-initiation of ibrutinib in 49 patients, with longitudinal biological analyses.

In **Paper I**, atrial fibrillation (AF), bleeding events, dose reductions, and treatment discontinuation were common during long-term ibrutinib treatment, highlighting the burden of continuous exposure. In **Paper II**, repeated SARS-CoV-2 vaccination during zanubrutinib treatment was associated with increased serum antibody levels, but humoral and mucosal responses remained limited, whereas spike-specific T-cell responses appeared relatively preserved. In **Paper III**, both acalabrutinib and zanubrutinib were associated with reductions in abnormal T-cell expansion, exhaustion-associated phenotypes, and inflammatory plasma markers, although immune remodeling was incomplete and abnormalities remained evident, particularly in the CD8+ compartment. In **Paper IV**, repeated response-guided interruption of ibrutinib was feasible in a selected subgroup. The first off-treatment phase had a median duration of 17 months, most patients remained off treatment for more than 12 months, and nearly all patients responded to treatment re-initiation. Treatment withdrawal was accompanied by coordinated immunologic, proteomic, and CLL-intrinsic biological changes, but reversibility was incomplete and some alterations persisted despite interruption.

BTKi therapy in CLL has effects beyond tumor control. Continuous treatment is effective but associated with long-term toxicity. Immune effects during BTKi therapy are compartment-specific and incompletely reversible. Response-guided interruption may be feasible in selected patients and provides a model for studying BTKi-associated changes that are treatment-dependent and persist beyond active drug exposure.

List of scientific papers

I. **Andersson ML**, Johansson H, Österborg A, Mansson-Broberg A, Hansson L, Palma M.
Incidence of cardiovascular and bleeding events and reasons for discontinuation in patients with chronic lymphocytic leukemia treated with ibrutinib – A retrospective analysis on consecutive patients from a well-defined region. Eur J Hematol. 2023 Nov;111(5):748-756.

II. **Andersson ML***, Wu J*, Wullimann D, Gao Y, Aberg M, Muschiol S, Healy K, Naud S, Bogdanovic G, Palma M, Mellstedt H, Chen P, Ljunggren HG, Hansson L, Sallberg Chen M, Buggert M, Ingelman-Sundberg HM, Österborg A.
Local and systemic immunity during five vaccinations against SARS-CoV-2 in zanubrutinib-treated patients with chronic lymphocytic leukemia. J Hematol. 2023 Aug;12(4):170-175.
* Equal first-author contribution.

III. **Andersson ML**, Lind O, Heimersson K, Berglöf A, Del Peso Santos T, Peña-Pérez L, Wang Q, Mulder TA, Zain R, Månsson R, Rosenquist R, Smith CIE, Österborg A, Palma M.
T-cell immunomodulation occurs with different time kinetics during acalabrutinib and zanubrutinib therapy in chronic lymphocytic leukaemia. Br J Haematol. 2025 Dec 25. doi: 10.1111/bjh.70293. Epub ahead of print. PMID: 41449785.

IV. **Andersson ML**, Palma M, Hauenstein J, Frengen N, Peña-Pérez L, Berglöf A, Lind O, Heimersson K, Wang Q, Cidh Ronge M, Mulder TA, Zain R, Hansson L, Kättström M, Uddevik A, Flogegård M, del Peso Santos T, Österholm Corbascio C, Rosenquist R, Smith CIE*, Månsson R*, Österborg A*, Lundin J*.
Clinical, cellular and molecular effects of suspended and repeated (on-off) ibrutinib treatment in patients with chronic lymphocytic leukemia in sustained partial remission. Manuscript 2026.
* Equal last-author contribution

Contents

List of abbreviations

ACE2	angiotensin-converting enzyme 2
AIM	activation-induced marker
APRIL	a proliferation-inducing ligand
ATAC-seq	assay for transposase-accessible chromatin using sequencing
BAFF	B-cell activating factor
BCL2	B-cell lymphoma 2
BCR	B-cell receptor
BLNK	B-cell linker protein
BTK	Bruton tyrosine kinase
BTKi	Bruton tyrosine kinase inhibitor
CDR3	complementarity-determining region 3
CLL	chronic lymphocytic leukemia
CR	complete remission
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
ddPCR	droplet digital polymerase chain reaction
del(11q)	deletion of chromosome 11q
del(13q)	deletion of chromosome 13q
del(17p)	deletion of chromosome 17p
EGFR	epidermal growth factor receptor
FACS	fluorescence-activated cell sorting
FCR	fludarabine, cyclophosphamide, and rituximab
FDA	Food and Drug Administration
HER2	human epidermal growth factor receptor 2
IGHV	immunoglobulin heavy chain variable region
ITAM	immunoreceptor tyrosine-based activation motif
ITK	IL-2-inducible T-cell kinase
iwCLL	International Workshop on Chronic Lymphocytic Leukemia
JAK3	Janus kinase 3
mAb	monoclonal antibody
MBL	monoclonal B-cell lymphocytosis
mRNA	messenger RNA
MRD	measurable residual disease
M-CLL	IGHV-mutated chronic lymphocytic leukemia
NF-κB	nuclear factor kappa B
NLC	nurse-like cell

NK	natural killer
OFF1	first off-treatment phase
OFF2	second off-treatment phase
OS	overall survival
PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death protein 1
PEA	proximity extension assay
PFS	progression-free survival
PI3Kδ	phosphoinositide 3-kinase delta
PKC	protein kinase C
PLCG2	phospholipase C gamma 2
PR	partial remission
R/R	relapsed/refractory
RBD	receptor-binding domain
RNA-seq	RNA sequencing
RLK	resting lymphocyte kinase
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SHM	somatic hypermutation
SLL	small lymphocytic lymphoma
SYK	spleen tyrosine kinase
TEC	tyrosine kinase expressed in hepatocellular carcinoma
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TLS	tumor lysis syndrome
TME	tumor microenvironment
TNF	tumor necrosis factor
TP53	tumor protein p53
Tregs	regulatory T cells
U-CLL	IGHV-unmutated chronic lymphocytic leukemia
uMRD	undetectable measurable residual disease

1 Introduction

Chronic lymphocytic leukemia (CLL) is a biologically heterogeneous B-cell malignancy in which tumor-cell survival depends on both cell-intrinsic factors and interactions with the tumor microenvironment (TME). A central component of CLL biology is B-cell receptor (BCR) signaling, which promotes the survival, proliferation, and trafficking of CLL cells. Bruton tyrosine kinase (BTK) is a downstream kinase in this pathway, and BTK inhibition has become a cornerstone of CLL treatment.

Covalent BTK inhibitors (BTKi), including ibrutinib, acalabrutinib, and zanubrutinib, have shown high clinical efficacy in CLL and are administered as continuous treatment. Prolonged exposure therefore raises questions regarding toxicity, immune effects, and the long-term clinical and immunological consequences of BTKi therapy. Beyond their antileukemic effects, BTKi influence several aspects of host immunity and disease biology, but the full clinical and biological implications of these effects remain to be clarified. This includes treatment-related toxicity in routine clinical practice, antiviral immunity and vaccine responsiveness during therapy, longitudinal immune remodeling, and whether interruption of covalent BTKi treatment is clinically feasible and associated with reversible biological changes. This thesis was undertaken to address these questions through four complementary clinical and translational studies.

2 Background

2.1 Chronic lymphocytic leukemia – definition

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature clonal B cells in the bone marrow, peripheral blood, and lymphoid tissues. The diagnosis requires the presence of ≥5 × 10^9/L monoclonal B cells in the peripheral blood, typically expressing CD5, CD19, and CD23, together with weak expression of either κ or λ immunoglobulin light chains (1). If CLL cells are detected in lymph nodes and/or the bone marrow but the number of circulating monoclonal B cells in the peripheral blood is <5 × 10^9/L, the disease is classified as small lymphocytic lymphoma (SLL). CLL and SLL represent different manifestations of the same disease entity and are managed similarly.

2.2 Epidemiology and etiology

CLL is the most common leukemia in the Western world and primarily affects older individuals, with a median age at diagnosis of approximately 72 years. The disease occurs more frequently in men than in women. In Sweden, approximately 550–600 new cases are diagnosed each year, and the prevalence has increased during recent decades. In 2015, the prevalence was 52 per 100,000, and it is expected to rise further as more effective therapies prolong survival (2).

The etiology of CLL remains largely unknown. However, familial clustering has been observed, and first-degree relatives of patients with CLL have an approximately 8.5-fold increased risk of developing the disease compared with the general population (3).

2.3 Pathogenesis

2.3.1 Monoclonal B-cell lymphocytosis

Monoclonal B-cell lymphocytosis (MBL) is defined by the presence of monoclonal B cells with a CLL-like immunophenotype in the peripheral blood at levels below the diagnostic threshold for CLL (<5 × 10^9/L), in the absence of lymphadenopathy, organomegaly, or cytopenias (4). MBL is considered a precursor condition to CLL, and its prevalence increases with age, occurring in approximately 5–20% of individuals older than 60 years (5). It can further be divided into low-count MBL and high-count MBL, defined as <0.5 × 10^9/L and 0.5–5 × 10^9/L monoclonal B cells, respectively. High-count MBL shares biological

features with CLL and is more likely to progress to CLL, with a rate of approximately 1–2% per year (5-7).

2.3.2 Genetic alterations

The heterogeneity of CLL is largely driven by genetic features, including chromosomal abnormalities, IGHV mutational status, and recurrent driver mutations, which have important implications for prognosis, risk stratification, and treatment resistance.

2.3.2.1 Chromosomal abnormalities and somatic mutations

Recurrent chromosomal abnormalities are present in the majority of CLL cases, most commonly del(13q), trisomy 12, del(11q), and del(17p). Together with other biological and clinical characteristics, these lesions remain important prognostic markers, with del(17p) generally conferring the highest risk and isolated del(13q) the most favorable outcome (8).

