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Targeted monoclonal antibody therapy in chronic lymphocytic leukemia

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Quality of life.

To be satisfied with life, after all.

Blandaren 1988

To the patients

ABSTRACT

Chronic lymphocytic leukemia (CLL) is still regarded as an incurable disease with a great need for developing new therapies and optimizing existing treatment options. The development of targeted therapy, i.e. therapy interfering with specific molecules needed for carcinogenesis and tumor growth, is rapidly evolving. The aim of this thesis is to delineate the clinical and immunological effects of targeted therapy with alemtuzumab in CLL patients.

In the first study, a long-term follow-up of patients who received alemtuzumab as first-line therapy was conducted. The results were compared with matched historic controls. Median time to treatment failure (TTTF) was 28 months for the alemtuzumab treated compared with 17 months for the control group (not significant). Additionally, our data showed that, despite long-lasting T cell suppression, alemtuzumab treated patients had comparable rates of infectious complications and incidence of Richter transformation as the matched controls.

In the second study, patients with advanced CLL, who all had severe transfusion-dependent and multi-agent refractory autoimmune hemolytic anemia (AIHA), received alemtuzumab as salvage therapy. All patients responded with a $\geq$ 2.0 g/dl rise in hemoglobin (Hb) concentration, in the absence of further transfusions, after a median time of 5 weeks. No further AIHA episodes were observed during long-term follow-up. CLL responses were achieved in all but one patient. These results suggest that alemtuzumab may be effective in the treatment of severe AIHA in patients with progressive CLL who have failed to respond to conventional therapy.

In the third study, the type, severity and duration of side-effects as well as efficacy of subcutaneous (SC) alemtuzumab, without dose-escalation, was evaluated in advanced-stage relapsed CLL patients. A starting dose of 30 mg SC was well tolerated and all but one injection-site reactions were grade 1/2. A 75% overall response rate (ORR) and long TTTF (median 20 months for responding patients) was obtained, suggesting that optimal selection of advanced-phase CLL patients for alemtuzumab therapy may result in a high response rate and durable remissions.

In the fourth study, T cell receptor B-variable (TCR-BV) gene usage in CD4 and CD8 T cells was assessed by real-time PCR, as well as complementarity-determining region 3 (CDR3)-length polymorphism, before and after therapy in patients with CLL who received alemtuzumab as first-line therapy. Our results indicate that perturbations of the T cell repertoire following alemtuzumab are complex and not reflected by changes in the total number of CD4/CD8 T cells only. A restricted CDR3 pattern present prior to therapy became even more restricted after treatment, followed by a normalisation of CD4 repertoire during long-term follow-up.

In the fifth study, we investigated the incidence and clinical relevance of subclinical virus reactivations and serological changes in CLL patients who received alemtuzumab as first-line therapy and compared the results with fludarabine-based combination therapy. Except for CMV, there was no increased incidence of virus reactivation compared with the fludarabine + cyclophosphamide +/- rituximab treated controls. All reactivations resolved spontaneously. The number of significant antivirus IgG decreases or increases did not differ significantly between the two treatment groups.

LIST OF PUBLICATIONS

I. **Karlsson C***, Norin S*, Kimby E, Sander B, Porwit MacDonald A, Nilsson B, Johansson E, Mellstedt H, Lundin J, Österborg A. Alemtuzumab as first-line therapy for B-cell chronic lymphocytic leukemia: long-term follow-up of clinical effects, infectious complications and risk of Richter transformation. *Leukemia, 2006, 20, 2204-2207*.

II. **Karlsson C**, Hansson L, Celsing F, Lundin J. Treatment of severe refractory autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab (humanized CD52 monoclonal antibody). *Leukemia, 2007, 21, 511-514*.

III. **Karlsson C**, Lundin J, Kimby E, Kennedy B, Moreton P, Hillmen P, Österborg A. Phase II study of subcutaneous alemtuzumab without dose escalation in patients with advanced-stage, relapsed chronic lymphocytic leukaemia. *British Journal of Haematology, 2009, 144, 78-85*.

IV. Rezvany MR, Jeddi-Tehrani M, **Karlsson C**, Lundin J, Rabbani H, Österborg A, Mellstedt H. Reconstitution of the T-cell repertoire following treatment with alemtuzumab (anti-CD52 monoclonal antibody) in patients with B-cell chronic lymphocytic leukaemia. *British Journal of Haematology, 2006, 135, 475-485*.

V. **Karlsson C**, Dahl H, Lundin J, Rossmann E, Brytting M, Mellstedt H, Linde A, Österborg A. Virus reactivations and serology patterns following first-line therapy with alemtuzumab or fludarabine-based combination therapy in patients with chronic lymphocytic leukaemia. (*Submitted*).

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
AIG	Autoimmune granulocytopenia
AIHA	Autoimmune hemolytic anemia
alloSCT	Allogeneic stem cell transplantation
ATM	Ataxia telangiectasia-mutated
β2-MG	β2-microglobulin
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
BM	Bone marrow
CCL	CC chemokine ligand
CD	Cluster of differentiation
CD40L	CD40 ligand
CDC	Complement-dependent cytotoxicity
CDR	Complementarity-determining region
CFAR	Cyclophosphamide/fludarabine/rituximab/alemtuzumab
CHOP	Cyclophosphamide, doxorubicin, vincristine, prednisone
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
CpG	Cytosine-phosphoguanine dinucleotide
CR	Complete response
CXCR4	Chemokine receptor type 4
D	Diversity
DAPK1	Death-associated protein kinase
DAT	Direct antiglobulin test
DLBCL	Diffuse large B cell lymphoma
EBMT	European group for Blood and Marrow Transplantation
EBNA-1	Epstein-Barr nuclear antigen-1
EBV	Epstein-Barr virus
ECOG	Eastern Cooperative Oncology Group
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EORTC	European Organization for Research and Treatment of Cancer
FA	Fludarabine/alemtuzumab
FACS	Flourescence-activated cell sorting
FC	Fludarabine/cyclophosphamide
FCA	Fludarabine/cyclophosphamide/alemtuzumab
FCO	Fludarabine/cyclophosphamide/oblimersen

FCR	Fludarabine/cyclophosphamide/rituximab
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
GC	Germinal center
GVL	Graft-versus-leukemia
HHV-6	Human herpesvirus 6
HLA	Human leukocyte antigen
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IGHV	Variable regions of the Ig heavy chain
IL	Interleukin
ITP	Immune thrombocytopenia
IV	Intravenous
IVIG	Intravenous immunoglobulin
IWCLL	International Workshop on Chronic Lymphocytic Leukemia
J	Joining
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
LPD	Lymphoproliferative disorder
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBL	Monoclonal B cell lymphocytosis
MRD	Minimal residual disease
MZ	Marginal zone
NCI	National Cancer Institute
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NLC	Nurse-like cell
OR	Overall response
ORR	Overall response rate
OS	Overall survival
PC	Proliferation center
PCR	Polymerase chain reaction
PD	Progressive disease
PFS	Progression-free survival
PI3Kδ	Phosphatidylinositol 3-kinase-δ
PLL	Prolymphocytic leukemia
PR	Partial response
PRCA	Pure red cell aplasia
PS	Performance status

RBC	Red blood cell
RIC	Reduced-intensity conditioning
RS	Richter´s syndrome
SC	Subcutaneous
SD	Stable disease
SHM	Somatic hypermutation
SLL	Small lymphocytic lymphoma
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TCR-BV	T cell receptor B-variable
TD	T cell-dependent
TGF	Tumor growth factor
TI	T cell-independent
TK	Thymidine kinase
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cells
V	Variable
VCA	Viral capsid antigen
VZV	Varicella zoster virus
ZAP70	Zeta-chain-associated protein kinase 70

CONTENTS

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a malignancy characterized by the accumulation of small B lymphocytes with a mature appearance in blood, bone marrow, lymph nodes or other lymphoid tissues [1]. In the latest WHO classification scheme CLL is considered as a mature B cell neoplasm and is not distinguished from small lymphocytic lymphoma (SLL) [2]. Although classifications of lymphoid malignancies have historically treated lymphomas and leukemias separately, this distinction is now appreciated as artificial [3].

CLL is extremely heterogenous in its clinical course, some patients live for decades with no need for treatment, whereas others have a rapidly aggressive clinical course with no or little response to therapy. During the past decade there have been major advances in understanding the pathogenesis of the disease and more efficient treatments have been developed. However, despite these advances, CLL remains incurable with standard therapies.

1.2 EPIDEMIOLOGY

CLL is among the most common lymphoid malignancies, accounting for approximately 11% of hematologic cancers in the Western world [4]. The annual incidence is 3-5 newly diagnosed patients per 100,000 [5], in Sweden this translates into approximately 500 new cases each year [6]. The disease is twice as common in males as females. Compared to the Whites, the disease is rarer among Blacks and much rarer in Asian/Pacific Islanders (75% and 23% that of Whites respectively) [7].

CLL is a disease mainly affecting the elderly and the median age at diagnosis is 72 years [7]. Although approximately three-quarters of all patients are older than 65 years, this group of elderly patients is heavily underrepresented in clinical trials [8]. In order to reduce the risk of severe side-effects, this imbalance needs to be considered before start of treatment, since there is a clear relationship between age and reduced tolerability to more intensive regimens [9].

1.3 ETIOLOGY

The etiology of CLL is still unknown. In most cases no obvious risk factor, except age, can be identified. However, for patients with a family history of CLL or other lymphoid malignancies, there is strong evidence that a genetic component contributes to the etiology [10]. Further, by identifying six CLL risk loci, a genome-wide association study have provided the first evidence for the existence of common, low-penetrance susceptibility, to a hematological malignancy [11].

The relationship with several environmental and occupational exposures has been investigated and some, but rather weak, associations have been found to; pesticides, magnetic fields, farming and animal breeding [12-14].

During the recent years, the hypothesis that an antigen-driven process contributes to CLL development has strongly been supported by evolving data concerning the genes responsible for B cell antigen specificity, as discussed in the following chapter.

1.4 PATHOGENESIS

The understanding of pathological mechanisms including the multiple events that are crucial in the transformation, progression and evolution of CLL, has helped to divide the disease into subgroups. This has had a profound impact on prognostication and treatment and has resulted in improved possibilities for individualized therapy.

1.4.1 The normal B cell counterpart

The cellular origin of CLL cells is not finally clarified [1]. However the current knowledge supports a derivation from an antigen-experienced B cell.

The B cell response to antigen (Ag) stimulation is mediated through the B cell receptor (BCR) in normal and malignant B cells. Each B cell displays a distinct BCR that is formed through variable combinations of variable (V), diversity (D) and joining (J) segments for the immunoglobulin (Ig) heavy chain and V and J gene segments for the light chain. In addition to the combinatorial diversity of different segments, the B cell repertoire is increased by the introduction of somatic hypermutations through the somatic hypermutation (SHM) process during the germinal centre (GC) reaction (reviewed in [15]). It has been demonstrated that CLL patients can be divided into two, approximately equal sized, subgroups characterized by the presence or absence of somatic hypermutation in the variable regions of the Ig heavy chain (IGHV) genes of the CLL clones [16-18]. The cut-off value underlying this separation with lesser or more than 98% sequence identity is arbitrary [19]. The two groups have different clinical course with poorer survival for patients with unmutated IGHV genes.

There is strong evidence that IGHV-mutated CLL cells (mostly) stem from antigen-experienced post-GC memory B cells [1]. First, when global gene expression profiles of CLL cells were compared to naïve, GC, memory and cord blood CD5+ B cells, it was shown that IGHV-mutated CLL cells are most similar in their gene expression patterns to memory B cells [20]. Second, the frequency of IGHV gene-mutated CLL cells that have mutated BCL6 (which is also targeted by the SHM machinery) is the same as the frequency of classical post-GC B cells with BCL6 mutations (30%) [21-23]. Third, a subset of CLL cells expresses IgG, indicating that these cases derive from class-switched B cells. These CLL cells mostly express IgG1 and IgG3 subclasses, which are typical of GC-associated class switching [24, 25]. Fourth, it has been shown that IgM+IgD+CD27+ B cells (as IGHV-mutated cells are suggested to be derived from) often are clonally related to classical class-switched memory B cells and derive from common CG B cell clones [26].

