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Targeted therapy and outcome in chronic lymphocytic leukemia

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"Nothing in the world can take the place of persistence.
Talent will not; nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not; the world is full of educated derelicts.
Persistence and determination alone are omnipotent".

Calvin Coolridge, President of the United States 1923-1929

To Petter

Abstract

Despite major advances, chronic lymphocytic leukemia (CLL) is still considered incurable. Fludarabine refractory patients and those with *TP53* disruptions have a particularly poor prognosis. Alemtuzumab is an established treatment option for these patients. However, its use is complicated by immunosuppressive side effects. New drugs targeting cellular pathways are currently developed but there are few manufacturer-independent comparative experiments.

The aim of this thesis is to gain an insight into the outcome of patients with advanced CLL and to explore new emerging drugs.

In the first study, the natural history of advanced CLL in a well-defined region without external referrals was retrospectively investigated. All patients diagnosed with CLL 1991-2010 were screened and those with bulky fludarabine refractory (BFR) and double (i.e. fludarabine and alemtuzumab) refractory (DR) CLL were identified. Subjects were primary referrals within the Stockholm region. The ORR to salvage therapy was 20% and the median time to treatment failure was 6 months, both significantly lower in BFR/DR patients than in non-BFR/DR patients. Median overall-survival (OS) was 18 months; 29 months in BFR vs 13 months in DR. Our results on consecutive patients in routine clinical practice may facilitate interpretation of non-randomized trials on novel drugs in advanced-stage CLL.

In the second study, the safety and efficacy of alemtuzumab, when used in routine health care in relapsed/refractory patients at experienced medical centres in a defined region, was evaluated. Records from patients diagnosed within the Stockholm region year 2000-2010 were retrospectively screened. Files from alemtuzumab treated patients were analysed in detail. Patients were heavily pretreated and the majority had fludarabine refractory disease. The median cumulative dose of alemtuzumab was 930 mg and median duration of therapy was 12 weeks. The ORR was 43% and a higher dose was associated with higher response rate. Median OS was 22.5 months. Compared with the results from previous reports, we observed a higher cumulative dose/longer duration of therapy, numerically higher response rates as well as longer survival in our regional, consecutive patients. Our results suggest that optimal patient management and patient identification may result in avoidance of early discontinuation of planned therapy and possibly better treatment outcomes.

In the third study, the safety and efficacy of lenalidomide in combination with alemtuzumab was explored in a phase I study and additionally, the capacity of low-dose lenalidomide in maintaining immune functions in advanced-stage CLL patients prior and during alemtuzumab was evaluated. Tumor flare reaction occurred in some patients but there was no evidence of tumor lysis syndrome. The combination showed an acceptable safety profile as well as clinical activity with an overall response rate of 42%. Median progression free survival was 5 months, exceeding 12 months in 3 patients. T-cell stimulation as well as a decreased number of regulatory T cells was evident on low dose lenalidomide. Our results provide the basis for an extended phase 2 trial on this combination of drugs as well as further studies on lenalidomide as an immune-enhancing agent.

In the fourth study, we provide a manufacturer independent, head-to-head comparison on sensitivity of CLL cells to a panel of emerging small targeted therapeutic molecules using high-throughput screening based on an automated fluorescence digital scanning system. Fresh CLL cells from patients with indolent or progressive disease were cultured in a unique primary cell-culture medium and the in vitro anti-tumor effects of 31 small therapeutic molecules were compared. The sensitivity to each drug showed considerable inter-patient variability. Highest mean direct killing was observed with one survivin inhibitor (YM-155), 2 BCL-2 inhibitors (ABT-199, ABT 737) and one selective CDK inhibitor (dinaciclib). Their killing capacity was similarly high in refractory and untreated patients and was significantly higher in cells with *TP53* disruptions. Sensitivity in bone marrow showed a high correlation to that in blood. Our results may help to identify drugs of particular interest for further clinical development.

List of publications

I. **Eketorp Sylvan S**, Hansson L, Karlsson C, Norin S, Lundin J, Österborg A. Outcomes of patients with fludarabine-refractory chronic lymphocytic leukaemia (CLL) – A population-based study from a well-defined geographic region. *Leukemia & Lymphoma, 55(8):1774-80, 2014*.

II. **Eketorp Sylvan S**, Lundin J, Ipek M, Palma M, Karlsson C, Hansson L. Alemtuzumab (anti-CD52 monoclonal antibody) as single-agent therapy in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) – a single region experience on consecutive patients. *Annals of Hematology, 93(10):1725-1733, 2014.*

III. **Eketorp Sylvan S**, Rossmann E, Mozaffari F, Porwit A, Karlsson C, Hansson L, Lundin J, Österborg A. Phase I study of lenalidomide and alemtuzumab in refractory chronic lymphocytic leukemia: maintaining immune functions during therapy-induced immunosuppression. *British Journal of Haematology, 159(5):599-613, 2012*.

IV. **Eketorp Sylvan S**, Skribek H, Norin S, Muhari O, Österborg A, Szekely L. Sensitivity of chronic lymphocytic leukemia (CLL) cells to small targeted therapeutic molecules: an in vitro comparative study. *Manuscript*.

List of abbreviations

ADCC	antibody dependent cytotoxicity
AIHA	autoimmune hemolytic anemia
AKT	anti-apoptotic protein kinase
APRIL	a proliferation-inducing ligand of the TNF family
ASCT	allogeneic stem cell transplantation
ATM	ataxia telangiectasia-mutated
BAFF	B-cell activating factor of the TNF family
BAX	BCL-2 associated X protein
BAK	BCL-2 antagonist/killer 1
BCR	B cell receptor
BLNK	B-cell linker protein
BFR	bulky fludarabine refractory
BIM	BCL-2 like 11
BIRC3	baculoviral IAP repeat containing 3 gene
BMSC	bone marrow stromal cell
BR	bendamustine, rituximab
BTK	Bruton's tyrosine kinase
CAR	chimeric antigen receptor
CCL	chemokine (C-C motif) ligand 3
CDC	complement dependent cytotoxicity
CDK	cyklin-dependent kinase
CDR3	complementarity-determining region 3
CLL	chronic lymphocytic leukemia
CMV	cytomegalovirus
CpG	cytosine-phosphoguanine dinucleotide
CR	complete remission
CT	computed tomography
CTLA-4	cytotoxic T-lymphocyte associated antigen-4
CXCR	chemokine receptor
DAT	direct antiglobulin test
DLBCL	diffuse large B cell lymphoma
DLT	dose limiting toxicity
DR	double refractory (i.e. fludarabine and alemtuzumab)
ECOG	Eastern Cooperative Oncology Group performance score
EMA	European Medical Agency
ERK	extracellular signal-regulated kinase
Fas-L	Fas ligand or CD95L
FC	fludarabine, cyclophosphamide
FCR	fludarabine, cyclophosphamide, rituximab
FDA	Food and Drug Administration
FISH	Fluorescent In Situ Hybridization
GC	germinal centers
GEP	gene expression profile
GVHD	graft versus host disease
GVL	graft versus leukemia

HDAC	histone deacetylase
HSC	hematopoietic stem cell
Ig	immunoglobulin
IGHV	heavy chain variable region of immunoglobulin
IRR	infusion related reactions
ITAM	immunoreceptor tyrosine based activation motifs
ITP	idiopathic thrombocytopenic purpura i.e. autoimmune thrombocytopenia
KE	killing efficiency
LDH	lactate dehydrogenase
LYN	Lck/Yes Novel tyrosine kinase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MAP3K14	mitogen-activated protein kinase 14 also known as NF-kappa-B-inducing kinase
MBL	monoclonal B cell lymphocytosis
MCL-1	myeloid cell leukemia sequence 1
MDACC	MD Anderson Cancer Center
miR	micro RNA
MOMP	mitochondrial outer membrane permeabilization
MRD	minimal residual disease
MSC	mesenchymal stromal cells
MYC	myelocytomatosis oncogene
MYD88	myeloid differentiation primary response gene 88
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next generation sequencing
NLC	monocyte-derived nurse-like cells
NK cell	natural killer cell
NOTCH1	Notch homolog 1, translocation-associated
nPR	nodular partial remission
NRM	non-relapse mortality
ORR	overall response rate
OS	overall survival
PBS	phosphate buffered saline
PD-1	programmed cell death 1 (CD79)
PET	positron emission tomography
PHA	phytohaemagglutinin
PD-L1	programmed death-ligand 1
PPD	purified-protein-derivative
PFS	progression free survival
PI3K	phosphatidylinositol-3-kinase
PLCγ2	phospholipase C gamma 2
PNP	paraneoplastic pemphigus
PR	partial remission
PTEN	phosphatase and tensin homolog
R-CHOP	rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone
RIC	reduced-intensity conditioning
ROR-1	receptor tyrosine-kinase-like orphan receptor 1

RPMI	Roswell Park Memorial Institute medium
RS	Richter syndrome
SD	stable disease
SF3B1	splicing factor 3B subunit 1
SHM	somatic hypermutation
sIg	surface membrane immunoglobulin
SLL	small lymphocytic lymphoma
SYK	spleen tyrosine kinase
TCL1	T-cell leukemia/lymphoma protein 1
TFR	tumor flare reaction
TLR	Toll like receptor
TLS	tumor lysis syndrome
TNF	tumor necrosis factor
T_{reg}	regulatory T cells
TTF	time to treatment failure
VCAM-1	vascular cell adhesion molecule-1
VEGF-A	vascular endothelial growth factor
VH	variable heavy (immunoglobulin chain)
VLA-4	very late antigen 4, the same as the α4β1 integrin CD49d
ZAP70	zeta-associated protein 7

Contents

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World and is mainly a disease of the elderly. It is characterized by a neoplastic clonal expansion of CD5+ and CD23+, morphological mature B-lymphocytes which accumulate in the blood, bone marrow and lymphoid tissues [1]. CLL is biologically and clinically a very heterogeneous disease where some patients never require therapy and some patients display an aggressive course with poor response to therapy and death within months. Despite recent major advances in understanding the pathogenesis and in the management of CLL, relapse ultimately occurs in almost all patients who require therapy and the disease remains incurable [2].

1.2 Epidemiology

CLL has a high incidence in Europe and North America, being the most common leukemia in the Western World but is rarer among people of Asian or African origin and with nearly twice the incidence in men as in women [1, 3]. In Sweden, it affects about 500 inhabitants per year [4]. CLL is mainly a disease of the elderly and the prevalence increases dramatically with age. In Sweden the median age of diagnosis is 71.

1.3 Etiology

The etiology of CLL remains unknown. The most obvious risk factor is age but there is strong evidence that a genetic component contributes as a family history of CLL or other lymphoid malignancies is stated a risk factor [5]. Regarding environmental factors, the relationship remains rather weak even though some associations to pesticides, magnetic fields, farming and animal breeding [6-8] and viruses [9, 10] have been found. There is evidence that CLL may be driven by both antigen [11] and cell-autonomous signaling [12, 13] which both will be discussed further in the following chapters.

1.4 Pathogenesis

1.4.1 Monoclonal B cell lymphocytosis (MBL)

MBL is considered an essential pre-leukemic state of CLL representing the asymptomatic proliferation of clonal B cells with circulating numbers < $5x10^9$/L and absence of lymphadenopathy or disease-related symptoms [14]. MBL is cytometrically indistinguishable from CLL and the prevalence is known to increase with age and ranges from < 1% to about 16% depending on the evaluated population and detection methods [14]. MBL may progress to CLL at a rate of 1-2% per year [15] and the vast majority of CLL patients exhibit such a precursor state for 6 months to 7 years [16]. Most MBL clones also exhibit a genotype similar to that of CLL [14]. Recent data suggest that certain driver mutations within subclones foster development to CLL [17].

1.4.2 Clonal evolution

Malignancies develop through multistep acquisition of critical mutations. Therefore the precursor cell in most cases needs a long life span to accumulate the required number of mutations. Next generation sequencing (NGS) techniques have demonstrated that somatic mutations could be accumulated within hematopoietic stem cells (HSC) and the number increases with age [18]. In addition, premalignant clones carrying somatic mutations have been detected in phenotypically normal HSC populations in acute myelogenous leukemia [19]. Thus, HSCs are considered to be the origin of several myeloid malignancies.

In contrast, CLL has been considered to originate from differentiated mature lymphocytes. However, HSCs from CLL patients have been shown to give rise to mono- or oligoclonal mature B cells, which simulate MBL, in mice. The generated B cell clones were independent of the original CLL clones with respect to VDJ genes. In addition, GEP analysis showed a skewed lymphoid gene expression compared with normal adult HSCs [20]. The first oncogenic event from MBL to CLL remains unknown but these findings and existence of oligoclonal B cell clones in both MBL and CLL suggests that the propensity to generate clonal B cells might have been acquired as far back as self-renewing HSC with germline IGH genes [20]. However, further studies are warranted to clarify the precise molecular mechanisms enabling HSCs to propagate malignant clones.

The complexity of CLL clonal architecture and the proof-of principle that genetically diverse subclones, not detectable by conventional methods, may be admixed with the dominant leukemic clone have been disclosed by recent genomic studies [21]. NGS has made detection of a small fraction of mutated clones (< 20%) possible. In a recent study subclonal *TP53* mutations were detected in 9% of untreated patients and these patients experienced the same clinical phenotype and poor survival as those with clonal *TP53* lesions. By time small *TP53* mutated subclones detected before treatment initiation became the predominant population and anticipated the development of chemorefractory CLL among patients requiring treatment [22]. This finding confirms the pathogenic effect of subclones and that clinically important mutations may be present at diagnosis but only become evident over time through selective pressure. In conclusion, CLL is not a static disease it has a clonal architecture that changes over time, influenced by selection pressures. Subclonal mutations in CLL may contribute to the variations observed in disease tempo and response to therapy [21, 22].

1.4.3 B cell receptor

Normal as well as malignant B cells, display B cell receptors (BCRs) on their surfaces. The BCR is composed of the antigen-specific surface membrane immunoglobulin M (sIgM) and the heterodimers CD79a and CD79b. In the normal B cell development process, activated B cells undergo massive clonal expansion, accompanied by the activation of somatic hypermutation (SHM) in the immunoglobulin (Ig) variable region genes, in the germinal centers (GC) [23]. B cells that have acquired BCR affinity-increasing mutations are selected by interaction with micorenvironmental cells through the $\alpha_4\beta_1$/VCAM-1 (vascular cell adhesion molecule-1) axis, others undergo apoptosis. Thereafter many selected B cells undergo class switch recombination of their Ig heavy chain constant region genes. Normal naïve B cells express functional sIgM and sIgD and after class-switch expression of other

classes of immunoglobulins may occur [23]. The process (proliferation, SHM and class switch) undergo multiple rounds until B cells differentiate in either memory B cells or plasma cells and leave the GC [1].

CLL cells have somatically mutated Ig variable region genes indicating that the cell of origin has passed through the germinal center [1] even though it is not completely clear whether SHM of B cells takes place only in the GC [24]. Characterized by the number of somatic hypermutations in the heavy chain variable region of immunoglobulin genes (IGHV), high or low, two major molecular subtypes of CLL can be distinguished. A ≥ 98% similarity of the IGHV gene with the corresponding germline sequence is termed "unmutated CLL" and < 98% homology is called "mutated CLL" [25, 26]. Approximately 50% of the patients belong to each group and clinical differences have been associated with IGHV mutational status [25-27]. Recent gene expression profile (GEP) analysis pointed to CD5+ B cells as the precursor population of CLL [28]. Stereotyped BCR, a characteristic of CLL, is also enriched in CD5+ B cells compared with conventional B cells. However, most CD5+ B cells are regarded as pre-GC lymphocytes with unmutated IGHV genes [29] however a small fraction co-expresses CD27, a verified marker of somatic mutations (i.e. a memory B cell marker) [28, 30]. CD5+CD27+ and CD5+CD27- B cells display a similar GEP which may indicate that CD5+CD27+ B cells derive from CD5+CD27- B cells that can undergo GC reactions [28].

Both normal CD27+ cells and mutated IGHV CLL carry BCL6 mutations, a genetic trait of a GC passage, in the same frequency as memory B cells [28, 31] supporting the hypothesis that IGHV mutated CLL derives from post-GC CD5+ memory B cells. Detailed GEP as well as the pattern of gene mutations indicate that mutated IGHV CLL may derive from CD5+CD27+ B cells and unmutated IGHV CLL derives from CD5+CD27- B cells [28, 32]. The distribution of stereotyped IGHV rearrangements, with lower frequency in the mutated CLL/mutated CD5+CD27+ B cell group compared to the unmutated CLL/ unmutated CD5+CD27- B cell group respectively, also supports this hypothesis [28].

Between the two subgroups of mutated and unmutated CLL, there are several biological differences (described in sections below) resulting in higher degree of signaling downstream the Ig receptor in unmutated CLL.

1.4.4 Antigens

The similarities between the normal memory B cell and the CLL cells, mutated as well as unmutated, indicate that CLL cells originate from antigen-experienced B cells [32]. This suggests that an antigen-driven process contributes to CLL pathogenesis. The hypothesis is supported by the finding that approximately 30% of the patients have strikingly similar BCRs and in 1% of the cases the Igs are nearly identical [33-36]. These, so called stereotyped, receptors are defined by highly homologous heavy and light chain complementarity-determining region 3 (CDR3) (the main determinant for antigen specificity) amino acid sequences as well as confined V-gene usage. Either presence or absence of somatic mutations strongly suggests that a set of common antigens contributes to CLL pathogenesis [33-35]. Stereotyped receptors are more frequent in unmutated than in mutated CLL cases and the presence of stereotyped BCRs also seems to affect prognosis, most convincingly demonstrated for the IGHV subgroup IGHV3-21 [33, 34, 37] (see 1.10.2). Mutated IGHV CLL

cases display oligo- or mono-reactive BCRs binding with high-affinity to restricted, more specific antigens whereas unmutated cases show poly-reactive BCRs with low-affinity binding to a broader range of self-antigens [38]. Several antigens have been suggested to drive the malignant process, exogenous as well as autoantigens. Examples of antigens are modified cytoskeletal proteins and oxidation-specific epitopes, i.e. molecular motifs on apoptotic cells, and polysaccharides from Streptococcus pneumoniae for example [39]. A link between Epstein-Barr virus (EBV) and cytomegalovirus (CMV) and utilization of IGHV4-34 gene has also been discussed [40].

1.4.5 Autonomous signaling

Despite indications that CLL is driven by an antigen mediated process, there are increasing evidence implicating BCR induced antigen-independent signaling in CLL. The BCRs may induce autonomous signaling dependent on the VH CDR3 structure and a specific epitope located there [13, 41]. Approximately 50% of CLL patients express such self-recognizing epitopes which cause a tonic, constitutive and autonomous signal of the BCR [13]. Recent data confirmed that CLL cells have a higher basal signaling than normal B cells and that basal signaling was particularly increased in IGHV mutated CLL cells [12]. Even though the relevance of autonomous interactions in vivo, where CLL cells are surrounded by high levels of serum Ig, is unclear it might contribute to CLL pathogenesis in concert with extrinsic ligands. In contrast to other B-cell malignancies, CLL cells do not have any activating mutations in BCR genes, suggesting that both autonomous signaling and external antigens contribute to the receptor stimulation [42].

1.5 Signal transduction in CLL

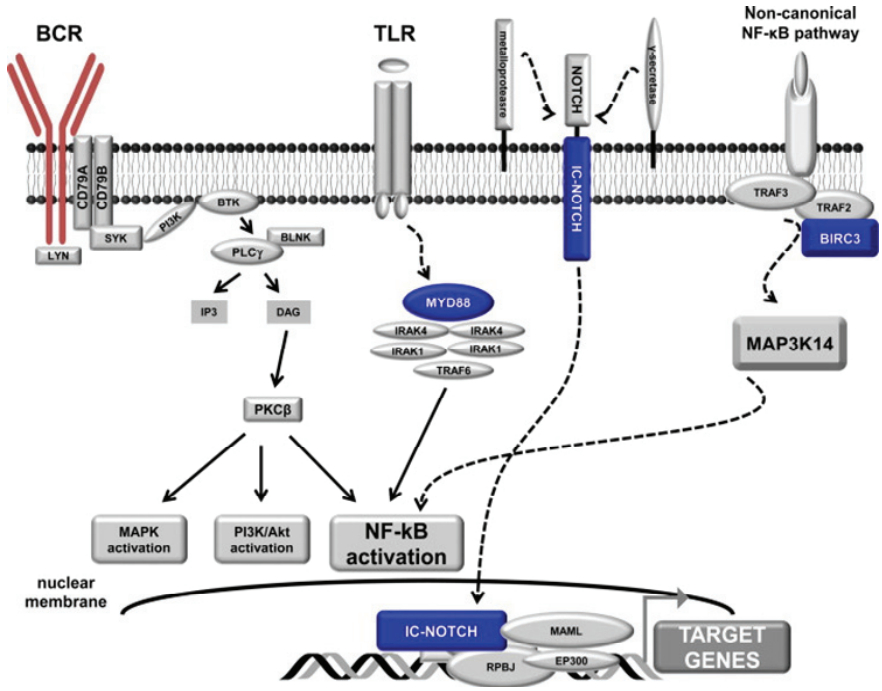

Figure 1. Molecular pathways involved in the pathogenesis of CLL. The BCR is composed of a membrane immunoglobulin bound to CD79A and CD79B. Antigen binding induces signaling initiation by the recruitment of SYK and LYN to the ITAM domains of CD79A/CD79B, and the subsequent activation of BTK and PLCγ leading to downstream activation of MAPK, PI3K/AKT or NF-κB signaling. Upon ligand binding of the TLRs receptor specific adapters, such as MYD88 are recruited leading to activation of NF-κB signaling. Upon ligand binding, NOTCH signaling is initiated by a series of proteolytic cleavages that lead to the formation of a complex that drives the transcription of target genes. NF-κB activation is achieved through either the canonical or non-canonical pathways. In the non-canonical pathway, for example BIRC3 is recruited to the active receptors, allowing the release and stabilization of MAP3K14 that, in turn, leads to the activation of NF-κB signaling.

Genes harboring somatic lesions in CLL are highlighted in blue.