TP53, a key tumor suppressor gene, is located on the short arm of chromosome 17. Consequently, del(17p) often involves loss of one TP53 allele. However, TP53 mutations may also occur in the absence of del(17p) and are likewise associated with an adverse prognosis (9, 10).

Next-generation sequencing has further identified recurrent mutations in genes involved in RNA splicing, DNA damage response, and B-cell-receptor, NOTCH, and NF-κB signaling, including *NOTCH1*, *ATM*, *SF3B1*, *XPO1*, *NFKBIE*, *POT1*, *BIRC3*, *MYD88*, and *EGR2* (11). Most of these mutations have been associated with a more aggressive clinical course, including shorter time to first treatment and inferior survival (12, 13). However, their prognostic impact appears to be context-dependent and may differ according to immunoglobulin heavy chain variable region (IGHV) mutational status (12).

2.3.2.2 IGHV mutational status and stereotyped B-cell receptors

The somatic hypermutation (SHM) status of the IGHV gene has both prognostic and predictive value in CLL. This difference may partly reflect the cell of origin. IGHV-mutated CLL (M-CLL) is thought to derive from post-germinal center memory B cells, whereas unmutated CLL (U-CLL) likely arises from naïve B cells. Unmutated CLL (U-CLL), defined by ≥98% sequence homology between the IGHV gene and the germline configuration, is associated with more aggressive disease biology and inferior outcome following chemoimmunotherapy. In the era

of targeted therapy, however, the prognostic impact of IGHV mutational status has become more treatment-dependent: continuous BTKi therapy appears to mitigate much of the adverse effect of U-CLL, whereas fixed-duration venetoclax-based regimens are still associated with shorter progression-free survival (PFS) in unmutated disease than in M-CLL (14-16).

Immunogenetic analyses have identified distinct groups of patients with CLL carrying highly similar, or stereotyped, B-cell receptor (BCR) immunoglobulins, representing approximately 30%–40% of cases (17, 18). These subsets are characterized by restricted immunoglobulin gene usage and highly similar sequence motifs, particularly within the complementarity-determining region 3 (CDR3), supporting a role for antigen selection in CLL pathogenesis (17). Several major stereotyped subsets have been associated with distinct biological and clinical features, including differences in disease course and treatment response. In particular, subset #2 has historically been linked to adverse prognosis and poor response to chemoimmunotherapy regardless of IGHV mutational status, whereas subset #8 has been associated with a more aggressive clinical course and an increased risk of Richter transformation (18-21). However, the predictive value of subset #2 in the era of targeted therapies remains to be established.

2.3.3 The B-cell receptor

B-cell receptor signaling is essential for the survival, proliferation, and migration of both normal and malignant B cells. The BCR consists of a membrane-bound immunoglobulin molecule associated with the signaling heterodimer Igα (CD79A) and Igβ (CD79B). Upon antigen binding, immunoreceptor tyrosine-based activation motifs (ITAMs) in CD79A/CD79B are phosphorylated by kinases such as spleen tyrosine kinase (SYK) and the LYN tyrosine kinase, initiating downstream signaling through B-cell linker protein (BLNK), Bruton tyrosine kinase (BTK), and phosphoinositide 3-kinase-δ (PI3Kδ). This results in calcium mobilization and activation of protein kinase C (PKC), the RAS–MAPK pathway, nuclear factor-κB (NF-κB), and PI3K–AKT signaling, thereby promoting B-cell survival and activation (Figure 1). The co-receptor CD19 further amplifies PI3K–AKT signaling (22).

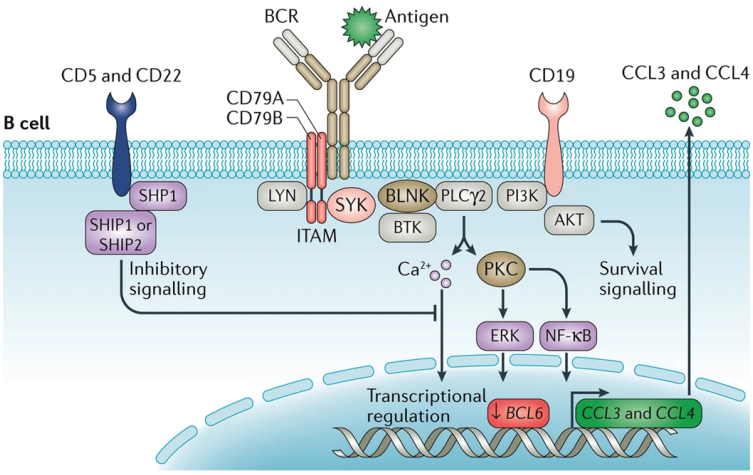

Figure 1. The B-cell receptor signaling pathway. Reproduced with permission from Burger JA, Wiestner A. Targeting B cell receptor signaling in cancer: preclinical and clinical advances. Nature Reviews Cancer. 2018;18:148–167. Springer Nature.

CLL cells can exhibit autonomous BCR signaling, i.e., independently of external antigen stimulation (23). In addition, antigen-dependent BCR signaling also appears to contribute to CLL pathogenesis. Although the exact antigens recognized by CLL BCRs remain incompletely defined, both self-antigens and exogenous antigens have been implicated. Activation of the BCR pathway promotes CLL cell survival and proliferation (22, 23).

2.3.4 Tumor microenvironment

The expansion and survival of CLL cells depend on a highly complex and multifaceted TME, including endothelial and mesenchymal stromal cells, monocytes/macrophages, natural killer (NK) cells, and T cells. When removed from this supportive microenvironment and cultured *in vitro* without stromal support, CLL cells rapidly undergo apoptosis (24, 25).

Although CLL is characterized by high numbers of malignant B cells in the peripheral blood, this compartment mainly contains resting tumor cells and plays a limited role in disease pathogenesis. In contrast, CLL cells home to secondary lymphoid tissues, particularly lymph nodes, where they receive critical microenvironmental signals that promote activation and proliferation (26, 27).

The migration and homing of CLL cells to lymphoid tissues are mediated in part by chemokines secreted by nurse-like cells, stromal cells, follicular dendritic cells, and high endothelial venules, including CXCL12, CXCL13, CCL19, and CCL21,

which bind to the corresponding receptors CXCR4, CXCR5, and CCR7 on CLL cells (28-30).

Within lymphoid tissues, CLL cells localize to proliferation centers, where they closely interact with nurse-like cells (NLCs), stromal cells, and T cells, which promote their retention, survival, and proliferation. NLCs express tumor necrosis factor (TNF) family ligands such as B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which provide survival signals through their corresponding receptors on CLL cells and activate NF-κB signaling (31). In addition, NLCs can enhance BCR signaling through antigenic and receptor-mediated interactions, thereby supporting CLL cell survival and proliferation (32). T cells in the bone marrow and lymph nodes are predominantly CD4+ helper T cells, and CD40–CD40L interactions between activated T cells and CLL cells further support proliferation and inhibit apoptosis (33).

2.4 Systemic immune defects

Immune dysregulation is a hallmark of CLL and is present from early stages of the disease. It affects both adaptive and innate immunity and involves impaired function of non-malignant B cells and plasma cells, quantitative and functional abnormalities in the T-cell compartment, and defects in other immune populations, including NK cells, dendritic cells, and myeloid cells. Clinically, these abnormalities contribute to increased susceptibility to infections, autoimmune complications, impaired vaccine responses, and a higher risk of second malignancies (34).

2.4.1 Humoral immune dysfunction

Hypogammaglobulinemia is one of the most common immune abnormalities in CLL and reflects impaired function of the non-malignant B-cell and plasma-cell compartments. It may be present at diagnosis but generally becomes more pronounced with disease progression (35). In addition to reduced immunoglobulin levels, qualitative defects in antibody production contribute to recurrent infections and poor responses to vaccination (36).

2.4.2 T-cell dysfunction

CLL is characterized by substantial alterations in the T-cell compartment, involving both quantitative imbalances and functional impairment. Along with the expansion of malignant B cells, both CD4+ helper T cells and, to an even greater extent, CD8+ cytotoxic T cells are increased in CLL. This results in an inverted

CD4:CD8 ratio, which has been associated with disease progression and shorter time to first treatment in untreated CLL (37). In parallel, there is a reduction in naïve T cells and an enrichment of antigen-experienced and highly differentiated memory T-cell subsets, suggesting chronic antigenic stimulation and ongoing immune dysregulation (38). T cells in CLL are not only quantitatively altered but also functionally impaired. They are often oligoclonal, indicating selection by restricted antigenic elements (39), and display defective immune synapse formation together with impaired cytokine production, degranulation, and cytotoxic activity (40, 41). In addition, both CD4+ and CD8+ T cells show features of exhaustion, including increased expression of inhibitory receptors such as programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (42-44).

At the same time, T cells may also support the leukemic clone. CD4+ helper T cells within the TME provide survival and growth signals to CLL cells, and in a xenograft model, autologous CD4+ T cells were required for efficient engraftment and clonal expansion of CLL cells (45). Thus, T cells in CLL appear to be both dysfunctional anti-tumor effectors and active contributors to the leukemic microenvironment (46).

Among CD4+ T-cell subsets, altered polarization has been reported, including expansion of regulatory T cells (Tregs) and Th17 cells, whereas data on Th1/Th2 dominance are less consistent (46).

2.5 Symptoms and indications for treatment

At the time of diagnosis, most patients with CLL are in an indolent, asymptomatic early phase of the disease. In this setting, treatment initiation is not beneficial, and patients are managed according to the “watch and wait” principle. Over time, however, approximately two-thirds of the patients will require treatment as the disease becomes active. According to the 2018 International Workshop on Chronic Lymphocytic Leukemia (iwCLL) guidelines, treatment should be initiated if at least one of the following criteria is met: bone marrow failure manifested by anemia and/or thrombocytopenia; massive and/or progressive lymphadenopathy or splenomegaly; autoimmune anemia and/or thrombocytopenia with poor response to standard therapy; constitutional symptoms, including unintentional weight loss, prolonged fever without evidence

of infection, and/or significant fatigue; or progressive lymphocytosis over a defined time period (47).