Surprisingly, there is evidence strongly arguing that IGHV-unmutated CLL cells also derive from antigen-experienced B cells that acquire features of memory B cells [1]. First, examination of unmutated IGHV CLL cells showed a gene expression pattern that was

more similar to post-GC memory B cells than to any other known B cell subset [20]. Second, there is evidence that GC reactions generate some memory B cells that lack somatic mutations [26]. Third, many IGHV-unmutated CLL cells express poly- and autoreactive antibodies [27-29], and the CLL cells show an activated phenotype [30].

Despite these findings, several issues regarding the origin of CLL cells with and without mutations in the IGHV genes remain. The possibility that the IGHV mutated cells might derive from B cells that accumulate SHM in a T cell-independent (TI) immune response, that does not involve the GC, or during a primary, Ag-independent BCR diversification process is considered [31]. It is also yet to be understood whether the IGHV unmutated CLL cells are activated as a part of TI or T cell dependent immune response and if they are conventional naïve B cells, CD5+ B cells or marginal zone-like B cells [1].

1.4.2 Monoclonal B cell lymphocytosis

Monoclonal B cell lymphocytosis (MBL) is defined as the presence of a population of monoclonal B cells, usually with a CLL phenotype, which comprise fewer than 5.0×10^9 cells/L with no evidence of tissue involvement [32]. MBL have been detected in approximately 3.5% of healthy individuals who have normal or slightly increased lymphocyte counts [33]. The occurrence of such clones increases with age and in first-degree relatives of patients with CLL [33, 34].

The hypothesis that CLL is typically preceded by MBL as a precursor state was tested in a population-based prospective study using prediagnostic blood samples from patients who were subsequently diagnosed with CLL [35]. In 44 of 45 cases MBL preceded CLL, indicating that MBL is a precursor state to CLL in virtually all cases.

Further, MBL clones often carry genetic lesions that are typical for CLL, such as the 13q14 deletion [36]. This shows that MBL is often not simply an expansion of normal B cells, but that the clones have already acquired initial genetic lesions, which are a hallmark of a pre-malignant tumor precursor cell.

Although several key features of MBL support the idea that it is a precursor stage of CLL it is important to stress that only a small fraction of MBL will ever progress to CLL, as the frequency of MBL is approximately 100-fold higher than the frequency of CLL and the risk of requiring treatment for progressive disease is approximately 1% per year [37, 38].

1.4.3 Genomic aberrations and gene mutations (see also 1.7.1)

In a few recurrently affected chromosomal regions, identified by fluorescence in-situ hybridization (FISH), approximately 80% of CLL cases show genetic aberrations [39]. The most frequent aberrations are deletions in 13q, 11q, or 17p, and trisomy 12.

Deletion of 13q14 affects two microRNA genes, mir-15a and mir-16-1, which might be implicated in the pathogenesis of CLL [40-42]. This is the most frequent aberration in early stage CLL (59%) [43].

Deletion of 11q22-q23 affects the ataxia telangiectasia-mutated (ATM) gene in almost all cases. The ATM protein kinase is a central component of the DNA damage pathway and

mediates cellular responses to DNA double-strand breaks. This deletion is more frequently found in refractory CLL than in early stage disease, 25% and 10% respectively [1]. ATM mutations have been shown to be present in 12% of all patients with CLL and in approximately one-third of the cases with a 11q23 deletion [44].

Deletion of 17p13 affects the tumor suppressor gene *TP53*. As with the 11q deletion this deletion is also more common in refractory CLL compared to early stage disease, 31% and 4% respectively [1]. It has been shown that there is a tight, but not absolute, correlation of 17p deletion and *TP53* mutation [45].

Trisomy 12 is present in 10-20% of the cases, with stable incidence in different disease phases [1]. The genes involved in the pathogenesis of CLL with trisomy 12 are unknown.

In contrast to other types of leukemia or B cell lymphomas, recurrent balanced translocations are rare in CLL [1].

1.4.4 Epigenetic alterations

As for other cancers, CLL demonstrates DNA methylation aberrations of multiple genes that may be affected at a transcriptional level and play an important role in tumorigenesis. An analysis of the global methylation profile found that 2.5-8.1% of the cytosine-phosphoguanine dinucleotide (CpG) islands in CLL samples were aberrantly methylated compared with controls. Such methylations are generally associated with stable silencing of the associated gene and it was suggested that a portion of these methylation events confer a selective advantage to the malignant cells [46]. A strong correlation between promoter methylation and transcriptional silencing of certain individual gene promoters, such as death-associated protein kinase 1 (DAPK1) and zeta-chain-associated protein kinase 70 (ZAP70) has been found [47, 48].

In contrast to genetic changes, epigenetic alterations in cancer cells can be reversed [49] and are therefore an attractive therapeutic target.

1.4.5 Microenvironment

In comparison to normal B lymphocytes, CLL cells show prolonged survival in vivo and therefore accumulate in peripheral blood, bone marrow and lymphoid organs [50]. On the other hand, when CLL cells are cultured, under conditions that support the growth of human B cell lines, they rapidly undergo spontaneous apoptosis [51]. This means that their apoptosis resistance, rather than being an intrinsic feature of the leukemic cells, depends on external signals [50, 52, 53]. However, CLL cells can be rescued from apoptosis ex vivo, if co-cultured with bone marrow-derived stromal cells, fibroblasts, dendritic cells, nurse-like cells (NLC) or T cells [54-59]. These cells provide a variety of survival stimuli for CLL cells, mediated via soluble factors, extracellular matrix components, and signals derived from cell-cell contact [60].

While circulating CLL cells are largely arrested in G0/G1 phase of the cell cycle, a small proportion of leukemic lymphocytes divide in the so-called “proliferation centres” (PC) [61]. PCs are focal aggregates of variable size found in lymph nodes and to a lesser

extent in the bone marrow (BM) [62, 63]. In the PCs, leukemic lymphocytes are in contact with numerous CD3+ T cells, most being CD4+ T cells that express CD40 ligand (CD40L) and can support the growth of CLL cells through CD40 ligation [61]. This ligation synergizes with BCR signaling [64] and in turn induces several anti-apoptotic signaling pathways, including survivin [65] and NF-κB [66].

Further, CLL cells seem not just to act as passive seeds that need to home into the appropriate soil but also as active players that create a supportive microenvironment. They, after BCR stimulation or exposure to NLCs, secrete chemokines, such as CCL22 [67], CCL3, and CCL4 [68, 69], which can attract other supportive cells, such as T cells. This indicates the possibility that the CLL microenvironment is created and maintained through a dynamic, interactive coevolution between leukemic and normal bystander cells [70].

1.4.6 Antigens and CLL pathogenesis

A picture of CLL as an antigen-driven disease is emerging. It has been shown that almost 30% of CLL patients share BCRs with restricted, quasi-identical Ig-sequences [71]. Thus, one in three patients carried a BCR which was almost identical to another unrelated CLL patient. These findings are very striking considering the extremely low probability (10^{-12}) of co-expression of identical BCRs.

Studies on CLL antibody/BCR reactivity have gradually provided clues as to which antigens it is that are involved in the formation of stereotyped BCR subsets and thereby may be involved in tumor development (reviewed by [72]). Examples of such antigens are molecular motifs on apoptotic cells (e.g. modified cytoskeletal proteins and oxidation-specific epitopes) and exogenous bacteria (e.g. Streptococcus pneumoniae polysaccarides). Interestingly, molecular evidence for a possible link between Epstein-Barr virus and cytomegalovirus persistence and the utilization of a specific BCR gene (IGHV4-34) was recently presented [73].

The extensive immunoglobulin analysis in CLL has lead to the hypotheses that selection by antigen seems to play an important part in the disease development and could even influence disease outcome [72].

1.5 CLINICAL MANIFESTATIONS

With the routine of blood testing, the number of CLL patients who are asymptomatic at diagnosis has increased to approximately 40%. The remaining 60% present with various symptoms, the most common complaint is fatigue, but this is generally not severe [74]. Other common features are enlarged lymph nodes or infection susceptibility. Splenomegaly may occur, but massive splenomegaly is usually only seen in patients with advanced phase disease. Hepatomegaly occurs less frequently than splenomegaly. Patients with very advanced disease may experience weight loss, night sweats and general malaise. Signs of BM failure, due to progressive BM infiltration in advance stage patients, are anemia, thrombocytopenia and neutropenia. Finally, autoimmune

phenomena are more common in CLL patients than in other lymphoid malignances and autoimmune hemolytic anemia is the most common one (see chapter 4).

1.6 DIAGNOSIS

The diagnosis of CLL requires the presence of more than or equal to 5×10^9/L B lymphocytes in the peripheral blood for the duration of at least three months [75]. The leukemia cells found in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. The clonality and phenotype of the circulating B lymphocytes needs to be confirmed by flow cytometry. Each clone of leukemia cells is restricted to expression of either kappa or lambda Ig light chains [76]. CLL cells coexpress the T cell antigen CD5 and B cell surface antigen CD19, CD20, and CD23 [75]. The levels of surface immunoglobulin, CD20, and CD79b are characteristically low compared with those found on normal B cells [76, 77].

A bone marrow aspirate or biopsy is not required at diagnosis of CLL. The diagnosis can easily be set by analyses of a blood sample.

1.7 PROGNOSTIC AND PREDICTIVE FACTORS

Since CLL is extremely heterogeneous in its clinical course it is hard to predict the disease-related morbidity and survival for an individual patient at important time points like when diagnosis is set or before start of treatment. For a long time the clinical staging systems of Rai and Binet, which classify patients according to tumor burden and hematopoetic impairment, have been used to stratify patients into different prognostic groups [78, 79]. However, there is still significant heterogeneity within the different stage groups. During the recent years several prognostic factors, and also one important predictive factor, have been identified.

1.7.1 Genomic aberrations and gene mutations (see also 1.4.3)

The presence of 17p deletion is predictive of the worst prognosis of CLL, characterized by rapid progression of disease and short survival [39]. It is also associated with poor response to chemotherapy with a median survival of less than two years from first treatment indication with alkylator and purine-analogue-based therapy (reviewed in [80]). There is evidence that the efficacy of “biologic” agents such as anti-CD-52 antibody alemtuzumab, lenalidomide and flavopiridol is independent of the genetic background of the disease [81-84]. Based on this, 17p deletion may be considered a predictive marker for non-response to chemotherapy-based treatment as compared to “biologic” agents such as alemtuzumab, lenalidomide and flavopiridol [80].

There are new data suggesting that the clinical behavior of cases with only the *TP53* mutation is very similar to cases with deletion of one allele and mutation of the remaining allele (reviewed in [80]). There is a tight, but not absolute, correlation of 17p deletion and *TP53* mutation. Among CLL cases which had monoallelic 17p13 deletions the majority

showed mutations in the remaining *TP53* allele (81%), whereas among cases without 17p13 deletions *TP53* mutations were much rarer (4.5%) [45]. However, the proportion of patients with *TP53* mutation without 17p deletion was higher (18%) in patients with fludarabine refractory CLL [85], suggesting that detection of 17p deletion may not be sufficient, but that also *TP53* mutation analysis might be important to identify all patients with a similarly poor prognosis [86].

The presence of 11q deletions is associated with extensive lymphadenopathy, rapid disease progression and shorter treatment-free and overall survival [39, 81, 87-89]. Notably, evidence from a recent clinical trial indicate that immunochemotherapy with FCR might overcome the adverse prognostic significance of 11q deletion [90].

Trisomy 12 has previously been associated with poor outcome, however this has not been confirmed [87, 88]. Finally, the presence of 13q14 deletion as a sole abnormality is characterized by a favourable clinical course [39].

1.7.2 B cell receptor, IGHV mutational status

The IGHV mutation status is currently a well recognized prognostic factor in CLL. Patients with unmutated IGHV follow an unfavorable course with rapid progression and earlier death [17, 18]. However, regardless of mutational status some V-genes in the VDJ rearrangement appear to be associated with distinct biological and clinical features. In a Swedish study it was shown that the overall survival among patients expressing the IGHV3-21 gene was significantly shorter than other mutated cases and similar to the unmutated cases, with a median overall survival of 83 months [91]. The role of the IGHV mutational status in guiding therapy is currently unresolved [1].

