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Targeted Therapy in Chronic Lymphocytic Leukemia

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**Karolinska
Institutet**

Stockholm 2020

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Printed by Eprint AB 2020

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ISBN 978-91-7831-756-1

TARGETED THERAPY IN CHRONIC LYMPHOCYTIC LEUKEMIA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Always pass on what you have learned.”

Yoda

Abstract

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature B lymphocytes in blood, bone marrow and lymphoid tissues. Historically, patients with *TP53* aberration and with refractoriness to chemoimmunotherapy had a dismal prognosis. During the past few years a paradigm shift has taken place in the treatment of CLL as new, targeted agents have been introduced.

The aim of this thesis was to explore targeted agents in patients with advanced CLL.

In the first study, the safety and efficacy of lenalidomide in combination with alemtuzumab was explored in a phase I-II trial. The rationale was that lenalidomide has its major effects in lymph nodes and alemtuzumab in the bone marrow. Furthermore, the capacity of low-dose lenalidomide in maintaining immune functions in advanced-phase CLL patients during alemtuzumab treatment was evaluated. The combination showed an acceptable safety profile as well as clinical efficacy with an overall response rate (ORR) of 58%. Median response duration was 12 months. Lenalidomide had a narrow therapeutic dose range, 2.5 mg/day was not efficient, and the maximum tolerated dose was 5 mg/day. Low-dose lenalidomide increased the frequency of proliferating CD8⁺ T cells but had no effect on the immune checkpoint marker, programmed cell death 1 (PD-1), on T cells. After combination treatment, granzyme B⁺ T cells increased. In conclusion, low-dose lenalidomide and alemtuzumab induced major changes in T cells, including increased proliferative activity and cytotoxic potential.

In the second study, the safety and efficacy of ibrutinib in combination with alemtuzumab was explored in a phase I trial. The rationale was that ibrutinib has its major effects in lymph nodes and alemtuzumab in the bone marrow. Further, the toxicity profiles differ. The treatment combination was efficient: 7 out of 8 patients responded to treatment and 7 achieved minimal residual disease negativity. Within 2 weeks, ibrutinib led to decreased proliferation of CLL cells and T cells. After 4 weeks of ibrutinib therapy, PD-1 expression was unchanged on T cells. Due to a high rate of opportunistic infections, the study was closed in advance and we recommend against the combination of ibrutinib and alemtuzumab.

In the third study, the safety and efficacy of ibrutinib, when used in routine health care, was evaluated. Ninety-five consecutive patients, treated in a compassionate use program, were analyzed. At a median follow-up of 10 months, the ORR was 84%, the progression free survival (PFS) rate was 77% and the overall survival (OS) rate was 83%. PFS and OS were significantly inferior in patients with *TP53* aberration. Atrial fibrillation occurred in 8% and Richter transformation (RT) occurred in 7% of patients. Half of the patients would not have met the inclusion criteria for the pivotal study of ibrutinib: this demonstrates the real-world representativity of the patients. The observed efficacy and toxicity of ibrutinib in the study were similar to that in pivotal studies.

In the fourth study, a long-term follow-up of the patients in the compassionate use program for ibrutinib was carried out. At 30-month follow-up, the ORR rate was unchanged at 84%, the PFS rate was 52% and the OS rate was 63%. Fifty-one percent of patients remained on treatment. In contrast to the early (10-month) report, *TP53* aberration had no negative survival impact. In multivariate analyses, OS was significantly associated with baseline comorbidities and PFS was associated with baseline comorbidities and number of prior therapies. Fifty-one percent of the patients had grade 3-4 infections and 13% had grade 3-5 opportunistic infections. Fifteen percent developed atrial fibrillation. RT occurred in 13%. Twenty-six percent of patients had dose reduction or temporary treatment breaks, which had no significant impact on the outcome. Four of 6 patients who had progressive disease while on ibrutinib were tested for mutation of Bruton's tyrosine kinase. All of them carried the most common mutation leading to ibrutinib resistance. In conclusion, ibrutinib was effective and well tolerated for long-term use. The observed efficacy of ibrutinib was somewhat inferior to that of pivotal studies. The observed frequencies of treatment discontinuation and dose reductions were greater than in clinical studies.

List of scientific papers

- I. **Winqvist M**, Mozaffari F, Palma M, Sylvan SE, Hansson L, Mellstedt H, Österborg A, Lundin J. Phase I-II study of lenalidomide and alemtuzumab in refractory chronic lymphocytic leukemia (CLL): effects on T cells and immune checkpoints. *Cancer Immunology Immunotherapy*, 2017;66(1):91-102.
- II. **Winqvist M**, Palma M, Heimersson K, Mellstedt H, Österborg A, Lundin J. Dual targeting of Bruton tyrosine kinase and CD52 induces minimal residual disease-negativity in the bone marrow of poor-prognosis chronic lymphocytic leukaemia patients but is associated with opportunistic infections - Results from a phase I study. *British Journal of Haematology*, 2018;182(4):590-594.
- III. **Winqvist M**, Asklid A, Andersson PO, Karlsson K, Karlsson C, Lauri B, Lundin J, Mattsson M, Norin S, Sandstedt A, Hansson L, Österborg A. Real-world results of ibrutinib in patients with relapsed or refractory chronic lymphocytic leukemia: data from 95 consecutive patients treated in a compassionate use program. A study from the Swedish Chronic Lymphocytic Leukemia Group. *Haematologica*, 2016;101(12):1573-1580.
- IV. **Winqvist M**, Andersson PO, Asklid A, Karlsson K, Karlsson C, Lauri B, Lundin J, Mattsson M, Norin S, Sandstedt A, Rosenquist R, Späth F, Hansson L, Österborg A. Long-term real-world results of ibrutinib therapy in patients with relapsed or refractory chronic lymphocytic leukemia: 30-month follow-up of the Swedish compassionate use cohort. *Haematologica*, 2019;104(5):E208-E210.

List of abbreviations

ADCC	antibody-dependent cellular cytotoxicity
AIHA	autoimmune hemolytic anemia
AKT	anti-apoptotic protein kinase
APRIL	a proliferation-inducing ligand
ATM	ataxia telangiectasia-mutated
BAFF	B-cell activating factor
BAK	BCL2 agonist/killer 1
BAX	BCL2 associated X protein
BCL2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCR	B cell receptor
BH3	BCL2 homology 3
BIRC3	Baculoviral IAP Repeat Containing 3 gene
BMX	bone marrow tyrosine kinase on chromosome X
BR	bendamustine, rituximab
BTK	Bruton's tyrosine kinase
CAR	chimeric antigen receptor
CCR	C-C motif chemokine receptor
CDC	complement-dependent cytotoxicity
CI	confidence interval
CK1 ϵ	casein kinase 1 epsilon
CLL	chronic lymphocytic leukemia
CLL-IPI	CLL International Prognostic Index
CMV	cytomegalovirus
CR	complete remission
CRF	case record form
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CYP	cytochrome P450
CSB	cell staining buffer
DLBCL	diffuse large B cell lymphoma
EBMT	European Group for Blood and Marrow Transplantation
ECOG	Eastern Cooperative Oncology Group
EGFR	endothelial growth factor receptor
EGR2	early growth response 2
EMA	European Medicines Agency
FC	fludarabine, cyclophosphamide
FCR	fludarabine, cyclophosphamide, rituximab
FDA	Food and Drug Administration
FISH	fluorescent in situ hybridization
HLA-DR	human leukocyte antigen - antigen D related
HR	hazard ratio
HSCT	hematopoietic stem cell transplantation
IGHV	heavy chain variable region of immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motifs

ITK	interleukin-2-inducible T cell kinase
ITP	immune thrombocytopenia
IWCLL	International Workshop on CLL
LYN	Lck/Yes novel tyrosine kinase
mAb	monoclonal antibody
MAPK-ERK	mitogen-activated protein kinase - extracellular signal-regulated kinase
MBL	monoclonal B-cell lymphocytosis
MCL1	myeloid cell leukemia 1
MRD	minimal residual disease
MSC	mesenchymal stromal cells
mTOR	mammalian target of rapamycin
NCCN	National Comprehensive Cancer Network
NCI	National Cancer Institute
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NK cell	natural killer cell
NLC	nurse-like cell
NOTCH1	Notch homolog1, translocation-associated
ORR	overall response rate
OS	overall survival
PBMC	peripheral blood mononuclear cells
PD	progressive disease
PD-1	programmed cell death 1
PD-L1	programmed death-ligand 1
PFS	progression free survival
PI3K	phosphatidyl-inositol 3-kinase
PJP	Pneumocystis jiroveci pneumonia
PLCγ2	phospholipase C gamma 2
PML	progressive multifocal leukoencephalopathy
PR	partial remission
PR-L	PR with lymphocytosis
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone
RCT	randomized clinical trial
RT	Richter transformation
SD	stable disease
SF3B1	splicing factor 3B subunit 1
SLL	small lymphocytic lymphoma
SYK	spleen tyrosine kinase
TCR	T cell receptor
TEC	tyrosine kinase expressed in hepatocellular carcinoma
Th1 cell	type 1 T helper cell
Th17 cell	type 17 T helper cell
Th2 cell	type 2 T helper cell
TLS	tumor lysis syndrome
Treg	regulatory T cells
WHO	World Health Organization
XLA	X-linked agammaglobulinemia

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PAPERS I-IV

1. CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 Introduction

Chronic lymphocytic leukemia (CLL) is characterized by a clonal proliferation of mature, CD5+ B lymphocytes, which accumulate in blood, bone marrow and lymphoid tissues. The disease mostly occurs in elderly patients and the clinical course of CLL is highly variable. Chemoimmunotherapy has been the standard first-line treatment for patients with CLL for many years ^[1]. With the advent of novel, targeted agents, the survival of CLL patients has improved and the treatment landscape is rapidly changing ^[2, 3].

1.2 Epidemiology

CLL has a high incidence in Europe and North America, intermediate in Africa and low in Asia ^[4]. The incidence of CLL in Sweden is 500 cases/year. Between the years 2000 and 2015, the prevalence of CLL in Sweden increased from 33/100 000 to 52/100 000 inhabitants ^[5]. The increase was due to the improvement of survival of CLL patients ^[5]. More men than women (2:1) are affected ^[6]. In Sweden, the median age at diagnosis is 71 years ^[6].

1.3 Etiology

The etiology of CLL is mainly unknown. The most apparent risk factor is age. Incidence is higher in persons with a family history of CLL. First-degree relatives of CLL patients have 8.5-fold elevated risk of developing the disease ^[7]. A limited number of other independent risk factors for CLL have been identified; exposure to pesticides/herbicides and exposure to hepatitis C virus have been associated with increased risk. Further, a protective effect of sun exposure on CLL risk has been reported ^[8].

1.4 Pathogenesis

The cell of origin in CLL remains a controversy. However, there is much evidence that the first genetic changes in CLL transformation occur at the hematopoietic stem cell stage ^[9]. The disease may be initiated by chromosomal alterations, e.g. del(13q) and trisomy 12. During the course of the disease, additional mutations may be acquired, and the leukemia becomes more and more aggressive and resistant to treatment. Del(11q) and del(17p) are usually acquired at later stages of the disease ^[10].