2.6 Principles of therapy

The therapeutic landscape of CLL has changed profoundly over the past decade, with a shift from chemoimmunotherapy to targeted therapies, primarily B-cell lymphoma 2 (BCL2) inhibitors and Bruton tyrosine kinase inhibitors (BTKis) (48-50). Current Swedish treatment recommendations for CLL are summarized in Figure 2.

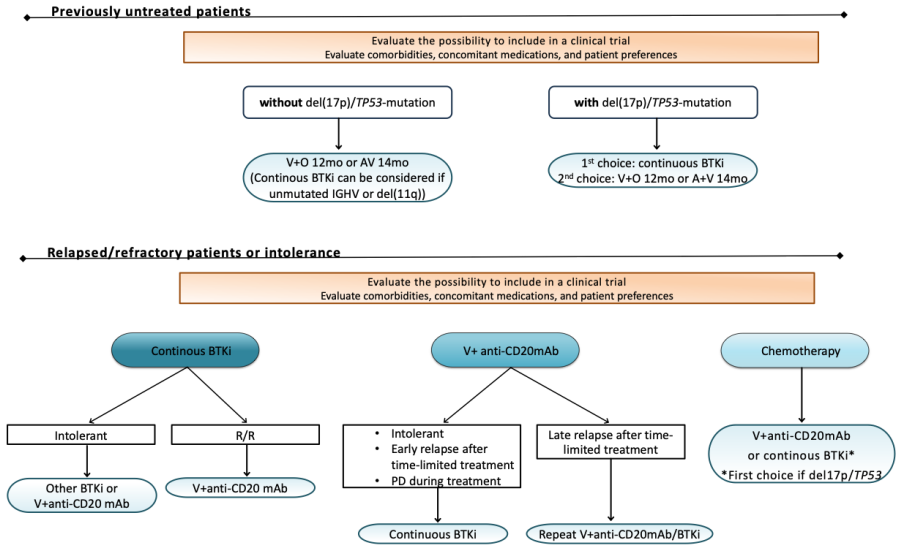

Figure 2. CLL treatment algorithm. Adapted from the national guidelines of the Swedish CLL Study Group, 2026. V, venetoclax; BTKi, Bruton tyrosine kinase inhibitor; A, acalabrutinib; mAb, monoclonal antibody; R/R, relapsed/refractory.

2.7 Venetoclax-based therapy

B-cell lymphoma 2 (BCL2) is overexpressed in CLL cells. Venetoclax, the only currently approved BCL2 inhibitor in CLL, selectively targets BCL2 and thereby induces apoptosis of CLL cells (51). Venetoclax was initially FDA-approved as monotherapy in 2016 based on a phase II study demonstrating substantial clinical activity in high-risk relapsed/refractory (R/R) patients with del(17p) (52). Subsequent studies established fixed-duration venetoclax-based combinations with anti-CD20 monoclonal antibodies (anti-CD20 mAb) and, more recently, BTKi as central treatment strategies in CLL.

2.7.1 Fixed-duration venetoclax in combination with anti-CD20 monoclonal antibodies

In the phase 3 CLL14 trial, fixed-duration venetoclax–obinutuzumab was established as a first-line option in previously untreated patients with durable remissions and sustained PFS on extended follow-up (53). Del(17p)/*TP53* mutation, unmutated IGHV, and lymph node size ≥5 cm were associated with shorter PFS (54). GAIA/CLL13 supported venetoclax–obinutuzumab-based treatment in fit patients and suggested superior efficacy for obinutuzumab- over rituximab-based venetoclax combinations in this setting (55). In R/R CLL, the phase 3 MURANO trial established fixed-duration venetoclax–rituximab as an effective regimen with durable long-term disease control (56). The most common toxicities of venetoclax-obinutuzumab treatment include neutropenia, infections, and infusion-related reactions to obinutuzumab (54, 55), although the regimen has generally been well tolerated in both fit and unfit patients (57). Early use of venetoclax treatment was associated with a risk of tumor lysis syndrome (TLS), particularly during treatment initiation, which led to the introduction of a stepwise dose ramp-up with strict prophylaxis and monitoring (58). In venetoclax–obinutuzumab regimens, obinutuzumab is administered before venetoclax initiation to achieve tumor debulking and thereby reduce TLS risk (59). Although this sequencing strategy may lower the risk of TLS, it may also increase the likelihood of infusion-related reactions in patients with a high tumor burden.

2.8 Covalent Bruton tyrosine kinase inhibitors

BTK is a key component of the downstream BCR signaling pathway. In CLL, BTK is overexpressed relative to normal B cells and plays an important role in survival, proliferation, and migration of the leukemic clone (60). These observations provided the rationale for the clinical development of BTKis. Covalent BTKis bind irreversibly to cysteine 481 within the ATP-binding pocket of BTK, thereby suppressing downstream BCR signaling (60-62).

The covalent BTKis currently approved for CLL are ibrutinib, acalabrutinib, and zanubrutinib. These agents are administered orally and have predominantly been used as continuous therapy until unacceptable toxicity or disease progression, although fixed-duration combination strategies have recently entered clinical practice. Compared with the first-in-class ibrutinib, acalabrutinib and zanubrutinib were developed to achieve greater selectivity for BTK and less off-target kinase inhibition, which may contribute to differences in adverse-event

profiles, treatment tolerability and possibly efficacy. In addition to BTK, ibrutinib inhibits several other kinases, including TEC family kinases (e.g., ITK, BMX and TEC), human epidermal growth factor receptor 2 (HER2), Janus kinase 3 (JAK3), and epidermal growth factor receptor (EGFR) (63). In contrast, acalabrutinib and zanubrutinib were designed to reduce off-target kinase inhibition, although their selectivity profiles are not identical (64, 65).

2.8.1 Efficacy

2.8.1.1 Ibrutinib

Ibrutinib, the first-in-class covalent BTKi, established BTK inhibition as an effective therapeutic strategy in CLL. In previously treated disease, the phase 3 RESONATE trial compared single-agent ibrutinib with ofatumumab in patients with R/R CLL/SLL, most of whom had high-risk genomic features, and demonstrated a marked and durable PFS benefit for ibrutinib. In the final analysis, with a median follow-up of 65.3 months, median PFS was 44.1 months with ibrutinib compared with 8.1 months with ofatumumab (66, 67). In the final analysis of RESONATE-2, with a median follow-up of 9.6 years in the ibrutinib arm, median PFS was 8.9 years with ibrutinib compared with 1.3 years with chlorambucil. A substantial benefit was maintained in patients with high-risk features, including unmutated IGHV, del(11q), *TP53* mutation, or complex karyotype, in whom median PFS was 8.4 years versus 0.7 years, respectively (68, 69). However, the use of chlorambucil as the control arm is questionable, and comparison with more effective standard regimens was warranted.

In the ALLIANCE trial, patients aged 65 years or older were assigned to ibrutinib, ibrutinib plus rituximab, or bendamustine–rituximab (BR). Both ibrutinib-containing arms prolonged PFS compared with BR, whereas the addition of rituximab did not confer further benefit. With longer follow-up, median PFS was 44 months with BR but was not reached in either ibrutinib-containing arm, and the corresponding 48-month PFS rates were 47% with BR and 76% in both ibrutinib-containing arms. This benefit was maintained irrespective of IGHV status and was also observed in patients with TP53 abnormalities, del(11q), and complex karyotype (70, 71).

In younger, fit patients, ibrutinib–rituximab was subsequently compared with fludarabine, cyclophosphamide, and rituximab (FCR) in the phase 3 E1912 and in the UK FLAIR trial. In E1912, ibrutinib–rituximab improved both PFS and overall

survival (OS) compared with FCR on long-term follow-up (72, 73). Similarly, in UK FLAIR, ibrutinib–rituximab prolonged PFS relative to FCR (74).

2.8.1.2 Acalabrutinib

Acalabrutinib is a second-generation covalent BTKi developed to achieve greater selectivity for BTK and less off-target kinase inhibition than ibrutinib. In the phase 3 ELEVATE-TN trial, treatment-naïve patients were randomized to acalabrutinib plus obinutuzumab, acalabrutinib monotherapy, or obinutuzumab plus chlorambucil. At 6 years of follow-up, both acalabrutinib-containing arms showed markedly prolonged PFS compared with obinutuzumab plus chlorambucil, with 72-month PFS rates of 78.0%, 61.5%, and 17.2%, respectively. OS was significantly longer with acalabrutinib–obinutuzumab, but not with acalabrutinib monotherapy, compared with obinutuzumab plus chlorambucil. In a post hoc analysis, the addition of obinutuzumab was associated with longer PFS and deeper responses compared with acalabrutinib alone, without a significant difference in OS (75, 76). In R/R disease, the phase 3 ASCEND trial showed superior PFS for acalabrutinib compared with investigator's choice of idelalisib–rituximab or bendamustine–rituximab and this benefit was maintained in the final analysis (77, 78).

Importantly, acalabrutinib was further evaluated head-to-head against ibrutinib in the phase 3 ELEVATE-RR trial, which enrolled previously treated patients with del(17p) or del(11q). Median PFS was identical in both arms at 38.4 months, establishing non-inferior efficacy for acalabrutinib. Its more favorable tolerability profile relative to ibrutinib is discussed below (79).