1.7.3 Surrogate markers

As the determination of IGHV mutation status is rather complicated, a search for parameters that are strongly correlated with IGHV mutates is ongoing. One such marker is the zeta-chain associated protein kinase 70kDa (ZAP70). Several studies have shown a strong association of high ZAP70 expression, as measured by fluorescence-activated cell sorting (FACS), and unmutated IGHV genes and BCR function [92-94]. However, discordance of ZAP70 expression and IGHV mutation status occurred in up to 25% of cases [93]. In a recent work, expression of human Fc receptor like molecules (FCRL, also analyzed by FACS) showed a strong association with IGHV-status [95]. FCRL2 demonstrated 94.4 concordance compared with 80.4% for ZAP70, indicating that this might be a reliable surrogate marker.

The expression of the surface marker CD38 is also correlated to IGHV mutation status. However, there are some important considerations, such as no consensus about the best cut-off value and that expression may vary over time [96], limiting the use of this surrogate marker.

1.7.4 Markers of tumor burden

Other parameters of disease activity and tumor burden, besides the Rai and Binet clinical staging systems, have been shown to be of prognostic relevance. A lymphocyte doubling time of 12 month or less identifies patients with poor prognosis [97]. Further, a number of serologic parameters such as thymidine kinase (TK) and β2-microglobulin (β2-MG) have been shown to provide information on outcome [98, 99].

In order to help patients and clinicians in decision making as well as facilitate clinical research a prognostic normogram including age, β2-MG, absolute lymphocyte count, sex, Rai stage and number of involved lymph node groups has been developed [100].

2 TREATMENT OF CLL

Historically, the goal for treatment of CLL has focused on reducing disease-related problems such as symptomatic lymphadenopathy or splenomegaly. Today, with access to more effective therapies it is possible to attain a complete response (CR) in a higher percentage of patients. However, physicians must select patients carefully when pursuing CR as their treatment goal. Risk of higher toxicity generally accompanies regimens that produce higher CR rates, making many patients poor candidates for more aggressive therapy. Multiple features, including the molecular characteristics of the patient's disease, clinical features of the patient's disease, characteristics of the patient and the therapy needs to be carefully considered before start of treatment.

The sections below will focus on treatment results mainly obtained in phase III clinical trials.

2.1 TREATMENT INDICATIONS

Conventional therapy for CLL is noncurative, and the standard care is to treat patients only when their disease is symptomatic or progressive. The criteria for active disease, defined in the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) guidelines for patients participating in study protocols [75], forms the basis for recommendation of treatment initiation in both clinical trials and in routine practice (Table 1).

Table 1. IWCLL criteria for definition of active disease [75].

Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia.
Massive (i.e., > 6 cm below the left costal margin) or progressive or symptomatic splenomegaly.
Massive nodes (i.e., > 10 cm in longest diameter) or progressive or symptomatic lymphadenopathy.
Progressive lymphocytosis with an increase of more than 50% over a 2-month period or lymphocyte doubling time (LDT) < 6 months.
Autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapy.
Constitutional symptoms, defined as any one or more of the following: • unintentional weight loss ≥ 10% in the previous 6 months; • significant fatigue (i.e., Eastern Cooperative Oncology Group (ECOG) PS ≥ 2; inability to work or perform usual activities); • fevers higher 38.0°C for ≥ 2 weeks without other evidence of infection; or • night sweats for ≥ 1 month without evidence of infection

It should be noted that in patients with initial blood lymphocyte counts of less than 30×10^9/L, lymphocyte doubling time (LDT) should not be used as a single parameter to determine treatment indication. In general, the absolute lymphocyte count should not be used as the sole indicator for treatment.

Since several studies have showed that the use of alkylating agents in CLL patients with early-stage disease does not prolong survival [101-103], "watchful waiting" is recommended for those without treatment indication. This often leaves patients feeling that they have a serious health problem but that "nothing is being done" to treat it. However, the results of ongoing clinical trials investigating early treatment in asymptomatic patients with high-risk molecular features may change this practice for selected patient groups.

2.2 FIRST-LINE THERAPY

2.2.1 Single-agent chemotherapy

Monotherapy with alkylating agents has served as initial therapy for CLL for a long time, and chlorambucil has been considered as the "gold standard" for several decades [101]. In the 1980s treatment with purine analogues was introduced and in the 1990s chlorambucil was challenged by fludarabine, the most studied purine analogue in CLL (Table 2). Despite higher response rates and a longer duration of remission and progression-free survival (PFS) for the fludarabine treated no improvement of overall survival (OS) could be demonstrated [104]. However, in a long-time follow-up analysis a survival benefit for the fludarabine treated could be shown [105]. An important objection to this and other similar studies is that the median age of the study population is below 65 years while three-quarters of the total CLL population are older than this age. Based on this imbalance the CLL5 study was designed [8]. Here, when patients above 65 years were randomized between chlorambucil and fludarabine, no difference in PFS or OS were observed (Table 2). This supports that chlorambucil still plays a role in treating frail patients, such as elderly with severe comorbidity, because it is convenient as an oral agent and has a low toxicity profile.

Bendamustine, a purine analogue/alkylator hybrid agent, has recently been tested (in patients up to 75 years of age) against chlorambucil [106]. The improved efficacy data lead to approval by the US Food and Drug Administration (FDA) in 2008 for the treatment of CLL (Table 2).

2.2.2 Monoclonal antibodies

Alemtuzumab is a recombinant, humanized, monoclonal antibody directed against CD52, a cell surface protein highly expressed on most normal and malignant B and T lymphocytes [107]. Alemtuzumab is traditionally given through intravenous (IV) infusion, which is the only approved route of administration. However, despite preventive measures with dosage escalation and premedication schedules, an administration related cytokine release syndrome affects most patients [82, 108]. The release of cytokines such as

TNFα, INFγ and IL-6 from e.g. natural killer (NK) cells have been related to this syndrome [109]. Frequent symptoms are rigors, fever, chills, nausea, vomiting, skin rash and urticaria. These adverse effects occur mainly during the first infusion. The intensity and frequency of the symptoms usually decrease during subsequent infusions [82, 108].

Acute administration-related events are less pronounced if alemtuzumab is administrated subcutaneously (SC) [81, 110] and despite local injection-site reactions a simplified administration schedule without dose-escalation may be used (Paper III).

Data from SC alemtuzumab studies have demonstrated at least equivalent efficacy compared with corresponding IV studies [81, 82, 108, 111] and recent management guidelines stated that SC administration may be used instead of IV [112].

When tested against chlorambucil, alemtuzumab treatment, as in a previous phase II trial [111], resulted in high response rates [82] (Table 2) and these results lead to approval as first-line therapy in the United States in 2007. Accumulating data have demonstrated that response to alemtuzumab is independent of the status of the high risk prognostic factor 17p deletion or *TP53* mutation and recent guidelines recommend that this drug should be considered as first-line treatment in selected patients, in particular those with 17p deletions [112].

Alemtuzumab treatment requires special infection-related considerations, since the antibody causes severe and prolonged lymphocytopenia that may last 6-18 months following first-line therapy [113]. Anti-infective prophylaxis treatment and weekly monitoring for cytomegalovirus (CMV) are recommended [112].

2.2.3 Combination chemotherapy

The repair of DNA interstrand crosslinks induced by alkylating agents can be prevented by the purine analogue fludarabine [114]. This provides a rationale for combining both classes of drugs together and the most extensively studied combination chemotherapy regimen for CLL is fludarabine plus cyclophosphamide (FC) [115]. Three randomized trials have compared fludarabine alone to the FC combination. All three demonstrated significantly higher OR, CR and PFS rates for the combination regimen [87, 116, 117]. However, no survival benefit were shown, not even in the largest of these studies where patients were randomly assigned to chlorambucil (given at higher doses than in previous trials) or fludarabine or fludarabine plus cyclophosphamide [87] (Table 2). It was stated that the absence of survival difference was related to the effect of second-line treatments since patients receiving the less effective treatment first are more likely to respond in the second-line situation [87].

In a recent randomized phase III trial there was a direct comparison between the two purine analogues fludarabine and cladrabine, each combined with cyclophosphamide, as first-line therapy against progressive CLL [118]. Both therapies showed to be equally effective and safe.

2.2.4 Chemoimmunotherapy

Since preclinical studies showed evidence for a synergy between rituximab and fludarabine [119], rituximab combinations with fludarabine-based regimens have been investigated in several trials. Recent data from the first randomized trial, FC +/- rituximab (FC versus FCR), showed higher OR rate, more CRs and longer median PFS for the FCR treated patients [90] (Table 2). Notably, for the first time this study was able to demonstrate that a specific first-line treatment for CLL resulted in a significantly improved OS. At three years after randomization, 87% were alive in the FCR arm versus 83% for the FC treated (p=0.01). Importantly, the population in this trial represents a selection of fairly young and physically fit patients. As a consequence, results should not be generalized to physically unfit and/or elderly patients with CLL. Further, the subgroup of patients with 17p deletion had the worst prognosis and there was no significant difference in OS between the two treatment arms for the patients with this genetic aberration. It has been commented that patients with 17p deletion should be identified before treatment and given alternatives to FCR [120]. In 2009 FCR was approved by European Medicines Agency (EMA) as first-line CLL treatment.

In order to validate the place of alemtuzumab in combination with FC a phase III study randomized previously untreated patients, less than 65 years old, with advanced CLL between FC plus alemtuzumab (FCA) and FCR. However, because of increased toxicity in the FCA arm, seven deaths (7/82), the trial was prematurely stopped [121]. Another trial in which a lower dose of alemtuzumab is combined with FC, is still actively recruiting (HOVON 68).

Table 2. Progress in the first-line treatment of CLL, phase III clinical trials.

Reference	Regimen	N	OR, %	CR, %	PFS, mo
Rai (2000) [104]	Chlorambucil	181	37	4	14
	Fludarabine	170	63	20	20
Eichhorst et al (2009) [8]	Chlorambucil	100	51	0	18
(elderly patients, > 65)	Fludarabine	93	72	7	19
Knauf (2009) [106]	Chlorambucil	157	31	2	8
	Bendamustine	162	68	31	22
Hillmen (2007) [82]	Chlorambucil	148	55	2	12
	Alemtuzumab	149	83	24	15
Catovsky (2007) [87]	Chlorambucil	366	72	7	20
	Fludarabine	181	80	15	23
	FC	182	94	38	43
Hallek (2010) [90]	FC	409	80	22	33
	FCR	408	90	44	52

2.2.5 Allogeneic stem cell transplantation

The crucial anti-leukemic principle for allogeneic stem cell transplantation (alloSCT) appears to be the immune-mediated anti-host activities conferred with the graft. Thus, alloSCT represents the initiation of permanently active cellular immune therapy in the recipient, thereby providing a treatment modality that is, in a biological sense, completely different to any other cytotoxic or immunological therapy [122]. In isolated cases this treatment perhaps may be curative. However, due to advanced age, comorbidity and indolent clinical course in the majority of CLL patients alloSCT can not be considered as a treatment option in most cases.

To identify situations where alloSCT might be considered as a preferred treatment option, the European group for Blood and Marrow Transplantation (EBMT) in 2006 worked out a consensus on indications for alloSCT in CLL. It was stated that alloSCT is a reasonable treatment option for eligible patients with previously treated, poor-risk CLL [123] (Table 3). Notably, according to the third EBM criterion alloSCT is indicated as part of first-line treatment for patients with 17p deletion and treatment indication.

Table 3. Criteria for poor-risk disease according to the EBMT CLL transplant consensus [123].