The activation of normal B cells can be dependent or independent of T helper cells. B cells, which are stimulated by T helper cells, will undergo affinity maturation, i.e. mutations will be introduced in the heavy chain variable region of immunoglobulin (*IGHV*) gene leading to an increase of the B cell receptor's (BCR) affinity for the antigen. Mutational status is measured by comparing the individual's *IGHV* gene to the germline sequence of *IGHV*. CLL with $\leq 98\%$ of the *IGHV* similar to the germline sequence is defined as mutated CLL and CLL with $> 98\%$ of the *IGHV* similar to the germline sequence is defined as unmutated CLL. To some extent the mutational status of the *IGHV*

gene reflects the existence of two different disease subtypes ^[11, 12]. The prevailing model implies that CLL with mutated *IGHV* genes derives from germinal center experienced B cells while CLL with unmutated *IGHV* genes derives from B cells that have differentiated in a germinal center independent path.

1.4.1 Genomic disruptions and gene mutations

Deletion of the long arm of chromosome 13 is the most frequent cytogenetic aberration in CLL occurring in approximately 55% of all patients ^[13]. Cases with isolated del(13q) have a favorable prognosis ^[13]. Del(13q) leads to down regulation of microRNA 15 and microRNA 16 and both microRNAs negatively regulate the anti-apoptotic gene BCL2 (B-cell lymphoma 2) at the posttranscriptional level, which may lead to increased resistance to apoptosis ^[14].

Del(11q) is found in about 10% of patients at diagnosis and in up to 25% of patients with chemotherapy-refractory disease ^[15]. It is usually a mono-allelic event and causes the loss of the *ATM* (ataxia telangiectasia-mutated) gene, which encodes ATM, a protein kinase that is activated by DNA damage and is an activator of p53 ^[16]. Thus, ATM works for the integrity of the genome. In contrast to patients with *TP53* aberration, patients with *ATM* alteration usually respond to chemotherapy ^[17].

Trisomy 12 is detected in 10-20% of CLL patients at diagnosis ^[13]. The genes behind the pathogenesis of CLL with trisomy 12 are largely unknown.

At diagnosis, 5-15 % of patients have 17p deletion ^[18]. The *TP53* gene is located on the short arm of chromosome 17 and deletion of 17p hence leads to a loss of the tumor suppressor protein p53. More than 80% of patients with del(17p) also carry an aberration in the remaining *TP53* allele ^[19], which implies just as bad a prognostic feature as deletion of 17p ^[18]. The terms *TP53* aberration and del(17p) can therefore be used interchangeably for a clinical purpose. The p53 protein, also known as “the guardian of the genome”, can activate DNA repair, hold the cell cycle and initiate apoptosis in response to DNA damage. Many chemotherapeutic agents, including alkylating agents and purine analogues rely on the pro-apoptotic and antiproliferative effect of p53. The lack of p53 activity results in resistance to chemotherapy ^[20]. *TP53* aberration is coupled with higher genomic complexity in CLL ^[21]. Aberration of *TP53* predicts a shorter time to progression with the novel targeted therapies as well, however the negative impact of *TP53* aberration is less prominent compared to chemoimmunotherapy ^[22-25].

Next generation sequencing (NGS) studies have provided a complete profile of mutations in CLL. Studies have shown an adverse prognostic impact of *TP53*, *SF3B1* (splicing factor 3B subunit 1), *MAPK-ERK* (mitogen-activated protein kinase - extracellular signal-regulated kinase), *EGR2* (Early growth response 2), *NOTCH1* (Notch homolog1, translocation-associated) and *BIRC3* (Baculoviral IAP Repeat Containing 3 gene) mutations ^[26-28]. However, *TP53* disruption is currently the only mutation affecting the treatment choice.

1.4.2 Heterogeneity and clonal selection

Many malignancies display intra-tumor-heterogeneity, i.e. genetic differences within one single sample. A subclone is defined as a group of cells with a mutation occurring only in

fraction of the malignant cells. With NGS technique it has been shown that CLL may be composed of heterogeneous malignant cells and that subclonal mutations can affect the clinical course of the disease [29]. The prevailing hypothesis is that a small clone of therapy resistant cells is present in the beginning of the disease and these resistant cells have an advantage of growth during therapy, a process known as clonal evolution [30]. The proportion of patients with *TP53* disruption increases from 5-15% at diagnosis [18] to approximately 40% in fludarabine-refractory patients [31]. In a longitudinal analysis, small clones of *TP53* mutated cells became the dominant clone at relapse, hence small *TP53* mutated subclones showed the same adverse prognostic impact as clonal *TP53* aberrations [32].

1.5 Microenvironment

CLL cells circulating in the blood are cell-cycle-arrested, while CLL cells located in the lymphoid tissue and the bone marrow are activated. CLL cells are subject to spontaneous apoptosis when removed from patients, indicating that the malignant cells are addicted to the microenvironment for proliferation and survival [33]. The proliferation of CLL cells occur within tissue sites known as proliferation centers, which exist in the bone marrow and in the lymph nodes. In the proliferation centers, the tumor cells interact with other cells: mesenchymal stromal cells (MSC), monocyte-derived nurse-like cells (NLC) and T cells [34]. NLCs were first reported as an in vitro phenomenon. NLCs differentiated from monocytes when co-cultured with CLL cells [35].

In CLL-patients, NLCs are found in the spleen and in the lymph nodes. NLCs secrete survival signals, including B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which results in higher expression of anti-apoptotic genes in cultured CLL cells [36].

NLCs induce a gene expression profile response in CLL cells with induction of genes in the pathways of the BCR and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [37]. MSC are found in the secondary lymphatic tissues of CLL patients and like the NLCs they secrete survival signals for tumor cells. Further, MSC secrete chemokines, of which C-X-C motif chemokine ligand (CXCL)12 is the most important, to guide the CLL cells into the tissue, a process known as homing. CLL cells overexpress the C-X-C motif chemokine receptor (CXCR)4 that allow the cell to sense and follow the level of CXCL12 [38].

CLL cells are in close contact with T cells in the microenvironment [34]. T cells can either suppress or promote B cell expansion, and their function depends on the activation status, T-cell subset, and micro-anatomical location. CD4+ and CD8+ T-cell numbers are increased in CLL patients [34]. There is a correlation of T-cell expansion and progressive disease [39], but the cause of the T-cell expansion is unknown. The CD8+ T-cell proportion is increased relative to the CD4+ T cells, leading to an inversion of the normal CD4:CD8 ratio [34].

Myeloid-derived suppressor cells have recently been identified. They seem to play a role in the CLL microenvironment by suppressing T cells and inducing regulatory T cells (T_{reg}) [40].

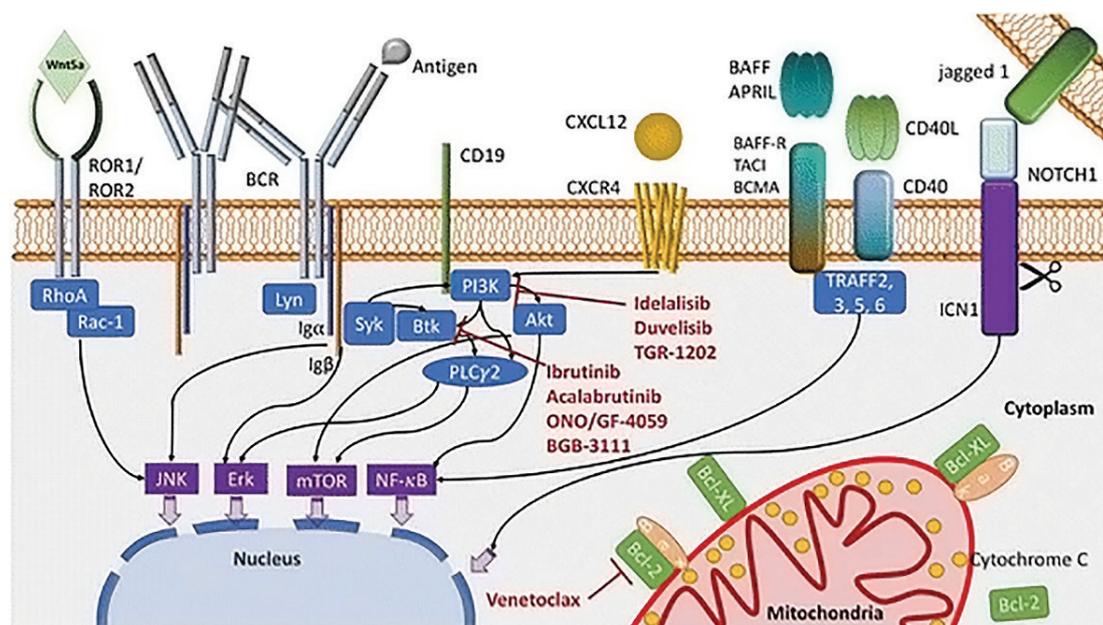


Figure 1. CLL main pathogenic pathways and target agents against BTK, PI3K and Bcl-2. BCR signaling is induced by the recognition of an antigen or by self-binding, Lyn promotes the phosphorylation of Ig α and Ig β that activates the spleen tyrosine kinase (SYK). SYK then triggers the formation of a multi-component ‘signalosome’, including BTK, AKT, PI3K and PLC γ 2 among others. BCR co-receptor CD19 is important for PI3K activation, which recruits and activates PLC γ 2, BTK and AKT. These lead to the activation of the c-Jun N-terminal kinase (JNK), MEK-extracellular signal-regulated kinase (ERK), mammalian target of rapamycin (mTOR) and NF- κ B signaling pathways. In addition, CLL cells activate these and other prosurvival, activatory pathways by their interaction with many soluble and surface factors. As an example: Wnt5a interact with the ROR1/ROR2 dimers promoting the activation of RhoA and Rac-1. CXCR4/CXCL12 engagement activates PI3K and downstream pathways, in addition to other molecules. The TNF receptors CD40, BAFF-R, TACI and BCMA interact with their ligands CD40L or BAFF and APRIL, inducing the activation of the canonical and alternative NF- κ B pathways depending on the TNF receptor-associated factor (TRAF). NOTCH1 signaling is initiated by the binding with one of the five ligands (e.g. jagged 1, Delta-like ligand 1 (DLL1)), followed by the release of the intracellular active portion (ICN1), enabling its migration into the nucleus. These pathways lead to the upregulation of anti-apoptotic molecules like Bcl-2, Bcl-XL and Mcl-1, sequestering the pro-apoptotic molecules BAX and BAK, and inhibiting the intrinsic apoptosis pathway. Inhibitors for PI3K, BTK and Bcl-2 are indicated in red.

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1.6 B cell receptor (BCR) and downstream signaling

The normal B lymphocyte receives survival and proliferation signals from the microenvironment and the BCR is a key factor in this interaction^[41]. CLL cells are capable of antigen-independent, cell-autonomous BCR signaling^[42]. The BCR is composed of a membrane bound immunoglobulin bound to Ig α and Ig β . BCR pathway signaling is activated through the antigenic stimulation of the extracellular domain of the BCR. Upon antigen binding, Lck/Yes novel tyrosine kinase (LYN) activates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic parts of Ig α and Ig β . Next, spleen tyrosine kinase (SYK) initiates the formation of a “signalosome” including Bruton’s tyrosine kinase (BTK), phosphatidylinositol 3-kinase (PI3K), anti-apoptotic protein kinase

(AKT) and phospholipase C gamma 2 (PLC γ 2). This leads to activation of downstream kinases including mammalian target of rapamycin (mTOR) and NF κ B (Figure 1). These events ultimately lead to a change in gene expression promoting survival and proliferation.

1.7 Diagnosis, clinical staging and manifestations

1.7.1 Diagnosis

According to the World Health Organization (WHO), classification the CLL diagnosis requires a circulating clonal B lymphocyte count of at least 5×10^9 cells/L. The CLL cells are morphologically mature and they express B-cell markers (CD23, CD19 and low CD20) and CD5^[43].