2.8.1.3 Zanubrutinib

Zanubrutinib, also a second-generation BTKi, exerts a higher selectivity for BTK and has fewer off-target effects than ibrutinib (65). In the phase 3 SEQUOIA trial, treatment-naïve patients without del(17p) were randomized to zanubrutinib or bendamustine–rituximab. In the 5-year follow-up analysis, median PFS was not reached with zanubrutinib and was 44.1 months with bendamustine–rituximab, with estimated 60-month PFS rates of 75.8% and 40.1%, respectively. Benefit was observed in both mutated and unmutated IGHV subgroups (80). Zanubrutinib was further evaluated head-to-head against ibrutinib in the phase 3 ALPINE trial in R/R CLL (81). In the final comparative analysis, after a median follow-up of 42.5 months, zanubrutinib maintained superior PFS compared with ibrutinib including in patients with del(17p) and/or TP53 mutation (82).

2.8.1.4 BTKi–venetoclax (± anti-CD20 antibody) combination therapy

Fixed-duration treatment strategies combining a covalent BTKi with venetoclax have emerged as an important therapeutic approach in CLL, aiming to integrate BTKi-mediated tumor debulking and redistribution from nodal compartments with the deep remissions achieved by venetoclax (56, 83). In practice-defining doublet studies, GLOW established fixed-duration ibrutinib–venetoclax as an effective first-line regimen in older and/or comorbid patients (56, 84), while CAPTIVATE demonstrated high rates of undetectable measurable residual disease (uMRD) and durable remissions in younger patients (85, 86). FLAIR extended this concept by applying MRD-guided ibrutinib–venetoclax and showed superior PFS, with an OS signal, compared with FCR (87). More recently, AMPLIFY showed that fixed-duration acalabrutinib–venetoclax, with or without obinutuzumab, significantly prolonged PFS relative to chemoimmunotherapy, thereby extending this strategy to second-generation BTKi-based combinations (88). Triplet regimens incorporating an anti-CD20 antibody have generally produced deeper remissions and higher uMRD rates, as shown in CLL2-GIVe and in the phase 2 AVO study, although a consistent long-term survival advantage over doublets has not yet been established (89, 90). Importantly, MRD has become a central endpoint in this field and is increasingly used to support treatment cessation, but it is not yet fully validated as a surrogate endpoint for long-term PFS across different BTKi–venetoclax platforms (87, 91). Early retreatment data are encouraging, but remain too limited to define standard retreatment strategies after prior fixed-duration BTKi–venetoclax therapy (92).

2.8.2 Toxicity

Although covalent BTKi have markedly improved the clinical outcome of patients with CLL, toxicity remains an important limitation and may lead to treatment discontinuation, particularly during long-term continuous therapy. This has been most evident with ibrutinib, for which adverse-event-related discontinuation rates of approximately 9–23% have been reported in clinical trials and long-term follow-up studies. The most clinically relevant toxicities of ibrutinib include atrial fibrillation (AF)/flutter, hypertension, bleeding, diarrhea, arthralgia or myalgia, rash, cytopenias, and infections (67, 69). Cardiovascular toxicity represents one of the clearest differentiators between first- and second-generation covalent BTKi. In long-term studies of ibrutinib, AF and hypertension have been reported in up to approximately 16% and 26% of patients, respectively, and the risk of AF appears to be higher in older patients and in those with pre-existing

cardiovascular comorbidity (67, 69). In the randomized ELEVATE-RR trial, acalabrutinib was associated with lower rates of AF/flutter, hypertension, and bleeding than ibrutinib; similarly, in the final ALPINE analysis, zanubrutinib showed lower rates of AF/flutter and fewer serious cardiac events than ibrutinib (79, 82, 93). Experimental work suggests that ibrutinib-associated AF is likely to be related primarily to off-target inhibition of C-terminal Src kinase rather than BTK itself, whereas the mechanism underlying BTKi-associated hypertension remains less clearly defined (94).

Bleeding events are also common during BTKi therapy. Most are low-grade mucocutaneous events, whereas major bleeding is less frequent but remains clinically relevant. Mechanistically, impaired platelet activation provides the most plausible explanation, as BTK and TEC family kinases contribute to signaling downstream of platelet transmembrane receptors, including glycoprotein VI and pathways involved in von Willebrand factor-dependent platelet adhesion (95). Major bleeding appears to occur at broadly similar rates across the covalent BTK inhibitors in head-to-head trials, despite clearer differences in cardiovascular toxicity (82, 93).

Other toxicity differences appear more agent-specific. Headache is reported more frequently with acalabrutinib, whereas neutropenia appears more prominent with zanubrutinib. By contrast, rates of serious infection do not appear to differ as clearly between agents as rates of cardiovascular toxicity, suggesting that infection risk is likely to remain multifactorial and influenced not only by kinase selectivity, but also by underlying immune dysfunction, prior treatment, and treatment-related cytopenias (93, 96). Overall, second-generation BTKi appear to retain efficacy while improving tolerability relative to ibrutinib, particularly with respect to cardiovascular adverse events, although cumulative toxicity remains an important concern during continuous treatment.

Overall, second-generation BTKis are associated with fewer treatment discontinuations due to adverse events than ibrutinib, together with lower rates of key cardiovascular toxicities and maintained efficacy (79, 81). However, continuous treatment with all BTKis remains a concern because of the cumulative risk of adverse effects.

BTKi-based combination therapy shifts rather than eliminates the toxicity profile. In fixed-duration BTKi–venetoclax regimens, BTKi lead-in reduces tumor burden and lowers the proportion of patients at high risk of TLS before venetoclax

introduction, while the toxicity profile shifts towards neutropenia and infections. In CAPTIVATE, an ibrutinib lead-in substantially reduced TLS risk before venetoclax ramp-up, whereas neutropenia was the most frequent grade ≥3 adverse event during combination therapy (85, 86). In AMPLIFY, grade ≥3 neutropenia was more frequent in the acalabrutinib–venetoclax–obinutuzumab triplet than in the doublet, illustrating that the addition of an anti-CD20 antibody may deepen remissions but at the cost of greater hematologic toxicity (88). Similar patterns have been reported in triplet studies such as CLL2-GIVe and AVO, in which cytopenias and infections were among the dominant high-grade adverse events, whereas major cardiovascular toxicity was less prominent (89, 90). Thus, a key trade-off of fixed-duration combination therapy may be reduced cumulative exposure to BTKi-associated long-term toxicity, but with greater short-term hematologic and immunologic toxicity during active treatment.

2.8.3 Resistance

Although BTKi induce durable remissions in most patients with CLL, progressive disease still develops in a subset during long-term therapy. Because ibrutinib, acalabrutinib, and zanubrutinib all bind covalently to the same cysteine-481 residue in BTK, on-target substitutions affecting this binding site remain the dominant molecular mechanism of resistance, most commonly *BTK* C481S, although other C481 substitutions have also been described (97). The second major class of resistance lesions comprises *PLCG2* gain-of-function mutations, which restore downstream signaling despite BTK inhibition (97, 98). Less frequent or emerging abnormalities, including non-C481 *BTK* mutations such as T474I, L528W, A428D, and E41V, as well as cooperating lesions outside the BTK–PLCG2 axis, have also been reported, but their prevalence and functional significance remain less well established than those of *BTK* C481 and *PLCG2* mutations (99, 100).

Clinically, resistance may present either as progressive CLL or as Richter transformation. In patients relapsing on ibrutinib, *BTK* and/or *PLCG2*

mutations can be identified in the majority, but not all, cases; with sensitive molecular testing, such lesions have been reported in approximately 65% of progressing patients, while around one-third remain mutation-negative (98, 100). Serial analyses have shown that resistance is often subclonal and branched, with multiple competing mutant populations emerging under treatment pressure, and

that resistance-associated mutations may be detectable months before overt clinical progression. In one longitudinal study, high-sensitivity testing identified such mutations up to 15 months before relapse became clinically apparent (98). These observations suggest that clinical progression often represents the result of a prolonged evolutionary process rather than a single late mutational event.

Available evidence does not suggest that acalabrutinib or zanubrutinib differ fundamentally from ibrutinib regarding the core biology of acquired resistance. In ELEVATE-RR, progression on acalabrutinib remained characterized predominantly by acquired B-cell receptor pathway mutations, again centered on *BTK* and *PLCG2*, supporting the view that second-generation BTKi share the same dominant resistance biology despite improved selectivity and tolerability (99). By contrast, relapse after fixed-duration BTKi–venetoclax may follow a different biological trajectory. In the molecular analysis of CAPTIVATE, no previously recognized resistance-associated *BTK, PLCG2,* or *BCL2* mutations were identified in evaluable patients relapsing after fixed-duration ibrutinib–venetoclax (101). Thus, classical BTK-mediated resistance appears to be a defining feature of continuous BTKi exposure, whereas relapse after time-limited BTKi-based combination therapy may be biologically distinct and may have different implications for subsequent treatment sequencing.

2.8.4 T-cell changes

All three approved covalent BTKis reshape and partially restore the T-cell compartment in patients with CLL. IL-2-inducible T-cell kinase (ITK) is a member of the TEC family tyrosine kinases. It is expressed on T-cell lineages and plays an important role in the development and function of T cells through downstream T-cell receptor signaling (102). Compared with acalabrutinib and zanubrutinib, ibrutinib inhibits ITK to a greater extent; acalabrutinib exerts minimal or no inhibition, whereas zanubrutinib exerts less inhibition of ITK. It is unclear to what extent T-cell changes are related to the inhibition of ITK rather than to the reduction of the tumor burden and therefore of the CLL-related immunosuppression.

Changes in the immune cell populations during treatment with ibrutinib have been extensively studied. However, the effects of the second-generation BTKis, especially zanubrutinib, have been less extensively investigated. In particular, long-term data are lacking.

The increased levels of CD4+ and CD8+ T cells seen in patients with CLL decrease during treatment with ibrutinib and normalize to levels similar to those of healthy controls within 6–12 months and are maintained at 4–5 years follow-up (103-107). A decrease in T-cell numbers has also been observed in patients treated with acalabrutinib (108). At 6 months of follow-up, no changes in CD4+ and CD8+ T cells were seen with zanubrutinib (109). All three covalent BTKis appear to partially reverse features of T-cell exhaustion, as the proportion of T cells expressing checkpoint inhibitors such as PD-1 and CTLA-4 decreases during treatment (103, 109).