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1.5.1 B Cell Receptor and downstream signaling

The BCR is a key factor in CLL [43-45] pathogenesis. As previously described, B cells expressing BCRs with high affinity are also those with an integrin-mediated signal sufficient to rescue them from apoptosis [46]. Antigen binding to the sIgM induces the formation of signalosomes associated with activation of immunoreceptor tyrosine based activation motifs (ITAM) in the tails of CD79a and CD79b by LYN (Lck/Yes Novel tyrosine kinase) and spleen tyrosine kinase (SYK) activation. B-cell linker protein (BLNK) propagates the signal further to the downstream signaling components Bruton's tyrosine kinase (BTK) and phospholipase C gamma 2 (PLCγ2) leading to calcium mobilization and the activation of several downstream pathways including mitogen-activated protein kinases (MAPK), anti-apoptotic protein kinase (AKT) and nuclear factor kappa-light-chain-

enhancer of activated B cells (NFκB) (Figure 1). These events in turn lead to activation of transcriptional factors and changes in gene expression promoting survival, proliferation and differentiation [42, 47]. LYN also phosphorylates the cytoplasmic domain of CD19 which downstream activates PI3K (phosphatidylinositol-3-kinase) and the subsequent pathway as well as promotes BCR aggregation. LYN has also a negative feed-back regulatory function on BCR signaling [42, 43]. BTK is a non-receptor tyrosine kinase, particularly expressed in B cells and BTK deficiency leads to low serum Ig levels and lack of peripheral B cells. BTK requires two signals for activation, a recruitment signal by PI3K and an activation signal by LYN/SYK. The activation of BTK is critical for the amplification of the BCR signaling and results in activation of transcription factors necessary for B cell proliferation and differentiation [42, 43, 45, 48, 49]. The expression of BTK is upregulated upon BCR activation as well as stimulation of toll like receptors or CD40 by CD40L on T cells [49]. In addition, BTK regulates BCR controlled integrin-mediated interactions such as those with VCAM-1 and fibronectin in the microenvironment as well as chemokine controlled signaling via chemokine receptor 4 (CXCR4) in vitro and in vivo thereby possibly also affecting adhesion and migration [48-50]. Gene expression of BTK is elevated in CLL compared to normal B cells.

In normal cells BCR signaling duration and intensity is tightly regulated by numerous mechanisms including receptor endocytosis and negative regulatory co-receptors (for example CD5) which modulate the functional outcome of the response [42]. The BCR downstream consequences depend on signal strength and the effectors of BCR activation are differently activated depending on the antigen ligated. Outcome ranges from a low level antigen-independent “tonic” signal essential for survival to a strong antigen-mediated signal which drives the cell toward proliferation via effects on key regulators such as myelocytomatosis oncogene (MYC), myeloid cell leukemia sequence 1(MCL1) and BCL-2 like 11 (BIM), survival or apoptosis [51-53]. Increased basal BCR signaling is a strategy of the immune system for silencing autoreactive B cells [54]. The level of basal cell autonomous signaling is higher in CLL cells compared to normal cells [12]. This indicates that CLL cells might be in an anergic state. CLL cells also express signs of constitutive changes in signaling molecules consistent with anergy [13, 55, 56]. This might explain why CLL cells appear largely unresponsive to stimulation irrespective of membrane Ig expression level [12]. However, circulating CLL cells generally express low levels of sIgM and sIgD. The features of anergy are more evident in cases with mutated IGHV genes. In this group sIgM expression is particularly low and is less responsive to triggering and the generated signal downstream the BCR is rather weak [55, 57]. In contrast, cases carrying unmutated IGHV genes have a higher sIgM expression and their poly-reactive BCRs are more responsive to triggering resulting in activation of robust intracellular signaling [57, 58]. There are also differences in basal signaling between the two CLL subgroups with a higher signaling in cells with mutated IGHV genes [12]. This difference may be due to a more efficient route to anergy in mutated CLL compared to the unmutated [12, 57]. The predominant response to BCR signaling in CLL being anergy might explain its generally chronic nature, characterized by a reduced apoptosis rather than a high proliferation, and the differences associated with mutational status suggest an association between more anergy and a favorable outcome [13, 55, 56].

In normal B cells, a chronic exposure to antigen leads to selective downregulation of sIgM [47]. This downregulation is dependent on continual antigen binding and is reversible following antigen removal. The precise mechanism triggering BCR activation in CLL, whether it is antigen dependent or autonomous, remains controversial as described above. However, exposure to antigen in proliferative centers (PCs) downregulates expression of sIgM, but not sIgD (a known feature of anergy) [57], and this together with local chemokine, downmodulate other receptors, such as CXCR4, leading to the exit of CLL cells into the blood. In the absence of stimulation there is a recovery of expression of both sIgM and CXCR4 which primes the cells for re-entry into tissues and antigen restimulation in further rounds [57]. The downregulation is dependent on the BCR signal strength where low level BCR engagement may enhance migration allowing the tumor cell to "search" for tissues rich in antigens where subsequent high-level BCR engagement inhibits migration allowing cells to effectively engage upon supporting interactions. However, a minor fraction of cells have been shown to express relatively high levels of sIgM and CXCR4 together with strong inducible anti-IgM signaling responses [59]. These cells seem primed for re-entry into tissues and for antigen response and might be particularly dangerous.

The downregulatory changes of sIgM after BCR stimulation and/or the rate of recovery in peripheral blood also differ depending on mutational status. Cases with unmutated IGHV genes are less sIgM downregulated [58] or maybe they recover more rapidly compared to mutated so that the balance point of expression of both sIgM and CXCR4 is higher than in cells with mutated IGHV gene [47].

The dominant fraction of the CLL clone (> 99%) is in a resting (G0) state of the cell cycle [60]. Even though most of these cells appear to be anergic and overexpress the apoptosis-inducing BIM, they are protected from death. This is probably due to the expression of the anti-IgM inducible, survival-promoting MCL1 protein and over-expression of BCL-2 whose principal function is to neutralize BIM [47]. CLL cells are highly dependent on the BCL-2 overexpression for survival which is supported by their sensitiveness to BCL antagonists [61] (see 3.2.3). BCL-2 overexpression also appears to be one mechanism by which CLL cells mitigate their requirements for B-cell activating factor of the tumor necrosis factor (TNF) family (BAFF). In summary, an unbalanced expression of pro and antiapoptotic molecules contributes to extended survival on anergic CLL cells.

Even though anergy may be the predominant response to BCR signaling there is a proliferative fraction in all CLL patients [45, 62]. Exposure of CLL cells to either foreign or autoantigen occurs most probably in lymphoid tissues and GEP studies demonstrated that BCR signaling is the key regulatory pathway activated in CLL cells in lymph nodes [63].

BCR associated pathways in CLL cells also show enhanced activation [63] with constitutively up-regulated LYN, SYK and BTK kinases [50, 64, 65] as well as the downstream effector molecules such as MYC [63]. This enhanced BCR activation was higher in cases with unmutated IGHV genes compared to the mutated cases whose activation was more heterogeneous [63]. A recent study noted a decrease in Ki67 (a marker of proliferation) positivity together with a reduced activity of BTK, PLCγ, ERK and AKT pathways which underscores the importance of BCR signaling-controlled cell proliferation in CLL [45]. The same study also supports the key role of cell proliferation in CLL and demonstrated that blocking cell proliferation via inhibition of BCR signaling is associated with clinical responses at the same time frame [45].

Although anergy may appear less dangerous than proliferation one has to bear in mind that this state is reversible. Therefore anergic CLL cells cannot be assumed to be harmless.

The balance between signaling leading to proliferation/survival and anergy and the proportion of CLL cells undergoing these distinct responses appears to determine the behavior of the disease. This assumption is illustrated by the prognosis differences related to mutational status where markers of anergy are more prominent in mutated- whereas markers of positive signaling are associated with unmutated IGHV genes.

In conclusion, signaling through BCR is a key factor for both CLL survival and proliferation. This statement is supported by the shown efficacy downstream inhibition [43, 66]. The pathway complexity and the multiple cross-talk mechanisms with other pathways suggest several options for targeted inhibition.

1.5.2 PI3K/AKT pathway

PI3Ks are divided into 3 classes where class I contains four catalytic isoforms (α, β, γ, δ) which differ in their tissue expression. PI3Kδ is restricted to hematopoietic cells and is highly expressed in lymphoid cells where it plays a critical role in B-cell homeostatsis and function [48]. The PI3Ks integrate and transmit signals from surface molecules, such as the BCR, but also from chemokine receptors (CXCR4) and adhesion molecules (CD40), thereby regulating the cell's growth, survival and migration. The most important outcome of PI3K activation is AKT signaling. Activated AKT phosphorylates proapoptotic proteins thereby promoting proliferation and survival [48]. In addition, PI3Kδ also activates mammalian target of rapamycin (mTOR) [67]. mTOR acts as a mediator of BCR signaling as well as a cell cycle regulator from G1 to S phase. PI3K is constitutively activated in CLL cells and the PI3K/AKT pathway appears to play a key role in CLL proliferation [66]. Recent data show that micro-RNA-22 (miR-22), overexpressed in CLL proliferating cells, maybe switch on the PI3K/AKT pathway by lowering phosphatase and tensin homolog (PTEN) expression [68]. PTEN is the main negative regulator of the PI3K/AKT pathway. When PTEN suppresses AKT activity genes promoting cell cycle arrest are upregulated [68]. Upon interactions between CLL cell and microenvironment engagement of CD40 induces overexpression of miR-22, which lowers PTEN, which in turns activates AKT resulting in inhibition of apoptotic mechanisms, upregulation of survivin (an inhibitor of apoptosis) and CLL proliferation through the NFκB pathway [68].

In summary, the PI3K/AKT pathway has a key role in CLL and the importance is underlined by the clinical effects of PI3K δ inhibitors [66].

1.5.3 NFκB pathway

NFκB transcription factors play an important role in proliferation, class-switching and survival in B lymphocytes. NFκB is a dimeric transcription factor normally kept inactive in the cytoplasm by interaction with inhibitors called IκBs. Activation is achieved through either the canonical or non-canonical pathways (Figure 1) [69]. The canonical pathway represents activation through antigen binding the BCR [42]. When BTK, PI3K or AKT is stimulated the IκB kinase causes phosphorylation and subsequent proteasomal degradation of IκB (the inhibitor of NFκB) allowing NFκB to translocate to the nucleus for gene transcription [43]. In the non-canonical pathway activated receptors, such as CD40 recruit a

protein complex, which negatively regulates mitogen-activated protein kinase 14 (MAP3K14), the central activator of NFκB in this pathway.

NFκB activation provides prosurvival signals to the CLL cells through dysregulation of several genes that regulate cell proliferation and survival (BCL-2, MYC) as well as aberrant expression of cytokines that regulate growth and proliferation (IL-6, CD40L) [69, 70]. Activation of the NFκB pathway is regarded as a mechanism of resistance to disease eradication in CLL as it is correlated with enhanced fludarabine resistance of CLL cells and poorer outcome [63].

1.5.4 Toll like receptor

Innate stimulation of normal B cells includes stimulation of toll-like receptors (TLRs). Upon ligand binding of TLR, receptor-specific adapters are recruited and induce a potent activation of the canonical NFκB pathway (Figure 1) [69]. Little is known about the repertoire and function of these receptors in CLL. However, activation of TLR promotes proliferation in CLL cells and protects them from spontaneous apoptosis [71] Some TLRs (TLR7 and TLR9) are believed to co-work with BCR in response to autoantigens. The TLR expression repertoire does not appear to be influenced by disease stage or mutational status but resembles the pattern found in antigen experienced B-cells [71]. MYD88 (myeloid differentiation primary response 88) protein is included in this adaptor complex and mutations of *MYD88* seem to be enriched in IGHV mutated CLL supporting the idea that different molecular mechanisms might be implicated in the development of mutated and unmutated IGHV CLL respectively [72].

1.5.5 MAPK pathway

Beyond being a part in the downstream signaling activating NFκB, SYK activation leads to downstream activation of MAPK (Figure 1) [42]. This pathway regulates a number of transcription factors such as the antiapoptotic proteins ERK1/ERK2 and MYC, important for proliferation and cell survival [43]. ERK1/ERK2 is constitutively phosphorylated in CLL and ERK1/ERK2 dependent induction of MYC might be important to CLL survival and proliferation [51].

1.5.6 WNT and ROR-1 pathways

WNTs are a family of glycoproteins with crucial roles in the regulation of proliferation, survival and migration of normal cells. Aberrant WNT signaling has been shown in many cancers. There are two WNT pathways, the canonical, dependent on varies forms of β-catenin, and the non-canonical which is β-catenin independent and regulates subsequent pathways associated with migration and cell polarity [73]. In CLL the WNT pathway, as a set, has shown a high mutation frequency although no individual gene has been significantly more involved [74]. The precise mechanisms by which a WNT stimulates the cellular responses through these pathways are not fully elucidated but probably involve distinct WNT receptors [73].

ROR-1 (receptor tyrosine-kinase-like orphan receptor family member 1) is a highly conserved receptor tyrosine kinase. The expression varies during embryogenesis but is

tightly downregulated after birth. Data indicates that RORs function as receptors in the noncanonical WNT pathway where RORs bind WNT regulatory proteins as ligands. ROR-1 is overexpressed and constitutively phosphorylated in CLL cells. WNT5a has been suggested to be a ligand for ROR-1 in tumor cells and they may physically interact with each other and activate NFκB [75]. WNT5a also seemed to maintain phosphorylation of ROR-1 and a significant correlation between disease activity and phosphorylation intensity of ROR-1 where a higher level of phosphorylation correlated with progressive CLL has been shown [75]. Targeting ROR-1 in vitro has resulted in selective apoptosis in CLL cells and therefore it might be an interesting clinical target. However, more data on ROR-1 and its significance in micorenvironmental interaction and intracellular signaling is warranted to advance clinical development.

1.6 Genomic aberrations, gene mutations and micro RNA

Genomic alterations are thought to constitute the basis for leukemic transformations and the variable clinical course of CLL is driven, at least partly, by the immunogenetic and molecular heterogeneity of the disease [76]. Genomic aberrations as assessed by fluorescence in situ hybridization (FISH) are present in more than 80% of cases [76]. The distinct prognostic implications of these aberrations suggest that each abnormality may reflect a distinct pathogenesis. At diagnosis a small proportion of patients have a high number of genomic aberrations but aberrations may accumulate during the course and lead to a more complex karyotype in the relapsed/refractory situation.

Deletion of 13q is the most common aberration at diagnosis presented as a sole abnormality in 35-45% [76].The function of at least one of the deleted genes is down-regulation of the anti-apoptotic gene *BCL-2* which may lead to an increased anti-apoptotic resistance [77]. Deletions of 13q vary markedly in size where larger aberrations are associated with a poorer prognosis [78].

Trisomy 12 is detected in 11-16% of the patients at diagnosis and does not increase at relapse or in refractory patients, possibly due to good response to treatment [77].

11q deletion is identified in 10-17% of patients with CLL at diagnosis. It is usually a mono-allelic deletion which includes the *ATM* (ataxia telangiectasia-mutated) gene. This tumor suppressor gene activates p53 and augments the DNA damage response. 11q deletion itself does not always result in mono- or biallelic silencing of *ATM* but is also associated with mutated *ATM* [79], unmutated IGHV genes and poor outcome [76]. *ATM* mutations in the absence of 11 q deletion is rarer compared with 11q deletion [79].

The presence of 17p deletion (including loss of the *TP53* gene) is predictive of the worst prognosis in CLL. The *TP53* gene induces cell cycle arrest and promotes DNA repair or apoptosis in case of DNA damage and is therefore a key regulator of the cell cycle [74, 80]. At diagnosis 17p deletion can only be detected in less than 3-7% of patients [76, 81] but the frequency increases up to about 1/3 in refractory CLL [82]. Deletions of 17p coincide with *TP53* mutations in the residual *TP53* allele in high frequency (80%), resulting in homozygous p53 inactivation, and patients displaying 17p deletion often harbor other poor-prognostic markers as well [74, 80, 83]. The frequency of *TP53* mutations in the absence of 17p deletion is much lower, 4-5% in the untreated setting [80] but increases with time up to about 18% in the refractory setting [84]. Mutated *TP53* has been associated to unmutated

IGHV status [85, 86] and a significant proportion of *TP53* mutated cases carries mutations in other genes as well. The genomic complexity associated with 17p and 11q deletions is most probably due to the fact that they have severely impaired cell repair capacity and thereby more easily acquire additional aberrations [77].

The genetic lesions identified by FISH do not fully recapitulate the molecular pathogenesis of CLL, nor do they entirely explain the development of chemorefractoriness. Massively parallel sequencing technology has provided the discovery of a range of mutated genes with well-established roles that underlie CLL. This allowed the reconstruction of genetic pathways in disease pathogenesis, such as the cell-cycle control for *TP53*, and clinically important features of CLL. Even though NFκB signaling is generally viewed as a consequence of microenvironmental interactions, it may also be activated through molecular lesions affecting genes in the pathways maybe making CLL cells independent from the microenvironment (Figure 1). A set of genes where mutations are associated with tumor genesis ("driver" mutations) have showed associations with standard prognostic markers suggesting that particular combinations of genetic alterations may act in concert to drive CLL development.

Besides *TP53*, the most extensively studied genes are *NOTCH1* (Notch homolog1, translocation-associated) and *SF3B1* (splicing factor 3B subunit 1) and they also seem to be the most frequently mutated ones [72, 87, 88]. Mutated *NOTCH1* is detected in approximately 5-20% of patients with increasing frequencies in advanced disease stages [72, 85, 87-90]. Mutations which are primarily a 2-bp deletion, generate a premature stop codon which results in a constitutively active *NOTCH1* [74, 87, 88] and further enhanced NFκB signaling, increased CLL cell survival and resistance to apoptosis [77, 88]. Mutated *NOTCH1* has been associated with unmutated IGHV [74, 85-88, 90] and trisomy 12 [74, 83, 85, 86, 91] but nearly exclusiveness of *SF3B1* mutation [83, 86] and isolated 13q deletion [85]. There is probably an association with *TP53* mutation as well [83, 86]. *NOTCH1* mutations have shown to have an adverse prognostic impact [86-89] and association with chemorefractoriness and Richter transformation even though data on the latter is a bit contradictive [83, 87, 89].

The *SF3B1* gene encodes a protein included in the spliceosome machinery. *SF3B1* regulates a splicing program of genes controlling cell-cycle progression and apoptosis. Mutations, primarily located within three hotspot exons [72, 91] may cause functional changes in the RNA splicing [74]. Aberrant splicing might be specific for neoplastic development in CLL since *SF3B1* mutations were not found in other lymphoid malignancies [72]. *SF3B1* mutations are present at 3-10% in newly diagnosed CLL but, maybe due to a subclonal selection, appear to increase over time with a frequency up to 20% in relapsed/refractory setting [72, 74, 85, 90-92]. The impact of *SF3B1* as an important driver mutation in CLL development is strongly supported by the clustering in evolutionarily conserved hotspots and the fact that mutated *SF3B1* appears to be an independent factor for more rapid disease progression [83]. *SF3B1* is associated with advanced clinical stage and 11q deletion [74, 85, 86] as well as other negative prognostic markers such as unmutated IGHV status, *TP53* mutation, CD38 positivity, absence of trisomy 12 and *NOTCH1* mutation [72, 74, 83, 86]. Patients primarily refractory to fludarabine showed a high percentage of *SF3B1* mutations [92] but in Richter transformation though, *SF3B1* mutations seem to play a minor role [92].

BIRC3 (Baculoviral IAP Repeat Containing 3 gene) encodes a member of the inhibitor of apoptosis protein (IAP) family which negatively regulates the non-canonical NFκB pathway through inhibition of the central activator MAP3K14 (Figure 1) [69]. Mutation of *BIRC3* leads to a constitutive activation of NFκB signaling. The *BIRC3* locus is located close to the *ATM* locus on 11q [93, 94] and mutations, mainly described as deletions or insertions, are selectively restricted to CLL [94]. Approximately 4% of the CLL patients display mutated *BIRC3* at diagnosis [93, 94] but mutations may emerge over time [79, 93, 94] to a frequency of 24% in the refractory setting maybe due to a clonal selection prior to treatment [94]. There is a high incidence of *BIRC3* mutations in cases with 11q deletion and a lack of *BIRC3* mutations in the absence of *ATM* mutations [79]. In contrast *BIRC3* mutations tend to distribute in a mutually exclusive fashion with *TP53* mutation [94]and IGHV mutated CLL [85, 94]. *BIRC3* mutations have been associated with fludarabine refractoriness [93, 94] being present in approximately 40% of fludarabine refractory CLL with *TP53* wild-type [94]. *BIRC3* mutation may therefore represent a large fraction of the chemo-resistant cases with wild-type *TP53*.

Various other recurrent mutations have been reported at frequencies below 10% [86, 88]. As data in CLL are limited their impact remains elusive. *MYD88* is such a mutation, coding for a critical adaptor molecule of the TLR signaling pathway (Figure 1). Mutations have been described as single nucleotide change, activating mutations of the NFκB pathway [74, 88]. Mutations of *MYD 88* are associated with sole deletion 13q [74, 85, 86]and with mutated IGHV status [74, 86-88] but with no other concurrent mutations [85].

The increased *TP53* mutation in *NOTCH1* and *SF3B1* mutated subgroups might indicate cooperating effects and the exclusivity of *NOTCH1* mutations and *SF3B1* mutations might indicate different pathogenic mechanisms and possibly distinct classes of driver mutations [83]. Larger studies are required to unravel the relative impact on co-localized mutations and deletions such as *ATM*- and *BIRC3* mutation and 11q deletion. In conclusion, associations between different mutations and other biomarkers are currently not fully explained clear and remain to be further investigated.

MicroRNAs (miRNAs) are small noncoding RNAs that encode tiny transcripts regulating the expression of multiple target genes. Mature miRNAs are loaded into the RNA-induced silencing complex which bounds to mRNA making it untranslated or degraded [95]. The extensive dysregulation of miRNA expression in CLL might contribute to the pathogenesis. Deletion 13q harbors miRNAs (miR-15a/16-1) that are negative regulators of BCL-2 [95]. The negative regulation of BCL-2 by these miRNAs might be a key mechanism in the CLL pathogenesis. Deletion of miR-15a/16-1 is inversely correlated with the expression of BCL-2 and deletion of miR-15a/16-1 appears to promote CLL in humans [95]. Numerous miRNAs with a distinct signature for CLL have been identified. Some are deleted in aggressive CLL (miR-34) [95], some seem to activate important pathways (miR-22 and PI3K/AKT pathway) [68] and others are suggested a role in an indolent clinical course (miR-29a) [95]. The mechanisms that cause aberrant miRNA transcriptional control are not completely clear. However, there is increasing evidence that epigenetic mechanisms play a role in the changes in regulation of miRNA transcription and promoters of some miRNAs have shown consistent hyper- or hypomethylation in CLL [96].

1.7 Epigenetic alterations

The importance of epigenetic control in tumor suppression is underscored by the recent discoveries of mutations in known epigenetic regulatory genes in human cancers [97]. Epigenetic mechanisms control the transcriptional availability of various parts of the genome through chromatin marking and packaging thus allowing genetically identical cells to achieve diverse stable phenotypes [97]. Epigenetic states can be stably maintained and persist through multiple cell divisions yet they can adapt to changing developmental or environmental needs and may exert powerful effects on cellular phenotype. Most probably these epigenetic changes cooperate with genetic mutations to mold tumor evolution [97].