Small lymphocytic lymphoma (SLL) is defined as lymphadenopathy and/or splenomegaly and a count of less than 5×10^9 /L CLL cells in the peripheral blood and the absence of cytopenia caused by clonal marrow infiltration. In the absence of extramedullary disease, a clonal B lymphocyte count of less than 5×10^9 /L is classified as “monoclonal B-cell lymphocytosis” (MBL)^[43]. MBL is classified into low-count MBL ($< 0.5 \times 10^9$ cells/L) and high-count MBL ($\geq 0.5 \times 10^9$ cells/L). Low-count MBL has an extremely low risk of progression and monitoring is not recommended, while high-count MBL has a 1-2 percent yearly risk for progression to CLL or SLL requiring treatment^[44].

1.7.2 Clinical staging

CLL has an extremely heterogeneous clinical course and therefore staging systems are helpful to facilitate management of patients. The most commonly used methods for predicting survival in CLL are the two similar staging systems: Rai^[45] and Binet^[46]. They are based on a clinical examination of lymph nodes, liver and spleen and the presence of thrombocytopenia and/or anemia. However, these staging systems do not fully reflect the heterogeneity of CLL since they do not take genetic and chromosomal characteristics into account. Several attempts have been made to improve prognostic models for CLL.

As cytogenetic techniques have developed, so has the information regarding prognostic factors. With the fluorescence in situ hybridization (FISH) technique, chromosomal disruptions in CLL and their prognostic implications have been explored. Patients with 13q deletion have the longest overall survival (OS) (median survival 133 months from the time of diagnosis). Trisomy 12 and normal karyotype carry intermediate risk^[13]. Patients with deletion of 17p have the poorest prognosis (median survival of 32 months from the time of diagnosis) followed by 11q deletion (median survival 79 months from the time of diagnosis)^[13].

Survival is shorter for patients with unmutated *IGHV* genes regardless of disease stage. Median survival for patients with low Binet stage and unmutated *IGHV* was 95 months compared with 293 months for patients with mutated *IGHV*^[11].

Recently a new prognostic index for treatment-naïve patients was developed, the CLL International Prognostic Index (CLL-IPI), which includes five independent prognostic factors^[47]. The CLL-IPI is based on *TP53* status (no abnormalities vs *TP53* disruption), *IGHV* mutational status (mutated vs unmutated), beta2-microglobulin (≤ 3.5 mg/L vs > 3.5 mg/L), clinical stage (Binet A or Rai 0 vs Binet B-C or Rai I-IV) and

age (≤ 65 years vs > 65 years). CLL-IPI identifies four risk groups showing different 5-year OS: low, intermediate, high and very high risk. The 5-year OS rate in the low risk group was 93% and in the very high risk group 23%. *TP53* disruption was the most important risk factor and patients with 17p deletion/*TP53* aberration always fell into the high or very high risk group. The CLL-IPI has not been validated in the era of new, targeted treatment.

1.7.3 Manifestations

Common symptoms of CLL include lymphadenopathy, hepatosplenomegaly, constitutional symptoms and cytopenia related to bone marrow infiltration. Patients may also suffer from infections, autoimmune hemolysis and transformation to high-grade lymphoma/Richter transformation (RT) [48].

Autoimmune complications

CLL is often complicated by autoimmune cytopenia. The most frequent autoimmune complication is autoimmune hemolytic anemia (AIHA) with an incidence of 5-10%, followed by immune thrombocytopenia (ITP) in 1-2 % of patients. Pure red cell aplasia and autoimmune neutropenia are unusual events [49]. AIHA is more frequent in patients with unmutated *IGHV*, with del(17p) and del(11q) and in heavily pretreated patients [50]. In both AIHA and ITP it is assumed that non-malignant B cells produce the autoantibodies since the antibodies are polyclonal [49]. Fludarabine monotherapy has a well-known association with autoimmune cytopenia [51]. When fludarabine is given in combination with rituximab the incidence of autoimmune cytopenia does not differ from the incidence in the general CLL population [52]. Compared to chemotherapy, triggering of autoimmune cytopenia is less common with novel targeted treatment [53]. If no other CLL symptoms are present, the first-line treatment for autoimmune cytopenia is corticosteroids [53].

Immune defects and infections

Infections account for 25-50% of deaths in patients with CLL [54]. Several factors have been reported to increase the susceptibility to infection; hypogammaglobulinemia, T-cell defects, natural killer (NK)-cell defects, neutropenia and defects in complement activation [55]. Further, the infection rate increases with duration of disease, with advanced stages and with CLL treatment [54].

Hypogammaglobulinemia is the most common immune defect with up to 85% of patients being affected [56]. Randomized trials conducted in the 1980s found that prophylactic immunoglobulin substitution decreased the rate of bacterial infections in CLL patients with hypogammaglobulinemia and recurrent bacterial infections, but there was no effect on survival [56]. The prophylactic use of immunoglobulin substitution is usually recommended in patients with severe hypogammaglobulinemia and recurrent bacterial infections [48, 57]. Vaccination against pneumococcus and the seasonal influenza are recommended for CLL patients [58], but the response to the vaccines might be inefficient [56]. Vaccination in the beginning of the disease course, before aggravation of hypogammaglobulinemia, can be beneficial [56]. The mechanism behind the development of hypogammaglobulinemia is not completely understood.

T-cell dysfunction is due to several factors, such as defective immunological synapse formation, impaired cell cytotoxicity and imbalance in cell subsets. T-cell activation through the T cell receptor (TCR) is regulated by co-stimulatory and inhibitory signals, including immune checkpoint receptors. In particular, the immune checkpoint receptor programmed cell death 1 (PD-1) is induced on activated T cells, and when bound to the ligand, programmed death-ligand 1 (PD-L1), expressed on tumor cells or cells in the tumor microenvironment, TCR signaling is reduced [59]. T cells, which have impaired effector functions due to chronic antigen stimulation, have an increased PD-1 expression and can be defined as “exhausted”. T cells in CLL patients displayed a high PD-1 expression [60-62].

In CLL, NK cells are defect with a lack of cytoplasmatic granules and a reduced ability of killing tumor cells [63]. It is largely unknown how the CLL cells cause the defect in NK cells. The size of the NK-cell compartment relative to the size of the CLL clone was significantly higher in CLL patients with lower stage disease and with mutated *IGHV* [64].

Richter transformation

Richter transformation is the development of a secondary aggressive lymphoma in the setting of underlying CLL. Two pathologic variants of these transformations are recognized: diffuse large B cell lymphoma (DLBCL) (90%) and more rarely Hodgkin lymphoma (10%) [65]. RT occurs at a median time of 2 years after diagnosis [65]. The clinical course is usually aggressive, and the prognosis is dismal. There are two major categories of RT – RT that is clonally related to the underlying CLL and RT that is unrelated to the underlying disease [66]. Clonally unrelated cases, which account for approximately 10% of cases, have a much better prognosis than clonally related [65]. The incidence rate of RT is estimated at 0.6% per year [67]. Patients treated with rituximab in combination with FC had a lower incidence rate, compared to patients treated without the addition of rituximab [65]. The rate of transformation in patients treated with ibrutinib, idelalisib or venetoclax seems to be the same as for historical controls treated with chemoimmunotherapy [65]; however, the follow-up time for the novel agents is limited.

The majority of patients with RT have unmutated *IGHV*. Further, the presence of adverse genomic disruptions and lymph nodes > 3 cm were correlated to RT [68]. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) is the most commonly used treatment for RT, but survival usually does not exceed 12 months [69]. In young patients (< 60 years), autologous or allogeneic hematopoietic stem cell transplantation (HSCT) can be effective, according to a retrospective study by the European Group for Blood and Marrow Transplantation (EBMT). At a follow-up of 3 years, the progression free survival (PFS) after autologous HSCT was 45%, but there was no plateau in the survival-curve. The PFS at 3 years after allogeneic HSCT was 27% [70].

2 TREATMENT OF CLL

2.1 Treatment indications

Until now, only patients with symptomatic disease require therapy^[71]. Eighty-five percent of patients do not require treatment at the time of diagnosis^[6] and they should be subject to “watch and wait”. Before treatment is initiated there should be signs of active disease i.e. the presence of cytopenia due to bone marrow failure, splenomegaly, hepatomegaly, symptomatic lymphadenopathy, a rapid doubling of the lymphocyte counts (< 6 months) or occurrence of constitutional symptoms (fatigue, fever, weight loss, night sweats). The lymphocyte count should not be the only criterion for initiation of treatment^[48].

Several randomized trials showed no benefit of early treatment with chemotherapy^[71]. Recent data indicate that ibrutinib prolongs PFS of patients with untreated, early stage CLL with increased risk of progression^[72]. However, no survival data have been reported and follow-up time is short, therefore it is still unknown if indication for treatment will change in the era of new, targeted agents.

2.2 Treatment evaluation and minimal residual disease (MRD)

The treatment response is evaluated using the International Workshop on CLL (IWCLL) criteria^[48]. Partial remission (PR) requires > 50% reduction of lymphocytosis and lymph node size. Complete remission (CR) requires absence of lymphadenopathy, hepatosplenomegaly, cytopenia and a lymphocyte count < 4 x 10⁹/L. In clinical trials, a bone marrow biopsy with less than 30% of nucleated cells being lymphocytes is required for CR. The tyrosine kinase inhibitors redistribute CLL cells from lymph nodes to the peripheral blood. As a consequence, the response criteria have been slightly modified introducing the concept of PR with lymphocytosis (PR-L), which is considered a response to such a treatment^[73]. Progressive disease (PD) is defined as > 50% increase of lymph node size, > 50% increase of lymphocytes or a significant decrease of hemoglobin or platelet count from baseline. Stable disease (SD) is defined as absence of PD and failure to achieve at least a PR. Patients not achieving at least PR or relapsing within 6 months after treatment initiation are classified as refractory to treatment. Relapse is classified as disease progression in a patient who previously achieved a response to treatment for 6 months or more^[48].

Minimal residual disease (MRD) negativity is defined as less than 1 CLL cell/10 000 leukocytes, measured by flow cytometry. MRD negativity independently predicts for longer PFS and OS in patients receiving chemoimmunotherapy in the frontline setting^[74]. Recent data suggest that MRD negativity is prognostic also for venetoclax treatment^[75]. The prognostic value of MRD negativity for tyrosine kinase inhibitors is unknown. MRD is not routinely measured in clinical practice, however many trials assess MRD level as an end-point.

2.3 Cytostatic agents

For decades, the only available agents with effect in CLL were the alkylating agents, chlorambucil and cyclophosphamide, which alkylate and cross-link DNA resulting in p53-dependent stress and apoptosis. Monotherapy with chlorambucil was the most frequent treatment [71]. Chlorambucil has low toxicity and cost, but low overall response rate (ORR) of 30-72% and CR 0-7% as well as short PFS of 8-18 months [76]. Chlorambucil did not prolong OS but might be used for symptom control [77, 78].

Bendamustine differs structurally from other alkylators by its benzimidazole ring. The drug appears to cause more extensive and more long-lasting DNA-breaks [79]. Bendamustine as monotherapy was found to yield higher ORR and longer PFS when compared to chlorambucil, but there was no significant difference in OS [80].

Fludarabine is a purine analog, which inhibits DNA polymerase and ribonucleotide reductase. In the 1980s, it was shown that purine analogues more frequently achieved CR than alkylator-based regimens [81]. However, fludarabine as monotherapy did not improve OS [82]. Fludarabine and cyclophosphamide in combination was well tolerated and improved PFS compared to fludarabine as monotherapy [83].