A reduction in both Th1 and Th2 helper T-cell subsets has been observed in patients treated with ibrutinib (106). The balance between Th1 and Th2 might be affected by ITK inhibition. *In vitro* studies have shown that Th2 differentiation depends on ITK while Th1 cells express resting lymphocyte kinase (RLK) which can provide compensatory signaling for activation and proliferation of Th1 and CD8+ cells (96). Further, when isolating CD4+ cells from CLL patients and exposing them to ibrutinib, they were skewed towards a molecular and phenotypic Th1 profile (96). A reduction of Th2-associated cytokines in plasma from ibrutinib-treated patients has been observed; however, reduction of Th1-associated cytokines has also been reported (103, 104, 110). A shift toward Th1 may favor anti-tumor and anti-viral immunity.

ITK also regulates Th17 versus Treg differentiation. *In vitro*, ibrutinib suppresses differentiation of CD4+ cells from healthy donors into Th17 cells. A reduction in elevated Th17 cells and associated cytokines as well as Tregs in patients with CLL have been observed during treatment (104, 110).

Beyond their direct anti-leukemic activity, BTKis also exert broader immunomodulatory effects. These include changes in T-cell number, phenotype, and functional exhaustion, and may also influence antiviral immune responses. However, the extent and kinetics of these effects may differ between individual BTKis and may also depend on treatment duration, disease burden, and prior therapy.

2.9 Non-covalent BTK inhibitors and BTK degraders

In addition to covalent BTKis, other forms of BTK-directed therapy are emerging in CLL. Non-covalent BTKis bind reversibly to BTK and do not depend on the C481 residue for target engagement, which allows activity in disease that has

become resistant to covalent BTKis through C481 mutations. Pirtobrutinib is the most clinically advanced agent in this class and has shown clinically meaningful activity in relapsed/refractory CLL after prior covalent BTKi exposure; it is now approved in Europe for relapsed or refractory CLL after prior BTKi treatment (111, 112). BTK degraders represent a distinct strategy in which BTK is targeted for proteasomal degradation rather than catalytic inhibition. Early-phase studies of agents such as BGB-16673 and bexobrutideg (NX-5948) have shown preliminary clinical activity in heavily pretreated CLL, including patients with prior BTKi exposure, but their place in therapy remains uncertain and current clinical data are still preliminary (113, 114).

2.10 COVID-19 and CLL

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus infection was declared a pandemic in 2020. The prevalence of SARS-CoV-2 positivity in patients with CLL is not higher than in the general population (115), though the risk of severe disease and mortality was dramatically increased in the first wave (116). In addition, humoral and cellular responses to SARS-CoV-2 vaccines are impaired (117-119). Seroconversion after messenger RNA (mRNA) vaccination against SARS-CoV-2 is particularly low in patients with CLL treated with either BTKis or venetoclax ± anti-CD20 antibody (119).

With regard to the vaccine-induced T-cell responses, these were observed in 28% of patients with CLL compared to 59% of healthy donors after 2 doses of mRNA vaccine (120). However, in a cohort of 29 patients with a history of COVID-19 who received three consecutive mRNA vaccinations, robust spike-specific CD8+ T-cell responses were observed, indicating that hybrid immunity, i.e. resulting from both infection and vaccination, is advantageous (121). Both in healthy individuals and in patients with CLL, memory T cells continue to provide cell-mediated immune recognition even of highly mutated emerging variants, hopefully compensating for the reduced serological responses observed in CLL (122).

3 Research aims

The overall aim of this thesis was to investigate the clinical and immunological effects of BTKi therapy in CLL. The included studies address treatment-related toxicity, antiviral immunity, T-cell immunomodulation, and the clinical and biological consequences of treatment interruption.

Paper I aimed to characterize the incidence of cardiovascular and bleeding events during long-term ibrutinib treatment in routine clinical practice, and to analyze treatment discontinuation, dose reduction, and clinical factors associated with AF.

Paper II aimed to investigate local and systemic immune responses to repeated SARS-CoV-2 vaccination in zanubrutinib-treated patients with CLL, with particular focus on humoral and T-cell-mediated immunity, and with comparison to ibrutinib-treated patients and healthy controls.

Paper III aimed to evaluate longitudinal T-cell immunomodulation during treatment with acalabrutinib and zanubrutinib, including changes in T-cell subset distribution, memory differentiation, exhaustion marker expression, and selected plasma biomarkers.

Paper IV aimed to assess the clinical feasibility of repeated response-guided interruption and re-initiation of ibrutinib therapy, and to investigate how suspended and resumed BTKi treatment affects immune cell subsets, plasma proteins, resistance-associated BTK mutations, and CLL-intrinsic transcriptional and epigenetic programs.

4 Materials and methods

4.1 Study design and study populations

This thesis is based on four studies investigating BTKi therapy in CLL using complementary clinical and translational approaches. The included studies comprised one retrospective real-world cohort study, two prospective longitudinal translational studies, and one prospective investigator-initiated multicenter phase 1b/2 trial.

Paper I included 134 consecutive patients with CLL treated with ibrutinib at Karolinska University Hospital between 2014 and 2021. Patients had received ibrutinib for at least 1 month, and follow-up continued until December 2022. Clinical data were collected retrospectively from medical records.

Paper II was a prospective study of SARS-CoV-2 vaccine responses in 9 zanubrutinib-treated patients with CLL. For comparison, 7 ibrutinib-treated patients and 7 age-matched healthy controls were analyzed after vaccine dose five.

Paper III was a prospective longitudinal immune-monitoring study including 34 patients with CLL, of whom 18 received acalabrutinib and 16 zanubrutinib. Age- and sex-matched healthy donors were recruited as controls, with 9 and 8 controls for the acalabrutinib and zanubrutinib groups, respectively.

Paper IV was an investigator-initiated multicenter prospective single-arm phase 1b/2 study of response-guided interruption and re-initiation of ibrutinib therapy in patients with CLL who had achieved at least partial remission during routine-care treatment. Forty-nine patients were enrolled across four Swedish centers.

4.2 Clinical data collection and endpoints

In **Paper I**, retrospective chart review was used to collect data on cardiovascular events, bleeding complications, dose reductions, treatment discontinuation, PFS, OS and time to treatment failure. Comorbidity burden was assessed using the Cumulative Illness Rating Scale.

In **Paper IV**, the major clinical objective was to evaluate the feasibility of repeated response-guided BTKi interruption. Patients discontinued ongoing ibrutinib treatment and were monitored off therapy until early asymptomatic progressive disease, at which point BTKi treatment was restarted. Time to first

BTKi re-initiation, response after restart, and outcomes during repeated on-off cycles were evaluated.

4.3 Sample collection and longitudinal follow-up

Paper II included longitudinal blood and saliva sampling before vaccine doses 3 and 5 and 2–3 weeks after doses 3, 4, and 5. Control cohorts were sampled after dose 5.

In **Paper III**, peripheral blood samples were collected at baseline and serially during treatment to allow longitudinal assessment of immune-cell dynamics and circulating biomarkers.

In **Paper IV**, peripheral blood samples were collected immediately before first treatment interruption, during the first off-treatment period, before treatment restart, and at later protocol-defined on- and off-treatment time points. When available, pre-treatment samples obtained before initial ibrutinib exposure were also analyzed. The study design and longitudinal sample collection are shown in Figure 3.

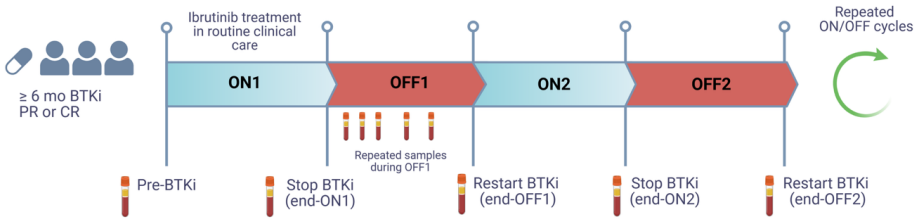

Figure 3. Study design and longitudinal sample collection in **Paper IV**, the response-guided on-off ibrutinib study. Abbreviations: BTKi, Bruton tyrosine kinase inhibitor; PR, partial remission; CR, complete remission.

4.4 Flow cytometry

Flow cytometry was a central analytical method in **Papers III** and **IV**. In these studies, peripheral blood immune cell subsets were analyzed longitudinally during treatment and treatment interruption. The analyses included major T-cell populations, differentiation states, and selected activation- or exhaustion-associated markers. In both studies, proliferative and resting CLL fractions were also assessed based on differential CD5 and CXCR4 expression. In **Paper III**, some flow cytometric analyses differed between the treatment cohorts, with zanubrutinib samples analyzed using PBMCs and acalabrutinib samples analyzed in whole blood.

4.5 Serological and cellular vaccine-response analyses

Paper II focused on vaccine-induced systemic, mucosal, and cellular immunity. Quantitative analyses of SARS-CoV-2-specific antibodies were performed in serum, including antibodies against the spike receptor-binding domain and nucleocapsid. Additional analyses of spike-specific IgG, spike-specific IgA, and ACE2 inhibition capacity were performed in both serum and saliva. T-cell responses were assessed in PBMCs using an activation-induced marker assay after stimulation with viral peptides.

4.6 Plasma protein profiling

Plasma biomarker profiling was performed in **Papers III and IV** using proximity extension assay (PEA; Olink). **In Paper III**, this was used to evaluate longitudinal changes in inflammatory and immune-related plasma proteins during treatment with acalabrutinib and zanubrutinib. In **Paper IV**, serial plasma samples from pre-treatment, on-treatment, and off-treatment phases were analyzed using panels covering inflammation, immune response, and oncology-related proteins.