- Non-response or early relapse (within 12 months) after purine analogue-containing therapy
- Relapse (within 24 months) after purine analogue combination therapy or treatment of similar efficacy
- *TP53* deletion/mutation (del 17p) requiring treatment

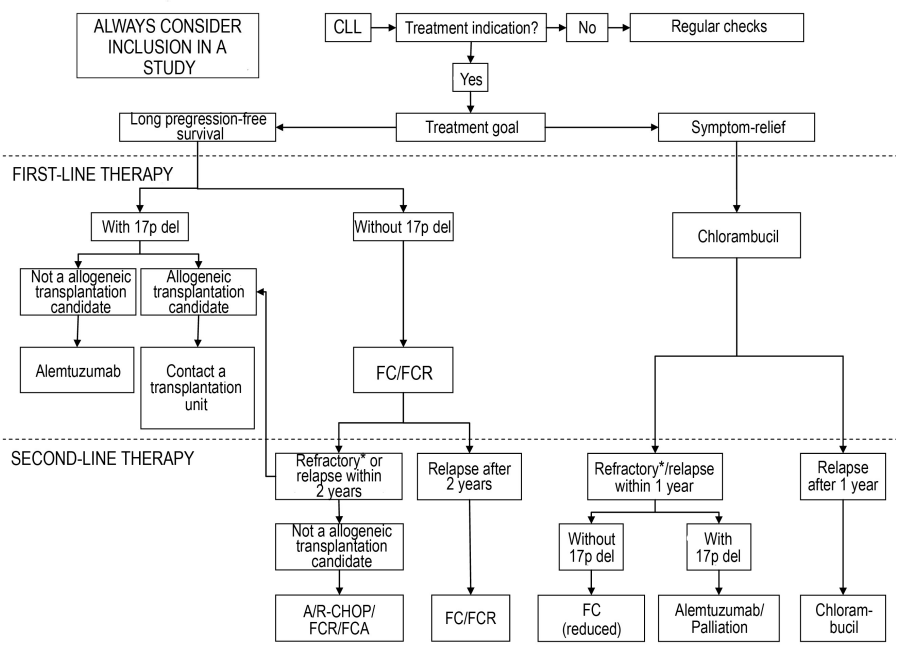
The approach of choice is usually reduced-intensity conditioning (RIC). By this shift from previously used myeloablative alloSCT the rate of non-relapse mortality have decreased from up to 44% to 15-25%. Efficacy data shows that long-time PFS can be achieved in 30-60% of patients by RIC alloSCT [122, 124].

2.3 SECOND-LINE THERAPY

When a patient relapses after first-line therapy, treatment selection depends on several factors such as genetic abnormalities within the CLL cells, disease-related manifestations, the general health status of the patient, type of initial therapy and the duration of the response. The physician has to reinvestigate the patient and consider collected data in order to present the best possible treatment/study suggestion for each individual. Generally this is an even more complex situation than before first-line treatment making it hard to transfer data from clinical studies into clinical praxis.

The Swedish CLL group has, in its National Guidelines, treatment recommendations for the second-line situation [125]. Here, the recommendations are dependent upon three main factors; treatment goal (related to patient general health), refractoriness to previous treatment and 17p deletion (Figure 1).

Figure 1. The Swedish CLL National Guidelines, updated version 2009-05-05 [125].



Abbreviations: CLL, chronic lymphocytic leukemia; FC, fludarabine and cyclophosphamide; FCR, FC and rituximab; A, alemtuzumab; R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone; FCA, FC and alemtuzumab.
*Refractory disease is defined as treatment failure or disease progression within 6 months to the last antileukemic therapy [75].

The results from a phase III-study, on second-line patients who had been randomized between FC and FCR, were recently presented [126]. Data regarding OR, CR and PFS were all superior for patients treated with FCR; 70% versus 58%, 24% versus 13% and 31 versus 21 months, respectively. After a median follow-up time of 25 months there was no statistically significant difference in survival between the two treatment arms. Notably, only a minority of the patients in this study had previously been exposed to fludarabine during their initial therapy. This is a clear limitation since most patients today, who are in good physical condition ("go-go" patients), are recommended fludarabine-based therapy as first-line treatment [115].

Preliminary results from a phase III-study where patients with relapsed or refractory CLL were randomized between combination treatment with fludarabine and alemtuzumab (FA) or fludarabine single-therapy showed significantly higher OR and CR rates (85% versus 68% and 30% versus 16%, respectively) and longer PFS (30 months versus 21 months) for patients treated with FA [127]. A limiting condition was that only 20% of the patients had previously been treated with fludarabine therapy. After a median follow-up of

17 months no difference in OS was observed. The rate of infectious complications were similar in the two gorups.

2.4 EMERGING THERAPIES

Despite advances in treatment, CLL remains incurable with standard therapies. Following successive relapses patients become increasingly resistant to treatment, and median survival is less than 1 year once patients are fludarabine-refractory [108, 128]. Thus, new agents are required and in response to this need several promising investigational agents are in clinical development. New agents with at least phase II study data are presented below.

2.4.1 Novel antibodies

Several second-generation anti-CD20 antibodies are in preclinical and clinical development among which ofatumumab has generated the most mature clinical data.

Ofatumumab is a human monoclonal antibody (mAb) targeting CD20. It binds an epitope on the CD20 molecule that is different from that of rituximab. In preclinical studies, ofatumumab demonstrated higher affinity for CD20 than rituximab, activated complement-dependent cytotoxicity (CDC) more effectively, and was superior to rituximab in killing B cell lines with low CD20 expression [129].

In a pivotal single-arm study, CLL patients with fludarabine- and alemtuzumab-refractory CLL (FA-ref, n=59) or with fludarabine-refractory CLL with bulky (> 5 cm) lymphadenopathy (BF-ref, n=79) were treated with ofatumumab as single-agent therapy [130]. For the FA-ref group OR, PFS and OS was 58%, 6 months and 14 months, respectively, and 47%, 6 months and 15 months in the FA-ref group, respectively. Importantly, similar response rates were seen irrespective of prior exposure to rituximab-containing treatments and also irrespective of refractoriness to FCR treatment, a standard regimen in earlier lines of CLL therapy. Ofatumumab was approved by FDA in 2009 for treatment of CLL that is refractory to fludarabine and alemtuzumab.

Lumiliximab is a genetically engineered (macacue variable regions, human constant regions) mAb targeting CD23, a transmembrane glycoprotein. CLL cells exhibit high expression of CD23, while non-CLL cells only minimally express this antigen. In preclinical studies, lumiliximab was shown to enhance the effects of fludarabine and rituximab [131]. Recently, data from a phase 1/2 study adding lumiliximab to the FCR regimen for patients with previously treated CLL were published [132]. The OR rate was 65%, with 52% of patients achieving a CR, which compares favorably with the CR rate previously reported for the FCR regimen alone in relapsed CLL. However, the following phase III study failed to show any advantage of the FCR plus lumiliximab combination compared to FCR and was prematurely closed [133].

A large number of other mAbs are currently in various phases of clinical testing against CLL (Figure 2).

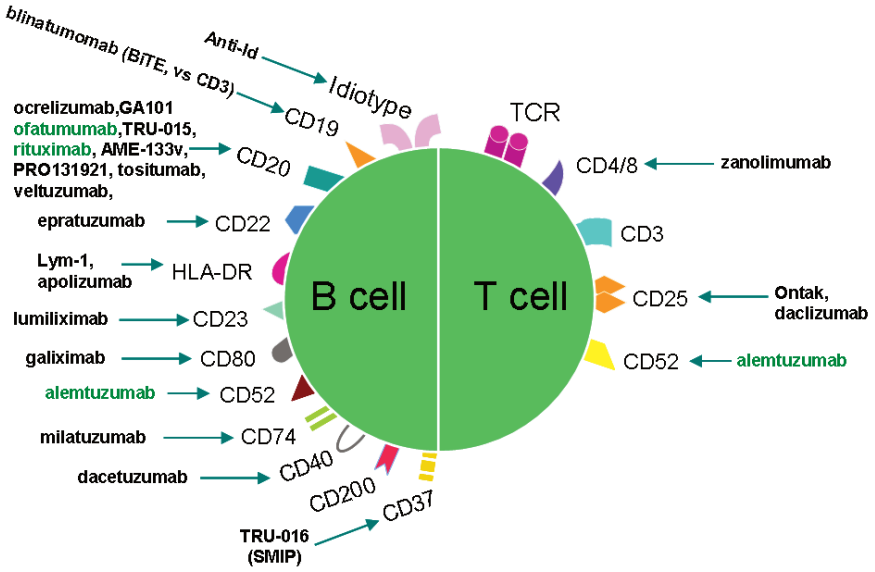


Figure 2. Antibodies tested against CLL.

Antibodies indicated in green text have reached approval by FDA and EMA.

2.4.2 Small molecules

Bcl-2, an antiapoptotic protein is overexpressed in CLL cells and results in resistance to chemotherapy. Several compounds affecting the apoptotic resistance mediated via the Bcl-2 family have showed activity against CLL. Of which, oblimersen has reached the phase III study level. Oblimersen is an antisense oligonucleotide, downregulating the Bcl-2 protein. Preclinically, it has demonstrated single-agent activity against CLL cells, and potentiated cytotoxic activity of agents such as fludarabine, rituximab, and alemtuzumab [134]. In a phase III trial 241 patients who had relapsed after or become refractory to fludarabine-based therapy were randomly assigned to FC or FC plus oblimersen [134]. The rate of CR/nodularPR was significantly higher for patients treated with FC plus oblimersen, 17% versus 7%, respectively. Among patients who achieved CR/nodularPR the response duration was significantly longer in the FC plus oblimersen arm, however, no significant difference remained if the intention-to-treat populations were compared.

Flavopiridol is a broad cyklin-dependent kinase inhibitor that induces apoptosis of CLL cell lines and is not dependent on p53 for its activity. Sixty-four patients with relapsed CLL, all previously treated with purine analogue therapy, were treated with single-agent flavoperidol [135]. In order to suppress cytokine release syndrome prophylactic

dexamethasone was added, this amendment improved treatment tolerability, compliance, and response. The rates for OR and CR were 53% and 2% respectively, and median PFS for all patients was 9 months. Notably 12 of 21 patients (57%) with 17p deletion responded. However, there is a problem with tumor lysis syndrome (TLS) in relation to flavopiridol therapy and three patients (5%) required transient hemodialyses with their first treatment.

A variety of other small-molecules are currently investigated in early phase CLL studies.

2.4.3 Immunomodulating drugs

Lenalidomide is a derivate to thalidomide and belongs to a class of drugs known as immunomodulating drugs (IMiDs). Although it has multiple effects on the immune system the mechanism of action in CLL treatment is not known. In two phase II trials evaluating lenalidomide for treatment of patients with relapsed or refractory CLL, the rates for OR and CR were 47% and 32%, respectively, and 9% and 7%, respectively [84, 136]. Of note, lenalidomide achieved the same OR rate in patients with 11q and 17p deletion as in patients without these high-risk abnormalities [136]. Problems with acute tumor lysis syndrome and tumor flare reactions were noted among the adverse effects.

Lenalidomide is currently being tested in 3 ongoing phase III trials in CLL.

3 IMMUNE DEFECTS AND INFECTIONS IN CLL

3.1 INTRODUCTION

Patients with CLL are all to a degree immunodeficient and infections are the major cause of death in between 25-50% of the individuals [137, 138]. This is mediated through impairment in humoral and cellular immunity inherent to the primary disease and in the further immunosuppression related to the treatment. Bacterial infection of the respiratory tract, skin or urinary tract is the most common problem [137] and the most frequent pathogens are Staphylococcus aureus, Streptococcus pneumonia, Haemophilus influenza, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa [139]. However, the use of purine analogues such as fludarabine, and monoclonal antibodies such as alemtuzumab has altered the spectrum of infections. Related to their T cell immunosuppressive effects opportunistic infections, like those caused by herpesviruses, Pneumocystis, Listeria, mycobacteria, Candida and Aspergillus, may occur [140].