2.4 Monoclonal antibodies

2.4.1 Anti-CD20: rituximab, ofatumumab and obinutuzumab

The CD20 antigen is expressed on the surface of B lymphocytes. The anti-CD20 monoclonal antibodies (mAbs) bind to the CD20 antigen and activate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) towards the B lymphocyte. Combinations of CD20 mAbs with chemotherapy or targeted agents are very effective treatments for CLL (see below) and CD20 mAbs are rarely used as monotherapy.

Based on their mechanisms of action, CD20 mAbs are grouped into type I and type II. Rituximab and ofatumumab are of type I; they induce both ADCC and CDC [84]. Ofatumumab targets a different epitope on the CD20 molecule and shows stronger affinity than rituximab [84]. In a pivotal study, ofatumumab alone induced a response in 43-49% of relapsed/refractory CLL patients, but the median time to disease progression was only 4.6-5.5 months [85]. In 2019, the European Medicines Agency (EMA) withdrew ofatumumab from the market at the request of the marketing authorization holder.

Obinutuzumab is a humanized, glycoengineered type II anti-CD20 mAb; it induces only ADCC but no CDC [84]. The glycosylation increases the activation of NK cells [86]. Obinutuzumab as monotherapy has proven to be an active drug for CLL with a median PFS of 10.7 months in relapsed/refractory patients [87].

2.4.2 Anti-CD52: alemtuzumab

Alemtuzumab is a humanized mAb directed against CD52, which is expressed by normal as well as malignant B and T lymphocytes. Important effector mechanisms for alemtuzumab are CDC and ADCC [88]. Alemtuzumab induced an ORR of 30-40 % and a median PFS ranging from 9-15 months in relapsed/refractory CLL patients [88, 89].

Alemtuzumab is effective in patients with high-risk genetic markers such as 17p deletion^[90] and the drug was previously considered the standard treatment of relapsed/refractory CLL patients. The main effect of alemtuzumab is in blood and bone marrow and its effectiveness in CLL with bulky lymphadenopathy is limited^[89]. Alemtuzumab depletes immune cells leading to an increased risk of infections, including opportunistic infections, in particular cytomegalovirus (CMV) reactivation^[91]. Alemtuzumab was withdrawn from the market in 2012 but is available through a free compassionate use program. Since the introduction of the novel, targeted agents, the use of alemtuzumab for CLL is very limited.

2.5 Chemoimmunotherapy

Until recently, a combination of fludarabine, cyclophosphamide and rituximab (FCR) was the gold standard frontline treatment regimen in most CLL patients younger than 65 years and without *TP53* aberration. FCR has shown ORR of 90%, CR rates of 44% and prolonged survival in CLL patients treated first-line^[1]. As previously mentioned, patients with *TP53* disruption are refractory to this treatment. Before the era of new, targeted agents, patients relapsing after FCR had a dismal prognosis with median OS of 51 months^[92].

A follow-up of 300 consecutive patients treated with first-line FCR (FCR300 trial) showed a median PFS of 6.4 years and a median OS of 12.7 years. At 13 years follow-up the PFS for mutated patients was 54 % and the PFS for unmutated patients was 8.7%. For patients with mutated *IGHV* there was a plateau on the PFS curve with no relapses beyond 10.4 years, implying that more than half of *IGHV* mutated patients had a long remission or cure with FCR^[93]. These results were confirmed in a long-term follow-up of the CLL8-trial in which 408 patients treated with first-line FCR showed a median PFS of 4.7 years. At 5 years patients with mutated *IGHV* had a superior PFS rate compared to patients with unmutated *IGHV* (67% vs 36%)^[94].

However, fludarabine-based regimens are not always easily tolerated. Myelosuppression with prolonged neutropenia and infections are common^[1]. Also, approximately 5% of patients develop secondary acute myeloid leukemia/myelodysplastic syndrome after FCR treatment^[93,95].

Results from a randomized comparison between FCR and bendamustine-rituximab (BR) in previously untreated patients showed that patients treated with BR had a significantly shorter PFS and to a less extent achieved CR as well as MRD negativity^[96]. However, in elderly (> 65 years) patients there was no significant difference in PFS between FCR and BR after a median follow-up time of 3 years. Further, treatment toxicities such as neutropenia and infections were more pronounced in elderly patients^[96]. This study led to the recommendation of BR to the majority of patients older than 65 years in Sweden^[58].

In the phase 3 CLL11-trial, 781 treatment-naïve patients with comorbidities were randomized to chlorambucil as monotherapy or in combination with either obinutuzumab or rituximab. Patients receiving obinutuzumab-chlorambucil had a longer PFS compared to those treated with rituximab-chlorambucil (median PFS 27 months vs 16 months)^[97]. Further, the proportion of patients reaching MRD negativity was significantly higher with obinutuzumab treatment (20% in the obinutuzumab-chlorambucil arm vs 3% in the

rituximab-chlorambucil arm). However, infusion-related reactions were significantly more frequent with obinutuzumab. The CLL11-trial established the combination of obinutuzumab-chlorambucil as first-line therapy for “unfit” patients [97].

2.6 Bruton’s tyrosine kinase (BTK) inhibitors

2.6.1 Ibrutinib

Ibrutinib is an oral, selective and irreversible inhibitor of BTK, a non-receptor tyrosine kinase that plays a critical role in the BCR signaling. The BTK gene was discovered in 1993 [98] and is named after Ogden Bruton, who described X-linked agammaglobulinemia (XLA) in 1952 [99]. Mutations in the BTK gene can cause XLA, a genetic disorder characterized by the lack of mature B cells and profound lack of immunoglobulins [100]. Ibrutinib binds covalently to the cysteine-481 (C481) amino acid of the BTK enzyme and thus inhibits the BCR signaling pathway [101] (Figure 1). In addition, BTK inhibition also leads to decreased CXCR4 surface expression and as a consequence, CLL cells are redistributed into the blood with rapid resolution of lymphadenopathy [102]. Among the majority of patients, the lymphocytosis resolved within 8 months, but prolonged lymphocytosis was not a sign of suboptimal response to therapy [103].

Ibrutinib is given orally once daily at a fixed dose of 420 mg and the treatment is continuous. After 4 hours of administration, the drug gives a complete occupancy of the active site of the BTK and the full occupancy is maintained for at least 24 hours. Ibrutinib is mainly metabolized by cytochrome P450 (CYP)3A4 and the use of strong CYP3A inhibitors increases ibrutinib exposure 20-fold [104]. Therefore, strong CYP3A inducers/inhibitors should be avoided. No dose adjustment is required in patients with moderate renal impairment since ibrutinib is mainly eliminated via faeces.

In addition to BTK, ibrutinib inhibits other tyrosine kinases including interleukin-2-inducible T cell kinase (ITK), endothelial growth factor receptor (EGFR), tyrosine kinase expressed in hepatocellular carcinoma (TEC) and bone marrow tyrosine kinase on chromosome X (BMX). Thus, ibrutinib has significant off-target effects.

The first phase 2 study (PCYC-1102) of ibrutinib included treatment-naïve as well as pretreated CLL patients. The relapsed/refractory group included 85 patients of which 89% responded to treatment. The response was independent of risk factors including *TP53* disruption and *IGHV* mutation [105]. A 5-year follow-up of PCYC-1102, which included patients who continued in an extension study (PCYC-1103), showed that the CR rate was modest in relapsed/refractory patients (10%). The median PFS in relapsed/refractory patients was 51 months, which is almost ten times longer than with previous standard of care salvage regimens such as ofatumumab [85]. The median OS was not reached. *TP53* disruption was an independent prognostic factor for inferior PFS and OS, patients with *TP53* disruption had a median PFS of 26 months and a median OS of 57 months. Outcome did not differ significantly between patients with mutated *IGHV* and unmutated *IGHV*. Complex karyotype (≥ 3 unrelated chromosomal abnormalities) was not established as an independent prognostic factor since the outcome was heavily influenced by the coexistence of *TP53* disruption [23]. However, this contrasted with another ibrutinib study that reported complex karyotype to be a stronger driver for treatment failure than *TP53* disruption [106].

In the pivotal phase 3 study (RESONATE), patients with previously treated CLL were randomized between continuous ibrutinib and fixed duration (24 weeks) ofatumumab [2]. The ORR in the ibrutinib arm was 91% and 11% of patients reached CR. Median PFS was significantly longer with ibrutinib than with ofatumumab (44 months vs 8.1 months). The PFS benefit with ibrutinib was preserved in the high-risk group (i.e. patients with *TP53* disruption, del(11q) and/or unmutated *IGHV*), which represented 82% of the patients [2, 107]. Median PFS in patients with del(17p) and/or *TP53* aberration was 41 months compared to 57 months for patients without del(17p)/*TP53* aberration. However, the difference was not statistically significant. The low number of patients in the subgroups was a limiting factor in the analysis. The median OS was 68 months in the ibrutinib arm, which was significantly longer than OS in the ofatumumab arm [107]. The RESONATE study led to Food and Drug Administration (FDA) and EMA approval of ibrutinib in the second line setting.

Discontinuation of ibrutinib therapy has been a matter of concern. In a 3-year follow-up of the phase 2 trial PCYC-1102/1103, 19% of treatment-naïve patients and 48% of previously treated patients discontinued treatment. Toxicity was the reason for 11% of patients [108]. In the 6-year follow-up of RESONATE, 78% discontinued treatment. Sixteen percent discontinued due to toxicity. The probability of ibrutinib discontinuation for non-relapse reasons has been reported to increase by 10 percentage points for each addition of prior therapy [109].

In summary, the abovementioned clinical trials in (mostly) relapsed/refractory patients reported an ORR to ibrutinib of approximately 90% with a modest CR rate of approximately 10%. The median PFS was 51 months in PCYC-1102/1103 and 44 months in RESONATE. *IGHV* status had no impact on survival time. Patients with *TP53* aberration had a shorter PFS and OS, however in the RESONATE trial the difference was not significant. Several other clinical trials have explored ibrutinib in patients with *TP53* aberration [110, 111]. A cross study analysis of the 230 patients with relapsed/refractory CLL and *TP53* aberration from 3 clinical studies (PCYC-1102/1103, RESONATE and RESONATE-17) showed 30-month PFS at 57% and OS at 69%, respectively [112].

Ibrutinib is generally well tolerated, even by elderly patients with comorbidities. Atrial fibrillation is a well-known side effect with ibrutinib with a reported incidence of 6-16% in clinical trials [2, 108, 110, 113-115]. Furthermore, the FDA reported ventricular arrhythmias in 0.2% of ibrutinib-treated patients [116]. Cardiac tissue expresses BTK, but it is not known if the suppression of BTK or other off-target kinases causes the arrhythmias. Another ibrutinib-associated adverse event is bleeding; mild bleedings have been reported in more than 50% of patients and major hemorrhages (\geq grade 3) in 8-9% [23, 108]. Bleedings are reported with the more selective, second-generation BTK inhibitors as well [117], implying platelet dysfunction induced by BTK inhibition. Rash and diarrhea are related to EGFR inhibition but tend to be resolved with time. Hypertension has been reported as a side effect of ibrutinib in up to 72% of patients [118]. In the case of adverse events, dose reduction of ibrutinib does not seem to mitigate the treatment efficacy [119-121].