The best-studied epigenetic modification is cytosine-phosphoguanine dinucleotide (CpG) methylation where methyl groups are added to CpG sites and regulate gene expression. The profile of CpG methylation differs among the prognostic subgroups of CLL. Genes involved in these aberrantly methylated loci are those involved in CLL pathogenesis (*BCL-2*, *ZAP70* (zeta associated protein 70) and *NOTCH-1*) as well as gene regulators and pathways involved in B cell signaling [98]. For example DNA methylation might have a role in regulating the expression of the integrin CD49d, where lack of methylated CpG was associated with CD49d expression [46]. One study was able to categorize patients into three distinct subgroups with differences in clinical outcome depending on methylation profile. In line with previous findings, unmutated IGHV genes were strongly related to naïve B cells and mutated IGHV genes aligned with mature B cells with a better outcome for the latter [99]. This suggests a methylation imprint corresponding to the putative cell of origin. The third subgroup consisted of mainly mutated IGHV genes but with a methylation signature more similar to naïve B cells and showed an intermediate clinical outcome [99]. It appears as differences in methylation patterns occur over time but not in absence of genetic changes which suggests a temporal hierarchy in which genetic alteration precede marked epigenetic changes [98]. In addition to DNA methylation, other epigenetic marks undergo broad changes, e.g. several miRNA promoters are aberrantly methylated which may affect the expression of miRNAs [96]. The epigenetic observations highlight the complex relationship between genetic and epigenetic features in CLL.

1.8 Microenvironment

CLL cells rapidly undergo apoptosis when removed from patients but this may be prevented by adding cytokines or other cell types [100] indicating that CLL cells are highly dependent on external stimuli for proliferation and survival.

New data point out lymph nodes as the predominant site for proliferation where exposure of CLL cells to either foreign or autoantigen probably occurs [63]. Besides BCR signaling, the tumor microenvironment that exists in the lymph nodes and bone marrow is important for CLL cell survival. The proliferation occurs within microanatomical tissue sites known as proliferation centers (PC). PC, also called pseudofollicles, is a hallmark histopathology finding in CLL and exist in the bone marrow and lymph nodes, where tumor cells interact with antigen and also have close contact to a host of accessory cells. These cells consist of mesenchymal stromal cells (MSC), monocyte-derived nurse-like cells (NLC) as well as T cells [42, 48].

The processes of extravasation and migration of CLL cells into secondary lymphoid tissues are mediated by adhesion molecules where circulating CLL cells interact transiently and reversibly with vascular endothelium through integrins (rolling). Integrins are heterodimeric glycoproteins which mediate cell-cell and cell-matrix adhesion. The α4β1 integrin CD49d (VLA-4) is a receptor for VCAM-1 and fibronectin which is expressed on cytokine-activated endothelium [48]. CD49d appears to be functionally linked to the chemokine receptor CXCR4, where triggering of CXCR4 upregulates the CD49d-VCAM-1 adhesion making a firm adhesion between CLL cells and stromal cells [46, 48]. In addition, CD49d seems to be functionally linked to the co-receptor CD38 where CD49d $^{+}$ cells overexpress chemokine C-C motif ligand 3 and 4 (CCL3/4)) upon CD38 triggering [46, 101]. High levels of CD49d and CD38 in CLL cells are associated with a higher migratory potential towards CXCL12 chemokine [42]. CD38, binding to CD31 on endothelial cells and NLC, also appears to potentiate a more efficient adhesion of CD49d^{+} cells [42, 46]. CLL cells have a high affinity for bone marrow stromal cells (BMSC) in vitro [100]. This firm adhesion favors upregulation of antiapoptotic proteins (BCL-2) and proto-oncogenes through PI3K/AKT or NFκB signaling in vitro and the firmer adhesion the stronger the signal to the antiapoptotic machinery [46, 48]. In conclusion, the integrin CD49d acts in a complex interplay with other surface receptors on CLL cells. The important role of integrins is underlined by the possibility to inhibit their function with the BTK inhibitor ibrutinib [48] and that expression of CD49d appears to have a significant prognostic impact [102].

After adhesion, chemokine gradients guide the CLL cells into the tissue, a process referred to as homing [48]. The CXCR4- CXCL12 axis appears to be a key pathway of CLL-microenvironment cross-talk where stromal cells in secondary lymphatic tissues attract CLL cells via secretion of chemokines of which CXCL12 is the most prominent. The chemokine receptor CXCR4 is highly expressed on CLL cells in peripheral blood which allow CLL cells to sense and follow levels of chemokine CXCL12, secreted by mesenchymal stromal cells, mediating chemotaxis and migration [103]. The surface expression of CXCR4 is normally downregulated by high levels of CXCL12 [103], proliferation and BCR signaling, all being present in tissues [48]. Stimulation of CXCR4 activates downstream pathways (PI3K and MAPK) providing a pro-survival effect [104]. High CXCR4 expression in CLL blood cells has been associated with an increased risk for organ infiltration and poorer outcome [105]. In conclusion, CXCR4 signaling appears to attract malignant cells as well as promote their survival. These effects can be inhibited by PI3K- [67], BTK- [50] and SYK [106] inhibitors leading to apoptosis as well as impaired migration of CLL cells.

Activated CLL cells also express and secrete CCL3/4, which are chemoattractants for monocytes and T cells, in response to BCR signaling [107]. Elevated plasma level of CCL3/4 has been associated with poor outcome [108]. The production CCL3/4 may partly explain the increased numbers of T cells in PC and this might be a way for CLL cells to induce trafficking and homing of accessory cells in the microenvironment, in particular T cells and monocytes [107, 108].

In addition to mediate chemotaxis, BMSCs seem to activate CLL cells by a prominent upregulation of the lymphoid proto-oncogene TCL1(T-cell leukemia/lymphoma protein 1) and BMSCs which in turn appear to be activated by the CLL cells via expression of protein kinase C and subsequent activation of NFκB in BMSCs [48].

NLC can be found in the spleen and lymphoid tissues. The mechanism through which NLC differentiate from blood monocytes is not completely clear but CLL cells appear to stimulate NLC differentiation in vitro by activating for example TLR-9 [42]. NLC attract CLL cells by secreting CXCL12 and CXCL13 and confine them in close contact via transmembrane chemokine receptors (CXCR4 and CXCR5). CXCL12 also acts as a co-stimulatory factor for the CD4+ T cells in CLL [109]. NLC activate the BCR signaling cascade in CLL cells confirmed by a GEP analysis showing characteristic induction of genes in the BCR and NFκB pathway [107]. In addition, NLC activate CLL cells via BAFF and a proliferation-inducing ligand (APRIL) which over-rides the pro-apoptotic function of BCL-2, thereby promoting survival and proliferation [47, 48]. Finally, NLC express CD31 which stimulates survival when binding CD38 on CLL cells [42].

In addition to antigen engagement, CD40L and cytokines (IL-4) provided by CD4+ T cells are critical for proliferation and survival in normal B cells by activating the NFκB pathway [42]. In PC there are an increased number of CD4+ T cells in close proximity to dividing malignant cells [110]. This might be due to chemokines (CCL3/4) secreted by CLL cells as a consequence of BCR signaling after NLC stimulation. These CD4+ T cells express CD40L [47, 48]. Recent data suggest that CD40-CD40L binding might induce activation of the PI3K pathway promoting survival and proliferation in CLL cells [68]. In summary, CD4+T cells appear to provide positive regulatory signals to CLL cells within tissue. In addition cytotoxic CD8+ T cells do not only have poor killing due to unfunctional immune synapses but also have an increased production of factors that might protect CLL cells from apoptosis [109].

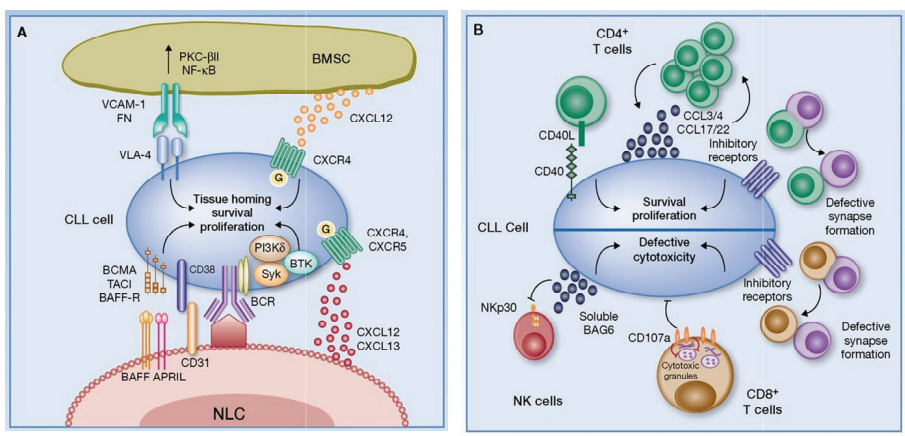

Figure 2. The CLL microenvironment.

A) In the CLL microenvironment, CLL cells interact with bone marrow stromal cells (BMSC) and nurse like cells (NLC) through adhesion molecules and chemokine receptors, expressed on CLL cells. These interactions, in addition to B-cell receptor engagement, promote CLL survival, proliferation, and homing to tissues.

B) $CD4^+$ T cells are recruited into the tissue microenvironment by CLL cell–derived chemokines, including CCL3 and CCL4, to support CLL cell survival and proliferation. Inhibitory receptors expressed by CLL cells induce defective immune synapse formation between CLL and T cells. Cytotoxic granule secretion by $CD8^+$ T cells is also defective, and production of soluble factors by CLL cells suppresses NK cell cytotoxicity, favoring immune evasion of CLL cells.

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In summary, there are several ways to activate the NFκB pathway in the lymphoid microenvironment including BCR signaling, TLR and CD40 stimulation (Figure 2A) [49]. The overall mix of chemokines contributes to co-localization of stromal cells, NLCs and activated CD4+ T cells. This altogether provides a niche favorable for CLL cell survival which keeps them away from immuno-chemotherapy resulting in drug resistance and disease persistence (Figure 2B) [48, 109, 111]. Agents inhibiting BCR downstream signaling induce a transient lymphocytosis indicating interplay between the BCR and tissue-homing events. The clinical effectiveness of these inhibitors provides compelling evidence for the importance of tissue-based antigen stimulation in CLL. Disruption of cross-talk in the microenvironment is an attractive novel strategy for treatment. Thus there is striking evidence that the neoplastic B-cells co-evolve together with a supportive tissue microenvironment which promotes the CLL cell survival, growth and drug-resistance.

1.9 Diagnosis, clinical staging and manifestations

CLL is defined by the World Health Organization [112] as a leukemic lymphocytic lymphoma which differs from small lymphocytic lymphoma (SLL) by its leukemic appearance. The diagnosis is set by analysis of blood count, blood smear and immune phenotype from a blood sample. The presence of more than or equal to $5x10^9$/L B lymphocytes in the peripheral blood is required [113]. The blood smear shows characteristically small, morphologically mature lymphocytes. The clonality and phenotype of the B lymphocytes need to be confirmed by flow cytometry. The CLL cells have weak sIg levels and co-express the T-cell antigen CD5, and the B-cell surface antigens CD19, CD20 and CD23. I contrast to normal B cells, the levels of CD20 and CD79b are characteristically low and CLL cells are usually negative for cyclin D1 and CD10. Each clone of leukemia cells is restricted to expression of either κ or λ light chains [114].

A bone marrow aspirate or biopsy is not required at diagnosis but is recommended in cases with cytopenia to distinguish whether it is of autoimmune cause or due to massive bone marrow infiltration. A bone marrow analysis is also recommended before initiating treatment [113].

Despite a common diagnostic immunophenotype CLL is characterized by extensive clinical heterogeneity. The classical staging systems according to Rai [115] or Binet [116] rely on physical examination and standard laboratory tests and identify 3 patient subgroups with distinct clinical outcomes.

Most patients are asymptomatic at diagnosis and are diagnosed incidentally while undergoing routine blood tests. Common symptoms include painless lymph node swelling, hepatosplenomegaly, constitutional symptoms and symptoms related to bone marrow failure [113, 115-117]. Patients may also suffer from recurrent infections and autoimmune complications [1].

1.9.1 Autoimmune complications

CLL is associated with autoimmune manifestations, both hematological, which are the most common, and non-hematological [118]. Epidemiological data on incidence and prevalence are limited but the overall risk of autoimmune disorders is estimated to be in the

range of 5 to 10% [119]. Examples of non-hematological autoimmune complications are paraneoplastic pemphigus, acquired angioedema and glomerulonephritis. These are very rare and will not be discussed further in this text.

The most common autoimmune complication is autoimmune hemolytic anemia (AIHA) reported in about 7% of CLL patients [120]. The second most common complication is autoimmune thrombocytopenia (ITP) reported in 2-3% of the patients. Autoimmune neutropenia and pure red cell aplasia are rare complications (< 1%) [120]. Cytopenias complicating CLL are classified into two groups: "simple" (stable CLL disease) and "complex" (concomitant CLL progression). The autoimmune cytopenias are particularly common in advanced disease but may occur at any clinical stage [120].

The underlying mechanisms for the development of autoimmune complications are only partially understood. In AHIA and ITP polyclonal, high-affinity IgG autoantibodies, produced by nonmalignant B-cells via a T-cell mediated mechanism attach and destroy the patient's own erythrocytes and platelets. The CLL cells seem to act as antigen-presenting cells, presenting peptides that are identified as foreign by helper T cells. The autoantibody-opsonized cells are subsequently destroyed via antibody dependent cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) [120]. Diagnosis of AIHA is based on laboratory features (LDH, DAT (direct antiglobulin test), haptoglobin, reticulocytes, and bilirubin). There are two main types, the warm (90%) and the cold (10%) type, where antibodies destruct the erythrocytes at normal and below normal body temperature respectively [120].

In ITP the diagnosis is in most cases based on a comprehensive evaluation of clinical findings such as a rapid, "unexplained" fall in platelet levels and rising levels after steroid or immunoglobulin treatment, absence of splenomegaly and an augmented megacaryocytes in the bone marrow [121]. AIHA and ITP may present together, a combination termed Evans syndrome [119, 120]. Some poor prognostic factors such as unmutated IGHV gene and ZAP70 expression have been associated with AIHA and ITP but autoimmune cytopenia does not necessarily confer to a poor prognosis [120, 121].

However, the most common cause of cytopenia is bone marrow failure (54%) and since autoimmune complications are not necessarily equal to advanced disease [119] a bone marrow sample is mandatory in order to distinguish autoimmunity from massive bone marrow infiltration.

Steroids are the first treatment option of simple autoimmune cytopenia with a response rate up to 80%. However, relapses are common and some patients develop life-threatening autoimmune cytopenia that is resistant to treatment [120, 122]. For non-responders or at relapse, intravenous immunoglobulins or splenectomy may be considered as second line treatment where the latter is more established in ITP. Alternative options for steroid-refractory autoimmune cytopenia are rituximab, immunosuppression with cyclosporine or azathioprine and for ITP thrombopoetin analogues may be an option as well [120].

In complex cytopenia, monotherapy with purine analogues and alkylating agents are associated with increased risk of severe autoimmune cytopenia but a combination of rituximab, cyclophosphamide, vincristine and prednisone or dexamethasone may be an option [2]. Single-agent alemtuzumab is effective in complex and therapy-related AIHA [122]. Alemtuzumab offers a combination of potent antitumor as well as profound immunosuppressive activity which may explain the effect in life-threatening hemolysis.

Therefore alemtuzumab may be considered for early use in the complex situation [122]. There are only limited data on alemtuzumab treatment against CLL related ITP.

1.9.2 Immune defects and infections

CLL cells have created strategies to escape from and to suppress the immune system. Immune dysfunction is a key feature of CLL highlighted by the increased susceptibility to infections and autoimmune complications. Infections are important causes of morbidity and mortality and constitute the major cause of death, in this group of patients. The impairment of the immune system may be further aggravated by treatment e.g. fludarabine and alemtuzumab [123]. These treatments have introduced a new spectrum of infectious complications caused by pathogens such as pneumocystis, listeria, candida and aspergillus and CMV. Infection prohylaxis with antimicrobial drugs against herpes virus and pneumocystis is routine in combination with alemtuzumab or fludarabine-based chemotherapy. Therapy-related immune defects persist after discontinuation of therapy. Therefore antimicrobial prophylaxis is continued for several months.

B-cell defects

Hypogammaglobulinemia is present in up to 67% of the patients and correlates with the duration and stage of the disease. It is an important factor contributing to infection risk in CLL and monitoring is recommended [124]. Patients with hypogammaglobulinemia and recurrent episodes of serious infections may benefit from immunoglobulin substitution [4, 125] even though intravenous immunoglobulin does not restore IgM or IgA deficiencies. Despite normal Ig levels some patients display reduced levels of IgG subclasses which may predispose them for infections. Screening for IgG subclass deficiency might be indicated for patients with recurrent infections [124]. The response to vaccines is generally inefficient and the more severe hypogammaglobulinemia the weaker vaccine response [126]. The mechanism underlying the development of hypogammaglobulinemia is not completely understood. The number of normal B lymphocytes is usually reduced maybe due to the decreased T-cell helper function and an excessive T cell suppression. A skewed cytokine production from T cells might also contribute. CLL T cells produce IL-4 which promotes upregulation of CD30. Upon CD30- CD30L binding the sensitivity to FasL-mediated cell death is increased and class-switching is impaired in non-malignant B cells [109]. In addition to hypogammaglobulinemia, CLL B cells have reduced expression of CD80 and CD86 and are functionally poor at antigen presentation. Even though CLL cells may respond to BCR triggering the overall picture is of extensive anergy [57].

T-cell defects

The T cell compartment is highly abnormal in CLL patients with increased total number of T-cells in the peripheral blood due to an increase in CD8+ T cells resulting in a fall in the CD4/CD8 ratio [48, 109]. In contrast, secondary lymphoid organs and bone marrow show increased numbers of CD4+ T cells [110].

Despite increased number, the circulating T cells in CLL have an abnormal phenotype with upregulation of activation markers (CD69 and HLA-DR) and downregulation of

CD28. This phenotype is consistent with chronic stimulation and it remains unclear whether this is directly related to CLL or if other factors, such as CMV, are involved [48, 109]. In addition, genetic T cell receptor studies have demonstrated expansions of clonal and oligoclonal T cells with an activated phenotype which provide further evidence of chronic activation [109]. In the normal setting, antigen exposure of naïve CD8+ T cells leads to differentiation to memory CD8+ T cells which can persist long term and proliferate rapidly at re-infection. In the course of chronic infections, CD8+ T cells become "exhausted" with poor effector function and the expression of multiple inhibitory receptors. CLL T cells have an increased expression of such inhibitory receptors (CD244, CD160 and PD-1 (programmed cell death 1)), called exhaustion markers [127]. Exhausted T cells fail to establish functional immune synapses which results in defective cytotoxicity and the CD8+ T cells become unable to effectively eliminate CLL cells [127]. CLL T cells differ from virally exhausted ones in having an increased ability to produce IFNγ and TNFα which can protect CLL cells from apoptosis and induce their proliferation [127]. In addition, CLL cells produce IL-6 which promotes T cells to produce IL-4 which in turn protects CLL cells from apoptosis by modulating levels of BCL-2. Increased IL-4 levels also promote upregulation of CD30 on T cells and upon CD30-CD30L interaction there is an increased production of IFNγ and TNFα [109]. GEP analyses of CLL T cells have shown profound alterations in expression of genes involved in cytoskeletal formation translated into a functional defect in filamentous actin polymerization resulting in defective immunological synapse formation with antigen presenting cells and in the CD8+ T cells also in impaired vesicle trafficking and cytotoxicity [109, 128] . Decreased actin polymerization also contributes to impaired integrin migration [109]. Further assays have identified additional receptors increased on CLL cells (CD200, CD270, PD-L1 (programmed cell death ligand1)) that may induce impaired immunological synapse function in T cells [129].

There is also a third-party effect mediated by increased numbers of regulatory CD4+CD25+T cells (T_{reg}). The number of T_{regs} increases with advanced disease. The high levels of T_{regs} might be mediated by increased expression of CD27 and CD200 on CLL cells. T_{regs} express the inhibitory receptor CTLA-4 (cytotoxic T-lymphocyte associated antigen-4) and T_{regs} from CLL patients have increased CTLA-4 expression. Thus it is likely that multiple inhibitory signaling axes induce defective T-cell function in CLL [48, 109].

Other immune defects

CLL cells downregulate NK-cell function. The mechanism is not known but might include soluble factors and/or direct contact with expression of certain molecules. This results in a functional defect with a lack of cytoplasmic granules and a reduced ability to lyse leukemia cell lines. NK cells also experience an impaired immunological synapse formation due to a defective actin polymerization.

NK-cells from patients with CLL had lower cytolytic capacity than those from MBL patients. Further, in later stages of CLL as well as in those with unmutated IGHV genes it was shown that NK cell numbers were decreased [48, 109].

There is some evidence for defects in monocyte and neutrophil function in CLL as well but this field is much less explored [109].

1.9.3 Richter syndrome

Among CLL patients 1-11% will experience transformation into a more aggressive lymphoma called Richter syndrome (RS) [130]. The vast majority of these transformations are diffuse large B cell lymphoma (DLBCL) but there are rare cases of transformation into Hodgkin lymphoma [130]. The median time from CLL diagnosis to RS is 1.8-5 years and therefore RS is not always a late event [130].

The molecular pathogenesis of RS is not completely understood. Among DLBCL 2 subgroups have been identified, those clonally related to CLL (80%) and those clonally unrelated to the underlying CLL (20%) [130]. The median overall survival (OS) differed significantly between these groups where patients with clonally unrelated RS representing de novo DLBCL had a better prognosis with a median survival of 5 years. In contrast, the median OS for the clonally related RS was approximately 1 year [130]. No single lesion or combination of genetic lesions or cellular pathways appear to be responsible for CLL transformation into RS however > 90% of the cases display combinations of lesions suggesting that RS transformation involves general regulators of tumor suppression, cell cycle control and cell proliferation rather than specific B cell signaling pathways [131, 132]. RS is associated with high risk cytogenetics (17p deletion, 11q deletion) [130, 133]. Approximately 60% of cases with RS display *TP53* mutations, an independent predictor of poor OS [133]. The second most frequent aberrations are *NOTCH1* and *MYC* mutations occurring in about 30% of the cases each [87, 133].

Several RS risk factors have been described. Heritable germline polymorphisms might predispose but their functional consequences in RS pathogenesis remain elusive [130]. The only clinical features associated with a higher risk of future RS are advanced stage and lymph nodes > 3cm on physical examination [134]. Unmutated IGHV status is associated with approximately 4-fold risk of RS relative to those with mutated IGHV genes [133, 134] and patients with stereotyped BCRs have a higher risk of RS independent of mutational status [134]. Conventional CLL therapy, alone or in combination, may be a risk factor but the impact remains unclear and little is known about the risk with new targeted therapies [130]. A single prognostic model for RS is not currently available but studies of a future model need to incorporate the clonal relationship together with genetic factors (*TP53* disruption, mutated *NOTCH*) and clinical features (performance status, lymph node size) [130].

Several signs and symptoms may indicate a possible transformation into RS but these may also be due to progressive CLL. They include rapidly enlarging lymph nodes, weight loss, high-grade fevers, hypercalcemia and elevated LDH. RS is diagnosed via excisional lymph node biopsy. A fine needle aspiration is not sufficient to establish or exclude RS. Since not all lymph node sites are affected by RS, the selection of site for biopsy is critical and PET-scan might be valuable to identify the most suitable site for biopsy [135].

R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) is the standard treatment of DLBCL RS and the response rates are about 50%. Several other combinations of chemotherapeutics have been evaluated but so far none has proven more effective than R-CHOP. However, randomized clinical trials are lacking. Ibrutinib monotherapy does not appear to be enough in RS however combinations with R-CHOP might become an option in the future [130]. Allogeneic or autologous stem cell transplantation might be an option for patients with limited comorbidities in remission [136]. However, few patients achieve adequate response to induction to proceed to transplant [130].