3.2 B CELL DEFECTS

3.2.1 Hypogammaglobulinemia

Hypogammaglobulinemia is the most obvious and well-known CLL-related immunodeficiency and is present in up to 85% of patients [138]. The incidence and severity of hypogammaglobulinemia is observed to be increased with advanced disease stage [141, 142] . All 3 classes (IgG, A, and M) are affected, although predominantly IgG3 and IgG4 [143]. The mechanisms underlying the decreased immunoglobulin synthesis is incompletely understood. A relation to cell-cell contact between plasma cells, which are the immunoglobulin-producing cells, and the malignant B cells and release of inhibitory cytokines by the latter have been suggested [143]. In vitro studies have shown suppression of IgG secretion to be secondary to inhibition by autologous CLL cells, dependent on cellular contact and proportional to the number of malignant B cells added. A role of the interaction between the Fas ligand (CD95L) on CLL cells and CD95 on plasma cells resulting in plasma cell apoptosis was demonstrated [144]. However, this has not been replicated by other groups and it seems more likely that the immunodeficiency stems from the interaction between B cells and T cells [137]. There is a direct correlation between low levels of IgG and the frequency and severity of bacterial infections, most commonly Streptococcus pneumonia and Haemophilus influenza [143].

3.2.2 Poor response to vaccination

Vaccination studies have demonstrated that overall antibody responses to vaccines in CLL patients are weak [145]. Protein vaccines have produced weak to moderate responses in up to 50% of patients, chiefly in early-stage patients with normal serum immunoglobulin levels, but responses to polysaccharide vaccines have been virtually

zero [146]. So far, there are no data regarding the efficacy of vaccination in preventing actual infections or their impact on survival.

3.3 T CELL DEFECTS

In most patients with CLL, T cell numbers are increased. This increase mainly affects CD8+ cells but CD4+ cells are also increased, though the CD4/CD8 ratio is reversed. It was also found that the T cell receptor (TCR) repertoire was skewed [147]. To elucidate the basis for the CD8+ T cell expansion in CLL the composition of the T cell compartment was investigated [148]. It was shown that the absolute numbers of CD3+ T cells was related to expansion of the CD8+ T cell population with a CD45RA+CD27- cytotoxic phenotype and that the expansion of these cells was related to CMV infection. Repeated antigenic stimulation in vivo as induced by chronic viral infections such as CMV was suggested as responsible for the disturbed balance in the composition of T cells.

Changes in normal CD4+ and CD8+ T cells can also be induced by CLL cells via direct cell-cell contact and soluble factors, in particular inhibitory cytokines such as IL-6, IL-10, TNF and TGF-β [149, 150]. These interactions induces a state of relative T cell anergy with poor responses in mixed lymphocyte reactions, poor delayed hypersensitivity reactions, Th2 polarization and reduced expression of CD154 [137].

Further, it has been shown that CLL patients have significantly increased number of CD4+CD25hi regulatory T cells (T_{reg} cells) [151]. Normally these cells play a central role in the maintenance of peripheral tolerance by suppression of autoreactive T cell populations. Whether the increase in T_{reg} cells in CLL patients contributes to the immune deficiency is unclear.

3.4 OTHER IMMUNE DEFECTS

Natural killer (NK) cell function is impaired in CLL patients. It has been found that, in contrast to normal controls, most of the patients are deficient in the azurophilic cytoplasmic granules that are thought to play a role in NK-mediated lysis [152]. Overexpression of human leukocyte antigen G (HLA-G), which counteracts the cellular immune response of T- and NK cells by several pathways, have in CLL patients shown to correlate with the degree of immunosuppression [153]. Neutrophils and monocytes have also shown to be defective in their phagocytic and bactericidal function as well as in migration and chemotaxis [154]. Finally, levels of different components of the complement pathway have been shown to be reduced and accompanied by aberrant activation and binding. These abnormalities were correlating with the stage of disease and were shown to be present in all patients with advanced disease [155].

3.5 INFECTIOUS PROPHYLAXIS

3.5.1 Antimicrobial prophylaxis

There are no standard guidelines for antimicrobial prophylaxis in CLL patients, and in general, most recommendations for these agents are derived from clinical trials and anecdotal reports [140]. Since fludarabine and alemtuzumab are highly immunosuppressive and frequently used, infection prophylaxis recommendations in relation to treatments including these agents are very important. Based on experiences from 92 patients with indolent lymphoid malignancies treated with fludarabine-containing combination therapy a model with six identified risk factors; age > 60, ≥ 3 previous therapies, previous fludarabine exposure, time from diagnosis to concurrent treatment > 3 years, performance status ≥ 2, and baseline ANC < 2.0 ×109/L, was developed [156]. Compared with patients with 0-2 risk factors, patients with ≥ 3 risk factors had higher infections rates, more grade 4 neutropenia, and more neutropenic septicemia. This model could be used to identify the population most appropriate for infection prophylactic treatment. However, when cyclophosphamide is added to fludarabine-based therapy (FC, FCR), the use of herpes virus prophylaxis with acyclovir and Pneumocystis jirovecii propylaxis with cotrimoxazole (or pentamidine inhalations, for cotrimoxazole–allergic patients) during and for 6 months after end of therapy, is common. Regarding recommendations for patients receiving alemtuzumab there are newly published guidelines recommending herpes virus and Pneumocystic prophylaxis treatments, see above, combined with mandatory weekly monitoring for CMV reactivation by quantitative PCR (during alemtuzumab therapy) for all patients [112].

3.5.2 Prophylactic immunoglobulins

The use of prophylactic intravenous immunoglobulin (IVIG) is controversial. Several clinical trials have demonstrated that it reduces the incidence of mild and moderate bacterial infections but none have shown a decrease in mortality [137]. Notably, the IVIG treatment does not restore deficiencies of IgM or IgA, low levels of which are implicated risk factors for infection. One study estimated that the cost of one quality adjusted life year was 6 million USD and concluded that prophylactic IVIG is extraordinary expensive in comparison with other treatments generally considered as cost effective [157].

In the Swedish CLL National Guidelines gammaglobulin prophylactic treatment is recommended for patients with serious and/or recurrent bacterial infections and a demonstrated hyopgammaglobulinemia with IgG concentration below the normal range [125]. Since low dose of IVIG showed to be efficient [158] a dose of 10g every three weeks is recommended. As an alternative, subcutaneous immunoglobulin therapy is mentioned. This approach over time does provide stable IgG levels and appears to provide adequate infection prophylaxis in patients with primary antibody deficiency syndromes [159] .

3.5.3 Vaccinations

There are no formal vaccination guidelines for CLL patients, pneumococcal and seasonal influenza vaccines are generally used [140]. Vaccination with live attenuated organisms should be avoided [137] because they may cause severe infections in immuncompromised individuals like CLL-patients.

4 AUTOIMMUNE COMPLICATIONS IN CLL

4.1 INTRODUCTION

Although autoimmune complications have been recognized as a complication of CLL for a long time, there are limited data on epidemiology, incidence and prevalence. However, recent studies suggest that the overall risk of autoimmune complications in CLL is approximately 5-10% (reviewed in [160]). Autoimmune hemolytic anemia (AIHA) is the most common autoimmune complication with a frequency of 5-10%. The reported risk for immune thrombocytopenia (ITP) and pure red cell aplasia (PRCA) ranges from 1-5% and about 1% respectively. Finally, autoimmune granulocytopenia (AIG) occur as a rare autoimmune cytopenia in CLL patients.

Non-hematological autoimmune complications of CLL such as paraneoplastic pemphigus, glomerulonephritis, C1 esterase deficiency and pernicious anemia are rare (reviewed in [160]) and will not be discussed in the following text.

4.2 DEFINITIONS

Strict diagnostic criteria for the CLL related autoimmune cytopenias are lacking. The diagnosis of AIHA is usually based on the following laboratory findings: normocytic or macrocytic anemia, reticulocytosis, low serum haptoglobin levels, elevated lactate dehydrogenase (LDH) level, increased indirect bilirubin level, and a positive direct antiglobulin test (DAT) with a broad spectrum antibody against immunoglobulin and complement [161]. Two main types of AIHA have been described and are defined according to the serologic characteristics of the autoantibodies. In warm-antibody AIHA, autoantibodies attach to and initiate the destruction of erythrocytes at temperatures of normal body temperature. In cold AIHA, autoantibodies become most active and attack erythrocytes only at temperatures well below normal body temperature. Warm AIHA is the type most commonly associated with CLL and accounts for about 90% of cases [162, 163]. For ITP the diagnostic criteria are less clear, and the platelet autoantibody tests lack sensitivity and specificity [143]. However, the occurrence of a rapid and deep “unexplained” fall in the platelet count, together with some adjuvant conditions, such as platelet rise after therapy with corticosteroids or high-dose intravenous immunoglobulins (IVIG), an augmented number of megacaryocytes in the bone marrow, or the absence of hypersplensim, have been used for ITP diagnosis in several reports [164]. In PRCA, examination of the bone marrow to assess the absence of hemopoietic precursors is essential and there is an absolute reticulocytopenia [143]. Diagnosis of AIG requires a BM study to evaluate neutrophil production and eliminate other possible causes [160]. Analyses for the detection of granulocyte-specific antibodies are used [165].

4.3 PATHOGENESIS

Autoimmune cytopenia affecting CLL patients is usually caused by polyclonal T cell dependent mechanisms that result from the loss of self-tolerance. In approximately 90% of cases of AIHA and ITP pathogenic high-affinity IgG antibodies directed against red blood cell (RBC) or platelet antigens are produced by non-malignant B cells (reviewed in [160]). These antibodies can ligate antigens on RBC and platelets and the opsonised cells are then destroyed via an ADCC mechanism mediated predominantly by macrophages in the spleen and liver [166]. In less than 10% of the autoimmune cytopenia cases the auto-antibodies are produced by CLL cells. In these cases the autoantibodies are monoclonal and usually of IgM-type directed against the I antigen causing AIHA by both complement-dependent cytotoxicity and ADCC (reviewed in [160]). In PRCA and AIG the autoimmune mechanisms are less well defined.

4.4 RISK FACTORS

Risk factors classically associated with autoimmune cytopenia are; male sex, older age, a high blood lymphocyte count and advanced disease [162, 164, 167, 168]. An association has also been found to unfavorable biomarkers including unmutated IGHV genes, high expression of CD38 or ZAP-70 and increased β2-MG [167].

In patients with AIHA, over 90% are positive for DAT [162, 163]. In the largest prospective trial in CLL to examine the incidence and prognostic impact of a positive DAT and AIHA in patients requiring therapy, there was a randomization between chlorambucil, fludarabine or fludarabine plus cyclophosphamide (FC) [169]. The incidence of DAT positivity for this population of previously untreated patients at study entry was 14% of which 28% developed AIHA in relation to therapy. DAT negativity, on the other hand, was a strong predictor (> 90%) for not developing AIHA after therapy. The association between CLL treatment and autoimmune cytopenia has been recognised since long and in this study patients treated with chlorambucil or fludarabine were more than twice as likely to develop AIHA as those receiving FC, the latter had a 5% incidence of AIHA, a level that is comparable with a 6.5% incidence reported after FCR treatment [170]. It was suggested that the FC combination might have a protective effect against the development of AIHA [169]. This, in contrast to fludarabine monotherapy, which was not just related to an increased incidence of AIHA, but also, to a more severe AIHA clinical course. However, in two other studies comparing F and FC there were no statistically significant difference in risk of developing AIHA [116, 117].

4.5 TREATMENT OF AUTOIMMUNE CYTOPENIA

The level of evidence regarding treatment recommendations for autoimmune cytopenias is not high [161]. Prospective randomized trials are lacking. The situation is also complicated by different level of concurrent CLL activity/severity and possible relation between the autoimmune cytopenia and previous treatment. Considering the latter

problems patients may be categorized into three groups; simple, complex and treatment related autoimmune cytopenia [160].

4.5.1 Simple autoimmune cytopenia

These patients have autoimmune cytopenia requiring treatment, but without signs of active CLL requiring antitumor therapy. In general these patients can be managed with therapies similar to those used to treat primary autoimmune cytopenias.

4.5.1.1 AIHA

In the Swedish CLL National Guidelines [125] single treatment with prednisolone is recommended as first-line therapy. The mechanism of action of corticosteroids in the treatment of autoimmune cytopenias is not fully understood. At least two mechanisms seems to be involved, i.e., decreased sequestration of RBCs in the spleen and decreased autoantibody synthesis [171]. Most patients respond rapidly to treatment, however, durable responses are only achieved in about one-third of patients (reviewed by [160]).