As previously mentioned, the susceptibility to infections is increased in patients with CLL. In CLL patients treated with ibrutinib, overall rates of infections are 10-15% in the first-line setting and 30-50% in relapsed disease [54]. There are several reports of opportunistic infections such as *Aspergillus*, *Cryptococcus* and *Pneumocystis jiroveci* pneumonia (PJP) during ibrutinib treatment [122-124]. BTK is an important activator of

macrophages and it has been suggested that BTK is needed for control of *Aspergillus* infection [125]. Moreover, BTK mediates pathways of immune response in neutrophils and monocytes [126]. A retrospective study of 560 patients reported a cumulative incidence of opportunistic infection at 4.7% after 5 years of treatment [127]. Invasive aspergillosis was the most frequent opportunistic infection and no case of PJP occurred. In a study of ibrutinib for primary central nervous system lymphoma, an incidence of *Aspergillus* infection of 39% was reported [125]. Ibrutinib has been reported to have an immunomodulating effect caused by the inhibition of BTK and ITK. PD-1 expression was markedly reduced during ibrutinib treatment [128, 129]. Regarding effects on T cell numbers, studies have reported disparate results [128, 129].

To date, it is unclear which is the most important reason for the infections seen during ibrutinib treatment. Reasons might be CLL, previous treatment or BTK inhibition. However, the infection risk is not increased when compared to treatment with alkylator-based regimens or CD20-mAbs [54].

Ibrutinib has indeed changed the treatment landscape for CLL and is now approved by the FDA and the EMA for all lines of treatment. However, ibrutinib resistance is a growing concern as more patients receive this treatment. One of the main mechanisms for acquired resistance is the development and selection of resistant clones. The most frequently described mutation leading to ibrutinib resistance is BTK cysteine to serine (C481S) mutation, resulting in a reversible binding of ibrutinib. The second most common mutation is a gain-of-function mutation in PLC γ 2, resulting in autonomous BCR activity [130]. In ibrutinib-treated patients with PD, mutations in BTK or PLC γ 2 were detected in 85% of patients and these mutations were detected at a median of 9.3 months before relapse [130]. In a French retrospective study, 30% of patients, who had been treated with ibrutinib for 3 years, had a BTK- and/or a PLC γ 2-mutation [131]. The mutations were significantly associated with subsequent CLL progression.

Real-world data for ibrutinib

“Real-world evidence” can be defined as information on health care that is derived from sources outside research settings [132]. Clinical trials are often conducted in selected populations that differ from patients in routine health care. An American real-world study included 616 ibrutinib-treated CLL patients, 88% from routine clinical practice and 12% from clinical trials. Eighty-seven percent of patients were previously treated. Thirty-nine percent had *TP53* aberration. The median PFS was 35 months and the median OS was not reached. There was no significant difference of PFS in first-line patients compared to PFS in previously treated patients. Further, there was no significant difference between outcomes for patients in routine clinical practice compared to those in clinical studies. In this real-world study, deletion of 17p had no significant impact on the outcome. However, complex karyotype predicted for shorter survival [133]. A Danish real-world study of 205 CLL patients reported 24-month PFS at 72%. Nineteen percent received ibrutinib as first-line and 75% had *TP53* aberration. [134]. In our Swedish real-world study, a median PFS of 37 months was reported [135]. Thus, the reported PFS times in the real-world studies are shorter than that previously described in the pivotal phase 3 study (RESONATE) with a median PFS of 44 months [107]. As previously mentioned, 27% of patients in RESONATE had *TP53* aberration [112] and in the cross study including only previously treated CLL

patients with *TP53* aberration, the reported PFS was similar to real-world results [112]. Thus, the perceived difference in outcome between the patients treated in routine clinical practice and those in clinical studies seems to be, at least partly, explained by the occurrence of *TP53* aberration.

At a median follow-up of 17 months in the abovementioned American real-world study, 41% of patients discontinued ibrutinib treatment. Twenty-one percent of patients discontinued treatment due to intolerance [133]. Another American real-world study included 1497 CLL patients who were treated with ibrutinib first-line. This study reported 16% discontinuation rate at 6 months [136]. The Danish real-world study reported that 42% of patients had discontinued their treatment at 21 months [134]. At 30-month follow-up of our real-world ibrutinib study, 49% of patients had discontinued treatment. Toxicity was the reason for 20% [121]. At 45-month follow-up, 63% had discontinued treatment [135]. In summary, real-world studies reported higher rates of discontinuation when compared to clinical trials, and in particular higher rates of discontinuation due to toxicity [108].

The American retrospective study reported that, at 17-month follow-up, 19% of patients required dose reduction due to adverse events. In our real-world ibrutinib study, 26% of patients required dose reduction at 30-month follow-up. In the clinical phase 2 study PCYC-1102/1103, 10% of patients required dose reduction at 36-month follow-up [108]. Thus, dose reductions seemed to be more frequent in routine health care than in clinical trials.

Phase 3 trials comparing ibrutinib with chemotherapy/chemoimmunotherapy

In a phase 3 study (RESONATE-2), previously untreated CLL patients, 65 years or older, without *TP53* disruption, were randomized to ibrutinib or chlorambucil. The ORR in the ibrutinib arm was 92%. CR rate increased from 11% at 18-month follow-up to 30% at 5-year follow-up. At a median of 5-year follow-up, the PFS in the ibrutinib group was 70% compared to 12% in the chlorambucil group. Five-year OS favored ibrutinib (83%) over chlorambucil (68%), despite 57% of patients in the chlorambucil group crossing over to ibrutinib [137]. These results demonstrated the improved survival with ibrutinib compared to chlorambucil first-line and ibrutinib monotherapy was established as an option for treatment-naïve, elderly (≥ 65 years) CLL patients.

The ECOG1912 trial compared ibrutinib-rituximab with FCR, which was the previous gold standard for young, fit patients. At a median of 4 years follow-up, ibrutinib-rituximab improved PFS significantly for patients with unmutated *IGHV*. In those with mutated *IGHV*, the small improvement observed with ibrutinib-rituximab was not significant [138, 139]. A longer follow-up time is needed for patients with *IGHV* mutation considering their excellent long-term response to FCR.

In the Alliance trial, treatment-naïve patients over 65 years were randomized to ibrutinib or ibrutinib-rituximab or BR. Both ibrutinib-containing regimens yielded superior PFS compared to chemoimmunotherapy regardless of cytogenetic features. However, there was no significant difference in OS. CR rates and MRD rates were modest in the ibrutinib arm; 7% reached CR and 1% reached MRD negativity. The addition of rituximab to ibrutinib did not impact PFS or OS [140].

In the Illuminate trial, ibrutinib-obinutuzumab was tested against chlorambucil-obinutuzumab in elderly and comorbid patients [141]. Ibrutinib-obinutuzumab showed a

significant PFS-increase. However, it was unclear if it is beneficial to add obinutuzumab to ibrutinib.

In summary, three phase 3 studies confirmed the superiority of ibrutinib compared to chemoimmunotherapy for all subgroups except for patients with mutated *IGHV*. For patients with mutated *IGHV*, the optimal front-line treatment is still not known.

2.6.2 Second-generation BTK inhibitors: acalabrutinib and zanubrutinib

The second-generation BTK inhibitors, acalabrutinib and zanubrutinib have higher selectivity for BTK compared to ibrutinib and they have shown promising early results and possibly also an improved toxicity profile^[142, 143]. Like ibrutinib, they bind irreversibly and covalently to C481 and thus, do not overcome common mechanisms of ibrutinib resistance. However, they may be useful in ibrutinib-intolerant patients^[144].

In a phase 2 trial, relapsed/refractory CLL patients were treated with acalabrutinib and PFS at 45 months was 58%. Thirteen percent of patients discontinued treatment and 7% showed atrial fibrillation^[145].

In a phase 3 trial, previously untreated CLL patients were randomized to acalabrutinib-obinutuzumab or acalabrutinib as monotherapy or chlorambucil-obinutuzumab. The 28-month interim results have recently been reported with a significantly improved PFS in both acalabrutinib arms. PFS rates for acalabrutinib-obinutuzumab, acalabrutinib as monotherapy and chlorambucil-obinutuzumab and were 93%, 87% and 47% respectively. Acalabrutinib was well tolerated; the incidence rates of atrial fibrillation were 3% and 4% in the acalabrutinib arms and 1% in the chlorambucil arm. The incidence rates of hypertension, grade 3 or higher, were not increased in the acalabrutinib arms. However, bleeding of all grades was more frequent in the acalabrutinib arms (43, 39% vs 12%). Thus, preliminary results report that acalabrutinib was efficient in treatment-naïve CLL patients. The toxicity profile was acceptable with less cardiac toxicity than for ibrutinib^[117], but with similar rate of bleeding toxicity. A phase 3 trial of acalabrutinib vs ibrutinib in previously treated CLL patients is ongoing (NCT02477696). Acalabrutinib is approved by the FDA for CLL treatment.

Zanubrutinib demonstrated less selectivity for BTK compared to acalabrutinib^[146]. In a phase 1 study, no patient progressed, at 12 months. Zanubrutinib demonstrated a low rate of major toxicities^[143]. A phase 3 trial on zanubrutinib vs ibrutinib in previously treated CLL patients is ongoing^[147]. Zanubrutinib was recently approved by the FDA for treatment of mantle cell lymphoma.

2.7 Phosphatidylinositol 3-kinase (PI3K) inhibitors

2.7.1 Idelalisib

PI3Ks are enzymes involved in many cellular processes including cell survival, proliferation and differentiation. The PI3Ks are divided into four classes (I-IV) and class I is subdivided into four isoforms: alpha, beta, delta and gamma. PI3K δ has a critical role in the BCR pathway mediating survival, proliferation, migration and adhesion^[148].

Idelalisib is an oral inhibitor of the delta isoform of PI3K (Figure 1) and it was investigated in a randomized phase 3 trial using rituximab in combination with either

idelalisib or placebo in relapsed CLL patients [149]. The study was closed at the first interim analysis due to the overwhelming advantage of the combination therapy over rituximab and this led to the approval of idelalisib in combination with rituximab by the FDA and the EMA in 2014. Idelalisib proved to be effective in patients harboring *TP53* disruptions; an extension study of all patients treated with idelalisib and rituximab in combination yielded a median PFS of 19 months in patients with *TP53* disruption and 21 months in patients without *TP53* disruption [24].

Further, in a phase 2 study of idelalisib in previously untreated CLL, serious autoimmune-mediated adverse events, such as transaminitis, hepatitis, pneumonitis and colitis were reported [150]. One hypothesis is that immune-mediated idelalisib-related toxicities are caused by inhibition of T_{reg} . T_{reg} express PI3K δ and inhibition of this enzyme leads to suppression of T_{reg} and thus activation of CD4+ and CD8+ effector lymphocytes. This hypothesis has been supported by clinical evidence since CD8+ T-cell infiltrates were seen in tissue biopsies of patients who experienced colitis or hepatitis [150, 151]. The activation of effector lymphocytes appears more pronounced in younger or treatment-naive patients or those treated concomitantly with another immunomodulating agent. These autoimmune toxicities generally respond to steroids [150].

Despite the reported autoimmune adverse events, three phase 3 trials (NCT01732913, NCT01732926, NCT01980888) investigating idelalisib in combination with rituximab vs chemotherapy in treatment-naive or early line patients were launched. In 2016, these 3 trials were prematurely stopped due to increased deaths and serious adverse events in patients receiving idelalisib. In particular, increased rates of opportunistic infections: fatal CMV infections and PJP were reported [152]. Therefore, prophylaxis for PJP and monitoring for CMV reactivation are now recommended during idelalisib therapy [152]. This demonstrates that idelalisib causes other immunomodulatory effects, besides autoimmune toxicity.