1.10 Prognostic and predictive factors

The staging systems of Rai [115] and Binet [116] use inexpensive, simple components. They have proved useful for estimating outcome since patients Binet A/Rai 0, Binet B/Rai I-II and Binet C/Rai III-IV show a median OS of > 10 years, 5-7 years and < 3 years respectively [137]. However, both staging systems lack precision in identifying prognostic subgroups, have limited ability to predict prognosis for individual patients [138] and provide no information on how patients will respond to treatment. Several prognostic biomarkers have been identified, some of these also have a predictive value, in order to improve discriminatory power and risk stratification.

1.10.1 Genomic aberrations and gene mutations

One of the most reliable molecular prognostic markers is the established hierarchical model of the prognostic impact of genomic aberrations (Figure 3).

The presence of 13q14 deletion as a sole abnormality is associated with a more indolent disease course [76] as well as a lower risk of transformation [139]. Trisomy 12 is associated with a less-favourable prognosis and deletion of 11q is associated with younger age at diagnosis, extensive lymphadenopathy, rapid progression and shorter treatment-free and overall survival [76]. However, the adverse prognostic significance of 11q deletion may be overcome with chemoimmunotherapy [81]. CLL patients with 17p deletions display the worst outcome with a rapid progression and short survival [76, 83].

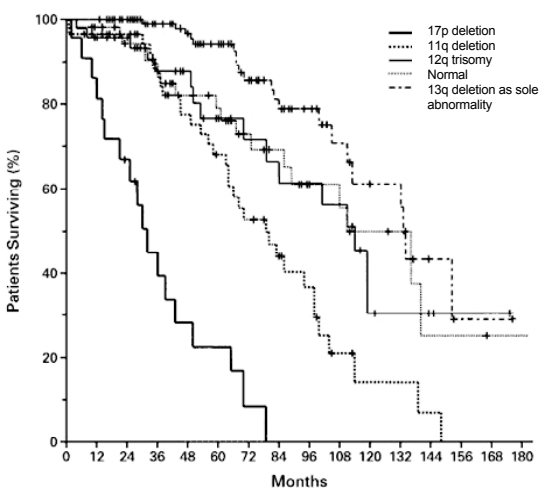

Figure 3. **Probability of survival from the date of diagnosis among patients in the five genetic categories**.

The median survival times for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as the sole abnormality were 32, 79, 114, 111, and 133 months, respectively.

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A *TP53* mutation is an independent prognostic marker related to short time to treatment, short progression free survival (PFS) and OS regardless of the presence of 17p deletion [80, 86, 140]. Notably, patients with *TP53* mutations carrying a mutated IGHV gene have shown a superior survival compared to those with unmutated IGHV. One recent study showed that OS for those with mutated CLL did not differ significantly from patients displaying normal karyotype/deletion 13q [93]. *TP53* disruptions (17p deletion and *TP53* mutation) are associated with poor response to chemotherapy [80, 83]. The mechanism of action of these agents relies on p53 induced cell death [76]. *TP53* disruptions are therefore predictive

markers which shall be evaluated before treatment initiation and patients with these aberrations should be recommended alternative less p53-dependent treatment alternatives [113]. Even subclonal *TP53* mutations, not detectable by tests currently used in clinical routine, may affect the response and chemotherapy might remove the incumbent clones in favor of a more aggressive one [21, 22].

NGS studies have pointed out novel genes frequently mutated in CLL [72, 74, 87-89, 92, 94].

Mutated *ATM* is associated with a shorter PFS and OS after first line treatment [141] and its presence might subdivide 11q deleted patients into a subgroup with poorer prognosis [79].

Mutated *NOTCH1* has been associated with both a shorter time to treatment and survival [85, 87-91] as well as chemorefractoriness [87, 89]. However, its independent prognostic impact is not completely clear. In recent studies, [83, 85, 86] mutated *NOTCH1* was not an independent prognostic marker for PFS [83], time to treatment or OS [86]. Mutated *NOTCH1* appears to be a predictive marker for decreased benefit from the addition of rituximab to FC (fludarabine and cyclophosphamide) [83] and might be a predictive marker for reduced benefit from the addition of ofatumumab when added to chlorambucil [142]. However, the biological mechanisms underlying these findings remain unclear.

Mutated *SF3B1* is associated with advanced disease and poor prognosis [72, 74, 83, 92] with a shorter time to treatment and OS [74, 88, 90-92] independent of other prognostic markers. However, in a multivariate analysis mutated *SF3B1* was only an independent marker for shorter PFS but not for OS [83]. Yet, it was still an independent factor for more rapid disease progression. Compared to the impact of the mutations status of the IGHV region the data is a bit more elusive [72]. *SF3B1* appears, after *TP53* mutation and IGHV mutational status, among the strongest prognostic markers in patients receiving the current standard first-line therapy but has no implication as a predictive marker [83].

Mutated *BIRC3* is associated with refractoriness to chemotherapy as well as short OS similar to that of CLL patients harboring *TP53* abnormalities [94]. Its independent effect is not definitely clear. Since *BIRC3* deletion in 11q deleted CLL tend to co-exist with *ATM* deletion [79, 93, 94] and the outcome for patients with *BIRC3* mutations and 11q deletions but without *ATM* mutations is comparable to those with only 11q deletion, *ATM* mutational status might be the most clinically informative genomic lesion in 11q deleted CLL [79]. *BIRC3* mutations have been associated with fludarabine refractoriness [93, 94] and mutations are seen in approximately 40% of fludarabine refractory CLL with *TP53* wild-type [94]. Therefore *BIRC3* mutation may represent a large fraction of chemo-resistant cases with wild-type *TP53* and might be valuable to include in routine analysis in order to detect cases that would not benefit from fludarabine. Further, recent findings in mantel cell lymphoma suggest that *BIRC3* mutations may predict refractoriness to the BTK inhibitor ibrutinib [143].

Mutated *MYD88* is associated with 13q deletion and patients harboring both defects might have an improved OS compared to wild-type patients [85, 93].

The identification of gene mutations has led to refined risk stratification and might help to identify patients that need closer follow-up and specific treatment recommendations. The influence of gene mutations may also vary according to the type and intensity of the regimen administered [83, 90], especially when taking into account that subclonal populations might be positively selected by treatments. Detection of tumor cell populations of very low clonal abundance might be highly sensitive predictive biomarkers of CLL in the future [22].

1.10.2 IGHV mutational status

Since stimulation through the BCR plays a preeminent role in CLL cells it is not surprising that the mutational status is constantly selected as a relevant prognostic marker [102]. IGHV mutational status can be examined by routine tests, is very stable and does not change during the course of the disease [26, 77]. Unmutated IGHV is associated with high-risk characteristics and shorter time to treatment [144] as well as survival [25, 26, 83, 86, 145]. Mutated IGHV cases had significantly superior outcomes compared to unmutated ones that had similar outcome to the cases carrying unfavorable genomic aberrations such as *TP53* mutations [85]. This was consistent even in low-/very-low-risk groups with isolated 13q deletion and/or no aberrations. However, there is heterogeneity within the IGHV mutated group where cases carrying IGHV3-21 genes display a poor outcome [146]. This gene is highly skewed in geographical distribution being more frequent in Scandinavia (6.5%) compared to other Western countries (2.6-4.1%) [147]. The IGHV mutational status and FISH aberrations may have a complementary role in predicting survival in CLL. Since the IGHV mutational status might influence the outcome of targeting BCR-associated kinases with inhibitory drugs it will perhaps be considered a predictive marker in the future [148].

1.10.3 Serum markers

Lymphocyte doubling time (LDT) is defined as the time in months it takes for the lymphocytes to double in absolute number. LDT is an independent prognostic marker [149]. A short LDT is related to a more aggressive disease and an LDT < 6 months indicates an active disease with treatment indication. A limitation with LDT is its inability to predict outcome at an early stage of the disease and the low predictable value. Three serum markers are used as prognosticators. LDH may be increased in CLL and a high level corresponds to poor prognosis and risk of transformation [77]. Similarly, high level of β2microglobulin indicates a more advanced clinical stage with a shorter PFS [77]. Finally, high levels of thymidine kinase are associated with a more aggressive disease, unmutated IGHV genes, high-risk genomic aberrations and short LDT [77]. Serum markers may have a role in CLL prognostication however focus is currently on factors that can provide more disease-specific information regarding survival and response to treatment.

1.10.4 Other prognostic markers

There is a myriad of molecules suggested as prognostic markers. This text focuses on the most relevant and evaluated ones.

A recent multicenter worldwide analysis showed that expression of CD49d, the α chain of the α_4 β_1 integrin heterodimer emerged as one of the most relevant biological predictors of OS along with 17p deletion [102]. Other well-established prognosticators, such as 11q deletion, CD38 expression and ZAP70 expression, turned out to be endowed by much lower prognostic power when either 17p deletion, IGHV mutational status or CD49d expression was concurrently available [102]. A possible explanation might be that there appears to be alterations in the NFκB signaling depending on differences in sensitivity to microrenvironmental stimuli between $CD49d^+$ and $CD49d^-$ CLL [46].

CD38 is expressed on cells of hematopoietic origin and can act as a receptor or as an enzyme regulating the intracellular calcium level. CD38 is highly expressed in activated B

and T cells as well as NK cells and dendritic cells and a high expression is associated with unmutated IGHV status, a poorer response to chemotherapy and a shorter OS [26, 145]. However, CD38 is difficult to use in clinical practice as the expression may vary during the course of the disease, its expression in CLL cells within a blood sample is heterogenic and a defined cut-off value for positivity is lacking [145].

ZAP70 is a tyrosine kinase associated with high responsiveness to BCR stimulation in CLL cells and enhances migration toward chemokines and response to survival stimuli from the microenvironment. Normal, as well as malignant B cells express ZAP70 at various differentiation stages. High ZAP70 expression has been associated with disease progression and short OS [144]. Since ZAP70 is an intracellular protein, detection with flow cytometry is complicated and mRNA measurement might be more reliable [77]. IGHV unmutated cases express higher levels of ZAP70 and CD38 compared to the mutated [144].

Expression of lipoproteinlipase (LPL), an enzyme suggested to play a role in cell adhesion, correlates to poor prognosis and is associated with poor-prognostic markers. LPL also seems to be able to distinguish patients with otherwise good prognostic markers and might be valuable in the prognostic evaluation in low-risk patients at diagnosis [77].

CLLU1 (CLL upregulated gene 1) is a gene uniquely expressed in CLL cells. High expression is associated with advanced disease, other poor-prognostic markers and a shorter time to treatment. However, it is neither an independent marker for OS nor a predictive marker. *CLLU1* may have a role as a prognostic marker in younger patients with favourable prognostic markers to identify who are likely to require treatment [77].

Several studies have revealed differences in miRNA expression between prognostic subgroups. Even though results have been discordant due to methodological differences and variations in patient material some miRNA may be used as prognostic markers [77].

Due to the limited information on the independent prognostic value, it is difficult to know how to interpret the different prognostic markers described above. Efforts are made to combine the "best" markers in a "CLL prognostic index". A recent model [140] based on a full array of genetic characteristics defined 4 hierarchically classified subgroups based on mutations of *TP53*, *BIRC3*, *NOTCH1* and *SF3B1* in addition to the established genomic aberrations [76]. However the model did not reach the C-statistic threshold ($c > 0.70$) necessary to have utility in the individual patient [150] and was not confirmed by others [86, 93]. Another attempt to develop a comprehensive prognostic index evaluated 23 clinical biological and genetic markers [138]. Their index ($c=0.75$) classifies patients into four risk groups based on 8 independent risk factors (deletion 17p, thymidine kinase, β2-microglobulin, unmutated IGHV, ECOG > 0, deletion 11q, male sex and age > 60 years). To be classified as very high risk, deletion of 17p was required. In this index, serum thymidine kinase and β2-microglobulin were considered as the second most important risk factors. However, the roles of novel mutations such as *NOTCH1*, *SF3B1* and *BIRC3* as well as CD49d expression need to be re-evaluated in the context of this model.

In addition to the difficulties in interpreting prognostic markers, knowledge about predictive relevance for the novel biomarkers transferrable to clinical practice is still very limited and there is an immediate need to identify biomarkers which can predict therapy response.

2 TREATMENT OF CLL

The most important patient subgroups in clinical practice and treatment decision making are the few patients in the very-high-risk group (4%) with a dismal 5-year survival of 19% and those in the low-risk group (25%) with a 95% 5-year survival and a median time to first therapy of > 10 years [151].

2.1 Treatment indications

There is no curative treatment for CLL with the exception of the very few patients who undergo allogeneic stem cell transplantation. Patients without symptoms shall be subject to "wait and watch" and where neither bone marrow biopsies nor CT scans are recommended. The use of prognostic markers is not recommended in this situation since these might turn the" watch and wait" into a "watch and worry" situation.

However treatment should be applied if the disease is active i.e. in the presence of cytopenias due to bone marrow failure, or if rapidly progressing lymphadenopathy or bulky lymph nodes (> 10cm), or if a rapid doubling of the lymphocyte counts (≤ 6months) or if severe constitutional symptoms (fever, night sweats, weight loss, fatigue) occur [4, 113]. It should be emphasized that the absolute lymphocyte count is not a criterion for initiation of treatment and that severe constitutional symptoms as the only criterion to start therapy are rare [152].

2.2 First line treatment

Before treatment initiation, a bone marrow biopsy and an evaluation of 17p deletion/*TP53* mutation is recommended. The latter is to be done due to the strong correlation with chemorefractoriness and early relapse [84, 113]. Biological prognostic markers such as mutational status, CD38, ZAP70 and CD49d are currently considered insufficient to precisely determine the clinical fate of individual patients and should not be used to make treatment decisions in CLL patients. The value of the new prognostic markers (*NOTCH1*, *SF3B1*, *BIRC3)* needs to be confirmed by prospective studies and reliable and reproducible detection methods need to be established before they can be used in clinical routine [152].

The treatment strategies shall be highly individualized and the clinical stage and genetic risk of the disease as well as the patient's fitness and the treatment situation (first- vs. second line; response vs. non-response to last treatment) shall be considered when selecting a treatment for CLL [137]. The patient's fitness will be influenced by age, organ function and comorbidities. Since CLL most often affects elderly people, individual expectations and quality of life should be considered when choosing treatments.

2.2.1 Good physical condition

Chemoimmunotherapy is established as the gold standard for first-line treatment of fit patients without 17p deletion or *TP53* mutation. A combination of fludarabine, cyclophosphamide and rituximab (FCR) has shown overall response rates (ORR) of about 90% and complete remission (CR) rates of 44% and has prolonged PFS (57.9 months) and

improved OS [81, 153]. FCR is a standard treatment in most patients younger than 65 years of whom some, especially those with mutated IGHV genes or with trisomy 12, may have very long remissions. Even though FCR has improved response rates and survival it is less well tolerated in patients > 70 years of age where severe neutropenia tend to be more frequent and patients remain susceptible to infections up to two years after discontinuation of therapy by exacerbating the immunodeficiency already present in CLL [152]. Therefore FCR is not a first-line treatment for all and it is generally too toxic in frail, elderly patients who constitute the majority of patients in the clinic.

For those with borderline fitness status a combination of bendamustine (90mg/m^2 on Days 1 and 2) with rituximab (BR) is an option. Bendamustine differs structurally from other alkylators by its benzimidazole ring. Like several other alkylating agents, bendamustine crosslinks DNA and causes cytotoxic single- as well as double-strand breaks. However bendamustine appears to cause more extensive and more long-lasting DNA strand breaks which may result from its different structure [154]. BR has been shown to achieve similar response rates (ORR 88%, CR 23.1%) but induces less neutropenia (19.7% vs 34%) and severe infections (7.7% vs 25%) than FCR [155].

Recent phase III data shows that FCR appears more efficient than BR as first-line treatment of fit patients as it shows a higher rate of CRs (47.4% vs. 38.1%) as well as longer PFS in patients < 65 years (not reached vs. 36.5 months). However these advantages might be balanced by a higher rate of toxicity, in particular neutropenia and infections, associated with FCR and the superior PFS for FCR disappeared in the elderly [156].

In the light of the new results, where the associated toxicities with FCR may sometimes overcome the benefits in the individual patient, no firm recommendation of one regimen over the other can be given at the present time regarding the first-line use in CLL patients with good physical fitness.

2.2.2 Impaired physical condition

Patients with an impaired physical condition, who are considered inappropriate for FCR or BR may be offered a mild regimen such as chlorambucil for symptom control. Chlorambucil is an orally available alkylating agent with low toxicity and cost but also with low response rates when used as monotherapy (ORR up 30-72% and CR 0-7%) and a median PFS of 8-18 months [157-159]. Bendamustine alone shows improved responses but greater toxicity and no survival benefit compared to chlorambucil alone and is therefore not recommended alone in first line treatment for frail patients [152, 159]. Until recently no treatment had proved to be superior to chlorambucil in this patient population [160]. Therefore more effective but tolerable treatment choices are needed. Recent data suggests a combination of chlorambucil and a CD20 antibody might be such an option [161, 162].

The chimeric type 1, CD20 antibody rituximab is widely utilized as first-line therapy in unfit patients due to its limited toxicity but there is no such indication in Europe. If used at standard doses, rituximab as a single agent is much less efficient in CLL than in follicular lymphoma, with a response rate of only 13% [163]. This might be due to the dim expression of CD20 on CLL cells [152]. Phase II studies combining rituximab with chlorambucil in elderly patients with comorbidities showed superior ORR (84% and 82.4%) as well as CR rates (10% and 16.5%) and PFS (23.5 and 34.7 months) than was previously achieved with

chlorambucil alone [157, 164]. Median OS was not reached with a median follow up of 30 and 34 months respectively. The combination was well tolerated in both studies with toxicities comparable to chlorambucil monotherapy.

Another CD20 antibody is ofatumumab, a fully human type 1 monoclonal antibody (mAb). It targets a unique epitope of CD20 with a higher binding affinity in comparison with rituximab. This results in a stronger complement-dependent cytotoxicity but similar ADCC and apoptosis induction and a slow release from the target compared to rituximab [165]. Ofatumumab is licensed by FDA (Food and Drug Administration) and EMA European Medical Agency) as monotherapy in double refractory ((DR) i.e. refractory to both fludarabine and alemtuzumab) (see 2.4.3) CLL. A phase III trial in the first-line setting with unfit patients, considered inappropriate for fludarabine-based treatment, showed that the addition of ofatumumab to chlorambucil is more efficacious without an increase of severe side effects (ORR 82% vs 69%, CR 12% vs 1% and PFS 22.4 vs 13.1 months) [162]. Of note, mutated *NOTCH1* might be a predictive marker for reduced benefit from the addition of ofatumumab to chlorambucil [142]. In contrast patients with *SF3B1* mutation appeared to benefit from ofatumumab addition [166]. In 2014 the combination of ofatumumab and chlorambucil was approved by FDA and EMA for the treatment of previously untreated CLL patients, for whom fludarabine-based therapy is considered inappropriate.

A third combination with chlorambucil in unfit patients is the one with obinutuzumab (GA101). Obinutuzumab is a humanized glycoengineered type 2 CD20 antibody, In preclinical studies it binds CD20 with a higher affinity and has increased ADCC activity and direct cell killing but has lower CDC than type I CD20 antibodies [167]. A phase III trial in untreated CLL patients with comorbidities investigated the addition of obinutuzumab to chlorambucil treatment compared with rituximab-chlorambucil and chlorambucil monotherapy. Patients receiving obinutuzumab-chlorambucil had a significantly better quality of response with higher CR rates (20.7% vs 7.0%), were more frequently minimal residual disease (MRD) negative (19.5% vs 2.6%) and had prolonged PFS (median PFS 27.6 vs 16.3 months) compared to those treated with rituximab-chlorambucil [161]. Benefit with regard to OS was seen but did not reach significance and the benefit from obinutuzumab was evident in all analyzed subgroups with the exception of those with 17p deletion. Adverse events (AE) were more common in the antibody groups and were most frequent with obinutuzumab where more infusion-related reactions were seen (20% vs 4%) but the rates of serious infections (11-14%) did not differ between the groups. Notably there were 14 cases of tumor lysis syndrome (TLS) in the obinutuzumab-chlorambucil group versus zero in the other groups. Obinutuzumab appears to be more potent and equally well tolerated as rituximab in combination with chlorambucil in unfit patients which led to FDA approval for this combination for untreated patients not eligible for more intensive therapy.

The patients in the studies evaluating chlorambucil in combination with either ofatumumab or obinutuzumab are not strictly comparable and a head-to-head comparison of both antibodies is warranted.

2.2.3 17p deletion or *TP53* mutation

Patients with 17p deletion or *TP53* mutation carry a very dismal prognosis. This group represents only about 5% at diagnosis but the group increases sharply up to 40-50% in the refractory situation [76, 80]. There is a small subgroup of patients with *TP53* disruptions who may have a benign clinical course over a prolonged time period [168, 169]. However the ORR is unsatisfactory and CRs are rare for the vast majority when treated with chemotherapy e.g. FCR or BR [81, 155]. Even if they respond, patients will usually relapse within a short time and when they do the median life expectancy is short [170]. There are limited data on the most efficacious first-line treatment for CLL patients with 17p deletion/*TP53* mutations since most trials include few patients with these abnormalities and randomized controlled trials have not been performed specifically for this subgroup. Current treatment approaches rely on agents able to induce apoptosis in a TP53 independent fashion.

Alemtuzumab, an anti-CD52 mAb (section 2.4.2) has been used as single agent in the first-line setting with an ORR of approximately 80% [158, 171]. Alemtuzumab is effective in patients with 17p deletion with no significant differences in response rates between patients with *TP53* abnormalities and those without [172] and neither 17p deletion nor *TP53* mutation had an impact on PFS or OS [172-174]. This suggests efficacy independent of the p53 signaling pathway and therefore alemtuzumab plays an important role in the treatment of these patients and can be considered upfront for patients with 17p deletion or *TP53* mutation [4].

However, given the improved toxicity profile of ibrutinib (see 3.2.1) over alemtuzumab, ibrutinib may represent the best available option for these patients [175] Emerging drugs, such as the BCL-2 inhibitor ABT-199 (GDC-0199) also provide hope for such patients (see 3.2.3).

In summary, there is currently no gold standard for the treatment of patients with 17p deletion or *TP53* mutation. In the absence of comparative trial data there are no firm recommendations but in clinical practice agents such as ibrutinib and alemtuzumab are usually the treatment of choice and allogeneic stem cell transplantation is often considered early. These patients still remain a challenge and should ideally be treated within clinical trials exploring new therapeutic agents. How to treat patients with subclonal *TP53* defects are not yet stated but since their prognosis is similar to those with clonal defects [22] it might be proper to manage them in the same way.

2.3 Treatment evaluation and minimal residual disease (MRD)

Definitions of response are stated in IWCLL guidelines [113]. Neither CT scans nor bone marrow or MRD evaluation are mandatory yet strongly recommended in clinical trials [113].