Rituximab has shown promising results as a therapeutic option against AIHA. How rituximab works in this situation is not fully understood. Depletion of B cells involved in antigen presentation, as well as, production of pathogenic autoantibodies has been discussed [172]. In a report on 14 patients treated with rituximab as second- or third-line therapy against CLL-related AIHA a response rate of 71% (10/14) was demonstrated, 3 patients reached CR [172].

In order to remove the primary site of red-cell trapping and destruction patients may be considered for splenectomy. Substantial data from studies on patients with CLL and simple autoimmune cytopenia are lacking. However, in a retrospective evaluation of 30 patients with DAT positive AIHA who underwent splenectomy patients with no associated systemic disease were separated. In this group splenectomy was an effective treatment approach, 11/12 patients responded [173].

High doses of IVIG may be effective against AIHA [174]. The blocking of RBC sequestration by macrophages is believed to be involved in the mechanism of action [175]. The response duration is usually short, however, IVIG treatment may stabilize patients with severe and poorly tolerated hemolyses i.e. before RBC transfusion and/or splenectomy.

For patients not responding to steroids or having AIHA recurrence on steroid withdrawal, the use of immunosuppressive drugs such as cyclosporine A, azathioprine, or low-dose cyclophosphamide is an option [75].

4.5.1.2 ITP

As in AIHA, corticosteroids are recommended as first-line therapy against ITP [125] and for about one-third of patients this treatment will be the only treatment required [163]. For responding patients who require long-term immunosuppression, immunosuppressive drugs such as cyclosporine A, azathioprine, or low-dose cyclophosphamide can be used as “corticosteroid-sparing agents” [160]. Splenectomy is traditionally considered as the

second-line treatment for relapsed ITP and up to 70% of patients may have a long-time response after surgery [143, 164].

Rituximab have shown promising efficacy in this condition. In a retrospective study on 89 patients with chronic ITP refractory to several treatments a response rate of 55% was reached [176]. Case-report data indicate that alemtuzumab may be a good alternative for the treatment of CLL-related ITP not responding to conventional therapy [177].

4.5.1.3 PRCA

The treatment approach is the same as for AIHA and treatment response can be monitored by assessing the reticulocyte count [143]. There are conflicting data from case reports regarding the efficacy of rituximab against PRCA [165, 178]. This may be related to the hypothesis that T cells are important in the pathophysiology of this condition [165].

4.5.1.4 AIG

The experience regarding management of this CLL-complication is limited. Immunosuppression is rarely effective, however spontaneous recovery is possible and it is important to support patients with appropriate infection treatment [163].

4.5.2 Complex autoimmune cytopenia

Patients with complex autoimmune cytopenia suffer from autoimmune cytopenia and progressive CLL simultaneously. Monotherapy with purine analogues and alkylating agents should be avoided in this situation. These therapies are known to increase the risk for autoimmune complications in CLL patients, and nucleoside analogues are additionally related to an increased risk of more severe autoimmune cytopenias [169, 179-181]. There are data suggesting that combination therapy including purine analogues such as FC [169], FCR [170] and pentostatin plus cyclophosphamide and rituximab (PCR) [182] do not increase the risk of autoimmune cytopenia, however, there are no data to support the use of these regimens in patients with active autoimmune cytopenia.

Optimal therapy for complex autoimmune cytopenia would selectively suppress the autoimmune cytopenia, be toxic to CLL cells and have a low risk of exacerbating the autoimmune process. Such a therapy has not yet been established. However, interesting data from two retrospective reports with similar chemoimmunotherapy combinations consisting of rituximab, cyclophosphamide, vincristine and prednisone (R-CVP) and rituximab, cyclophosphamide and dexamethasone (RCD), respectively, were recently published [183, 184]. Both studies showed high response rates against autoimmune cytopenia (R-CVP 19/20, RCD 7/7). Importantly, eighteen of the R-CVP treated patients had previously been treated for autoimmune cytopenia compared to none of the RCD treated. Despite high response rates, response duration was suboptimal both regarding autoimmune cytopenia (median time to next treatment for R-CVP was 22 months, median response duration for RCD was 26 months) and CLL (median time to next treatment for R-CVP was 28 months, not reported for RCD).

Interestingly, in this difficult situation, single-agent treatment with alemtuzumab has shown efficiency (Paper II, [185, 186]) and may be considered for early use in patients with complex autoimmune cytopenia.

4.5.3 Therapy-related autoimmune cytopenia

This refers to patients developing autoimmune cytopenia during or within 6-12 months of treatment for progressive CLL. Those who had a sufficient response to their CLL treatment can be treated for their autoimmune cytopenia using the same modalities used for "simple" autoimmune cytopenia [160].

By the same reasons as mentioned in the text about complex autoimmune cytopenia, therapy with purine analogues or monotherapy with alkylating agents are not recommended in this situation.

Although their suboptimal response duration, RCD, R-CVP or high-dose methylprednisolone and rituximab have been mentioned as safe therapeutical options for patients with therapy-related autoimmune cytopenia [160].

As in complex autoimmune cytopenia, single-agent alemtuzumab has, in small series of patients and case-reports, shown efficacy in therapy-related autoimmune cytopenia (Paper II, [110, 185, 187, 188]).

5 RICHTER´S SYNDROME

5.1 INTRODUCTION

The term Richter´s syndrome (RS) was introduced in 1964 [189] and named after Maurice N. Richter who in 1928 described the simultaneous occurrence of a "reticulum-cell sarcoma" and CLL [190]. Initially the definition was restricted to the development of a large-cell lymphoma in a patient with preexisting CLL. Although the broad definition of RS subsequently included other lymphoid malignancies that develop in patients with CLL, such as Hodgkin´s disease, prolymphocytic leukemia (PLL), lymphoblastic lymphoma and hairy cell leukemia, classical RS describes the progression of CLL/SLL to diffuse large B cell lymphoma (DLBCL) [191].

CLL transforms to RS in 2.2-16.2% cases [192] at a median time from CLL diagnosis of 23-48 months and with DLBCL as the most common subtype [193, 194]. A major factor affecting the variability in the RS transformation risk and the time from CLL diagnosis to RS is the heterogeneity in biopsy policies among different institutions. This can be exemplified by a study, which had a clearly defined and intensive biopsy policy, who demonstrated a high rate of RS and short time from CLL diagnosis, 16.2% and 23 months respectively [193]. The clonal relatedness of CLL and RS have been investigated by characterization of the immunoglobulin heavy and light chain gene rearrangements and approximately two-thirds of cases are evolved from the initial CLL clone, the remainder consisted of independent second malignancies [195].

5.2 RISK FACTORS

Despite the abundance of biological markers available for predicting CLL progression, only few have been shown to be useful to RS prediction. In a consecutive series of 185 previously untreated CLL cases lymph node size $\geq$ 3 cm was identified as the sole clinical risk factor of RS [193]. In the same study CD38 expression, IGHV4-39 usage and absence of del13q were identified as independent risk factors of transformation. However, in an observational cohort study using the Mayo Clinic CLL Database no statistically significant association was observed between risk of second lymphoproliferative disorder (LPD) and CD38, ZAP-70, IGVH mutation status or cytogenetic abnormalities among 962 CLL patients [196].

CD38 has a genetic polymorphism, characterized by a C > G variation in the regulatory region of intron 1. A study investigating the distribution of CD38 alleles in 248 CLL patients showed that G allele carriers had a significantly higher incidence of transformation than CC homozygotes and is was stated that the G allele may represent an independent risk factor for RS [197]. In a series of 401 CLL patients telomere length (TL) was tested as a risk factor of RS and it was found that the risk of RS was significantly higher for patients with shorter telomere length [198].

Purine analogues are highly immunosuppressive and could possible increase risk of RT. Data from 962 CLL patients in the Mayo Clinic CLL Database demonstrated an

increased risk of secondary lymphoproliferative disorder (5.2%) in patients treated with purine analogues compared with patients who had not received this kind of drugs (1.9%; P=0.008) [196]. However, no significantly increased risk was shown in long-term follow-up data from 2014 patients with CLL or hairy cell leukemia treated on National Cancer Institute (NCI) Group C protocols or in the three-armed prospective multicenter LFR CLL4 trial where 777 CLL patients were randomized between; fludarabine plus cyclophosphamide, fludarabine and chlorambucil [87, 199].

Information on the immunosuppressive effect of monoclonal antibodies and the development of RS are limited. In a first-line phase III study comparing alemtuzumab with chlorambucil no case of RS was reported after a median follow-up of 24.6 months [82]. However, longer follow-up time is needed to better investigate the role in RS transformation as in the long-term results from a first-line phase II study with FCR therapy showing a 2.5% risk of RS at a median follow-up of 6 years [200] or as in our study on first-line alemtuzumab and matched historic controls with a 16% and 12% risk of RS, respectively, at a median follow up of over 5 years (Paper I).

5.3 CLINICAL PRESENTATION AND DIAGNOSIS

Richter´s transformation is characterized by the development of systemic symptoms (e.g., fever, weight loss, and/or drenching night sweats), sudden clinical deterioration, and, usually, a rapid increase in the size of a lymphoid mass at one site. Bulky retroperitoneal adenopathy and massive splenomegaly are common presenting features [201]. Extranodal spread may occur in RS and sites of involvement include the central nervous system, eye, gastrointestinal system, nose, skin, face, bone and bronchus (reviewed in [202]). Patients with Hodgkin´s variant of RS generally present at a more advanced stage than do patients with true Hodgkin´s disease [194].

The most common laboratory finding is an elevated lactate dehydrogenase (LDH) level, a marker of tumor growth. Approximately 80% of patients with transformation to diffuse large-cell lymphoma have marked elevations of serum LDH disproportionate to that anticipated in uncomplicated CLL in the absence of hemolysis [203].

The RS transformation diagnosis requires a histological proof and should be distinguished from both CLL progression and CLL refractory to therapy [75]. Surgical excision or large incision biopsy is recommended since with fine needle aspiration CLL cases with numerous proliferation centers are indistinguishable from diffuse large B cell lymphoma (DLBCL) [192].

In order to guide decisions on which lymph nodes or masses should undergo tissue sampling investigation with 2-^{18F}fluoro-2deoxy-D-glucose positron emission tomography/computed tomography (FDG PET/CT) can be useful. A retrospective study on 37 CLL patients who had underwent FDG PET/CT showed that this investigation can detect Richter transformation to DLBCL with a high sensitivity (91%) and a high negative predictive value (97%) [204]. Nine patients had false-positive FDG PET/CT; other malignancy (n=3, one case of Hodgkin´s lymphoma), accelerated phase of CLL (n=2),

refractory CLL with extensive bone marrow involvement (n=3) or atypical pneumonia (n=1) affecting the specificity and positive predictive values, 80% and 53% respectively. The role of PET remains a research tool in early detection of RS [96].

5.4 PROGNOSIS AND THERAPY

The clinical outcome of RS is generally poor. An analysis of 130 patients treated with chemotherapy, with or without rituximab, over a 30-year period at M.D. Anderson Cancer Center (MDACC) demonstrated a median survival of 8 months. From five adverse factors predicting shorter survival, identified by multivariate analysis in the same study, a RS score was developed to predict an individual patient's risk of death: Zubrod (WHO) performance status of more than 1, LDH levels higher than 1.5 × the upper limit of normal, platelet counts lower than 100×10^9 /L, tumor size ≥ 5cm, and more than one prior therapy. According to number of presenting risk factors, patients could be assigned to one of the following risk groups: low risk (score, 0 or 1); low-intermediate risk (score 2); high-intermediate risk (score 3); high risk (score 4 or 5). Despite this division, the median survival in the low-risk group was only 1.12 years [205].

There is no consensus on the best therapeutic approach for RS patients [96]. Regimens that have been utilized for classical RS treatment reflect the evolution of therapy observed in the last few years in the treatment of aggressive NHL. The role of treatment combinations containing rituximab was retrospectively evaluated in the case-control study from MDACC [205]. Combinations of rituximab with Hyper-CVXD (a fractionated cyclophosphamide, vincristine, liposomal daunorubicin, and dexamethasone regimen) variants or CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) induced responses in 47% of patients compared with a 34% response rate seen with chemotherapy alone (P = 0.2). Notably, the number of patients treated with chemotherapy and rituximab combinations (n = 47) compared with those treated with chemotherapy alone (n = 79) was relatively small and a further study with a larger sample size was suggested.