Idelalisib in combination with rituximab is now approved for patients who have received at least one prior therapy, or as first-line treatment in patients with *TP53* disruption who cannot be treated with any other therapy.

2.7.2 Duvelisib

Duvelisib is an oral inhibitor of PI3K δ (Figure 1) and, in contrast to idelalisib, also a strong inhibitor of PI3K γ . The value of inhibiting PI3K γ in CLL is unknown. Duvelisib has proven to be effective in relapsed/refractory CLL patients. A phase 3 randomized clinical trial (DUO) included patients with relapsed/refractory CLL that were randomized to either duvelisib or ofatumumab [153]. The ORR was higher for the duvelisib group compared to the ofatumumab group (74% vs 45%), with nearly all responses being PR. The median PFS was 13 months in the duvelisib arm vs 10 months in the ofatumumab arm. Adverse events grade 3 occurred in 87% in the duvelisib arm compared to 48% in the ofatumumab arm. Adverse events during duvelisib treatment included neutropenia, infections, diarrhea, and colitis, which occurred as frequently as during idelalisib treatment [154]. Pneumonitis and transaminitis seemed less frequent with duvelisib. Based on this study, the FDA approved duvelisib for relapsed/refractory CLL patients having had at least 2 lines of prior therapy.

2.8 B cell lymphoma 2 (BCL2) inhibitor: venetoclax

In CLL, the prosurvival intracellular protein BCL2 is highly expressed, which is one of the main mechanisms of pathogenesis. Apoptosis is regulated by a complex interplay of proapoptotic and pro-survival proteins triggered by intracellular and extracellular signals. The most important effectors of apoptosis are the proteins BCL2 associated X (BAX) and BCL2 agonist/killer 1 (BAK). Both proteins permeabilize the outer membrane of the mitochondrion leading to a release of cytochrome C into the cytoplasm with a resulting activation of caspases and apoptosis [155]. This action is initiated by the proapoptotic BCL2 homology 3 (BH3) -only family of proteins. Under normal cellular circumstances the BH3-only family proteins are bound to pro-survival protein, such as BCL2, myeloid cell leukemia 1 (MCL1) and B-cell lymphoma-extra large (BCL-XL), preventing them from binding to BAX and BAK [156]. Venetoclax is an oral selective inhibitor of BCL2. Venetoclax binds to the BH3-binding pocket on BCL2 thereby displacing pro-apoptotic proteins resulting in the activation of BAX and BAK and finally apoptosis (Figure 1).

In the pivotal phase 2 study in relapsed/refractory patients with *TP53* disruption, venetoclax induced response in 77% of patients including 20% CR. The 24-month PFS rate was 54% [157]. In patients failing ibrutinib therapy, venetoclax showed a response rate of 65% and a median PFS of 25 months [158].

Venetoclax is generally well tolerated. The most important adverse event reported in a phase 1 trial was tumor lysis syndrome (TLS), which in one case was fatal [3]. To minimize the risk of TLS a ramp-up-schedule was introduced. Hematologic toxicity was neutropenia occurring in 31-51% of patients (grade 3-4) and thrombocytopenia occurring in 15-29% (grade 3-4). Common non-hematologic adverse events included diarrhea, upper respiratory tract infection and cough.

Acquired resistance to venetoclax is an increasing concern. The most common mechanism of resistance is a G101V mutation in the BCL2 binding site for venetoclax and the mutation has been detected after 19-42 months of treatment [159, 160]. This mutation results in a 180-fold decreased affinity of venetoclax for BCL2 [160]. Due to the concerns of acquired resistance with targeted therapies, clinical trials aimed at reaching deep responses with time-limited combination therapies.

2.8.1 Combinations using venetoclax and anti-CD20 antibodies

Venetoclax-rituximab

In the randomized phase 3 trial (MURANO), relapsed/refractory CLL patients were treated with fixed-duration venetoclax-rituximab or BR. The 3-year PFS rate was significantly higher in the venetoclax-rituximab group (71%) than in the BR group (15%). MRD negativity was achieved in 62% of patients treated with venetoclax-rituximab and 13% of patients treated with BR. MRD negativity at end of treatment predicted for longer PFS. The study established a time-limited use of venetoclax and rituximab in relapsed/refractory CLL patients [22, 75].

Venetoclax-obinutuzumab

A phase 3 study including 432 previously untreated patients with coexisting morbidities were treated with fixed-duration venetoclax-obinutuzumab or chlorambucil-obinutuzumab. Approximately 80% of the patients in the venetoclax-obinutuzumab completed treatment and 50% reached CR. The PFS rate at 2 years was significantly higher in the venetoclax-obinutuzumab group (88% vs 64%) [161]. The study led to the approval of venetoclax in combination with obinutuzumab as a first-line treatment by the FDA and EMA.

In summary, combinations of venetoclax and CD20-antibodies have yielded unprecedented CR rates in relapsed/refractory CLL patients. Venetoclax is approved by the FDA for all lines of therapy and by the EMA for the treatment of relapsed/refractory patients (as monotherapy or in combination with rituximab) and for treatment-naïve patients (in combination with obinutuzumab). No randomized comparison with a BTK inhibitor has been performed.

2.9 Other immunotherapy

2.9.1 Immunomodulatory drug: lenalidomide

Lenalidomide has been explored in clinical trials for CLL treatment. It is an immunomodulatory drug with an ORR of 32-47% in patients with relapsed/refractory CLL [162-164]. However, CLL patients have shown problems tolerating the lenalidomide treatment, with TLS and neutropenia being the best-known toxicities [163]. The mechanisms of action for lenalidomide in CLL are not entirely understood, but might include a direct tumor cell killing effect, immune modulation and alteration of the tumor microenvironment [165]. Lenalidomide has been reported to diminish PD-1 expression of T cells in previously treated CLL patients, thus reversing the “exhaustion” of T cells [166].

In phase 3 studies, lenalidomide has proven to be efficient as maintenance therapy after chemoimmunotherapy [167, 168]. However, with the introduction of BTK inhibitors, the role of lenalidomide as maintenance therapy is unclear. Lenalidomide is currently not approved for CLL treatment and its possible use in this disease is not established.

2.9.2 Allogeneic hematopoietic stem cell transplantation

Until recently, allogeneic HSCT was regarded as the only curative treatment of CLL. According to the EBMT, the current indication for allogeneic HSCT requires high-risk disease and failure on at least one BCR pathway inhibitor [169]. Allogeneic HSCT should be performed when the disease is in remission. Chemoimmunotherapy was used historically, but retrospective data support the use of venetoclax or idelalisib in combination with rituximab for induction and bridging to allogeneic HSCT [170, 171]. Estimated PFS at 10 years after allogeneic HSCT was 28% and OS was 35%. Non-relapse mortality was 40% [172].

2.10 Selecting the right treatment

Treatment of CLL patients is becoming more individualized, since there is no single, first-line or salvage regimen that is widely agreed upon. For treatment-naïve patients, ibrutinib

has turned out to be superior to other drugs and drug combinations in all subgroups, except for fit patients younger than 65 years with mutated *IGHV* and without *TP53* aberration. For this group of patients FCR could give a very long remission and possibly even cure [93]. However, no drug has proven to be significantly better than any other drug for this group and there is still no agreement on how to best treat these patients. The choice of treatment depends on age, performance status, comorbidities and del(17p)/*TP53* mutation status.

The U.S. National Comprehensive Cancer Network (NCCN) treatment guidelines recommend ibrutinib as first-line treatment of all patients with CLL without del(17p)/*TP53* mutation. For patients with del(17p)/*TP53* mutation, participation in a clinical trial should be considered. For patients not participating in clinical trials, ibrutinib is recommended [173].

Ibrutinib is not subsidized by the Swedish national health care system for first-line treatment and national guidelines recommend clinical studies to be considered for all CLL patients. For patients not participating in clinical trials, chemoimmunotherapy (FCR, BR or chlorambucil-rituximab) is recommended for patients without del(17p)/*TP53* mutation and ibrutinib for patients with del(17p)/*TP53* mutation [58]. If ibrutinib is considered inappropriate, venetoclax or idelalisib in combination with rituximab could be used.

For relapsed patients, no treatment strategy is widely agreed upon. The Swedish national guidelines recommend clinical studies for these patients [58]. NCCN recommends ibrutinib or venetoclax-rituximab [173].

2.11 Emerging therapies

2.11.1 Reversible BTK inhibitors: vecabrutinib, ARQ-531 and LOXO-305

Reversible BTK inhibitors bind non-covalently and do not rely upon interaction with the cysteine residue (C481). They might be an alternative treatment for patients with ibrutinib resistance. Recent data from a phase 1B/2 study on vecabrutinib demonstrated that among 23 CLL patients who were refractory to a BTK inhibitor, none responded to therapy. The next group included in the study will receive a higher treatment dose [174]. Early phase 1 data on ARQ-531 demonstrated that out of 9 patients with C481S BTK mutation and ibrutinib refractoriness, 8 responded to treatment (all PR) [175]. Early phase I data on LOXO-305 reported efficacy in CLL, 10 of 13 evaluable patients responded to treatment (all PR), including those with BTK or BCL2 mutation [176, 177]. For the reversible BTK inhibitors, longer follow-up is needed to evaluate safety and efficacy.

2.11.2 New CD20-antibody: ublituximab

Ublituximab is a type I, chimeric, glycoengineered anti - CD20 recombinant mAb with improved ADCC. Ublituximab in combination with umbralisib has shown promising results in relapsed/refractory CLL [178], but its role in the spectrum of treatments of CLL remains to be further explored.

2.11.3 New PI3K inhibitor: umbralisib

Umbralisib is an oral PI3K δ inhibitor with a different molecular structure than idelalisib and duvelisib. The change in molecular structure leads to inhibition of casein kinase 1

epsilon (CK1 ϵ) and it may change the toxicity profile. The role of CK1 ϵ in CLL is unclear but it has been suggested that inhibition of CK1 ϵ may hinder suppression of T_{reg} mediated by PI3K δ [179]. Thus, umbralisib may have less immune-mediated toxicities than other PI3K δ -inhibitors and might lead to a renaissance for the PI3K inhibitors in CLL. Single agent umbralisib proved to be efficient and well tolerated in relapsed/refractory CLL in a phase 2 trial for patients who were intolerant to BTK- or PI3K δ -inhibitors [180]. A phase 3 study (UNITY-CLL) comparing umbralisib-ublrituximab to chlorambucil-obinutuzumab in treatment-naïve and relapsed/refractory CLL patients is ongoing (NCT02612311).

2.11.4 Combination of ibrutinib and chemoimmunotherapy

In the phase 3 HELIOS study, 578 previously treated patients without 17p deletion were treated with either ibrutinib or placebo in combination with BR. At a median follow-up of 35 months, the median PFS was not reached for the ibrutinib-BR arm while it was 14 months for the placebo-BR arm. Concerning OS, the hazard ratio (HR) was 0.65 for ibrutinib vs placebo. Thus, the study reported improved survival with ibrutinib-BR compared to BR in relapsed CLL [181]. However, the advantage of adding ibrutinib to chemoimmunotherapy has been debated since, in the ibrutinib-BR group the PFS at 36 months was 68%, being very close to the PFS at 30 months of 69% reported with ibrutinib as monotherapy [108].