The high CR rate with FCR raised the question of the value of achieving MRD negative status as a treatment end-point. Methods to study MRD in CLL include allele-specific oligonucleotide polymerase chain reaction (PCR) for the clonal IGHV gene and multicolor flow cytometry which can reach the 0.01% sensitivity recommended in IWCLL guidelines [113]. The use of NGS techniques might become an additional way of detecting MRD in the future [176]. MRD status from blood and bone marrow may be discordant where blood might be a less sensitive, especially during and for several months after treatment with monoclonal antibodies. This might be due to the importance of the microenvironment

in CLL cell growth and survival. Therefore bone marrow is the site recommended for MRD evaluation in the absence of definitive data, but remains an issue for study [177, 178].

Low level of MRD in bone marrow or peripheral blood appeared to be associated with improved OS irrespective of the treatment of choice [179]. Further studies also showed that MRD negative status in blood or bone marrow was independently associated with longer PFS and OS after first-line treatment with FCR [180, 181]. It has even been suggested that early MRD eradication may prompt treatment discontinuation since there was no significant difference in PFS among patients achieving MRD negativity regardless of the number of courses, and patients who were MRD negative and continued treatment did not seem to improve PFS [180]. Such a strategy might reduce associated complications such as infections, important in elderly, and second malignancies, important in young patients. The level of MRD also appears to be associated with PFS where the higher MRD level the shorter PFS [181]. Mutated IGHV status and trisomy 12 were independently associated MRD negativity where the latter might be explained by a higher level of CD20 expression associated with trisomy 12 maybe favoring a greater sensitivity to rituximab [180]. MRD negativity appears to be of prognostic significance in patients with *TP53* abnormalities as well [181, 182]. However, patients with *TP53* mutations have a relatively short PFS even if MRD negative [80] which suggests that both *TP53* abnormalities and MRD response independently predict for PFS.

2.4 Treatment of relapsed/refractory CLL

Relapse and disease progression in patients treated with chemotherapy may occur because of clonal evolution of CLL cells. In the relapsed situation, mutations affecting p53 function are more frequent resulting in chemoresistant disease [1, 84]. However, *TP53* abnormalities only explain a fraction of these cases. The approach to relapsed CLL is similar to the one before first-line therapy and treatment initiation should not occur until patients are symptomatic and bone marrow and cytogenetics should be analyzed [113]. However, apart from the recommendation to repeat the first-line therapy in the case of a relapse > 24 months after initial treatment provided by the European Society of Medical Oncology (ESMO) [183] little is known about which therapies to use in the case of an earlier relapse.

Progression within 24 months after chemoimmunotherapy is regarded as unsatisfactory [178] and is associated with a low proportion of responses and short survival times when chemoimmunotherapy is used [170, 184]. Failure to achieve at least a partial remission (PR) or a relapse within 6 months after last treatment is the current definition of refractoriness in CLL [113]. Approximately 80% of fludarabine refractory patients harbor multiple molecular alterations of which *TP53* is the most common. The lesions are to some extent are mutually exclusive [94]. Patients refractory to fludarabine have a poor prognosis and usually show limited response to salvage chemotherapy with response rates ranging between 22% and 34% and even those who respond usually experience a very short remission duration with a median OS ranging between 9 and 19 months [172, 185, 186]. These subgroups, patients being refractory to fludarabine or experiencing a short remission after intense therapy (e.g. FCR), are considered “ultra-high-risk” together with the ones harboring *TP53* abnormalities [170]. High-risk CLL is defined by 11q deletion, unmutated IGHV gene or advanced stage.

It is difficult to compare safety and efficacy of different regimens in relapsed or refractory CLL. Characteristics of patients enrolled in studies differ widely and second-line therapies after first-line FCR appears very heterogeneous [153]. Specific treatment recommendations for relapsed patients are therefore lacking. However, the ORR after second-line therapy will depend on the length of initial remission which should influence the choice of relapse treatment and consolidation approaches.

The treatment options for ultra-high-risk CLL are limited as well and recommendations are not based on comparative trial data. Allogeneic stem cell transplant shall be considered and participation in clinical trials testing novel agents is particularly recommended.

2.4.1 Chemoimmunotherapy

FCR appears more efficacious than FC in the relapsed/refractory setting [187] and has been extensively tested. The ORR is about 70% with 25-30% CR and a PFS of approximately 20-30 months. However, the outcome is affected by the type of previous treatment [184] with a poorer outcome for those refractory to fludarabine [185, 188].

A study investigating FCR as salvage therapy showed an ORR of 73% with 25% CR whereas in the subgroup of fludarabine refractory patients the ORR was 58% with CR rate of 6% [188]. Another study only including fludarabine-sensitive patients at relapse after first line treatment also achieved higher response rates (ORR 69.9% CR 24.3%) [187]. The poorer outcome of those refractory to fludarabine might be due to enriched *TP53* abnormalities in this subgroup. Results for FCR in patients with 17p deletion are no good. Response rates are markedly poor with almost no complete remissions and short PFS and OS of less than one year [184, 187, 188]. The most common toxicity of FCR is myelosuppression where about two thirds of the patients experience ≥ grade III neutropenia [184, 188] and approximately 16% experience ≥ grade III infections [184, 187, 188]. Many patients, sometimes the majority and especially those > 70 years of age, are not able to receive 6 courses of FCR due to toxicity [184, 187, 188]. FCR combined with bevacizumab, a humanized mAb targeting VEGF (vascular endothelial growth factor), was recently investigated. [189]. The results showed no benefit on PFS or OS of the addition of bevacizumab and patients with 17p deletion displayed a poorer outcome. Notably, the proportions of high-risk features such as 17p deletion were significantly higher in the FCR-bevacizumab group compared to the FCR group.

Bendamustine is only partially cross-resistant with other alkylating agents and fludarabine and is less toxic than FCR. When used as monotherapy in the relapsed/refractory setting in doses ranging from 70mg/m^2 to 100mg/m^2 (day 1 and 2) the ORR was about 50% with some CRs [190]. However no patients with 17p deletion were investigated in this trial. BR as salvage therapy achieved ORR and CR rates of 59% and 9% respectively in a higher risk population and the durations of remissions were about 1 year [191]. There are subgroups that seem to benefit much from BR. Most patients with 11q deletion or trisomy 12 responded (ORR 92.3% and 100%) as well as those sensitive to fludarabine (ORR 60.5%) whereas those with 17p deletion or refractory to fludarabine displayed poorer responses (ORR 7.1% and 45.5%). When comparing BR to FCR in the relapsed/refractory setting the adverse effects of BR compare favorably (neutropenia ≥ grade III 21.3% vs 42-62%).

Bendamustine in combination with ofatumumab has been investigated in relapsed/refractory patients as well. Recent data from a phase II multicenter trial [192] compared favorably to BR showing an ORR of 72.3% with 17% CR [191]. However *TP53* disruptions, present in 22% of the study population, were significantly associated with lower response rates (ORR 30%) as well as shorter PFS. Bulky lymphadenopathy was also associated with lower response rates. Myelosuppression was the most common toxicity with ≥ grade III neutropenia developed in 61.7% i.e. higher than observed with BR [191] but similar to that reported with FCR [188] (23% and 66% respectively). Despite the significant proportion of neutropenia, infections ≥ grade III were only recorded in 6% which is comparably low to BR and FCR (12.8 and 16% respectively. There was no difference in response rate or toxicity with respect to age.

2.4.2 Alemtuzumab

Alemtuzumab is a recombinant, humanized mAb. It is directed against CD52, a glycoprotein of unknown function, expressed at high density on most normal as well as malignant B- and T-lymphocytes but not on hematopoietic stem cells [193]. Binding of alemtuzumab to CD52 is considered to cause cell death by ADCC, CDC and possibly also by direct cytotoxicity [194]. New preclinical data suggest that antibody-mediated macrophage phagocytosis might be a major mechanism of alemtuzumab-dependent cellular cytotoxicity [195]. Alemtuzumab is mainly used as treatment for relapsed or refractory CLL with an overall response rate of about 33%, a response duration of around 9 months [194, 196] and an OS of approximately 19 months [197] when used as monotherapy.

Alemtuzumab shows profound activity in clearing blood and bone marrow disease but its effectiveness in bulky (> 5cm) lymph nodes is limited [171, 194, 196, 198]. An option to overcome this shortcoming may be the addition of corticosteroids which induce apoptosis in a p53-independent way. Alemtuzumab in combination with methylprednisolone (CamPred) achieved an ORR of 88% with 36% CRs in patients with 17p deletion/*TP53* mutation [182]. In this study neither lymphadenopathy nor age had any significant influence on response or survival even though the infection rates among the older patients were higher. However, the duration of response was short with a median PFS and OS of 11.8 and 23.5 months respectively. Combining alemtuzumab with dexamethasone (CamDex) also appeared feasible with high response rates (ORR 79% CR 4%). However, PFS and OS were short (10.3 and 21.3 months respectively) with this combination as well [199].

Full dose alemtuzumab combined with FC or FCR appears to improve response rates but is accompanied with considerable toxicity. Neither the addition of cyclophosphamide to CamPred nor alemtuzumab combined with FCR or mitoxantrone were feasible due to higher toxicity and excess mortality due in particular to infections [152].

However a lower dose of alemtuzumab might be feasible in combination with chemotherapy. Low-dose alemtuzumab in combination with FC (FCA) in the first-line setting showed an increased PFS in patients younger than 65 years compared to FC [200]. Unfortunately there was no benefit of FC in the subgroup with 17p deletion. A recent trial combining low dose of alemtuzumab (30mg in 3 days every third week) with gemcitabine in relapsed/refractory high-risk CLL patients [201] had no safety problems. This study

showed an ORR of 65% including 29.6% CR with a median PFS and OS of 15.4 and 24 months respectively. Bulky disease was associated with a shorter OS.

Another option is to combine alemtuzumab with bendamustine. Phase I/II data on this combination in relapsed/refractory CLL showed an acceptable safety profile with an ORR of 68% including 24% CR and a median PFS of 17.3 months. Responses were consistent (57%) in patients with *TP53* disruptions but the response duration was shorter [202].

The major side effect of alemtuzumab is a profound, long lasting T- and NK-cell depletion leading to an increased risk of infection, in particular reactivation of CMV [203, 204]. Prophylaxis with valaciklovir and cotrimoxazole, or equivalent, is therefore mandatory and monitoring for CMV should be done liberally [4]. About 35-40% of patients experience ≥ grade III infections despite prophylaxis [172, 198]. Infusion-related symptoms have been overcome by a switch to subcutaneous administration without a loss of efficacy [171, 172].

In summary alemtuzumab-based regimens can yield a substantial proportion of CRs in high-risk patients, although of short duration. Combining alemtuzumab with bendamustine appears as effective as a combination with FC but safer. It might be an option in patients who have previously received FCR, particularly if pancytopenia is present due to a heavily infiltrated bone marrow, or as a bridge to transplant in patients with *TP53* disruptions. Alemtuzumab for the treatment of CLL was withdrawn from the market in Europe and the US but is still available through a free, named-patient program.

2.4.3 Ofatumumab

In the initial phase II study conducted in patients with relapsed/refractory CLL the response rate was 50% but the median time to disease progression was only 3.5 months [205]. A phase II multicenter study was then conducted in patients who were refractory DR or had bulky disease refractory to fludarabine (BFR). Ofatumumab was well tolerated by the majority of the patients. The most common AEs were infusion–related reactions which predominantly occurred during the first and second infusions. Response rates were 47-58% but the remission duration of all patients was short and the majority relapsed while being on therapy (median PFS 5.9 -5.7 months respectively) [206]. Even though patients with 17p deletion responded, the results in this subgroup as well in those with bulky disease were poorer. However, the results from this trial promoted the initial registration of ofatumumab as single agent for these patient groups in Europe and the US. Ofatumumab monotherapy is also effective and well-tolerated in patients previously treated with rituximab [207]. A recent retrospective follow-up of ofatumumab in the relapsed/refractory clinical setting showed an ORR of 23% and a median PFS and OS of 5 and 12 months respectively. The safety profile was consistent with that observed in clinical trials [208].

2.4.4 Allogeneic stem cell transplantation

Allogeneic stem cell transplantation (ASCT) is still the only curative treatment option in CLL with an OS of 30-70% [209]. ASCT is recommended if evidence of *TP53* abnormalities, no response or relapse within 12 months after purine analogues or relapse within 24 months after purine analogue based combinations, by the European Group for Blood and Marrow Transplantation (EBMT) [209]. The crucial component of ASCT is the graft-versus-leukemia (GVL) effect in which donor hematopoietic cells mount an immune response against the

malignant clone. The mechanistic basis of GVL is complex but is known to be primarily T cell mediated [210]. The importance of GVL is supported by several findings. First is the phenomenon that relapse incidence seems to decrease with time showing a plateau in disease-free survival. Second there is a difference in long-term molecular responses between allogeneic and autologous transplantation. Third, myeloablative therapy alone is generally not sufficient to cure CLL and reduced-intensity conditioning (RIC) may provide the same cure rate. Finally there are reports on the efficacy of donor lymphocyte infusion [209, 211]. Myeloablative treatment is generally not tolerated in elderly and comorbid individuals. However, even though no randomized trial comparing RIC with myeloablative conditioning is published, RIC appears sufficient as conditioning before ASCT and has expanded the eligible population including patients older than 70 years [209, 212]. Even with RIC, ASCT is associated with relevant non-relapse mortality (NRM). Grade III-IV infections occur in up to 60% even though only a few are life threatening. NRM mounts up to 15% to 25% during the first two years after transplantation largely due to complications of acute and chronic graft versus host disease (GVHD) which affects up to 20%. Due to the high NRM, ASCT is only recommended for those with a good performance status [209].

A challenge treating *TP53* disrupted patients is that the response achieved prior to transplantation appears to be an important factor influencing long term success [209, 213]. Consolidation with ASCT has shown ability to overcome the impact of 17p deletion and long term remissions have been documented for these patients (OS 57% at 5 years and 59% at 4 years) [212, 213]. Neither *TP53* nor *SF3B1* nor *NOTCH1* mutations appear to have an impact on long term disease control [209]. Retrospective data showed higher relapse rates after in-vivo T cell depletion of the graft with alemtuzumab during conditioning for ASCT [214] and for this reason a 3 months wash-out phase after alemtuzumab may be considered.

In conclusion, consolidation with ASCT is a treatment option to strongly consider in patients with *TP53* abnormalities. There is no consensus regarding conditioning before ASCT due to lack of data but the outcome is better in absence of bulky lymphadenopathy and if the patient is in remission at the time of transplant [212, 213].

In summary, fit patients who are not refractory to fludarabine and without *TP53* abnormalities may benefit from FCR as salvage therapy especially if they are not too heavily pretreated [184, 187, 188]. Due to the favorable toxicity profile BR might be a preferable salvage option in the elderly without *TP53* disruptions especially for patients with trisomy 12. Phase II study results suggest that bendamustine in combination with ofatumumab might be a good option in frail patients. For patients harboring *TP53* disruptions, alemtuzumab as single agent or in combination with high-dose steroids (if bulky disease) is an option as well as ofatumumab. Outside clinical trials alemtuzumab in combination with high-dose steroids and ASCT as consolidation, due to the otherwise short duration of remission, is an option to consider for fit patients harboring *TP53* abnormalities [215, 216]. Combination therapy with alemtuzumab or ofatumumab is to be investigated further.

However, on the basis of recent results ibrutinib (see 3.2.1) might be the best salvage therapy for relapsed CLL irrespective of genomic risk.

3 NEW AND EMERGING THERAPIES

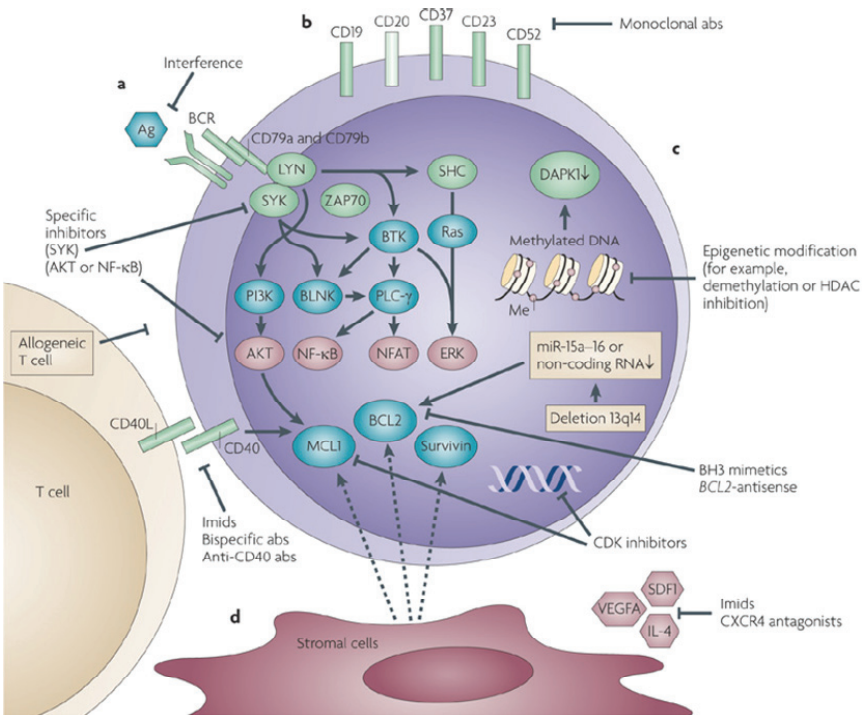

Figure 4. **CLL pathogenic mechanisms and examples of targeted treatment options**.
a) The BCR with downstream signaling of SYK, LYN and BTK are targets for small molecules. b) Multiple epitopes on the CLL cell are targets for antibody-based therapies. c) The function of at least one of the genes deleted in 13q deletion is down-regulation of the anti-apoptotic gene *BCL-2* and the deletion harbors miRNAs that are negative regulators of BCL-2. Epigenetic changes might play a role in regulation of miRNA transcription. Small molecule BH3 mimetics or HDAC inhibitors can target these changes.
d) Stromal and T cell interactions contribute to CLL pathogenesis. Lenalidomide was shown to target the interaction with T cells. Chimeric antigen receptor T cells or allogeneic T cells may provide an effective immune response to CLL cells. The crosstalk between CLL cells and accessory cells up-regulates anti-apoptotic proteins, such as survivin and BCL-2 which may be targeted by small molecules.
Ag, antigen; CDK, cyclin-dependent kinase; HDAC, histone deacetylase; Me, methyl group; NFAT, nuclear factor of activated T cells; PLC-γ, phospholipase C-γ; SDF1, stromal cell-derived factor 1;VEGFA, vascular endothelial growth factor A.

3.1 Novel antibodies

Monoclonal antibodies are approved for use in CLL in the first line mainly in combination with chemotherapy (rituximab, ofatumumab, obinutuzumab) as well as single-agent therapy in the refractory setting (alemtuzumab (until recently), ofatumumab). In addition a new generation of anti-CD20 antibodies has recently become available for preclinical and clinical studies and antibodies directed against new targets are currently investigated (Figure 4b).

Anti-CD20

Type I and II mAbs differ in their CD20 binding and activation of cell death pathways. Type I CD20 antibodies are associated with a stronger CDC but only low levels of direct cell death. The occurrence of resistance to type I mAbs seems to be associated with the loss of CD20 by "shaving" or antibody internalization. Type II CD20 antibodies have a lower CDC effect but can induce increased direct cell death. Less resistance to therapy can be expected due to less antigenic internalization which may prolong the presence of the antibody on the cell surface which likely increases ADCC [167].

Rituximab is a type I chimeric anti-CD20 antibody which exerts its function via CDC and ADCC [217].

Obinutuzumab represents the first humanized glycoengineered type II mAb. Preclinical studies showed a superior killing activity and a greater B-cell depletion than rituximab [167]. A recent phase I/II trial of obinutuzumab monotherapy in relapsed or refractory CLL investigated the safety at different doses in phase I and efficacy at an established dose (1000mg) in phase II. The overall end-of-treatment response was 62% (all partial) the median PFS was 10.7months [218]. The toxicity profile was acceptable (transient neutropenia, thrombocytopenia) with a dose dependent incidence of AEs. Nearly all patients experienced infusion reactions. As significantly elevated cytokine levels were observed together with a rapid and profound B-cell depletion, cytokine release from malignant cells might be the reason for the high frequency and intensity of infusion related reactions (IRR). However, lymphocyte counts and lymphadenopathy were no strong predictors of IRR In the absence of validated risk factors, all patients have to be closely monitored during the first infusion. Cases of TLS have been observed as well [218]. In summary obinutuzumab monotherapy appears active in advanced CLL but shows a higher toxicity than rituximab.

Tumor burden appears to contribute to the clearance of obinutuzumab in the way that those patients with a higher tumor burden have a faster clearance of obinutuzumab [218]. The optimal dose, schedule of administration and duration of treatment is still elusive and further studies are warranted. An ongoing study in the first-line setting compares single agent obinutuzumab at doses 1000 or 2000mg in untreated CLL patients (NCT 01414205).

Given the encouraging results with obinutuzumab combined with chlorambucil first-line [161] other combinations are investigated. A non-randomized study in previously untreated patients investigates obinutuzumab in combination with either FC or bendamustine. Preliminary results show ORR and CR of 90% and 20% respectively in the FC arm and 62% and 10% respectively in the bendamustine arm [219]. Approximately 90% in each arm experienced IRR but the combinations were rather well tolerated otherwise. At a median follow-up of about 1 year none of the patients had experienced disease progression. Ongoing studies investigate obinutuzumab in combination with otlertuzumab, PI3Kδ inhibitors or ABT-199(GDC-0199) [167].

There are several anti-CD20 mAbs under development.

Ocaratuzumab is a humanized anti-CD20 type I antibody designed for effective ADCC at very low concentrations. Preclinical results showed more ADCC compared to rituximab or ofatumumab and at low concentrations similar ADCC to obinutuzumab. The possible lower doses might facilitate future sub-cutaneous administration, potentially reducing the costs and at the same time being more convenient to patients [217].

Ocrelizumab is a humanized anti-CD20 type I antibody which binds to a different CD20 epitope than rituximab with improved ADCC and reduced CDC and improved Fcγ-binding. Clinical responses have been achieved in follicular lymphoma but no clinical results from CLL are available [217].

Veltuzumab is also a humanized anti-CD20 type I antibody which binds to a different CD20 epitope than rituximab with improved ADCC and reduced CDC and improved Fcγ-binding. Early studies in non-Hodgkin lymphoma (NHL), including CLL have shown responses and further clinical trials in NHL are ongoing [217].

Ublituximab (TG-1101) is a chimeric anti-CD20 type I antibody with improved Fcγ-binding which induced higher ADCC activity against CLL cells compared to rituximab at lower concentrations. However, the ADCC was lower in CLL cells from patients with 17p deletion [220]. Ublituximab has shown promising results in a first-in-human phase I/II clinical trial with 45% ORR in advanced CLL at a relatively low dose [221]. A phase I/II study of ublituximab monotherapy in relapsed/refractory B cell lymphoma (including CLL) showed an ORR of 67% in CLL and no dose-limiting toxicity (DLT) [222].