4.7 BTK mutation analyses

Papers III and IV included analyses of acquired *BTK* resistance-associated mutations. In both studies, hotspot *BTK* mutations were analyzed in genomic DNA extracted from PBMCs using droplet digital PCR. In **Paper III**, the analyses included *BTK* p.C481S c.1442G>C, *BTK* p.C481S c.1441T>A, and *BTK* p.L528W c.1583T>G, whereas **Paper IV** included the hotspot *BTK* p.C481S-encoding mutations c.1442G>C and c.1441T>A. The assay sensitivity was 0.025%.

4.8 Cell sorting and sequencing analyses

In **Paper IV**, sequencing-based analyses were performed. Fluorescence-activated cell sorting was used to isolate highly enriched CLL cell populations from cryopreserved PBMCs. The sorted cells were subsequently analyzed by RNA sequencing and assay for transposase-accessible chromatin using sequencing (ATAC-seq) to investigate transcriptional and epigenetic changes associated with BTKi exposure and treatment interruption.

4.9 Statistical analyses

Statistical analyses were adapted to the design of each study. In **Paper I**, descriptive statistics, group comparisons, Kaplan–Meier estimates and

competing-risk regression were used. In **Papers II and III**, non-parametric methods were applied for longitudinal and between-group comparisons. In **Paper IV**, time to first BTKi re-initiation was analyzed using Kaplan–Meier estimates, log-rank tests, and Cox proportional hazards models, and plasma proteomic data were analyzed using Wilcoxon signed-rank tests with correction for multiple testing. For RNA-seq and ATAC-seq analyses, differential expression and differential accessibility analyses were performed using DESeq2, with patient as a covariate.

4.10 Ethical considerations

All studies included in this thesis were approved by the Swedish Ethical Review Authority and conducted in accordance with the Declaration of Helsinki. For the interventional study of intermittent ibrutinib treatment, the protocol further stated compliance with ICH Good Clinical Practice guidelines and required ethics approval before study initiation and before implementation of protocol amendments.

The prospective parts of this work involved both patients with CLL and healthy controls. Participants received oral and written study information describing the purpose of the research, study procedures, and possible risks and benefits. Participation was voluntary, and participants were informed of their right to withdraw at any time. Written informed consent was obtained before any study-related procedures were performed.

Papers II–IV included repeated blood sampling and, in **Paper II**, also saliva sampling. These procedures involve limited but relevant burdens, primarily discomfort, pain, bruising, and the inconvenience associated with additional study visits. To reduce this burden, blood volumes were limited and sampling was coordinated, when possible, with routine clinical visits. In the interventional on-off ibrutinib study, participants were monitored closely during off-treatment periods through scheduled visits, laboratory assessments, and adverse-event reporting, reflecting the need for careful safety surveillance when evaluating treatment interruption.

The studies also involved handling of personal and sensitive health data, including information retrieved from medical records. Participant confidentiality was protected through coded data handling, with restricted access to the code key. The study protocol for **Paper IV** further specified protection of subject

privacy in accordance with local regulations and described requirements for secure retention of study records and source documentation.

Particular ethical attention was required for **Paper IV**, which evaluated response-guided interruption and re-initiation of ibrutinib. Although all patients had already achieved a sustained partial remission on treatment, treatment interruption could theoretically involve risks such as disease progression, rebound phenomena, or delayed detection of intolerance or resistance. For that reason, the protocol defined eligibility criteria, close follow-up, adverse-event reporting procedures, and safety oversight, including reporting of serious adverse events and notification of new significant risks to the ethics committee and regulatory authorities when required. In addition, an early prespecified interim analysis was conducted to assess the initial safety and clinical feasibility of the first off-treatment phase before further enrolment.

4.11 Ethical approvals

Paper I:
2013/952-31/3, amendments: 2015/1025-32, 2021-04660.

Papers II and III:
00-135, amendment: 2010/1479-32
00-138, amendment: 2010/1478-32, 2016/2506-32, 2018/2565-32, 2019-05661, 2021-01107, 2023-07585-02.
2018/76-32
2019/01902
Paper IV:
2017/1297-31/2, amendments: 2018/265-32, 2019-00916, 2021-00731, 2021-01198.

5 Results

5.1 Paper I

Paper I examined cardiovascular and bleeding events, treatment modifications, and clinical outcomes in a real-world cohort of 134 patients with CLL treated with ibrutinib. Baseline cardiovascular comorbidity was common.

Among patients without prior AF, 24.5% developed AF during treatment. The cumulative incidence was 11.9% at 1 year, 14.4% at 2 years, and 22.7% at 5 years, with most cases occurring during the first year of treatment (Figure 4).

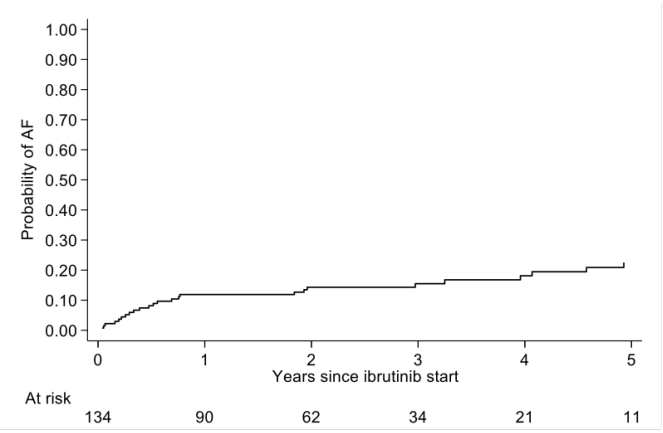

Figure 4. Cumulative incidence of atrial fibrillation (AF) during ibrutinib treatment. Estimated cumulative incidence (95% CI), adjusted for competing risks including toxicity, allogeneic stem cell transplantation, death, and other events.

Bleeding events were frequent and occurred in 66.4% of patients, although most were grade 1–2. Grade 3–4 bleeding was observed in 7.4%. Treatment modification was also common: 47% of patients underwent at least one dose reduction and 68% permanently discontinued ibrutinib, most often because of toxicity.

Clinical outcomes differed by treatment setting. In first-line treatment, median PFS and OS were not reached. In the relapsed/refractory setting, median PFS was 3 years and median OS 5 years.

5.2 Paper II

Paper II investigated systemic, mucosal, and cellular immune responses to repeated SARS-CoV-2 vaccination in zanubrutinib-treated patients with CLL,

with comparison to ibrutinib-treated patients and age-matched healthy controls. Nine zanubrutinib-treated patients were sampled longitudinally before and after vaccine doses 3 to 5, while the comparator cohorts were analyzed after dose 5. All zanubrutinib-treated patients were seronegative before dose 3, and the median treatment duration before the first vaccination was 28 months.

Repeated vaccination was associated with increased serum spike-specific antibody levels in zanubrutinib-treated patients. Compared with baseline, total spike-specific antibody titers increased significantly after doses 3, 4, and 5. However, 44% of patients remained seronegative after dose 3, 50% after dose 4, and 43% after dose 5. After dose 5, median total spike-specific antibody levels were 2.7 U/mL in zanubrutinib-treated patients, compared with 21,648 U/mL in healthy controls. Antibody levels in ibrutinib-treated patients were also significantly lower than in healthy controls (Figure 5).

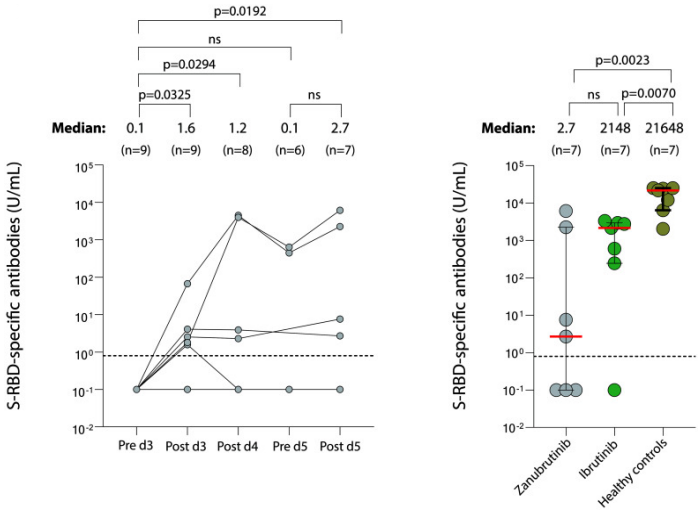

Figure 5. Serum antibody responses after repeated SARS-CoV-2 vaccination.
Antibodies against the SARS-CoV-2 spike receptor-binding domain (RBD) were measured longitudinally in zanubrutinib-treated patients and after dose 5 in ibrutinib-treated patients and healthy controls.

Functional humoral responses were also limited. In zanubrutinib-treated patients, serum ACE2 inhibition correlated strongly with spike-specific IgG levels, indicating that detectable antibodies were functional. In contrast, mucosal immunity remained poor. Salivary spike-specific IgG increased modestly, but spike-specific IgA remained unchanged in both serum and saliva, whereas healthy controls exhibited higher serum IgA levels after dose 5.

T-cell responses were better preserved than humoral responses. No significant differences in spike-specific CD4+ or CD8+ T-cell responses were observed between BTKi-treated patients and healthy controls after dose 5. In the zanubrutinib-treated cohort, Omicron-specific CD8+ T-cell responses increased significantly after dose 3, with no further increase after doses 4 and 5.

Collectively, these findings showed that repeated SARS-CoV-2 vaccination during zanubrutinib treatment was associated with preserved T-cell responsiveness but persistently impaired humoral and mucosal immunity.