2.11.5 Combinations of BCR inhibitors and BCL2 inhibitors

Preclinical studies demonstrated a synergistic effect of venetoclax and ibrutinib in combination [182]. The safety of this drug combination was first demonstrated in a phase 2 trial of high-risk CLL patients. Ibrutinib was given as monotherapy for 3 months and then in combination with venetoclax for 24 months. If MRD negativity was achieved, treatment was discontinued. This study reported 88% CR and 61% CR with MRD negativity [183, 184], which was higher than for each agent as monotherapy. The safety profile of venetoclax and ibrutinib in combination was similar to known toxicity for both agents separately. Another phase 2 study (CAPTIVATE), with a similar design, confirmed these findings [185, 186]. The combination has also shown promising results in relapsed/refractory CLL patients. In the VISION study, MRD negativity was achieved in 55% of patients and in the CLARITY trial, 36% of patients attained MRD negativity [187, 188].

In summary, venetoclax and ibrutinib in combination yielded a high frequency of CR and MRD negativity and the treatment was well tolerated. However, the follow-up times of these studies are still short. Two phase 3 studies with the combination of ibrutinib, venetoclax and obinutuzumab aiming at deeper responses are ongoing [189, 190].

2.11.6 Checkpoint inhibition: pembrolizumab

Immune checkpoint inhibition targeting PD-1 has been explored in CLL. In a phase 2 trial of a PD-1-blocking antibody, pembrolizumab, responses were reported in 0 out of 16 refractory CLL patients and in 4 out of 9 patients with RT [191]. Pembrolizumab is now explored in combination therapy in CLL and RT [192].

2.11.7 Chimeric antigen receptor (CAR) T-cell therapy

T cells bearing a chimeric antigen receptor (CAR) are produced by genetic engineering and they are designed to direct the patient's T cells against the tumor cells. At present, the most advanced CAR T-cells are directed against CD19. A review summarized the results of trials on CAR T in 134 heavily pretreated CLL patients between the years 2011 and 2018. CR was obtained in 20-30% of patients and at 18 months, PFS was 25%. The effect of CAR T-cell therapy on RT was partial and transient ^[193]. The most frequent complications to CAR T-cell therapy are cytokine release syndrome and neurological toxicities ^[194]. Fewer tumor cells at the time of injection lead to less cytokine release and thus to less toxicities ^[194].

Recent data on the CD19-targeted CAR T-cell therapy, lisocabtagene-maraleucel (liso-cel), which is given in a predefined composition of CD4+ and CD8+ T lymphocytes, were promising. In 23 relapsed/refractory CLL patients, 82% responded to treatment. Most patients had deepening responses with time. However, the follow-up time was only 11 months ^[195].

Efficacy of CAR T seems lower in CLL than in acute lymphoblastic leukemia and DLBCL. This could in part be due to the T cell exhaustion seen in CLL ^[196]. Data suggest that the combination with ibrutinib might reduce this problem ^[197].

3 AIMS

The aims of this thesis are to explore safety and efficacy of targeted agents in patients with advanced-phase chronic lymphocytic leukemia.

More specifically:

- I. To obtain knowledge about the safety and efficacy of lenalidomide and alemtuzumab in combination therapy and to examine whether low-dose lenalidomide could stimulate and maintain immune functions in very advanced-phase CLL during therapy with alemtuzumab.
- II. To obtain knowledge of the safety and efficacy of ibrutinib and alemtuzumab in combination therapy in previously treated CLL patients.
- III. To obtain knowledge about the efficacy and safety of ibrutinib in consecutive patients with advanced-phase CLL when used in routine health care.
- IV. To obtain knowledge about the long-term efficacy and safety of ibrutinib in consecutive patients with advanced-phase CLL when used in routine health care.

4 MATERIAL AND METHODS

4.1 Evaluation of response and toxicity

Treatment response was classified according to the IWCLL response criteria of 2008 with the exception that lymphocytosis was not the sole criterion for disease progression [73, 198]. Response evaluation in patients with SLL was based on the 2007 International Working Group Criteria for non-Hodgkin lymphoma [199]. CR required evaluation by computed tomography scan and bone marrow examination. Cumulative Illness Rating Scale (CIRS) was used to define comorbidities at baseline [200].

Treatment toxicity was evaluated using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 except for anemia, thrombocytopenia and neutropenia, which were graded according to the IWCLL grading scale for hematologic toxicity in studies [198]. In Paper I and II, adverse events of all grades were recorded. In Paper III, adverse events of grade 3 or higher were recorded, whereas any grade of adverse events was reported for hemorrhage, diarrhea, arthralgia, atrial fibrillation and blood counts. In Paper IV, adverse events of grade 3 or higher were recorded, with the exception of atrial fibrillation for which all grades were recorded.

4.2 Study procedures

4.2.1 Paper I and II

Consecutive patients with chemotherapy-refractory CLL were included in the studies. Adequate organ function and Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 were required. All patients had bacterial and viral prophylaxis and, if needed, G-CSF.

4.2.2 Paper III and IV

All patients in the compassionate use program for ibrutinib in Sweden were identified and included in this retrospective real-world study. The compassionate use program was open for inclusion between May 15, 2014 and May 31, 2015 and all the included patients were identified. Patients from 27 hospitals were included. Data were extracted from the medical files of the patients and entered into case record forms (CRF) by each treating physician. The data from individual patient files were crosschecked by the academic study team.

Patients were eligible for the compassionate use program if they had chemotherapy-refractory CLL or *del(17p)/TP53* aberration, adequate organ function and ECOG performance status ≤ 2 and had not undergone an allogeneic HSCT within the last 6 months. Baseline comorbidities were evaluated by CIRS score.

4.3 Laboratory methods

4.3.1 Paper I

Immune monitoring was performed at baseline, at week 4, 16, 30 and 45. Laboratory methods are described in detail in paper I.

Flow cytometric analysis of whole blood lymphocytes

After lysis of red blood cells, cells were washed and stained according to the manufacturer's recommendation using fluorochrome-coupled antibodies. Flow cytometric analysis was done for CD19, CD3, CD4, CD8, CD16, CD56, CD52, CD45RA, human leukocyte antigen-antigen D related (HLA-DR) and C-C motif chemokine receptor (CCR)7.

Flow cytometric analysis of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood by using Ficoll gradient density separation. Cells were freshly used or stored in liquid nitrogen until use. The PBMC for flow cytometry were mixed with appropriate amount of fluorochrome-coupled antibodies for PD-1, PD-L1, CCR6, CXCR3, CD25 and HLA-DR.

For intracellular staining, cells were resuspended in fixation/permeabilization buffer, incubated for 30 min at 4 °C and washed with permeabilization buffer. Antibodies (FOXP3, granzyme B, perforin and Ki67) were added to the resuspended cells and incubated for 30 min at room temperature. Excess of antibodies was removed by washing twice with permeabilization buffer. The cells were then resuspended in cell staining buffer (CSB) and analyzed by flow cytometry.

4.3.2 Paper II

Immune monitoring was performed at baseline, at week 2, 4, 10, 16, 21 and 32. Laboratory methods have previously been described in detail ^[61].

Flow cytometric analysis of whole blood lymphocytes

After lysis of red blood cell, cells were washed and stained for CD19, CD16, CD56, CD4, CD3, CD8 and CD45. After incubation and washing, cells were resuspended in CSB and analyzed by flow cytometry.

Flow cytometric analysis of PBMC

PBMC were isolated from fresh blood by using Ficoll density gradient separation. Cells were freshly used or stored in liquid nitrogen until use. Flow cytometric analysis of PBMC was done for CD19, CD4, CD5, CD3, CD8, CD45RA, PD-1, CD69, Ki67, HLA-DR, CD52, CCR6, CCR4, CD127, CXCR3, CCR7, PD-L1, Ror1 and CD20 and the appropriate isotype controls.

To assess Ki67 expression, freshly isolated PBMC cells were fixed with 1.6% paraformaldehyde and permeabilized with 80% methanol. The cells were then stained with both the appropriate surface antibodies and the anti-Ki67 or isotype control.

4.3.3 Paper IV

The testing for BTK mutation in 4 of the 6 patients who had PD while on ibrutinib treatment was done with Sanger sequencing.

4.4 Statistical analysis

4.4.1 Paper I and II

Survival analysis was done with the Kaplan-Meier method. Comparison of marker expression at different time points was done with the Wilcoxon signed-rank test for related samples. All tests were two-sided and $p < 0.05$ was considered significant.

4.4.2 Paper III and IV

Time to response was defined as the time from start of ibrutinib until the date of fulfilling criteria for PR-L, PR or CR. PFS was defined as the time from start of treatment until PD, the start of new anti-cancer treatment or death from any cause. Patients, who proceeded to allogeneic HSCT or had donor lymphocyte infusion after having responded to treatment, were excluded from PFS analysis. OS was defined as the time from start of ibrutinib treatment to death or latest follow-up.

Response rates were presented with 95% confidence intervals (CI). Survival (PFS, OS) was estimated by the Kaplan-Meier method. Univariate and multivariate analyses of time to failure were estimated using the Cox proportional regression hazards model. Results are presented as HR with 95% CI.

In exploratory analyses, characteristics associated with PFS and OS were identified. Patients were grouped according to each characteristic and groups were compared by means of log-rank test.

4.5 Ethical aspects

All studies were approved by the Regional Ethics Committee. Patients who were included in the studies in Paper I and II provided informed consent. Since the studies in Paper III and IV were retrospective, no informed consent was required. The studies in Paper I and II were approved by the Medical Products Agency.

5 RESULTS, DISCUSSION AND CONCLUSIONS

5.1 Paper I

Phase I-II study of lenalidomide and alemtuzumab in refractory chronic lymphocytic leukemia (CLL): effects on T cells and immune checkpoints

Winqvist et al. Cancer Immunol Immunother, 66(1):91-102, 2017

When this study was initiated, there was no consensus on how to treat refractory/relapsed CLL patients. Alemtuzumab was the most frequently used drug in this clinical situation, but treatment results of alemtuzumab as monotherapy were dismal and resulting opportunistic infections were of great concern. The rationale for combining alemtuzumab and lenalidomide was based on the assumption that lenalidomide might stimulate immune functions, thus preventing the negative effect on T cells seen with alemtuzumab. Furthermore, lenalidomide has its major effects in lymph nodes and alemtuzumab in the blood and bone marrow. A phase I trial, in which 12 refractory CLL patients were included, explored the safety and efficacy of lenalidomide and alemtuzumab in combination [201]. The present study was an extension of the phase I study and included 11 additional refractory CLL patients.

In total, 23 consecutive patients were enrolled. They were treated with lenalidomide alone during the first 4 weeks and alemtuzumab was added at week 5. Maximum treatment period was 16 weeks of lenalidomide and 12 weeks of alemtuzumab.

The combination showed an acceptable safety profile and clinical activity with 58% ORR. Median PFS was 6 months and median OS was 17 months. The median response duration was 12 months (range 1-29). Lenalidomide had a narrow therapeutic dose range; 2.5 mg/day was not efficient and the maximal tolerated dose was 5 mg/day. The most common grade 3-4 adverse event was neutropenia (84% of patients), which was also the dose limiting toxicity. Febrile neutropenia occurred in 30% of patients and CMV reactivation requiring valganciclovir occurred in 30%. Grade 3-4 thrombocytopenia occurred in 55%.

Immune stimulation was apparent after 4 weeks of low-dose lenalidomide. It was indicated by an increased frequency of proliferating (Ki67+) CD8+ T cells accompanied by an increased frequency of activated (HLA-DR+) T cells. Furthermore, the frequency of antigen-experienced CD8+ effector memory cells increased at week 4, while effector cells decreased in both the CD4+ and CD8+ subsets.