Anti-CD37

CD37 is a transmembrane protein expressed on normal mature B cells. It is upregulated in CLL cells but is expressed at very low density on monocytes, macrophages, neutrophils, T cells, plasma cells and dendritic cells.

Otlertuzumab (TRU-016) is a CD37-specific protein consisting of antibody-derived single chain variable fragments linked to Ig constant domains. It is similar to an antibody in its functionality and pharmacokinetic properties but is smaller and shows superior NK cell mediated ADCC compared to rituximab [217]. A phase I study evaluating safety in CLL patients did not identify any maximum-tolerated dose (MTD). The most frequent AEs (IRR, fatigue, nausea and diarrhea) were not dose related [223]. However, as a single agent it appears to have modest clinical activity in CLL (PR 23% CR 0%) and lower response rates in 17p deleted patients (16%) [223]. Responses were more common in untreated or less treated patients. Preclinical data indicated additive or synergistic activity with rituximab and bendamustine [224]. The combination otlertuzumab and bendamustine compared to bendamustine alone was investigated in relapsed CLL [225] and appeared tolerable with an ORR of 80% (20% CR) compared to 42% (4% CR) for bendamustine alone. However no patients with 17p deletion responded. In addition, whether otlertuzumab combined with bendamustine is better than BR remains unknown. Otlertuzumab combined with rituximab is investigated in previously untreated CLL and seems feasible but to date there are no data on response rates [226].

Anti-CD23

CD23 is expressed on B cells and on most CLL cells. It is a B-cell activation marker and CLL cells typically overexpress CD23 compared to normal B cells [217]. Lumiliximab is a chimeric anti-CD23 antibody which mediates cell death through ADCC and CDC. Preclinical studies showed antitumor activity against CLL cells. However, a phase III trial comparing FCR combined with lumiliximab to FCR closed prematurely owing to the low benefit in relapsed CLL and manufacturers will not pursue further development [217].

Anti-CD40

CD40 is a transmembrane protein expressed on normal as well as malignant B cells. CD40L, produced by T helper cells and NK cells, appears to be coexpressed with CD40 in CLL cells. This finding suggests an autocrine loop in CLL which results in activation of PI3K as well as NKκB pathway and inhibition of apoptosis [217].

Lucatumumab is a fully human anti-CD40 antibody which triggers ADCC and inhibits CD40L induced proliferation in vitro [217]. However, phase I data in the relapsed setting showed mainly stable disease (SD) as best response [227].

Anti-CD19

CD19 is a transmembrane protein with expression restricted to B cells and acts as a co-receptor enhancing signaling in response to BCR antigen stimulation [217].

MEDI-551 is an anti-CD19 antibody which destroys CLL cells by ADCC. A phase II study evaluates safety/efficacy in advanced B cell malignancies (NCT 00983619). In combination with bendamustine MEDI-155 appears tolerable showing a different toxicity profile compared to rituximab with more infusion-related reactions but less neutropenia and nausea [228].

XmAb5574 is a humanized CD19 antibody with enhanced binding to FcγR. ADCC is mediated by NK cells through a granzyme B dependent mechanism [217]. A phase I study in CLL patients is completed but results are not yet published (NCT 01161511).

Blinatumomab (MT103/MEDI538) is a so called bispecific T cell engager (BiTE). It is a mAb with specificity for both CD19 and CD3 and exerts its effect by tether resting T cells to CD19 positive CLL cells and cause tumor lysis [229]. High response rates were obtained in lymphoid malignancies but there are no clinical data in CLL.

Anti-CD49d

CD49d, an integrin with variable expression in CLL appears to be an important prognostic marker [102]. It plays a role in leukocyte migration, activation, survival and in leukocyte trafficking. In addition, activation of CD49d results in signaling which upregulated BCL-2. Natalizumab is an anti-CD49d mAb which has been shown to decrease migration of CLL cells in vitro [230]. However there are no clinical data in CLL yet.

3.2 Small targeted molecules

3.2.1 Targeting the B cell receptor and downstream SYK, LYN and BTK

BCR activation is a central mechanism in CLL [43, 44]. The downstream activation of BCR–associated kinases (LYN, SYK BTK and PI3Kδ) is also involved in other pathways such as chemokine and integrin signaling, cell adhesion and chemokine secretion [42, 48]. These BCR downstream kinases can be targeted by small molecule inhibitors (Figure 4a).

SYK and LYN inhibitors

SYK and LYN are non-receptor kinases activated upon BCR ligand binding. They activate signaling pathways downstream the BCR, and modulate cell adhesion and chemotaxis of B cells and are therefore critical for survival and maintenance [42, 46, 48].

Fostamatinib is an orally reversible, relatively selective SYK inhibitor. In vitro its bioactive form is called R406. To date it is the only SYK inhibitor in clinical use. In a phase I/II clinical trial it was well tolerated and partial responses achieved [231]. An initial lymphocytosis was probably due to disrupted adhesion factors in the lymph nodes and bone marrow [232]. However, further development has focused on rheumatoid arthritis [42].

In vitro data on GS-9973, another SYK inhibitor, in combination with the PI3Kδ inhibitor idelalisib has indicated synergistic growth inhibition and reduced CCL3/CCL4 production in CLL cells [233]. Clinical phase II data on this combination demonstrated that 70% of patients had a > 50% reduction of lymph node size. However the study was terminated early due to the development of severe, steroid responsive, pneumonitis [234]. Early phase II data on single GS-9973 reported a > 50% reduced tumor bulk in 64% of the patients and the treatment was well tolerated even though reversible grade III and IV elevation of transaminases occurred in 4.5% [235].

Dasatinib is a reversible pan-Src kinase inhibitor which also inhibits a variety of other kinases such as LYN and BTK. In vitro data demonstrated inhibited BCR signaling and reduced survival of CLL cells as well as reduced chemotaxis and adhesion to stroma even in the presence of microenvironmental stimuli [42]. However, phase II data relapsed/refractory patients indicate modest results even in combination with fludarabine [236]. The low response rate compared to other kinase inhibitors targeting BTK or PI3Kδ might be due to the function of LYN as a negative regulator of BCR signaling [42].

Bafetinib (INNO-406) is an orally available LYN kinase inhibitor. When evaluated in relapsed and refractory CLL in the elderly there were no objective responses. A higher dose is under investigation [237].

BTK inhibitors

Ibrutinib (PCI-32765) is the first human BTK inhibitor. It binds irreversibly and covalently to a cysteine residue of BTK (Cys481), which inhibits BTK's enzymatic activity. Ibrutinib blocks BCR signaling in vitro [238] and in vivo [45] also inhibiting PLCγ2 and downstream signaling molecules (AKT, ERK). Both in vitro and in patients, ibrutinib impairs microenvironment-induced survival [238] and proliferation [50] as well as release of and migration towards tissue homing chemokines [50, 239, 240]. It also impairs integrin signaling and thereby CLL cell adhesion [239].

The inhibition of adhesion/migration probably explains the CLL cell redistribution, seen with ibrutinib, which is characterized by a rapid and sustained decrease in lymphadenopathy accompanied by transient lymphocytosis [241, 242]. Lymphocytosis is asymptomatic, normalizes after a median of 6.2 months and the degree of it is not linked with response [243]. In vitro [50] as well as in vivo [45] data argues that BTK inhibition targets cell proliferation but ibrutinib has only a modest degree of apoptosis in vitro [50, 238]. Finally, ibrutinib might have an immune-modulating potential by affecting the repertoire of CD4+ T cells [244]. However this has to be explored further in future studies.

Ibrutinib is given orally once daily at a fixed dose of 420 mg continuously until disease progression or toxicity. Ibrutinib achieved an ORR of 60%-71% with a median PFS of 13.6 months and estimated 26 months PFS of 75% as a single agent in relapsed or refractory CLL [241, 245]. The OS was 83% at 26 months [245]. Response was independent of clinical and genomic risk factors with an ORR of 68% in cases with 17p deletion [245]. These results led

FDA to approve single-agent ibrutinib for relapsed CLL patients in February 2014. A phase III randomized trial compared ibrutinib with ofatumumab in relapsed/refractory CLL/SLL [246]. Responses, as assessed by response criteria specifically adapted for this trial and not the commonly used IWCLL criteria, were significantly more common with ibrutinib (43%) than with ofatumumab (4%). At a median follow-up of 9.4 months, PFS as well as OS was significantly improved with ibrutinib. For patients harboring 17p deletion median PFS in the ibrutinib arm was not reached as compared with 5.8 months in the ofatumumab arm. As first-line treatment ibrutinib monotherapy yielded an ORR of 71% with 13% CR in untreated elderly patients [247]. Combination therapies with ibrutinib are investigated to shorten the drug-induced lymphocytosis and to increase CR rates. Preclinical early data showed that a combination with anti-CD20 monoclonal antibodies might be less effective [248]. However, when investigated in patients with either 17p deletion or a short PFS after first-line, the combination of ibrutinib and rituximab achieved an ORR of 95% even though the CR rates were still low (8%) [240]. Combination therapy with ofatumumab or BR in non-controlled trials in relapsed/refractory patients yielded response rates of 100% and 93% (including 17% CR) respectively [249, 250].The initial lymphocytosis appears less frequent with combination therapy [240, 249]. However, the relevance of clearing the peripheral blood remains unknown. Data on combination therapy from randomized trials is lacking but trials are ongoing (NCT 01611090 (ibrutinib-BR), NCT01886872 (BR vs ibrutinib-rituximab vs ibrutinib), NCT02048813 (ibrutinib in combination with rituximab vs FCR) and NCT01722487 (ibrutinib vs chlorambucil in elderly)). Phase III trials in the combination with obiniutuzumab are about to start soon.

Ibrutinib is generally well tolerated as single agent and in combinations even in a heavily pretreated and elderly population with comorbidities [240, 245-247, 249, 250]. The most common AEs are ≤ grade II non hematologic toxicities (diarrhea, fatigue, nausea, myalgia and upper respiratory tract infection). Hematologic toxicity ≥ grade III is uncommon and infections ≥ grade III are rare [240, 245-247]. Adverse events of ≥ grade III usually include diarrhea and atrial fibrillation [246]. Atrial fibrillation of any grade might occur at a higher rate with ibrutinib [246]. Potential reasons for this are being explored. Serious bleeding, including subdural hematoma, not related to thrombocytopenia has also been observed [241, 245]. Even though major bleeding was rare this led to current recommendations for avoidance of concomitant warfarin therapy and interruption of ibrutinib for 3-7 days before and after invasive procedures. Lower grade (≤ grade 2) bleeding-related AEs, most commonly petechiae, are frequent [245, 246]. Recent studies indicate that ibrutinib induces a dysfunction in platelet aggregation [251, 252] probably because BTK is a critical mediator of platelet signaling and collagen binding.

Resistance to ibrutinib generally occurs in the context of Richter transformation and less commonly, in CLL progression [253]. Generally resistance involves mutation of the cysteine residue where binding occurs [254]. This mutation prevents irreversible drug binding leading to increased BCR signaling [255]. Another resistance mechanism is mutation in PLCγ2, immediately downstream of BTK, which potentially allow BTK independent BCR activation [254].

In summary, ibrutinib represents a promising drug in CLL. However, it does not seem to represent a "cure" as true CR [240, 245, 247] and MRD negativity appears rare [240] and continuous treatment seems to be needed. Furthermore, despite no significant difference in

response rates patients with 17p deletion appear to have weaker response as well as a shorter PFS [240, 245, 256-258] and most events associated with disease progression appear to occur in these patients [245, 247].

There are other BTK inhibitors in clinical trials. CC292 is an orally available, highly selective and irreversible inhibitor of BTK. It binds to the same cysteine residue as ibrutinib but is more specific. In a phase I study of relapsed or refractory CLL, it was well tolerated and the ORR was about 60% [259]. Even though not significant, patients with 17p deletion displayed a lower response rate (25%).

ONO-4059 is also an orally available BTK inhibitor. It binds covalently to BTK and is more specific than ibrutinib. A recent phase I study of relapsed or refractory high risk CLL, ONO-4059 achieved an ORR of 90% (including modified PR with lymphocytosis) despite that the majority displayed *TP53* disruptions [260]. ONO-4059 was well tolerated and no dose-limiting toxicities occurred.

3.2.2 PI3K inhibitors

Signaling from several major transduction pathways converge on PI3K which has a key role in regulating B-cell function and survival [48].

Idelalisib (CAL-101) is an orally available, highly specific and reversible inhibitor of PI3Kδ [261]. Idelalisib causes inhibition of AKT activation which decreases MCL-1 (myeloid cell leukemia sequence 1) expression and promotes apoptosis in CLL cells in vitro as well as in patients [262, 263]. Conversely, it shows minimal cytotoxicity against normal T and NK cells [262]. Idelalisib inhibits chemotaxis toward chemokines, pro-survival cytokines and secretion of chemokines (CCL3/4) from CLL cells in vitro as well as in treated patients [67, 262, 263]. Idelalisib also decreases CLL adhesion by interfering with CD49d/VCAM-1 binding [264] which might explain the redistribution of CLL cells observed in treated patients [163, 263]. Finally, idelalisib might have immune modulating capacity as inhibition of regulatory T cells has been shown in vitro [265].

The recommended maximum starting dose is 150 mg twice daily due to an observed plateau in plasma and consistent clinical response in lymph nodes with higher doses [263]. A phase I study investigated idelalisib in heavily pretreated patients where 80% had bulky lymphadenopathy and 24% had *TP53* disruptions [263]. No DLT were observed. The ORR was 72%, and median PFS was 15.8 months but patients who received a dosage of ≥ 150mg twice daily showed a longer PFS than those treated with lower doses (31.9 vs. 6.6 months). As single treatment in elderly previously untreated patients, idelalisib yielded objective responses in 96% (including CRs) and a 93% PFS rate at 24 months [266].

Preclinical data on idelalisib in combination with monoclonal antibodies is contradictive. Some in vitro data showed no interference with ADCC [262], while other suggests that idelalisib inhibits the cell-mediated effector mechanisms of CD20 monoclonal antibodies [248].

The only fully published combination study investigated rituximab combined with either idelalisib or placebo as part of salvage therapy [163]. The study was closed prematurely after 24 weeks at the first pre-specified interim analysis because the combination therapy demonstrated a significantly higher ORR (81% vs 13%), PFS rate

(93% vs 46%) and OS rate (92% vs 80%) at 12 months. Phase I data on combinations with either rituximab or ofatumumab showed similar response rates and a median PFS of 26 months [267]. Idelalisib combined with rituximab or ofatumumab appears effective in patients harboring *TP53* disruptions in untreated [266] and relapsed patients [267, 268]. In relapsed patients idelalisib monotherapy achieved an ORR of 54% and a PFS of 5 months in those with *TP53* disruptions compared to 72% and 41 months respectively for those without [263].

Idelalisib has shown to sensitize CLL cells for bendamustine, fludarabine and dexamethasone in stromal cocultures [67]. When combined with BR or chlorambucil the ORR was 90% with similar response rates in both cohorts [269]. Several studies evaluating combinations with BR (in first line as well as in the relapsed setting) or ofatumumab (NCT 01980888, NCT 01569295 and NCT 01659021) are ongoing.

Idelalisib is generally well tolerated as monotherapy as well as in combination with mAbs or bendamustine [163, 263, 267, 269, 270]. The most common toxicities are non-hematologic ≤ grade II (fatigue, rash, pyrexia and cough). However, several unique AEs have been reported. High rates (up to 30%) of pneumonia and pneumonitis have been observed. The mechanisms for these side effects remain unclear. Diarrhea, mostly ≤ grade II, has been reported in about 20-30% of the patients and may infrequently also be associated with colitis [163, 263, 266]. Hepatic transaminitis is the third side effect requiring special attention. It has not resulted in clinically significant liver dysfunction and upon drug withdrawal it seems to improve rapidly [163, 263, 266]. However, close monitoring of serum transaminases appears important. Adverse reactions necessitating discontinuation of treatment have most frequently been hepatotoxicity and/or diarrhea/colitis [163, 263, 266].

Preclinical data showed a synergistic effect of the combinations of idelalisib and GS-9973, a SYK inhibitor [233]. However, one clinical trial was terminated early due to the development of severe, steroid responsive, pneumonitis [234].

Idelalisib was approved by FDA 2014 for relapsed CLL as a single agent and in combination with rituximab in patients for whom rituximab alone would, according to US therapeutic traditions, be considered an appropriate therapy. Idelalisib is approved in the EU in combination with rituximab for patients who have received at least one prior therapy; or as first-line treatment in patients with *TP53* disruptions unsuitable for chemoimmunotherapy.

In summary, idelalisib appears to be active in high-risk patients. It is generally well tolerated but is associated with certain side effects that require special attention. Preclinical data indicates that idelalisib might be a treatment to explore further in patients with progressive disease during ibrutinib treatment [255].

Another PI3K inhibitor, IPI-145 (INK-1147) is currently investigated in CLL. It is an orally available PI3K inhibitor inhibiting both the δ and γ isoforms. Results from a phase I study in patients with advanced CLL showed an acceptable safety profile [271]. The ORR was 52% (including CRs) and patients with *TP53* disruptions had similar response rate. A transient treatment-related lymphocytosis along with decreased lymphadenopathy was observed. The most common side effects were transient neutropenia and elevated transaminases. A phase III study of IPI-145 versus ofatumumab in relapsed refractory patients (NCT 02004522) as well as combination studies with FCR or BR (NCT 02158091 NCT 01871675) is currently ongoing.

3.2.3 BCL-2 inhibitors

BCL-2 family members contribute to CLL cell survival. It includes more than 20 members with either anti-apoptotic or pro-apoptotic activity. The anti-apoptotic proteins (BCL-2, BCL-xL, MCL-1) share sequence motifs termed B1 to B4. The pro-apoptotic proteins are divided into two subgroups where the first group (BAX (BCL-2 associated X protein) and BAK (BCL-2 antagonist/killer 1)) displays three BH3 domains and the second group (BIM, BAD) shares sequence homology only with the BH3-domain (BH-3 only proteins). BAX/BAK leads to the formation of pores resulting in mitochondrial outer membrane permeabilization (MOMP) and apoptosis. BH3-only proteins act as activators of BAX/BAK by displacing them from being bond to anti-apoptotic proteins The anti-apoptotic proteins (BCL-2, BCL-xL, MCL-1) bind the BH3 domains of pro-apoptotic proteins thus preventing activation of BAX/BAK [272]. Many pro-apoptotic BH3-only proteins are *TP53* transcriptional targets, thus this balance appears even more skewed toward survival in CLL cells with defect *TP53* [273]. Inhibiting anti-apoptotic BCL-2 proteins might be a way to tip the balance between survival and apoptotic signals and to circumvent a requirement for functional *TP53*.

BH3 mimetics (Figure 4c) are small molecules which bind to anti-apoptotic inhibitors thereby allowing the pro-apoptotic activity of BH3-only molecules. Navitoclax (ABT-263) is an orally available BH3-mimetic, targeting preferentially BCL-2 and BCL-xL proteins. As monotherapy in relapsed/refractory patients in a phase I study it yielded 35% PRs and was equally effective in patients harboring 17p deletion [274]. Dose-dependent thrombocytopenia was the dose-limiting event and many patients did not tolerate adequate drug concentrations. This side effect was somehow expected since BCL-xL is the primary survival factor in platelets and preclinical data show that inhibition of BCL-xL has caused a dose dependent thrombocytopenia [275]. Early phase I data on navitoclax in combination with BR showed promising results (ORR up to 81% including CRs) [276]. The study has been completed (NCT 008 68413) but is to date not published.

ABT-199 (GDC-0199) is a very specific BCL-2 inhibitor with higher affinity to BCL-2 compared to BCL-xL [61]. ABT-199 inhibits growth of BCL-2 dependent tumors in vivo and, consistent with its lower BCL-xL inhibition, spares platelets [61]. A recent phase I study of ABT-199 in relapsed/refractory patients reported an ORR of 77% with a CR rate of 23% and with a 2 year PFS of 59%. The response rates in high-risk patients were similar and MRD-negativity was observed in several patients achieving CR, including those with 17p deletion [277]. The DLT has been hyperacute, TLS but it seems to be manageable by using a ramp-up dosing scheme combined with aggressive prophylaxis and monitoring [277].

Neutropenia was the most common severe (≥ grade III) side effect, affecting at least one third, however febrile neutropenia was not frequent (6%). Non-hematological side effects (diarrhea, nausea and fatigue) affected about one third of patients [277, 278].

Early data from a phase Ib study of ABT-199 in combination with rituximab in relapsed/refractory patients showed objective responses in 84% including 36% CRs as well as observed MRD negativity [278]. The combination appears well tolerated with no new toxicities identified. However, a case of fatal TLS amended the dosing regimen.

Further trials with ABT-199 as a single agent as well as in combinations are ongoing. (NCT 01889186 phase III study of ABT-199 in relapsed/refractory patients harboring 17p

deletion and NCT 02005471 phase III study of ABT-199 combined with rituximab vs BR in relapsed /refractory patients).

3.2.4 CDK inhibitors

Cyclins are important regulators of the cell cycle. Normally their expression fluctuates regularly and they require the presence of a cyclin-dependent kinase (CDK) to do their regulatory work. There is a large variety of cyclins and CDKs involved in cell cycle progression where some play key roles in RNA transcript production of anti-apoptotic proteins [279]. CDKs can be targeted by several inhibitors and the inhibition results in a decrease of anti-apoptotic proteins, and thereby induction of programmed cell death in CLL cells in a p53-independent way [280, 281].

The broad CDK inhibitor flavopiridol (alvocidib) is the most comprehensively studied compound in this category of drugs. In early clinical trials it induced an ORR of 45-53% with similar response rates in patients with 17p deletion and/or bulky lymphadenopathy [282, 283]. In combination with rituximab higher response rates were achieved but this study included other lymphomas as well [284]. A study investigating the combination with lenalidomide is ongoing (NCT 00735930). As flavopiridol is a broad inhibitor targeting several CDKs its therapeutic window is narrow. A dose escalation approach seems to be needed since the limiting factor in clinical trials have been acute TLS [282, 283].

The pan-CDK inhibitor dinaciclib (SCH 727965) has shown potent pre-clinical activity against CLL cells independently of high-risk genomic features by downregulating messenger RNA and protein expression of MCL-1 [281]. However, it appears ineffective at overcoming the protective effect between CLL cells and stromal cells [281]. Dinaciclib achieved an ORR of 58% and a PFS of 16 months in patients with relapsed or refractory CLL in a phase I/II trial [285]. The ORR for patients with 17p deletion was similar (57%).

P1446A is a novel orally active CDK inhibitor which has shown pre-clinical activity in CLL. However, samples carrying 17p deletion showed decreased sensitivity [286].

Pre-clinical data on CDKI-73, a CDK9 inhibitor, showed higher induction of apoptosis compared to flavopiridol [287]. It was equally effective in cells from high-risk patients and showed synergy with fludarabine. This makes CDKI-71 interesting for further exploration.

The most frequent related toxicity appears to be myelosuppression and the associated increased risk of tumor lysis syndrome [282, 283, 285].