5.3 Paper III

Paper III investigated longitudinal T-cell immunomodulation during treatment with acalabrutinib and zanubrutinib in 34 patients with CLL, including 18 treated with acalabrutinib and 16 with zanubrutinib, with age- and sex-matched healthy donors as controls. Both drugs were associated with marked changes in circulating lymphocyte populations during treatment. Total CD19+ B cells declined significantly in both cohorts, with earlier reductions in the acalabrutinib group than in the zanubrutinib group.

At baseline, both CD4+ and CD8+ T-cell counts were elevated compared with healthy donors. During therapy, both subsets decreased significantly. CD4+ T cells normalized by month 2 in the acalabrutinib cohort and by month 6 in the zanubrutinib cohort, whereas CD8+ T cells remained above healthy donor levels at 2 years in both groups. Expression of the exhaustion markers PD-1 and TIGIT on both CD4+ and CD8+ T cells was also increased at baseline and declined during treatment, again with earlier changes in the acalabrutinib cohort. However, exhausted CD8+ T cells remained elevated at 2 years (Figure 6).

Helper and memory T-cell subsets also changed during treatment. Th1 cells and regulatory T cells were expanded at baseline and decreased during therapy, with earlier normalization in the acalabrutinib cohort than in the zanubrutinib cohort. Central memory CD4+ and CD8+ T cells were elevated at baseline and declined during treatment, whereas effector memory subsets progressively decreased towards healthy donor levels. In contrast, naïve CD8+ T cells remained stable, while naïve CD4+ T cells declined during treatment.

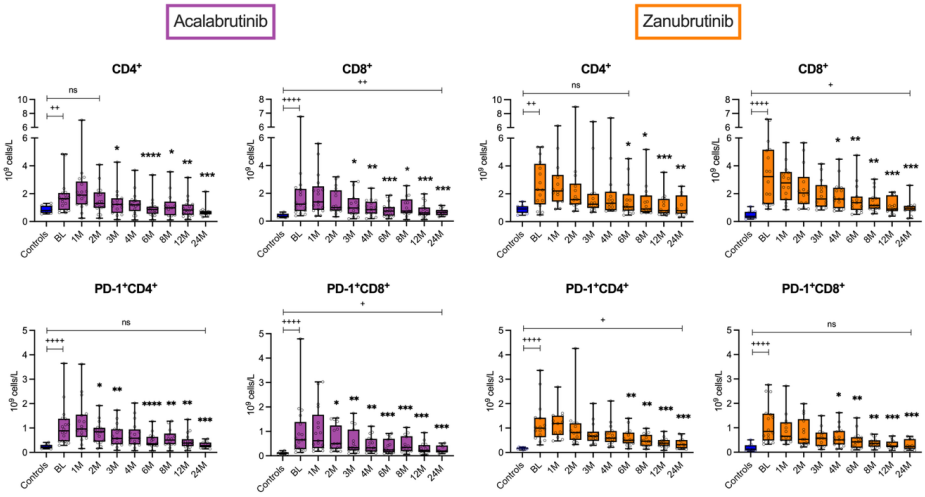

Figure 6. Longitudinal changes in CD4+ and CD8+ T cells and PD-1-expressing CD4+ and CD8+ T cells during acalabrutinib and zanubrutinib treatment. Asterisks (*) indicate statistically significant differences between baseline and the respective time point, and plus signs (+) indicate statistically significant differences between patients and healthy donors. "ns" denotes no significant difference.

Plasma protein profiling showed a broad reduction in inflammatory mediators during treatment. Of 92 analyzed inflammatory biomarkers, 17 decreased significantly in both cohorts, including the CLL-associated proteins CCL3, CCL4, CD5, and CD6. In addition, distinct treatment-specific biomarker changes were observed, with acalabrutinib and zanubrutinib showing partly overlapping but non-identical plasma protein signatures. Hotspot *BTK* mutations also emerged in both cohorts, primarily in R/R patients.

5.4 Paper IV

Paper IV investigated the clinical feasibility and biological effects of repeated response-guided interruption and re-initiation of BTKi therapy in 49 patients with CLL who had achieved at least partial remission during long-term ibrutinib treatment in routine clinical care. Median follow-up from the first treatment cessation was 47 months.

The median duration of the first off-treatment phase (OFF1) was 17 months. Overall, 73% of patients remained off treatment for more than 12 months, 37% for more than 24 months, and 5 patients for more than 36 months. At the time of analysis, 40 patients had restarted BTKi therapy, and 39 of 40 (98%) responded to treatment re-initiation. Twenty patients entered a second off-treatment

phase (OFF2), with a median duration of 10 months at data cut-off. The longitudinal treatment course of each patient is shown in Figure 7.

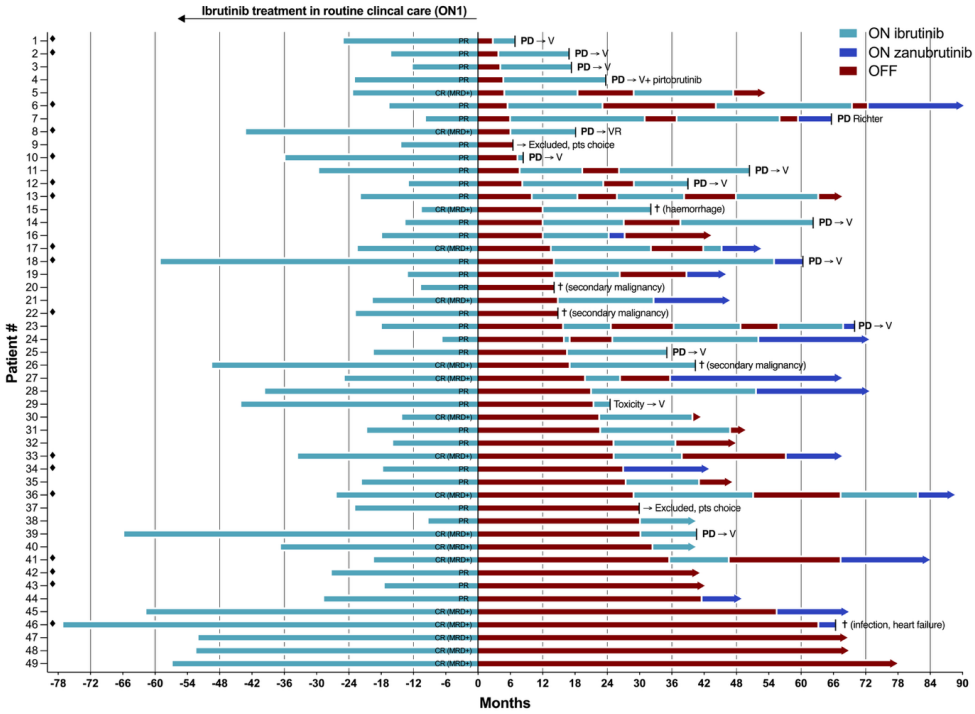

Figure 7. Individual treatment courses during repeated response-guided interruption and re-initiation of BTKi therapy. BTKi, Bruton tyrosine kinase inhibitor; ON, on-treatment phase; OFF, off-treatment phase; PR, partial remission; CR, complete remission; ♦patients with del(17p)/*TP53* mutation, PD, progressive disease; V, venetoclax; †, death (cause of death).

The duration of OFF1 differed between clinical subgroups. Patients with mutated IGHV genes had longer OFF1 than patients with unmutated IGHV genes (median 64 vs 14 months). Longer OFF1 was also observed in patients who were in complete remission at study entry and in those with longer prior exposure to ibrutinib, whereas no significant differences were observed according to age or del(17p)/*TP53* status.

BTK p.C481S hotspot mutations were analyzed in 36 evaluable patients at end-OFF1. Mutations were detected in 7 patients (19%), including 5 with low variant allele frequencies and 2 with high variant levels.

Treatment interruption was accompanied by marked biological changes. During OFF1, the proliferative fraction of CLL cells increased rapidly, whereas the resting fraction decreased. PD-1 and Ki-67 expression on CLL cells also increased during

OFF1 and declined after treatment re-initiation. In parallel, CD4+ and CD8+ T-cell counts increased at the end of OFF1, driven mainly by expansion of central and effector memory subsets, and exhaustion-associated markers on T cells increased during the off-treatment phase and decreased again after treatment restart.

Plasma protein profiling showed broad treatment-associated modulation of inflammatory mediators. Many proteins suppressed during BTKi treatment increased during OFF1 and approached pre-treatment levels at the time of treatment restart, whereas a subset remained persistently altered. Sequencing-based analyses of FACS-sorted CLL cells from six patients showed that many transcriptional and epigenetic changes induced during BTKi treatment reverted during OFF1, but not all changes returned fully to the pre-treatment state.

6 Discussion

This thesis examined clinical and immunological consequences of BTKi therapy in CLL across four complementary studies. Taken together, the findings show that BTKi therapy affects more than disease control alone. It is also associated with toxicity, host immunity, and treatment-associated biological states. The work therefore supports a broader view of BTKi therapy in CLL, in which continuous exposure, immune remodeling, and the feasibility of treatment interruption need to be considered alongside antileukemic efficacy.

6.1 Continuous BTKi therapy is effective, but long-term tolerability remains a central issue

In **Paper I**, AF, bleeding, dose reductions, and permanent treatment discontinuation were common during long-term ibrutinib treatment in routine clinical practice. The proportion of patients who discontinued treatment was higher than in randomized trials (123, 124), which may in part reflect the older age and greater comorbidity burden of patients in the present cohort. These findings highlight the cumulative clinical burden of continuous ibrutinib exposure outside trial-selected populations.

The introduction of second-generation covalent BTKis has partly improved this situation. Comparative studies have shown that acalabrutinib and zanubrutinib retain efficacy while reducing some adverse events associated with ibrutinib, particularly cardiovascular toxicities, and are also associated with lower rates of treatment discontinuation due to adverse events (79, 82). Nevertheless, adverse events remain an important clinical issue during continuous covalent BTKi therapy, and prolonged treatment exposure continues to raise questions regarding long-term tolerability and treatment burden.