After combination treatment all the lymphoid subsets decreased dramatically. T cells shifted towards a type 2 T helper cell (Th2) profile. The frequencies of type 17 T helper cells (Th17) and T_{reg} were reduced. The proportion of granzyme B+ T cells increased at 30-week follow-up, indicating an enhanced cytotoxic potential. PD-1 expression was unaffected at all analyzed points of time.

In conclusion, lenalidomide and alemtuzumab is an active combination in refractory CLL, but with novel targeted therapies the treatment combination is of very limited use. Further, low-dose lenalidomide and alemtuzumab had major effects on T cells including increased proliferative activity and cytotoxic potential.

5.2 Paper II

Dual targeting of Bruton tyrosine kinase and CD52 induces minimal residual disease-negativity in the bone marrow of poor-prognosis chronic lymphocytic leukemia patients but is associated with opportunistic infections - Results from a phase I study

Winqvist et al. Br J Haematol, 182(4):590-4, 2018

The safety and efficacy of ibrutinib in the combination with alemtuzumab was explored in this phase I study. The rationale for the combination was that ibrutinib has its major effects in lymph nodes while alemtuzumab has effects in the blood and bone marrow. Moreover, the drugs have different mechanisms of action and different toxicity profiles. Eight patients with advanced-phase CLL were included. They were treated with ibrutinib alone during the first 4 weeks and alemtuzumab was added at week 5.

Seven patients responded to treatment (4 had CR and 3 had PR). All but one reached MRD negativity in the bone marrow. One patient developed progressive multifocal leucoencephalopathy (PML) and two patients developed *Aspergillus* pneumonia.

During the monotherapy with ibrutinib the percentage of proliferating (Ki67+) T cells decreased and the proportion of proliferating (Ki67+) CLL cells became undetectable. The balance of type 1 T helper cells (Th1) and Th2 was unaffected, as well as the PD-1-expression on T cells. Other studies have reported a decrease in PD-1 expression [128, 129]. The divergent results could be explained by the limited time of ibrutinib monotherapy in our study. Moreover, the expression of CD20 and CD52 on CLL cells decreased.

After combination therapy, the frequencies of Th17 cells and T_{reg} were significantly reduced. Further, there was a shift to a Th2 profile. These findings confirmed the findings of Study I. PD-1 expression on T cells increased with combination therapy. This finding was contrary to the findings in Study I. Therefore, control experiments were done to determine the effects of the single agent alemtuzumab. These experiments indicated that the increase of PD-1 on T cells was attributable to alemtuzumab. Immune profiling was consistent with immunosuppression, as defined by increase of PD-1 expressing T cells and the Th2 shift.

The study was closed in advance due to the high rate of opportunistic infections. We advise against the combination of ibrutinib and alemtuzumab.

5.3 Paper III

Real-world results of ibrutinib in patients with relapsed or refractory chronic lymphocytic leukemia: data from 95 consecutive patients treated in a compassionate use program. A study from the Swedish Chronic Lymphocytic Leukemia Group.

Winqvist et al. Haematologica, 101(12):1573-80, 2016

Randomized controlled trials (RCT) are the standard prerequisite for treatment guidelines. Discrepancies of results between clinical trials and routine health care are commonly observed in oncology/hematology, as shown in CLL patients with the FCR regimen [1, 202]. Clinical trials are conducted in selected populations that differ from patients in routine health care. Clinical trials in CLL more frequently enroll younger patients with less comorbidity than patients in routine health care. Further, clinical trials use extensive monitoring and specialized research staff to ensure adherence to a standard protocol.

Hence, the internal validity of clinical trials is high, but often at a cost of less generalizability (external validity). Thus, real-world studies may provide important data on treatments in a wider population, reflecting patients in routine health care. Real-world studies may also generate data on concomitant comorbidities and medications. Further, real-world studies are generally more cost-effective and rapid than RCTs. However, real-world studies generally are affected by lower internal validity and susceptibility to selection bias.

Several new, targeted agents in oncology/hematology are subject to the FDA accelerated approval program, which allows a faster approval of drugs targeting serious illnesses with an unmet clinical need. Real-world evidence can play a role to support accelerated approval and the FDA has expressed their interest in more frequent use of real-world evidence ^[132].

In Paper III, 95 consecutive patients in a compassionate use program for ibrutinib were identified and included in the retrospective study. The median age was 69 years. Sixty-three percent had *TP53* aberration. At a median follow-up of 10.2 months the ORR was 84%, the PFS rate was 77% and the OS rate was 83%. PFS and OS were significantly shorter for patients with *del(17p)/TP53* aberration, no other factor turned out to be significant in the Cox proportional regression model.

Twenty-four percent of patients discontinued ibrutinib due to PD (n=10), adverse events (n=10) or due to other reasons (n=3). Ibrutinib was well tolerated. The most common non-hematologic grade 3-4 adverse event was infection. Hematomas occurred in 46% of patients, all of them grade 1-2. Eight percent had atrial fibrillation. Seven patients showed RT occurring after a median time of 9.5 months.

Half of the patients would not have fulfilled the inclusion criteria for the pivotal ibrutinib trial (RESONATE). This fact demonstrates the real-world attributes of the patients. The results from this study show that efficacy and toxicity of ibrutinib in routine health care was similar to that of pivotal trials.

5.4 Paper IV

Long-term real-world results of ibrutinib therapy in patients with relapsed or refractory chronic lymphocytic leukemia: 30-month follow-up of the Swedish compassionate use cohort.

Winqvist et al. Haematologica, 104(5):E208-E10, 2019

Paper IV is a long-term follow-up of the 95 patients in the compassionate use program for ibrutinib. At 30-month follow-up, the ORR was unchanged compared to the 10-month follow-up. The CR rate was not evaluable since bone marrow examinations were only carried out in a few patients. The PFS rate was 52% and the OS rate was 63%. In contrast to the early (10-month) report there was no negative survival impact of *TP53* aberration. In multivariate analyses, PFS was significantly associated with baseline comorbidities and number of prior therapies, while OS only with baseline comorbidities. As mentioned above, 44% of the patients in the study did not meet the inclusion criteria for the pivotal ibrutinib trial, RESONATE. The most frequent criterion that would have excluded the patients in was ECOG performance status ≥ 2 . OS was significantly shorter for patients who did not match the RESONATE inclusion criteria.

The observed PFS and OS in our study were shorter than in the pivotal phase 3 study, RESONATE. The explanation for the inferior PFS and OS in our study when compared to the pivotal study, RESONATE, could in part be attributed to a larger percentage of patients with *TP53* aberration (27% in RESONATE vs 63% in our study), since these patients seem to have a shorter survival on ibrutinib. Moreover, “real-world-factors” such as comorbidity and performance status probably partially explain the difference.

Fifty-one percent of the patients had grade 3-4 infections and 13% had grade 3-5 opportunistic infections. Fifteen percent developed atrial fibrillation at 30-month follow-up. This is, in comparison with the cumulative incidence of atrial fibrillation of 8% at 10-month follow-up, implying that the risk of atrial fibrillation is substantial during long-term treatment. RT occurred in 13 % after a median time of 14 months.

In our study, 49% discontinued treatment. The reasons for treatment discontinuation were toxicity (n=19), RT (n=11), CLL progression (n=6), allogeneic HSCT (n=4), second malignancy (n=3), need of dual platelet therapy (n=3) and sudden death (n=1). Thus, 20% of patients discontinued due to toxicity. The most common toxicity leading to treatment interruption was infection. In a 3-year follow-up of the phase 2 study PCYC-1102/1103, 28% of patients interrupted treatment and 11% discontinued due to toxicity ^[108]. Thus, discontinuation of treatment, and particularly due to toxicity was more frequent in our study. Other real-world studies also reported higher rate of discontinuations in comparison to clinical studies ^[133, 134, 136].

Twenty-six percent of patients experienced dose reductions or temporary treatment breaks. Survival (PFS and OS) was not inferior for these patients. The observed frequency of dose reductions in our study was greater than in clinical trials ^[108].

Four of six patients who had PD while on ibrutinib were tested for mutation of BTK and all of them carried the C481S mutation, which is in line with the abovementioned findings from a French real-world study ^[131].

In conclusion, ibrutinib is effective and well tolerated for long-term treatment in routine health care. Nevertheless, non-tolerability and progressive disease are recurring events.

6 FUTURE PERSPECTIVES

During recent years, a remarkable change in treatment of CLL has occurred. There is a shift towards targeted therapies in routine health care and in a near future chemoimmunotherapy might not be an alternative among the standard treatments of CLL. In the era of targeted treatment, many questions concerning clinical applications remain unanswered.

The “watch and wait” strategy might be challenged by new therapies. Especially, for high-risk patients, start of therapy at an earlier stage of disease might be considered ^[190, 203].

Ibrutinib has shown unprecedented results, especially in high-risk patients. However, this therapy raises concerns about residual disease, acquired resistance and continuous treatment. Our work and the work by others show that non-tolerability and discontinuation are recurrent events during ibrutinib treatment ^[121, 133, 134]. It is uncertain if the treatment can be discontinued safely and if so, at what depth of remission or length of therapy. At present, our group is carrying out an academic study to search for an answer. Further, second-generation BTK inhibitors, such as acalabrutinib and zanubrutinib, might limit off-target toxicity and reversible (non-covalent) BTK inhibitors such as vecabrutinib and LOXO-305 may overcome the problems of acquired resistance due to BTK point mutation. However, BTK inhibitors rarely lead to CR or MRD negativity.

Venetoclax in combination with CD20-mAbs have resulted in deep remissions and a high rate of MRD negativity ^[161]. The relative efficacy of ibrutinib vs venetoclax in combination with a CD20-mAb in the first-line setting is still unknown and a phase 3 study (CLL17) will seek the answer to this question. The results might give insight in the optimal sequencing of targeted therapies.

It was demonstrated that MRD negativity translated into longer remissions ^[75]. Therefore, studies are now aiming at even deeper remission with the aim of achieving long treatment-free intervals or even cure. A time limited therapy with a triple-combination including a BCR inhibitor, a BCL2 inhibitor and a CD20-mAb seemed to yield unprecedented MRD negativity rates ^[204]. Besides, the combination showed good tolerability. Such triple-combinations are likely to be the future approach in CLL therapy. However, more clinical data is needed before it could be given in routine practice. Two phase 3 studies (NCT03701282, NCT03737981) investigate the effect of venetoclax as a supplement to obinutuzumab-ibrutinib in the first-line setting.

The future perspectives for CLL patients look positive.

7 ACKNOWLEDGEMENTS

I would like to thank all those who have contributed to the completion of this thesis. In particular, I wish to thank:

First and foremost, the *participating patients*.

Jeanette Lundin, my main supervisor, for introducing me to the field of CLL and her guidance through each stage of the process with patience, commitment and encouragement.

Anders Österborg, my co-supervisor, for your broad scientific knowledge, wise inputs and support.

Marzia Palma, my co-supervisor, for teaching everything about laboratory methods, and for enthusiasm and for supportive comments.

Lotta Hansson, my co-supervisor, for being supportive of my work and for giving good advice.

Kia Heimersson and *Fariba Mozaffari* for teaching me so much about laboratory work.

Leila Relander for being so helpful and skillful at editing manuscripts.

All the other members of the *Österborg-Mellstedt* group.

All the *co-authors* of the papers.

All *superiors* who over the years have given me time for research.

All *colleagues and friends* at the Hematology Department. I am looking forward to our further collaboration at the clinic.

Finally, I would like to express my deepest gratitude to my *family and friends*. This thesis would not have been possible without your warm love and support. In particular, I am grateful to my mother, *Karin Winqvist*, for sharing her statistical and linguistic expertise.

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