In conclusion, CDK inhibitors appear effective both as single agents and in combination with chemotherapy in relapsed and refractory CLL, including cases with *TP53* disruptions. However recent limited retrospective data analysis showed that ibrutinib surpassed CDK inhibitors both at inducing response and prolonging median PFS and OS [256]. Still, both ibrutinib and CDK inhibitors were more effective than other standard or investigational regimens. Therefore the place of CDK inhibitors remains to be defined.

3.2.5 HDAC inhibitors

Histone deacetylases (HDACs), in several isoenzyme classes, are overexpressed in CLL [288, 289]. HDACs mediate the epigenetic silencing of certain miRNAs which are critical tumor suppressors. Inhibition of HDACs in vitro has been shown to induce expression of these miRNAs followed by a declined level of MCL-1 and induction of cell death [289] i.e.

HDAC inhibitors have the ability to reverse epigenetic alterations associated with CLL cell survival (Figure 4c). These results suggest that patients exhibiting epigenetic silencing may benefit from HDAC inhibitors. HDAC inhibitors may also induce autophagy, a "self-eating" process, which is a pro-apoptotic mechanism in CLL [290].

Mocetionstat (MGCD0103) is an orally available HDAC inhibitor that demonstrated efficacy in vitro in human cancer cell lines [291]. It modulated the expression of critical autophagy genes which possibly contributes to autophagy impairment in CLL cells [290]. However, the clinical effectiveness in CLL appears to be limited, with no responses in a phase II trial in CLL high-risk patients [292]. Since HDAC isoenzyme levels in CLL may be of more than one class [288] a combination of therapies [293] or a broader targeting was thought to be needed to get a clinical effect. However, treatment with the pan-HDAC inhibitor vorinostat did not result in clinical responses [294].

3.3 Other immunotherapy

One of the paramount ways malignancies establish themselves is through suppressing both the innate and acquired immune systems. In CLL effective antitumor immune responses are lacking due to the capacity of the malignant cells to induce a state of profound immune dysfunction [109].

Circumventing this with allogeneic stem cell transplantation can be effective but is also associated with high morbidity and mortality [213]. Strategies to restore autologous antitumor immune responses, also tolerable for the elderly, are emerging. These strategies interfere with either inhibitory signaling axes or CLL cell stimulating niches.

3.3.1 Lenalidomide

Lenalidomide belongs to the immunomodulatory class of drugs. Its mechanism of action appears disease specific and since it is not directly cytotoxic in vitro [295], its clinical activity in CLL has been presumed to be secondary to its immune modulating activity.

Lenalidomide improves T cell function by; repairing the functional synapse defects of T cells, downregulating expression of T cell inhibitory molecules (such as PD-1) and enhancing T cell motility [128, 129, 296-298]. In addition, lenalidomide inhibits T_{regs} and normalize functional T cell subsets and induces T helper (Th17) cells [299, 300] which further antagonizes inhibitory signaling axes.

Lenalidomide has important direct effects on CLL cells as well. In vitro, CLL cells appear to be activated as they express higher levels of immunostimulatory molecules (CD80, CD86, HLA-DR, CD95 and CD40) and lower levels of inhibitory molecules [301-303]. This might enhance their capacity to engage T cells in cognate interaction and maybe lead to immune activation by tumor associated antigens. Lenalidomide may also reverse hypogammaglobulinemia by upregulation of CD154 on CLL cells which in turns may co-stimulate normal B cells to produce antibodies [302]. Further, B cells treated with lenalidomide showed to downregulate the expression of PD-L1 thereby possibly reducing the inhibitory impact on T cells [129]. NK cells appear to be affected as well showing enhanced cytotoxicity [128]. Moreover, treatment with lenalidomide appears to affect the levels of cytokines, such as IL-2, IFNδ and TNFα [299, 304] and in vitro lenalidomide appears

to reduce the survival support from nurse-like cells and to modulate the migratory potential of CLL cells (Figure 4d) [305].

Even though the major effects of lenalidomide in CLL might be through the microenvironment new data suggest a direct antiproliferative effect [306]. Lenalidomide binds cereblon, a component in a complex responsible for target recognition and binding of proteins to be degraded. This results in increased expression of the CDK inhibitor $p21^{WAF1/Cip1}$ (p21), a negative regulator of cell cycle progression, leading to cell cycle arrest. Lenalidomide is also suggested to promote an enhanced cereblon-mediated degradation of transcription factors (Ikaros and Aiolos,) thereby enhancing T-cell and NK-cell activation and function [307]. However additional studies are required to evaluate whether this is a mechanism of action in CLL. Altogether, lenalidomide represents one of the few immune restorative therapeutics used in CLL and may be of special interest since its function appears p53-independent.

As a single agent, lenalidomide induced objective responses in 32% - 47% in heavily pretreated patients with relapsed or refractory CLL [304, 308]. When further developed as initial treatment for elderly patients [298, 309] response rates of up to 65%, including 10% CRs, were reported, with an OS of 86% at 4 years follow up [310]. However, a randomized controlled trial (NCT 00910910) comparing the safety and efficacy of lenalidomide versus chlorambucil as first-line therapy for elderly patients was recently halted by FDA because of significant safety concerns due to an imbalance in the number of deaths in lenalidomide-treated patients [311].

A previous study reported that lenalidomide could reduce CD20 expression in vitro and thereby maybe mitigate the activity of rituximab if given in combination with lenalidomide [312]. However, another study reported that lenalidomide enhanced NK-cell mediated ADCC in relation to rituximab treatment [313]. In clinical trials with previously untreated or relapsed patients, the combination of rituximab and lenalidomide achieved response rates of 88% and 66% (including 15% and 12% CR) respectively [303, 314]. The estimated 3 year OS, in relapsed/refractory patients, was 71% [314]. The median PFS in the first line setting was 19 months being similar to median time to treatment failure in the relapsed/refractory patients (17.4 months). PFS was longer in patients who achieved CR but elderly patients did not display any shorter PFS/time to treatment failure. In addition, only a modest decline in CD20 levels was observed after lenalidomide monotherapy [303].

Lenalidomide combined with ofatumumab in relapsed/refractory patients yielded an ORR of 48%, all PR, and a median OS of 21.5 months [315]. Another study of the same combination with a more intense schedule is ongoing and early data indicate response rates of 68% including 24% CR [316].

Even though lenalidomide appears to act in a p53 independent manner the ORR with lenalidomide monotherapy was only 31% in relapsed/refractory patients with 17p deletion [304] and when lenalidomide was used as initial treatment in elderly there was no responses in this group of patients [309]. In a preliminary report from a study comparing lenalidomide single to lenalidomide plus rituximab 17p deletion was significantly associated with inferior survival [317]. However, early results from a phase II trial investigating single agent lenalidomide in relapsed/refractory patients with or without *TP53* mutations showed similar outcomes in both groups [318].

There are some troublesome side-effects with lenalidomide of which the most notable ones are specific for CLL. TLS, resulting in some deaths, was reported when the starting dose was 25 mg [308] but did not occur when the starting dose was lowered to 10 mg in the relapsed/refractory setting. However, TLS was observed in treatment-naïve patients [298] and this prompted an even more careful approach with starting doses of 2.5 mg. Prophylaxis with allopurinol, hydration and careful laboratory monitoring together with a low starting dose followed by slow dose escalation has successfully mitigated the risk of TLS. The MTD of lenalidomide remains unclear.

Tumor flare reaction (TFR) manifested by acute swelling of involved lymph nodes sometimes associated with fever, localized erythema or rash is a side-effect occurring in CLL patients [319]. Onset may start within hours after the first dose and even if observed more frequently during the first cycle, it may be recurrent for months [298]. TFR is associated with upregulation of CD40, CD80 and CD86 on CLL cells as well as T-cell activation [301]. First-line studies reported higher rates of TFR (52-88%) [298, 309] than studies in relapsed patients [304, 308]. This might suggest a greater tendency for immunostimulation in treatment-naïve patients. For patients treated according to a dose-escalation schedule TFR is usually of low grade if it occurs and manageable with nonsteroidal anti-inflammatory or steroid therapy [298, 303, 314]. TFR has been associated with response but data is contradictive [303, 315, 319]. Neutropenia is the most common hematologic adverse event and even on low doses the incidence of severe (≥ grade III) neutropenia is up to 83% [298, 303, 304, 308, 309, 314]. Severe thrombocytopenia is less common (up to 45%). Non hematologic toxicity such as fatigue, gastrointestinal symptoms and skin rash is predominantly of low grade (I-II) if an appropriate dosing schedule is applied [303]. The increased risk of thromboembolism described in first studies appears to be manageable with aspirin prophylaxis [298, 303, 304, 308].

In summary, the clinical efficacy of lenalidomide in CLL appears to include both T-cell antitumor immune responses and increased CLL cell "visibility" to the activated immune system as well as cytostatic mechanisms. Lenalidomide has clinical activity in CLL as single agent [298, 304, 308, 309] as well as in combination with rituximab [303, 314]. Side-effects are manageable but require special attention. As consolidation after chemo-immunotherapy, it appears to result in improved quality of remissions as well as prolonged time to salvage therapy [297]. In line with previous studies this shows that deeper responses appear to require a long duration of therapy [298, 314]. However, patients with 17p deletion appear to benefit less [309, 310, 314] and the place for lenalidomide in treating patients with *TP53* disruptions is currently unclear.

3.3.2 CAR-T cells

Cure is achievable by breaking immunological tolerance in the context of ASCT. This underscores the powerful therapeutic effect of the T cell immune response in eliminating CLL cells [212, 213]. Another approach to circumvent tolerance to tumor-associated antigens is to modify the patient's own T cells to target the malignant cells (Figure 4d). This offers an opportunity to produce a GVL without GVHD. In addition, ex vivo manipulation of T cells outside the inhibitory microenvironment may also offer a possibility to reverse the T-cell dysfunction. This has been achieved by the use of the single-chain variable fragment form an antibody molecule fused with an internal signaling domain such as CD3ζ to from a

chimeric antigen receptor (CAR). CAR-T cells may redirect the immune responses through their immune receptors with tumor associated antigen specificity. Genetic manipulation of T cells for introduction of a CAR transgene often relies on transduction using recombinant retrovirus but non-viral approaches such as electroporation have been investigated [320]. A non-viral approach has the advantage of limiting the theoretical risk for insertional mutagenesis, causing autonomous T cell proliferation. However, no genotoxicity has been apparent so far [320]. The optimal design of CAR-T cells remains to be determined. First generation CARs were activated only through CD3ζ but problems with in vivo persistence and thus anti-tumor effect led to development of second generation CAR-T cells containing a co-stimulatory molecule, such as CD28, in addition to CD3ζ thus enabling CAR-T cells to respond to and kill CLL cells despite lack of costimulatory molecules on the malignant cells [321]. Third generation CAR-T cells contain three signaling domains. The most commonly targeted tumor-associated antigen in CLL is CD19 which is a suitable target since it is only expressed on B-cells and is not shed into the circulation [320]. Other antigens, for instance ROR-1, are explored in trials.

Anti-CD19 CAR-T cells have shown impressive early results in CLL. The first trial treated 3 patients with relapsed/refractory CLL [322]. All patients responded (2 CR and 1 PR) despite bulky lymphadenopathy (3/3) and 17p deletion (2/3). In a recent update 14 relapsed/refractory patients of whom 6 had 17p deletion have been treated. The ORR after a median follow-up of 9.4 months was 57% (21% CR) [323]. Responses were independent of age, number of prior therapies and *TP53* abnormalities and there does neither seem to be a dose:response nor a dose:toxicity relationship at the dose ranges of CAR-T cells used so far. The CAR-T cells expanded in vivo were able to persist for at least 3 years [323, 324].

Most patients with an anti-tumor effect experienced infusion-related toxicity (fever, hypotension and occasionally, deterioration in mental status and neurological changes). These side-effects seem to arise from the synchronous activation of infused CAR-T cells by bulky deposits of CD19+ CLL cells leading to a surge in cytokines, such as TNF and IFNγ, and activation of macrophages [322, 323]. In addition, clinical management can be complicated by TLS. Anti-inflammatory agents and systemic steroids must be dosed to mitigate toxicity without immediately compromising the anti-tumor effect mediated by CAR-T cells. To avoid these toxicities patients should probably have a reduced tumor burden before infusion of CAR-T cells. So far the vast majority have received chemotherapy prior to infusion which also might be a way to achieve better responses [323]. An effect of undesired on-target toxicity was the elimination of normal B cells and profound lymphocytopenia [324]. Patients probably benefit from intravenous immunoglobulin until B-cell immunity recovers.

Reasons for CAR-T cells failure is not completely understood but may be related to downregulation of tumor associated antigens, or inability of CAR-T cells to recycle effector functions which may be due to an influence from tumor microenvironment [320]. Many challenges remain including better efficacy, acceptable toxicity and ability to produce CAR-T cells in a large commercial scale thus providing a more favorable cost profile, and to achieve good efficacy with acceptable toxicity.

3.3.3 Targeting the CXCR4 –CXCL12 axis

The stromal cell-mediated drug resistance may, at least partly, be reversed by CXCR4 antagonists who inhibit CLL cell activation by CXCL12 [48]. Plerixafor, is a selective, reversible inhibitor of the chemokine receptor CXCR4. It inhibits CXCL12-mediated chemotaxis and binding. It is approved by FDA in lymphoma and multiple myeloma for mobilization of HSC [48]. Early phase II data from relapsed CLL patients treated with plerixafor in combination with rituximab demonstrated a dose-dependent mobilization of CLL cells from tissues into the blood as well as some responses [325].

An alternative approach is to target the chemokine CXCL12 (the ligand of CXRC4). NOX-A12 is an RNA oligonucleotide that binds and neutralizes CXCL12. It effectively inhibits CXCL12 induced chemotaxis of CLL cells and enhances their sensitivity to fludarabine and bendamustine in preclinical models [326]. A phase IIa clinical trial evaluates NOX-A12 in combination with BR in relapsed CLL (NCT 01486797).

3.3.4 PD-1/PD-L1 axis

An important mechanism for the failure of immune responses in CLL appears to be the suppression by inhibitory pathways. PD-1 is an inhibitory receptor predominantly expressed on exhausted T cells. After binding its ligand PD-L1, PD-1 inhibits the T cell receptor-mediated lymphocyte proliferation and cytokine secretion. In the normal setting this is an important function for maintenance of peripheral tolerance [127]. In CLL T cells and CLL cells display an increased expression of PD-1 and PD-L1 respectively [327]. CLL T cells show enhanced expression of PD-1 [127]. PD-1 appears involved in tumor-mediated immunosuppression by binding its ligand PD-L1. T cells in CLL patients display increased expression of PD-1 and CLL cells have an increased expression of PD-L1 [327]. Blockade of this interaction might restore the function of anergic T cells. Results in solid tumors with PD-1 blocking have been promising [328]. However, there are few clinical trials in hematological malignancies. Phase I data on an anti-PD1 antibody in CLL showed SD but no CR/PR [329].

3.3.5 CD200

CD200 is a transmembrane IgG cell surface ligand that functions as a negative immune regulator. By blocking the interaction between CD200 and its ligand the tolerance to tumor antigens might be prevented [330]. CD200 is upregulated in CLL is used as a marker to distinguish CLL from mantle cell lymphoma [331] and plasma levels of CD200 appeared elevated in CLL patients compared with healthy controls and plasma levels were significantly correlated with CLL disease stage [332]. Preclinical data indicates that CD200-blockade stimulates T cell responses against CLL cells and decreases the number of T_{reg} [333]. However, to date clinical data in CLL are limited.

4 AIMS OF THE THESIS

The aims of this thesis are to gain a deeper insight into the outcome of patients with advanced-phase chronic lymphocytic leukemia (CLL) and to explore new emerging drugs.

More specifically:

I. To obtain routine clinical practice outcome data on consecutive chemotherapy-refractory CLL patients from a well-defined geographical region.

II. To obtain knowledge about the efficacy and safety of alemtuzumab in previously treated consecutive patients with advanced CLL when used in routine health care at experienced centers in a defined region, and to compare the results with those from multicenter trials and other previous reports.

III. To study safety 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 patients prior to and during therapy with alemtuzumab.

IV. To examine, in a manufacturer-independent analysis, the in vitro sensitivity of CLL cells to a panel of small targeted therapeutic molecules and whether there is a difference in their individual in vitro cytotoxic capacity and between patients with progressive or indolent CLL and also to study associations between clinical characteristics and in vitro drug sensitivity.

5 MATERIAL AND METHODS

5.1 Evaluation of response and toxicity

Response evaluation of CLL therapy (Paper I, II and III) was performed using the IWCLL guidelines response criteria [113]. In paper II we compared with the NCI-WG criteria [334].

Adverse events were graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In Paper I and Paper II only AEs ≥ grade III were recorded, with the exception of CMV reactivations and herpes infections, which were always recorded even those considered ≤ grade II.

5.2 Definition of refractory CLL

Fludarabine refractoriness was defined as failure to achieve at least PR following a minimum of one cycle (if progression on therapy, otherwise at least two cycles were required) of fludarabine-containing regimen or disease progression within 6 months of completion of therapy. Bulky nodal disease was defined as lymphadenopathy with lymph node(s) ≥ 5 cm in largest diameter, assessed by physical examination or CT scan. Alemtuzumab refractoriness was defined as failure to achieve at least PR following a minimum of 12 doses of alemtuzumab, or disease progression within 6 months of completion of therapy.

5.3 Study procedures

5.3.1 Paper I and II

The Regional Cancer Registry in Stockholm was used to identify all patients diagnosed with CLL year 1991-2010. Their medical files were reviewed to identify a) those being refractory to fludarabine, including those who in addition were BFR or DR and b) those treated with alemtuzumab in the relapsed or refractory setting. The medical records were reviewed commencing from the date of first diagnosis until death or until the end of the study period (April 2010), whichever came sooner. Demographic and treatment outcomes were recorded in CRF. A record of death was validated against the Swedish Cause of Death Registry [335]. In Paper I patients with fludarabine refractory CLL were further classified into 3 subgroups for subsequent analyses: BFR, DR and a non-BFR/DR group consisting of patients who were refractory to fludarabine but never met the criteria for BFR or DR. Patients in the DR and BFR groups were further divided into 2 smaller subgroups, depending on whether or not they matched all the inclusion criteria stipulated for a pivotal ofatumumab trial [206] (this agent was approved by EMA on phase II study results without a control group). In Paper II patients were considered evaluable for safety/efficacy of alemtuzumab if they had relapsed/ refractory CLL and had received at least one dose of alemtuzumab. Both intravenous and subcutaneous administrations of alemtuzumab were included. Patients were classified into three subgroups for subsequent analyses. Those who were refractory to purine analogues comprised the Refractory Group. Those who had relapsed after treatment with purine analogues but not fulfilling the refractory criteria

comprised the Purine analogue relapsed Group. Finally, those who relapsed after treatment with agents other than purine analogues comprised the Relapsed/Other Group.

5.3.2 Paper III

Consecutive patients diagnosed with refractory CLL or considered inappropriate for fludarabine-based therapy were included. Adequate organ function was required and patients had to have a performance status of ECOG score ≤ 2. All patients had viral and bacterial prophylaxis and, if needed, G-CSF. Lenalidomide alone was given weeks 1-4 according to a stepwise dose escalation schedule with a starting dose of 2.5 mg/d. Week 5 alemtuzumab s.c. was added and the combination was given for 12 weeks. Patients were regularly monitored regarding TFR and TLS. Immunomonitoring was performed at baseline, at the end of the first cycle, i.e. week 4, and at the end-of treatment.

5.3.3 Paper IV

Blood samples were acquired in connection with routine tests from consecutive CLL patients at the Department of Hematology, Karolinska University Hospital.

Patients were classified into 2 groups; indolent CLL, with no treatment indication, and progressive, symptomatic CLL, requiring treatment irrespectively of previously treated or untreated [113]. Some patients with progressive CLL also provided bone marrow samples

5.4 Laboratory methods

5.4.1 Paper III

Peripheral blood mononuclear cells (PBMC) were isolated from fresh blood by using Ficoll gradient density separation**.** PBMC were used for the subsequent experiments which included flow cytometry and T-cell response to purified-protein-derivative of tuberculin (PPD) and phytohaemagglutinin (PHA).

The PBMC for flow cytometry were mixed with appropriate amount of antibodies; CD4, CD8, CD3, CD16 & CD56 in prospective tubes for surface staining. After washing and permeabilization of the cells, perforin & granzyme B were added and incubated for 30 minutes. Foxp3 staining was performed using a T_{reg} staining kit. After a final wash, cells were resuspended in PBS (phosphate buffered saline) and run by LSR11 flow cytometer. All data were analyzed using the FlowJo software .Expression of activation markers (CD154, CD28, CD25, CD69, CD40, HLA-DR), co-stimulatory molecules (CD80, CD86), proportions of memory and naïve T cells (CD45RA, CCR7), apoptosis molecules (Fas-L, CD95) and chemotaxis (CXCR4) as well as CD38, CD52, CD83, CD95 and CD16 were also evaluated.

For the functional tests, semi-purification by nylon wool separation of PBMC was made if the number of lymphocytes was > $10x10^9$ /ml in the sample. The T-cell response to PPD and PHA was assessed during a 5 day culture system on microtiter plates where 6 wells were loaded with PPD 5 µg/ml and PHA 10 µg/ml respectively. Each well was loaded with 100 µL cell suspension containing approximately 200 000 cells in cell culture medium (RPMI (Roswell Park Memorial Institute) + 10% AB^+ serum). As controls, 6 wells were loaded with PBMC alone. During the last 18 hours of incubation, 1µCi/well

thymidine was added and incorporated radioactivity was measured by Wallac 1450 MicroBeta® TriLux. Results were displayed as ΔCPM, calculated mean radioactivity (CPM) of 6 replicates of experimental wells subtracted by the background value (cells with medium alone), and as stimulation index (SI) calculated by mean CPM divided by the background value.

5.4.2 Paper IV

Laboratory methods are described in detail in Paper IV. Briefly, blood and bone marrow samples were collected in EDTA- or heparinized glass tubes and immediately transported to the laboratory. The blood was diluted with PBS and PBMC were isolated using Ficoll gradient density separation. The drug sensitivity of primary CLL cells was assessed using a 72h cell culture system (in vitro viability (iVV) assay) on 2 different 384-well microtiter plates loaded with 31 small therapeutic molecules and 29 cytostatic agents. The killing-capacity of each drug was measured at 4 different concentrations in triplicate. Each well was loaded with 30 µL cell suspension containing approximately 4000 cells in a primary cell culture medium (OmniSanguine, Qantascope Biotech, Stockholm, Sweden) which mimics the in vivo environment. Two types of controls were used on each plate, wells containing culture medium alone and wells containing culture medium and PBMC but no drug.

To verify the robustness of the system, cells were also analyzed after 24 and 48 hours and an additional analysis where CLL cells from the same patient were cultured in both OmniSanguine and RPMI medium was made.

After 3 days of incubation, live and dead cells were differentially stained by fluorescent VitalDye. An automated digital fluorescent scanner HexascopeHaema quantified the precise number of living and dead cells. The image capturing control software, the digital image analysis software package and the clinical report generating computer program was applied as previously described [336]. The assay has been validated on CLL cells using conventional cytostatics [336] and received a European in vitro diagnostical approval (IVD) for Europe in July 2014.