OFAR (oxaliplatin, fludarabine, cytarabine and rituximab) is a regimen of immunochemotherapy specifically devised for patients with RS or refractory CLL. Twenty patients with RS were enrolled in a phase I / II trial with increasing doses of oxaliplatin. The response rate was 50% and the 6-month survival rate was 59%. The median response duration for the whole study population, including 30 fludarabine-refractory CLL patients, was 10 months [206].

Allogeneic stem cell transplantation (alloSCT) is a therapeutic option for selected patients with RS. In seven patients from the MDACC-study who received alloSCT after a CR, CRu, or PR there was a 3-year estimated cumulative survival of 75% [205].

The role of radiation therapy is generally palliative because RS is dissiminated at the time of diagnosis [191].

Patients with the Hodgkin´s transformation of CLL usually respond to standard therapies for Hodgkin´s disease [191].

6 AIMS OF THE THESIS

I. To conduct a long-term follow-up of clinical effects, infectious complications and risk of Richter transformation in CLL patients who received alemtuzumab as first-line treatment.

II. To study the effect of alemtuzumab for the treatment of severe, refractory autoimmune hemolytic anemia in CLL patients.

III. To evaluate type, severity and duration of side-effects as well as efficacy of subcutaneous alemtuzumab, without dose-escalation, in advanced-stage relapsed CLL patients and to prospectively analyze if careful selection of patients can lead to increased overall efficacy.

IV. To analyze the T cell repertoire following first-line alemtuzumab treatment in CLL patients.

V. To study virus reactivation and serology patterns following first-line alemtuzumab treatment in CLL patients.

7 MATERIALS AND METHODS

This research project is based partly (Paper I, IV, V) on our phase II trial of alemtuzumab given to previously untreated but symptomatic CLL patients [111] in which trial the majority of patients achieved clinical remissions but also severe depletion of T cells [113].

Response evaluation at end of CLL therapy (Paper I, II, III, V) was performed using the NCI-IWCLL response criteria [207].

Paper I. The Swedish Cancer Registry was used to identify matched historical control patients. Threehundred fifty-one patients with CLL, diagnosed during the period 1990-1997 at four hospitals in Stockholm, were identified. Seventy-five of these patients matched the inclusion criteria of the phase II trial [111], but had recieved other first-line treatments, and were included as controls. The above mentioned time interval was choosen to reduce bias related to inclusion in the alemtuzumab study, which started 1997. Data were collected through a detailed case record review. The two-sided Chi-square test was used to test differences in proportions. The Wilcoxon´s Gehan exact test was used to test differences between curves representing time to treatment failure (TTTF) or time to Richter transformation.

Paper II. Patients with severe refractory autoimmune hemolytic anemia (AIHA) were recruited and treated at Karolinska University Hospital Solna. All known cases with CLL and AIHA at the Department of Hematology, treated with alemtuzumab, were included. AIHA response was defined as the elimination of the need for red blood cell transfusions and a $\geq$ 2.0 g/dl rise in Hb concentration independent of transfusions.

Paper III. Patients were recruited at three centers, one in Sweden and two in United Kingdom. Consecutive patients who met the inclusion criteria were recruited. However, since it had previously been noted that efficacy in patients with bulky disease was less marked [208], no such patient was included, although this was not a exclusion criteria.

Based on the experiences of the injection site reactions from the first-line SC study [111] a new subscale, to assess erythema and oedema in detail, was invented by the investigators. Additionally, monitoring of subjective adverse events was conducted by patients at 6-12 hour intervals using a diary. Objective skin reactions were graded by a trained and experienced health care professional every 24-48 hour until completely resolved. For statistical analysis of differences in lokal skin reactions Binominal test was used.

Paper IV. Blood samples, systematically stored before and during alemtuzumab treatment, as well as during long-term follow-up, from five patients from the phase II study [111], were investigated. The analyses were restricted to this reduced number of patiens due to limitations in the number of frozen blood samples and the extremely

labour-intensive techniques applied. T cell receptor B-variable (TCR-BV) gene usage in CD4 and CD8 T cells was assessed, by real-time PCR, as well as complementary-determining region 3 (CDR)-length polymorphism, before and after therapy. The relative changes and the expression diversity (number of peaks) in B-variable gene expression comparing pretreatment (T0) and post-treatment (T1, T2, T3, …) for each patient were assessed by the two-tailed Wilcoxon signed rank test. Comparison of peak numbers for all patients at T0 and T1 was performed using the Mann-Whitney *U*-test.

Paper V. Systematically stored blood samples, from the phase II study patients (n=18) [111] and from consecutive control patients (n=27) treated with fludarabine-based combination therapy at three centers in Stockholm, were investigated. The number of alemtuzumab treated patients was reduced due to limitations in the number of frozen blood samples. The laboratory virus and serological analyses, from samples taken before, during and after therapy, were done at the Swedish Institute for infectious Disease Control in Solna. Quantitative real time PCR was used to detect and measure the presence of CMV, EBV and HHV-6. Qualitative real time PCR was used for Parvovirus B19 detection. The presence of serum immunoglobulin G (IgG) antibodies against CMV, VZV, morbillivirus and Epstein-Barr nuclear antigen-1 (EBNA-1) protein of EBV were detected by enzyme-linked immunosorbent assay (ELISA) and against EBV viral capsid antigen (VCA) IgG by immunofluorescence (IF). Fisher exact test (two-tailed) or Chi-square test was utilized for comparison of the incidence of virus reactivations, changes of IgG levels and adverse events in the two treatment groups. For comparison of different cell counts between the two treatment groups and between two subgroups of alemtuzumab treated (with and without virus reactivation), nonparametric independent Mann-Whitney signed-rank test was used. All statistical tests were two-sided.

8 RESULTS, DISCUSSION AND CONCLUSIONS

8.1 PAPER I

Alemtuzumab as first-line therapy for B-cell chronic lymphocytic leukemia: long-term follow-up of clinical effects, infectious complications and risk of Richter transformation
(Karlsson et al, Leukemia 2006;20:2204-2207)

A long-term follow-up of a phase II study on CLL patients who received alemtuzumab as first-line therapy was conducted. The following variables were studied; time to progression, time to treatment failure (TTTF), incidence of early and late infectious complications as well as frequency of and time to Richter transformation. The results were compared with consecutive matched historic controls who had been treated with other first-line treatment regimens.

The median follow-up time from the start of first-line therapy was, for the 38 evaluable alemtuzumab-treated and the 75 control patients, 64 (13-102) and 61 (4-132) months, respectively. The median TTTF for all alemtuzumab-treated patients was 28 months. Among these, 33 were responders and their median TTTF was 32 months. Seven patients reached CR and had the longest median TTTF, 77 months. In the control group the median TTTF was 17 months (p=0.07 versus alemtuzumab).

During therapy, no grade 4 infectious complications were observed in the alemtuzumab group. Grade 3 infections were observed in four patients (10%) and consisted of cytomegalovirus (CMV) reactivation that caused fever without pneumonitis in three patients and one Pneumocystis jiroveci pneumonia (in a patient with no prophylaxis due to allergy to cotrimoxazole). In the matched controls, grade 3 or 4 infections were observed during first-line treatment in 14 patients (19%) and included; fever of unknown origin (n=5), pneumonia (n=4, one fatal), septicemia (n=2), CMV (n=1), herpes zoster (n=1), dental infection (n=1) and skin infection (n=1). The difference in incidence of infections between alemtuzumab-treated patients and controls was statistically not significant.

During long-term unmaintained follow-up, 7/38 alemtuzumab-treated patients (18%) experienced totally 10 episodes of reversible grade 3 infections. No grade 4 or fatal infection was observed. In the control group, for which the median TTTF was shorter, the incidence of grade 3 or 4 infections was 8/75 (11%). The difference was statistically not significant.

Sixteen percent (6/38) of the alemtuzumab-treated patiens developed Richter transformation. The corresponding incidence for the controls was 12% (9/75, not significant). The median time from CLL diagnosis to transformation was 44 months (range, 20-75) for patients treated with alemtuzumab, and 41 months (range, 14-94) for the matched historical controls. The median time from the start of CLL therapy to diagnosis of transformation was 16 months (range, 3-32 months) in the six alemtuzumab-treated

patients and 36 months (range, 1-84 months) in the nine matched historical controls (not significant).

The current work represents the first study of long-term follow-up of patients who have received first-line therapy with alemtuzumab for CLL. In the absense of randomized trials, retrospective historic comparisons may provide meaningful preliminary information with proper considerations. Despite a high proportion of patients with advanced stage disease, a significantly higher overall response rate was observed in the alemtuzumab group than in the historical control group (p=0.01). Notably, our data on response rate, PFS and TTTF for the alemtuzumab treated patients were almost identical as for the CLL patients (n=147) randomized to the alemtuzumab arm in a subsequent first-line multi-center phase III trial, CAM307 [82].

It is also important to compare the incidences of CMV reactivation and its consequenses to alemtuzumab treated patients in our study and in the CAM307 study. In the latter trial patients were tested for CMV during therapy by weekly PCR, in contrast to our patients who where only tested if they were symptomatic. The systematic screening in the CAM307 trial lead to the detection of positive CMV PCR results in 77 (52%) asymptomatic patients and 23 (16%) symptomatic patients. Six (4% of all alemtuzumab treated) of the asymptomatic patients with positive CMV PCR were admitted to hospital for treatment with IV ganciclovir, and this was recorded as grade 3 events. Among the symptomatic patients six (4% of all alemtuzumab treated) were recorded as grade 3 events. In our study three (8%) symptomatic grade 3 CMV-reactivations were recorded. In both studies all grade 3 reactivations resolved on antiviral treatment. Taken together, our strategy, i.e. CMV testing with PCR only if early symptoms occur, instead of weekly monitoring, seems reasonable. In Paper V the incidence and clinical relevance of subclinical virus reactivations are further investigated.

The incidense of Richter transformation/development of Richter´s syndrome (RS) was high among both the alemtuzumab-treated and the matched historic controls in our study, 16% and 12% respectively. This might partly be explained by the circumstances that all patients had active disease with treatment indication and a relatively long follow-up time of 64 and 61 months, respectively. Further, a larger proportion of patients treated with alemtuzumab in our studypopulation had advanced disease, Rai stage III-IV, compared with historic controls, patients included in CAM307 and other studies [209]. However, an increased incidence of RS, related to the immunosuppressive effect of alemtuzumab can not be excluded. Although data from CAM307, showing no case of transformation after a median follow-up of 24 months, does not indicate such a risk. A long-term analysis of the randomized CAM307 study is still pending.

In conclusion, this long-term follow-up of our previously reported efficacy data [111] demonstrated, despite severe and long-lasting T cell suppression, that alemtuzumab therapy appears to be safe as first-line therapy, with comparable rates of infectious complications and incidence of Richter transformation as for matched controls.

8.2 PAPER II

Treatment of severe refractory autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab (humanized CD52 monoclonal antibody)
(Karlsson et al, Leukemia 2007;21:511-514)

This is a report on the effect of alemtuzumab treatment on severe, multi-agent refractory CLL-related autoimmune hemolytic anemia (AIHA).

Five patients with advanced CLL, all with severe transfusion-dependent AIHA, resistant to conventional therapy, received alemtuzumab at the standard dose. All 5 patients responded with a ≥ 2.0 g/dl rise in hemoglobin (Hb) concentration, in the absence of further transfusions, after a median time of 5 weeks (range, 4-7 weeks). The mean Hb increased from 7.2 g/dl at baseline to 11.9 at end of treatment. All patients remained stable , without further AIHA episodes, after a median unmaintained follow-up time of 12 months with a mean Hb of 12.5 g/dl. Clinical responses (as defined by NCI criteria [207]) were seen in 4 patients (when the study was published one was still on therapy and response data were not available) of which three reached a CR.