These observations also align with the rationale for the response-guided interruption strategy explored in **Paper IV**. The results support the feasibility of this approach in a selected subgroup of patients who had already achieved durable disease control during long-term ibrutinib therapy, but they do not support broad generalization to all patients receiving covalent BTKi. This should be viewed in the historical context in which the study was initiated. At that time, continuous covalent BTKi treatment was an established strategy in CLL, whereas fixed-duration BTKi–venetoclax regimens had not yet entered routine clinical practice. In that setting, a response-guided interruption strategy was clinically

provocative but rational: if prolonged exposure contributes to cumulative toxicity and treatment burden, carefully selected treatment pauses might reduce exposure while also helping to clarify which effects are closely linked to active BTKi treatment.

Since the study was initiated, the therapeutic landscape in CLL has changed, and time-limited targeted therapy has become established in CLL management, including fixed-duration venetoclax-based regimens and BTKi–venetoclax combinations in first-line treatment algorithms (50). More recently, CLL17 has provided prospective randomized evidence that fixed-duration targeted therapy can achieve disease control comparable to continuous ibrutinib in previously untreated CLL (125), although follow-up remains relatively short and longer-term data are still needed. **Paper IV** should therefore not be discussed as an alternative to current fixed-duration combination regimens, but rather as an earlier and distinct strategy addressing a related clinical problem: whether treatment burden during prolonged covalent BTKi exposure can be reduced in selected patients, and what treatment withdrawal reveals about disease and host biology.

Regarding the duration of the off-treatment phases, OFF1 was longer than OFF2, with median durations of 17 and 10 months, respectively. The shorter duration of OFF2 likely reflects, at least in part, both shorter preceding BTKi exposure and a less deep remission before the second treatment stop. The study therefore provides stronger support for the feasibility of an initial response-guided interruption than for a fully established repeated on-off strategy. This is also relevant when interpreting off-treatment duration in relation to newer fixed-duration regimens, in which treatment-free intervals extending over several years have been reported in a considerable proportion of patients, although longer follow-up is still needed and follow-up maturity differs between studies (84, 88, 125).

6.2 BTKi therapy is associated with compartment-specific immune remodeling

Paper II suggests that vaccine-induced immunity during BTKi therapy is compartment-specific. Repeated SARS-CoV-2 vaccination during zanubrutinib treatment was associated with some increase in serum antibody levels, but humoral and mucosal responses remained limited. In contrast, spike-specific T-cell responses appeared to be relatively preserved. The study did not

demonstrate a difference between zanubrutinib- and ibrutinib-treated patients. Instead, the findings suggest that humoral and cellular responses may be affected differently during BTKi therapy. This interpretation should, however, be made with caution, since the T-cell analyses were based on relative frequencies rather than absolute cell counts. Notably, the recent randomized IMPROVE trial showed that a 3-week pause of covalent BTKi around SARS-CoV-2 booster vaccination did not improve antibody titers, neutralization, or cellular responses (126). Together with the present findings, this suggests that vaccine responsiveness during BTKi therapy is influenced by factors that are more complex than ongoing kinase inhibition alone.

Paper III extends this observation beyond vaccine responses and addresses the broader effects of second-generation covalent BTKis on the peripheral T-cell compartment. Both acalabrutinib and zanubrutinib were associated with reductions in abnormal T-cell expansion, exhaustion-associated phenotypes, and inflammatory plasma markers, suggesting that part of the immune dysregulation in CLL is at least partly reversible during effective targeted therapy. At the same time, the findings do not support complete immune normalization. In particular, abnormalities within the CD8+ compartment remained evident at later time points, including persistently increased CD8+ T-cell counts and exhaustion-marker expression, indicating that immune remodeling during therapy is partial rather than complete.
These findings should, however, be interpreted within the limits of the study design. The analyses were confined to peripheral blood and primarily describe phenotypic changes rather than direct functional recovery. They therefore do not establish whether corresponding changes occur in lymphoid tissues, where key tumor–host interactions in CLL take place, or whether the observed remodeling translates into restored immune competence. Another observation was that these changes appeared with different time kinetics in the two cohorts, with earlier changes in the acalabrutinib-treated patients than in the zanubrutinib-treated patients. This should be interpreted cautiously. **Paper III** was not designed as a formal head-to-head comparison, cohort sizes were limited, and some flow-cytometric analyses differed methodologically between the cohorts, including the use of whole blood in the acalabrutinib group and PBMC preparations in the zanubrutinib group. The most robust conclusion is therefore not that one agent is immunologically superior to the other, but that

the peripheral immune effects of second-generation covalent BTKis may not be temporally identical.

6.3 Response-guided interruption provides a model for studying reversibility of BTKi-associated changes

Paper IV extends the thesis from observation of BTKi-associated changes during treatment to direct assessment of their reversibility during treatment withdrawal. Clinically, repeated response-guided interruption of ibrutinib was feasible in a selected subgroup of patients with sustained benefit from prior therapy, with prolonged first off-treatment periods in many patients and preserved sensitivity to BTKi re-initiation in almost all evaluable cases. Beyond the clinical findings, the study also provided an informative model for examining which biological effects were closely linked to active BTKi exposure. During OFF1, coordinated changes were observed across several compartments: the proliferative CLL fraction expanded, checkpoint-marker expression increased, and many plasma protein changes moved back towards the pre-treatment state. Sequencing-based analyses likewise showed that many transcriptional and epigenetic changes induced during BTKi therapy shifted during interruption. However, this reversion was incomplete. Some plasma proteins remained persistently altered, not all gene-regulatory changes returned fully to the pre-treatment state, and *BTK* p.C481S mutations remained detectable in a subset of patients at the end of OFF1. Taken together, these findings suggest that BTKi-associated changes are heterogeneous in their reversibility. Some appear closely treatment-dependent, whereas others persist despite drug withdrawal, indicating that BTKi exposure may leave biological effects that do not immediately disappear when treatment is stopped.

6.4 Strengths and limitations

A strength of this thesis is the integration of four complementary studies addressing clinically and biologically linked questions within the same therapeutic framework. Together, they combine real-world clinical toxicity data, prospective immune monitoring, vaccine immunology, and longitudinal clinical-translational analyses during treatment interruption. Additional strengths include serial sampling, clinically anchored longitudinal designs, the inclusion of both clinical and laboratory endpoints, and the use of multiple analytical platforms spanning flow cytometry, proteomics, ddPCR, RNA sequencing, and ATAC-seq.

The limitations should also be acknowledged. **Paper I** was retrospective and single center. **Papers II** and **III** were limited by modest cohort sizes, and **Paper III** was not designed for formal comparative evaluation between acalabrutinib and zanubrutinib. In **Paper II**, interpretation of preserved T-cell responsiveness is complicated by the use of relative rather than absolute response measures. In **Papers III and IV**, the biological analyses were based largely on peripheral blood and therefore do not fully capture processes within lymphoid tissues, where key tumor–microenvironment interactions in CLL occur. In **Paper IV**, several analyses were performed in relatively small subsets, and the mutational analysis was limited to two *BTK* p.C481S-encoding hotspot variants and was further complicated at some time points by very low circulating CLL-cell counts despite the use of a highly sensitive ddPCR approach. In addition, the interruption strategy was applied to a selected population of patients who had already achieved sustained benefit from long-term ibrutinib treatment, which limits generalizability.

7 Conclusions

BTKi therapy in CLL has important consequences beyond tumor control. Long-term ibrutinib treatment was associated with frequent cardiovascular and bleeding complications, dose reductions, and treatment discontinuation in routine clinical practice. Repeated SARS-CoV-2 vaccination during zanubrutinib treatment induced limited humoral and mucosal responses, whereas spike-specific T-cell responses were relatively preserved. Treatment with acalabrutinib and zanubrutinib was associated with longitudinal T-cell remodeling with different time kinetics. Repeated response-guided interruption and re-initiation of ibrutinib was feasible in selected patients and showed that several BTKi-associated immune and tumor-cell changes were treatment-dependent and partly reversible.

8 Points of perspective

The treatment landscape in CLL has changed, and targeted treatment options have expanded. Fixed-duration targeted regimens have become established, and recent randomized data support that time-limited treatment can achieve disease control comparable to continuous ibrutinib in the first-line setting. An important next step will therefore be to define more precisely which patients are likely to require prolonged BTK-directed therapy.

The growing number of BTK-directed therapies also raises questions about how covalent BTKi should be sequenced relative to non-covalent BTK inhibitors and, potentially, BTK degraders. Pirtobrutinib is the first non-covalent BTK inhibitor to receive regulatory approval in CLL/SLL after prior covalent BTKi exposure, making treatment sequencing within this class increasingly relevant. Early-phase studies of BTK degraders have also shown clinical activity in heavily pretreated disease, but their role remains uncertain. Future studies will need to determine how these different forms of BTK-directed therapy should best be sequenced, how prior treatment exposure influences later response, and whether they select for distinct resistance patterns or address different resistance states.

The present thesis also highlights an important unresolved question in the field. Tumor control and immune recovery should not be regarded as equivalent outcomes. The findings suggest that BTKi-associated immune effects are compartment-specific and only partly reversible, and that treatment withdrawal can help distinguish changes that are closely linked to active exposure from those that persist beyond it. Future studies should therefore examine immune recovery as a distinct clinical and biological objective, using not only peripheral blood phenotyping but also functional immune assays and, when feasible, tissue-based analyses. This may be important both for infection prevention and for understanding which immune changes during BTKi therapy are biologically and clinically relevant.

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10 Declaration about the use of generative AI

The AI-assisted tool ChatGPT (OpenAI, GPT-5.2) was used in the writing of the comprehensive summary of the thesis for language refinement purposes. Specifically, it was used to improve grammar and overall text flow. No scientific content, data interpretation, or conclusions were generated by the AI tool.

I take full responsibility for the content of the thesis.

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