The 31 small molecules were obtained from a single distributor to ensure comparable quality. The drugs were either in clinical use or in early clinical/preclinical testing. An equimolar concentration range was applied for the small targeted molecules in order to optimize head-to-head comparison.

As controls, 29 cytostatic agents were tested in the same way as previously described, where the drug concentration was selected by approximating from concentrations achieved in vivo [336].

The drug sensitivity was calculated as a single value of killing efficiency (KE%) from triplicate measurements at four different dilutions after 72 hours of incubation. KE%, a value graded from 0 to 100, was calculated for each drug and is a weighted sum of dose dependent killing-capacity. A comparison of KE% in OmniSanguine and RPMI was made as well.

5.5 Statistical analysis

5.5.1 Paper I and II

The Kaplan-Meier method was used to calculate survival from the start date of first salvage therapy (Paper I) or alemtuzumab therapy (Paper II) to the date of death or date of last follow-up and from the date of diagnosis of CLL to the date of death or last follow-up. Patients who received an ASCT after salvage therapy/alemtuzumab therapy were not included in survival analysis. In Paper II, the Landmark method [337] was used to calculate survival as well. Differences in survival distributions were compared using the log-rank test. Categorical and continuous data were compared using the Fisher's exact and χ 2 tests (Paper I), as appropriate and using Fisher's exact and Wilcoxon rank-sum test (Paper II), as appropriate. Cox regression analysis was used for univariate and multivariate models of survival while log-rank test was used to test for equality over strata. All reported p values were two-sided.

5.5.2 Paper III

Wilcoxon signed-rank test for paired samples was used for comparison between the baseline and end of treatment of laboratory values. Spearman correlation was used for the dose-dependent reduction of relative proportion of T_{reg} in the CD4+ T cell population during cycle 1. All reported p values were two-sided.

5.5.3 Paper IV

Independent two-sample *t* test was used to compare drug response and clinical data between patient subgroups and survival between OmniSanguine and RPMI medium. Cluster analysis with laboratory (KE%) and clinical data using custom developed analysis and visualization. Heatmaps were generated using custom built plugins in ImageJ [338]. Discontinuous clinical data were represented by defined values on a 0-100 scale. Binary categories were represented by 0 and 100 respectively whereas discrete categories (e.g. Rai or Binet stage) were shown by values normalized for hundred grade scale. Hierarchical clustering was done using Euclidian distance and complete linkage. Pearson´s correlation coefficient was calculated for every drug sensitivity and clinical data. All reported p-values were two-sided.

5.6 Ethical aspects

All studies were approved by the Regional Ethics Review Committee (www.epn.se). As Paper I and II were retrospective observational studies no informed patient consent was required. Patients participating in studies reported in Paper III and Paper IV provided informed consent. The study reported in paper III was performed in accordance with Good Clinical Practice (GCP) and studies reported in Paper III and Paper IV were performed in accordance with Guidelines on Good Laboratory Practice (GLP).

6 RESULTS, DISCUSSION AND CONCLUSIONS

6.1 Paper I

Outcomes of patients with fludarabine-refractory chronic lymphocytic leukemia – a population-based study from a well-defined geographic region

Eketorp Sylvan et al. Leukemia & Lymphoma, 55(8):1774-80, 2014

The natural history of advanced CLL in a well-defined region without external referrals was investigated retrospectively by screening the medical records of patients diagnosed with CLL 1991-2010 within the Stockholm region. In total, 1479 patients were diagnosed with CLL within the time period. The medical files from 1301 (88%) patients were available for review and identified 92 patients as fludarabine (or cladribine (n=6)) refractory with 22% classified as DR, 40% as BFR and 38% as non-BFR/DR. Median age was 69 years and 67% had Rai-stage III/IV with median 3 prior therapies. The number of previous treatment lines was fewer in BFR and non-BFR/DR patients than in DR being 2, 2 and 4 respectively. ORR was 20%, significantly lower in BFR (8%) and DR (20%) than in non-BFR/DR (31%). Median time to treatment failure (TTF) was 6 months, significantly longer in non-BFR/DR (9.2 months) than in BFR/DR (5.3/4.4 months) and significantly longer in antibody-treated patients (9.1 months) compared to other regimens (5.2 months). Early-death occurred in 5% and ≥ grade III infections in 20%. Median OS was 18 months, 29 in BFR vs 13 in DR (p =0.054). Male sex was the only prognostic factor on OS.

Patients with DR or BFR CLL are groups where new anti-CLL agents are often evaluated in non-randomized phase II trials and conditioned approval of new drugs are currently often based on data obtained in such trials. Patients selected for clinical trials are rarely representative of the general disease population. Thus there is a need for data from "real-world" clinical care to compare and complement data obtained from pivotal trials. The Stockholm area with its Regional Cancer Registry provides an opportunity to obtain comprehensive data on patients treated in a well-defined geographical region without external referrals, thereby minimizing selection bias. The results of this study provide data which could be instrumental in judging the safety/efficacy of new agents before subsequent phase III randomized trials are completed. An example was provided by the comparison to the results from the pivotal trial of ofatumumab [206] with a subgroup in our study matched those included in the trial. This comparison showed that corresponding patients in this study had a lower response rate (10-15%) to salvage therapy compared to those in the pivotal trial (47-58%). However, TTF and OS were comparable and the incidence of early deaths and major infections similarly low. The comparison showed that even though the trial [206] had relatively broad inclusion criteria, still 25% of the BFR/DR patients would not have been includable highlighting that trial patients were not truly representative of patients in routine clinical care. Compared to a previous study operating in a different healthcare environment (MD Anderson Cancer Center (MDACC)) [186] the patients in that study were 10 years younger and had received more prior lines of therapy. However neither the ORR nor TTF was very different. Both studies show a shorter OS for DR patients compared to BFR patients confirming the great need for new treatment options in this patient group. Our study was also able to point out that BFR patients have a particularly low response rate as

well. However, there were some striking differences between the two studies. The risks of early death as well as the incidence of major infections were lower in our study (3-5% and 16-20% vs. 10-16% and 45-60%) and the OS was longer (DR 13 vs. 8 months, BFR 29 vs. 14 months). The reasons may be multifactorial but might reflect a more ill and heavily pretreated group of patients in the MDACC study [186].

An interesting finding was the differences in outcome following patients treated with antibodies, where the MDACC study reports poor effects (no responders and short OS) whereas in our study the corresponding group appeared to perform better in most outcome analyses. A further evaluation of the effectiveness of antibody treatment in our region was made in Paper II. Another striking finding was that male sex was the only baseline prognostic factor that reached statistical significance. This finding remains unexplained but might have been influenced by the fact that there were twice as many male than female subjects in the analysis. The lower statistical power in this study may have contributed to the inability to confirm findings from previous regression analyses [186] but does probably not explain all the differences. Limitations of our study are its retrospective nature and the relatively long time period during which regimens used might have changed. The same problems were evident in the MDACC study. However, no differences in survival were found in patients treated in the first decade compared to subsequent years in any of the studies which is in contrast to the improved OS measured from initial diagnosis [81] and further underlines the great need of new treatment options for patients with fludarabine refractory CLL.

In conclusion, our results on "real-world" consecutive patients may facilitate interpretation of non-randomized trials on novel drugs in advanced-stage CLL.

6.2 Paper II

Alemtuzumab (anti-CD52 monoclonal antibody) as single-agent therapy in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) – a single region experience on consecutive patients

Eketorp Sylvan et al. Annals of Hematology, 93(10):1725-1733, 2014

The safety/efficacy of alemtuzumab, when used in routine health care at experienced medical centers in a well-defined region (Stockholm), was evaluated in patients with relapsed/refractory CLL. Records from patients diagnosed year 1991-2010 were retrospectively screened and files from patients treated with alemtuzumab were analyzed in detail. The ability to review the majority of records is a key strength of our study as selection bias is minimal.

In total, records from 1301 patients identified 56 includable patients. The median age was 69 years, 84% had advanced Rai stage, and at least 23% had bulky lymphadenopathy. The median number of prior lines of therapy was 3. Most patients (86%) had received treatments with purine analogues with 73% and 13% being in the Refractory and Purine analogue relapsed groups respectively. The median cumulative dose of alemtuzumab was 930 mg, higher for responders (1063 mg) compared to non-responders (643 mg). As expected, a higher dose was associated with higher response rate. The ORR was 41%, including 7% CR/nPR (nodular partial remission), and the median duration of therapy was

12 weeks. The response rate was significantly associated with subgroup being 32.5% in the Refractory, 50% in the Purine analogue relapsed and 87.5% in the Relapsed subgroup. There was also a trend towards a survival effect by group. When new drugs are evaluated in clinical trials and published, results from patients are often pooled as one combined relapsed/refractory (R/R) cohort. However, the results in our study indicate that separate analyses of relapsed and refractory patients are recommended. In line with previous data [194], a better performance status was predictive of response. The median TTF was 7.8 months. Major infections (≥ grade III) occurred in 36% of the patients. Opportunistic infections affected 21%, CMV reactivation being the most common and with a frequency in line with previous reports [194, 339]. Median OS was 22.5 months. Responders had a longer survival than non-responders, although the difference when using the Landmark method [337] was not significant, confirming that survival in relation to response must be analyzed with caution. Predictive factors for longer survival were better performance status and fewer prior treatment lines. We also observed a trend towards better survival for patients without bulky lymphadenopathy. In previous reports, the outcome with alemtuzumab treatment appeared poorer in routine clinical care [198, 339] than in clinical trials [172, 194]. However, despite aggravating baseline characteristics, the ORR in our study was at least as good as in previous reports [172, 174, 194, 198, 339] and survival was comparably long. A possible explanation might be the higher cumulative dose/longer duration of therapy observed in our study. A higher dose being significantly associated with response rate supports this hypothesis. Patients with advanced CLL in our region are usually treated by physicians with a great interest in CLL and we have a long-lasting experience in the use alemtuzumab, starting already in the early 1990s. Other data have shown better outcome for patients treated by physicians specialized in CLL [340]. Possibly by optimal identification of patients and management of predictable toxicities, such physicians may be able to avoid early discontinuation. The limitations of our study include the relatively small number of patients, a great number of unknown cytogenetics and the relatively long observation period (1991–2010) during which regimens used prior to alemtuzumab have changed. Due to the retrospective nature of the study, it was difficult to grade adverse events with certainty and not all records were found. However those not found were predominantly from outside the hospitals where alemtuzumab is not used.

In conclusion, our results suggest that optimal patient management and patient identification may result in avoidance of early discontinuation of planned therapy and possibly better treatment outcomes.

6.3 Paper III

Phase I study of lenalidomide and alemtuzumab in refractory chronic lymphocytic leukaemia: maintaining immune functions during therapy-induced immunosuppression

Eketorp Sylvan et al. British Journal of Haematology, 159:599-613, 2012

The safety and efficacy of lenalidomide in combination with alemtuzumab was explored in a phase 1 study and the capacity of low-dose lenalidomide in maintaining immune functions in advanced-stage CLL patients prior and during alemtuzumab was evaluated. Twelve consecutive patients with refractory CLL were enrolled and treated with

lenalidomide alone the first cycle and lenalidomide/alemtuzumab in combination cycle 2-4. Most patients were elderly, had advanced-phase, unmutated CLL, large lymph nodes and poor-prognosis cytogenetics. Due to early progressions on a starting dose of 2.5 mg lenalidomide, 6 patients started on 5 mg/d after protocol amendment. Half of the patients completed planned lenalidomide and 75% completed planned alemtuzumab treatment. Reasons for early withdrawal included progressive disease and neutropenia.

The combination showed an acceptable safety profile as well as clinical activity with an ORR of 42%, all PR. Responding patients received a median lenalidomide-dose of 495 mg (range 42–625) and non-responders 162 mg (range 50-532). Altogether these results suggest that a relatively high lenalidomide starting dose is required to avoid early progression, as previously reported [309]. Median PFS was 5 months, exceeding 12 months in 3 patients. TFR occurred in 4 patients, who were all responders, supporting that TFR may precede clinical responses [308, 309, 319]. There was no evidence of TLS. The most common grade III-IV adverse-events were neutropenia, which was also a dose-limiting toxicity, and thrombocytopenia (75% each). The maximum tolerated dose of lenalidomide in this combination was 10 mg/d, but most side effects occurred early on low-dose lenalidomide alone.

Immune stimulation was already apparent after 4 weeks on low dose lenalidomide, indicated by a significant increase in the proportion of activated (HLA-DR+) CD4 and CD8 T cells. A sustained activation was revealed by HLA-DR co-expression on both T cell subsets at the end of treatment.

T cells showed a significantly increased proportion of CD28-expression and T cell proliferation (PPD/PHA stimulation) showed a non-significant increasing trend at week 4. We observed a gradual decrease in the proportion of T_{regs} not only during week 1–4 but also over the course of the combined treatment. The magnitude of change at week 4 correlated with cumulative dose of lenalidomide. A numerical, but not significant, increase in absolute numbers of CD4 and CD8 T-cells and NK-cells was observed at week 4 and at the end of treatment the CD4 counts appeared higher than after alemtuzumab alone [194, 203]. However, no relationship between T-cell activation and clinical response was observed but the number of patients was too small to draw a conclusion.

In contrast to others [301, 302, 309], we found neither any upregulation of CD80/86/40 or Fas-L nor increased immunoglobulin levels but a trend in the opposite direction for immunoglobulins. Possible explanations for discrepancies might be different doses of lenalidomide as well as length of therapy and also because patients in our study were heavily pretreated with advanced disease and therefore immunocompromised.

In conclusion, our results provide the basis for an extended phase 2 trial on this combination of drugs as well as further studies on lenalidomide as an immune-enhancing agent.

6.4 Paper IV

Sensitivity of chronic lymphocytic leukemia (CLL) cells to small targeted therapeutic molecules: an in vitro comparative study

Eketorp Sylvan et al. Manuscript

For drugs in early clinical testing it is of interest to analyze their in vitro killing capacity in head-to-head comparative experiments. We provide such a manufacturer independent, academic comparison on sensitivity of CLL cells to a panel of emerging small targeted therapeutic molecules using high-throughput screening based on an automated fluorescence digital scanning system. The in vitro anti-tumor effects of 31 emerging small therapeutic molecules were investigated on fresh CLL cells, thereby minimizing possible artifacts, from 42 consecutive patients with indolent (n=22) or progressive (n=20) CLL. Most progressive patients were relapsed and half of them were also fludarabine refractory. Culturing in a unique medium, OmniSanguine, prevents early apoptosis of CLL cells thus allowing long enough culturing (72 hrs.) to evaluate the full effects of the investigated drugs, including those with a less pronounced cytotoxicity. The pattern of effectiveness for drugs at 24 and 48 hours remained similar at 72 hours and the comparison with RPMI medium showed a significantly better survival of CLL cells in OmniSanguine altogether supporting the preserved viability of the CLL cells in this medium.

The sensitivity to each drug showed considerable inter-patient variability. Highest mean direct killing was observed with one survivin inhibitor (YM-155), 2 BCL-2 inhibitors (ABT-199, ABT 737) and one selective CDK inhibitor (dinaciclib) showing a mean KE% of 67, 70, 70 and 67, respectively. These 4 molecules were effective also on samples from clinically more advanced patients. Regarding BCL-2 inhibitors and dinaciclib, our results are in line with previous studies [61, 285] which indicated high anti-tumor activity despite poor clinical prognostic markers. A pairwise comparison of drugs with similar molecular targets showed strong correlation but interestingly, the survivin inhibitor YM-155, showed no clear-cut correlation with any of the other drugs. YM-155 has also the most equal effectiveness in progressive and indolent patients, in heavily pretreated and untreated, as well as in fludarabine-resistant and -sensitive patient samples. Little is known about YM-155 but it has been shown to overcome microenvironment mediated cell protection independently of prognostic markers [341]. Our results suggest that clinical trials may be warranted on this compound. Clinically promising drugs such as ibrutinib and idelalisib had a KE% of less than 12. This finding is in line with previous observations [243, 262] where induction of apoptosis with these drugs was modest. Their major clinical effect may not be due to direct cytotoxicity but, at least partly, by modulating the interaction between CLL cells and the microenvironment.

Control experiments using cytostatic agents confirmed previous findings with a high killing capacity for daunorubicin [336]. An unexpectedly low KE% of bendamustine led to supplementary analysis with a stepwise drug titration up to 200 times higher concentration. However, when recalculating the previously described values into KE%, the effect in our system was comparable. In contrast to other drugs tested, the sensitiveness to corticosteroids did not appear to be dose-dependent which is somehow contradictious to clinical results with high dose steroids [182]. The KE of small molecules was, in contrast to most cytostatics, similarly high in refractory and untreated patients and for the four most

effective small molecules it was significantly higher on cells with *TP53* disruptions, a finding that needs to be confirmed in an extended analysis.

To evaluate KE in different compartments, the sensitivity of bone marrow was compared to cells from the blood. Since stromal cells were not separated from CLL cells in the bone marrow the microenvironment was, at least partly, preserved. Sensitivity in bone marrow showed a high correlation to that in blood and the drugs with the highest KE showed no difference between compartments.

In conclusion, even though direct killing may not be the only therapeutic effector function in vivo, our results may help to identify potent drugs with multi-compartment activity of particular interest for further clinical development.

7 FUTURE PERSPECTIVES

Extensive basic research has unraveled multiple factors underlying CLL. This has created prerequisites for development of drugs targeting key pathways, such as the BCR and antiapoptotic pathways, or redirecting the immune system towards the malignant cells. The evolution of increased therapeutic options in combination with the heterogenetic clinical course demands routine health care data for comparison with phase II study results. Patients in clinical trials are rarely representative of those in routine health care which is supported by our comparison in Paper I. The choice of treatment in routine health care is indeed driven by effectiveness but also convenience and cost. The choice of comparative treatment in trial may be affected by company interests, thus not necessarily and fully reflecting the treatments that are often used in clinical practice. Comprehensive, "real-world" data might help to interpret the cost-effectiveness of new drugs and our studies in Paper I and II add to the understanding of advanced CLL. An extended study investigating all patients in Sweden diagnosed with CLL 2007-2014 is ongoing. This study will evaluate treatments first-line as well as in the relapsed situation and additionally analyze whether type of health care unit and adherence to national guidelines influence patient outcome.

The watch and wait strategy might be challenged by new therapies. In patients who harbor ever-so-tiny *TP53* defective subclones at diagnosis early intervention with chemotherapy might select resistant clones. These patients shall when treatment is imminent, achieve therapies effectively targeting the unfavorable subclones. Early-intervention trials need to be carefully assessed as the standard endpoint PFS may not be fully relevant in this setting. Our published (Paper I and II) and future planned studies may help to interpret such data. However, the situation may be different with new drugs. On one hand, an early intervention with new agents might be able to prevent disease onset or development thereby possibly avoiding the progressive accumulation of dismal genetic alterations during disease progression. On the other, early treatment may increase the risk of inducing resistance mechanisms, such as shown with kinase inhibitors. The low CR rate with BCR pathway inhibitors and the constant decline in PFS and OS curves suggest that very long-term remissions are unlikely with single agent therapy. Even though ABT-199 might be effective on small subclones harboring *TP53* lesions [61] and resistance to ibrutinib may be overcome by other BCR inhibitors such as CC292 [259], SYK- or PI3Kδ inhibitors [255] resistance might occur if single agent therapy is used. Carefully designed studies of combinations of drugs with different mechanisms of action are needed to circumvent the issues related to clonal diversity and to achieve long lasting remissions or perhaps even cure.

"Sequential triple T" (tailored, targeted, total eradication of MRD) is a recently suggested strategy of combining therapies [342]. Briefly it consists of three steps; debulking, induction and maintenance. Debulking is indicated when high lymphocyte counts (> 30,000/μL) or bulky lymphadenopathy is present. It is thought to be a short therapy (1-2 months) with mainly chemotherapy. However, chemotherapy might select aggressive subclones and the effect would probably be limited in patients with *TP53* disruptions. Drugs targeting the BCR signaling might be a better option for these patients and the favorable toxicity profile of these drugs makes combinations, which reduce peripheral lymphocyte counts, interesting to investigate. Induction, part two in "sequential triple T",

may consist of a combination of antibodies and a kinase- or a BCL-2-inhibitor. This phase is supposed to last as long as the remission continues to improve (4-6 months) and MRD assessment is performed 3 months after CR. Ibrutinib combined with rituximab was effective and tolerable but without impressive CR rates. However the effectiveness of single agent rituximab in CLL is limited. Combining ibrutinib with a more potent, compartment-selective monoclonal antibody might render better responses, even with MRD negativity. A phase I/II study investigating ibrutinib in combination with alemtuzumab is planned by our group. The effectiveness of alemtuzumab in clearing the blood and bone marrow in combination with the effect of ibrutinib on lymph nodes has the potential of longer and deeper responses. The CD20 antibody obinutuzumab might be an alternative considering the achieved MRD negativity [161]. Combinations with BTK or PI3K inhibitors and other small molecules appear attractive for further investigation as well. Our study in Paper IV provides some suggestions for such combinations (dinaciclib, ABT-199, or YM-155). In vitro the combination of ABT-199 and ibrutinib showed synergistic apoptosis in B cells, but no effect on normal T cells [343]. However, our study (Paper IV) is unable to completely evaluate the impact of the microenvironment. Therefore we plan an extended study, where lymph node specimens will be incubated with and without stromal cells, for evaluation of small molecules CLL killing capacity. The third part (Triple T) is maintenance with the aim to maintain a very good remission. Immunotherapy might be an attractive option in this part as tumor burden is supposed to be low. Lenalidomide is immunstimulating even in low doses, as shown in Paper III. A large randomized placebo-controlled study is investigating it as maintenance but preliminary data are not yet available (NCT 00774345). Lenalidomide might also be effective as a vaccination adjuvant and we have an ongoing trial in previously untreated patients with slowly progressive CLL but without treatment indication, who receive lenalidomide in a dose-escalation schedule together with autologous tumor-loaded DCs. Early data showed an acceptable tolerability for low-dose lenalidomide as immune adjuvance and induced specific immune responses [344]. A combination with an anti-PD-1 antibody or ibrutinib might enhance the immunostimulatory effect. Which drug, or combination, to use for maintenance is not stated but single agents with favorable toxicity profiles, such as lenalidomide, antibodies or kinase inhibitors appears reasonable. However, long-term use of kinase inhibitors might possibly favor resistance mechanisms. Targeted therapy against ROR-1, an antigen selectively expressed on B-cell CLL but not on normal B cells and other major adult tissues might be a future treatment option using either monoclonal antibodies [75] or a kinase inhibitor [345]. Also CAR-T cells against ROR-1 have been developed and preclinical data appears promising [346]. Monitoring of the maintenance is performed by MRD assessment and treatment would probably last for 1-2 years even though the duration remains to be clarified.

In summary, the future CLL-therapy algorithms will offer an increased number of possibilities and result in decreased morbidity and mortality. Even though cure still seems to be far ahead, an emerging transition of CLL from a disease with an often fatal outcome, to a truly chronic disease is in sight of the horizon. However, this demands further and intensified translational research in order to reach effective personalized cancer medicine.

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