AIHA is a frequent and serious complication in patients with CLL [160]. Before treatment is initiated the level of concurrent CLL activity/severity and possible relation between the AIHA and previous treatment has to be considered. Addressing this, a categorisation of patients with autoimmune cytopenia into three groups have been suggested [160]. Thus, AIHA may be defined as simple, complex or therapy-related.

All five patients in our report may be categorised as being affected by both complex and therapy-related AIHA. They had progressive CLL and AIHA concomitantly and, additionally, had received treatment for progressive CLL within 6-12 months. This is a challenging situation with no established treatment recommendations. However, alemtuzumab, with a well documented antitumor efficacy [112] and profound immunosuppressive activity [113], constitutes a potential therapeutic option.

All patients responded with elimination of AIHA and a marked increase in hemoglobin concentrations. In parallel, CLL responses were achieved in all but one patient.

In conclusion, the results from this report suggest that alemtuzumab may be highly effective in the treatment of severe AIHA in patients with progressive CLL who have failed to respond to conventional immunosuppressive therapy, such as corticosteroids, splenectomy, rituximab and chemotherapy.

8.3 PAPER III

Phase II study of subcutaneous alemtuzumab without dose escalation in patients with advanced-stage, relapsed chronic lymphocytic leukaemia
(Karlsson et al, British Journal of Haematology 2009;144:78-85)

A phase II study was conducted evaluating the type, severity and duration of side-effects as well as efficacy of subcutaneous (SC) alemtuzumab, without dose-escalation, in advanced-stage relapsed CLL patients. Alemtuzumab 30 and 3 mg was administered SC simultaneously, in opposite thighs, day 1, followed by 30 mg three times per week.

The first doses of 30 mg and 3 mg produced injection-site-reactions (all but one were grade 1/2) in 13/20 and 9/20 patients, respectively. The second dose on day 3 resulted in skin-reactions in 10/20 patients and the third, fourth, fifth and sixth injection produced reactions in 6/20, 1/20, 2/20 and 0/20 patients, respectively. Mild "flu-like" symptoms occurred during week 1 in 10/20 patients. All side-effects had subsided by the sixth dose.

15/20 patients (75%) responded (12 partial responses, three complete responses) with a median time to treatment failure (TTTF) of 20 months.

Intravenous administration is still a frequently used (and the only approved) route of delivering alemtuzumab to patients with CLL [82, 108]. However, one study in previously untreated CLL patients [111], and one study in fludarabine-refractory CLL patients [81], have suggested that SC injection may result in a marked reduction of the "first-dose" flu-like reactions that frequently occur after IV administration. SC injections may induce transient skin reactions, which in previously untreated patients may sometimes be quite extensive [111].

Our findings have demonstrated that patients with previously treated, advanced-phase CLL can be treated with the maximum dose of alemtuzumab 30 mg SC upfront without unacceptable toxicity. Local skin reactions at the injection site were generally of grade 1 or 2 and transient, and appeared to be less intense than those seen with SC alemtuzumab as first-line therapy in patients with CLL [111]. Even though the first alemtuzumab 30 mg SC injection resulted in slightly more injection-site reactions than the 3 mg SC control injection (65% versus 45%), most skin symptoms had subsided within 2 days, and the reactions induced by continued alemtuzumab administration were less intense and of shorter duration.

Furthermore, by upfront careful selection of patients, a high overall response rate (ORR) (75%) and long TTTF (median 20 months for responding patients) was obtained, even though all but three patients had relapsed after, or were refractory to, purine analogue-based therapy and 60% of the patients had Rai stage III or IV disease. One possible major reason for the high response rate may be that only patients with WHO performance status (PS) grade 0 or 1 were recruited as it has previously been suggested that a good PS is closely linked to successful therapeutic outcome with alemtuzumab [210]. Another and possibly linked explanation for the high ORR in our study may be that most patients completed therapy as planned; the median length of therapy was 12 weeks. Finally, none of

the patients had bulky lymph nodes, i.e. ≥ 5 cm, which also contributed to the high ORR. Established effective treatments for CLL patients with bulky lymph nodes who have failed purine analogue-based therapy are currently lacking.

In conclusion, a starting dose of 30 mg SC was well tolerated. The SC route of administration for alemtuzumab may confer major advantages once the injection skin reactions have subsided, both in terms of practical aspects for the patients as well as the burden on health care providers. Our results also suggested that optimal selection of advanced-phase CLL patients for alemtuzumab therapy may result in a high response rate and durable remissions, the results can be regarded as preliminary only due to the small size of our study (n=20). Important success factors, to be confirmed in extended trials, may be a good performance status, absence of bulky lymph nodes, and avoidance of early discontinuation of planned therapy by appropriate management of predictable toxicities.

8.4 PAPER IV

Reconstitution of the T-cell repertoire following treatment with alemtuzumab (anti-CD52 monoclonal antibody) in patients with B-cell chronic lymphocytic leukaemia

(Rezvany et al, British Journal of Haematology 2006;135:475-485)

In this pilot study, T cell receptor B-variable (TCR-BV) gene usage in CD4 and CD8 T cells was assessed by real-time PCR, as well as complementarity-determining region 3 (CDR3)-length polymorphism. Analyzes were performed on samples taken before and after first-line alemtuzumab therapy, in five patients with CLL.

A decline in expression of most BV family genes in both CD4 and CD8 T cells was observed after alemtuzumab treatment, which was followed by a gradual increase in most BV families during long-term follow-up. After treatment, CDR3-length polymorphism showed an even more restricted pattern in CD4 T cells compared with pretreatment, with a shift towards a monoclonal/oligoclonal pattern. The clonally restricted pattern was significantly reduced in CD4 ($P < 0.01$) but not in CD8 T cells. This was followed by a gradual increase in the number of peaks within the CDR3 region of the different TCR-BV families, i.e. a polyclonal repertoire, during long-term follow-up.

Despite being clinically effective, concerns were raised in early trials with regard to the risk of infections associated with alemtuzumab therapy in heavily pretreated patients with advanced B cell malignancies [211, 212]. The introduction of valaciclovir and cotrimoxazole prophylaxis resulted in significant reduction, but not elimination, of the risk of serious infections [81, 82, 108, 111, 213-215]. Further, because of an increased risk of CMV reactivation, recent alemtuzumab management guidelines recommend weekly monitoring for CMV reactivation during therapy [112].

It has been recommended that the length of anti-infective prophylaxis should be based on the actual number of CD4 T cells, and that such prophylactic treatment should be continued until the CD4 T cell count exceeds 0.2×10^9 cells/L [216]. However, this recommendation does not take into account the recovery pattern of the complex elements of the T cell immune system (repertoire in particular) following alemtuzumab treatment.

In this pilot study, we analyzed in depth the usage and clonality patterns of TCR-BV gene families in CD4 and CD8 T cells before and after first-line treatment with alemtuzumab in CLL patients. The TCR-BV pattern and T cell recovery during follow-up were different in CD4 T cells compared with CD8 T cells. CD4 T cells showed a more clonal/oligoclonal pattern than CD8 T cells shortly after treatment compared with pretreatment. Because a broad CD4 T cell repertoire is mandatory for protective immunity against various pathogens [217], it is likely that the highly restricted repertoire, as described here, along with low total numbers of CD4 T cells, contributes significantly to the increased frequency of viral (i.e. CMV) and opportunistic infections that may occour during and after alemtuzumab therapy [218]. Our finding, that the T cell repertoire

normalised during long-term follow-up corresponded to the very few late infections observed in our patients [219].

In conclusion, these results indicate that perturbations of the T cell repertoire following alemtuzumab are complex and not reflected by changes in the total number of CD4/CD8 T cells only. The restricted CDR3 pattern present prior to therapy became even more restricted after treatment, followed by a normalisation of CD4 repertoire during long-term follow-up.

8.5 PAPER V

Virus reactivations and serology patterns following first-line therapy with alemtuzumab or fludarabine-based combination therapy in patients with chronic lymphocytic leukaemia
(Karlsson C et al, Submitted)

In this study we systematically investigated the incidence and clinical relevance of subclinical virus reactivations and serological changes in CLL patients who received alemtuzumab as first-line therapy [111], then compared the results with those of patients who received fludarabine-based combination therapy.

Virus reactivations were rare, but significantly more common in alemtuzumab-treated patients; 6/440 analyses were positive versus 0/455 ($P < 0.05$) and 6/18 patients had at least one virus reactivation versus 0/27 ($P < 0.01$). No significant difference remained when CMV was excluded from the comparison. All virus reactivations resolved spontaneously. Alemtuzumab-treated patients with virus reactivation had significantly higher lymphocyte subset counts ($P < 0.01$ - < 0.05) at end of treatment than those without virus reactivation.

Sixteen of the 18 alemtuzumab-treated patients were evaluable with regard to differences in the IgG levels between baseline, end of treatment and 6-12 months post-therapy, except for EBV p107 IgG analysis, which included samples from 15 patients. Seventeen of the fludarabine combination-treated patients had their serology results evaluated in the same way as the alemtuzumab-treated patients. A significant decrease of at least one IgG titre was observed in 4/16 and 3/17 of the patients, respectively (not significant). Increasing titres were observed in some patients.

Results from this study demonstrate a low but statistically significantly higher incidence of virus reactivation in the alemtuzumab group, expressed both as total number of reactivations and number of patients with at least one reactivation, compared with the fludarabine combination-treated patients irrespectively if the latter were first or later-line patients. However, this difference was mainly attributable to CMV and was not significant if the other viruses were considered separately. Besides CMV, only one other therapy-related reactivation – an asymptomatic HHV-6 reactivation at 1 month during therapy – was detected, compared with no virus reactivation in the control group. All reactivations resolved spontaneously without discontinuation of alemtuzumab therapy. This indicates that CMV testing with PCR on symptomatic patients, instead of weekly monitoring, might be considered during alemtuzumab monotherapy in the first-line setting at experienced centres.

The incidence of serological changes was higher in the alemtuzumab group, but the differences were not significant. However, some titres decreased significantly in up to 25% of the patients, which raises the question of whether such patients might need specific preventive measures to avoid reinfection in terms of health care contacts and otherwise. Especially VZV-infections may become very severe even in healthy adults, and control of

serology status, at least after exposure, should be recommended. Notably, decreasing titres were also observed in some FC(R) treated patients. However, this study also shows that spontaneous recovery of IgG reactivities after alemtuzumab therapy is possible: one patient (A8) had four significant decreases of IgG titres, against CMV, VZV, measles and EBV p107G. This patient was later included in the control group (C9), and comparison of the IgG levels at baseline 1, before alemtuzumab, with levels at baseline 2, before fludarabine combination treatment, did not show any remaining significant decrease; the IgG levels had spontaneously recovered. Further, the IgG increase in patient (A18) had a clinical correlate, a reinfection with VZV 10 months after end of treatment. This patient had very low detectable titres at baseline but generated such IgG titres following the infection.

At end of therapy, the number of analyzed lymphocyte subsets was significantly lower in the alemtuzumab group. Interestingly, patients in the alemtuzumab group who had had a subclinical virus reactivation had significantly higher amounts of CD4+/CD3+, CD8+/CD3+ and CD3+/CD56+ cells at end of therapy, and for CD8+/CD3+ cells also at 8-12 months post-therapy, compared with patients from the same group without virus reactivation. This could correlate to an antiviral immune response and can be related to previous findings of expansions of CMV-specific CD8+ and CD4+ T cells in CLL patients [148, 220].

In conclusion, except for CMV, there was no increased incidence of subclinical or clinical virus reactivation following first-line SC alemtuzumab compared with the FC(R)-treated controls. All reactivations resolved spontaneously, indicating that CMV testing with PCR on symptomatic patients, instead of weekly monitoring, might be considered during alemtuzumab monotherapy in the first-line setting at experienced centres. The number of significant antivirus IgG decreases or increases did not differ significantly between the two treatment groups; however, the titre decreases noted in individual patients raises the question of whether such patients might need special infection-preventive measures to avoid reinfection. Patients ability to respond with titre and lymphocyte subset increases following clinical infection as described in this paper suggest that vaccination could be successful if proper vaccines/adjuvants are used.

9 FUTURE PERSPECTIVES

The evolution of new therapeutic options for CLL patients is promising. Monotherapy with alemtuzumab has been established in the first-line (selected patients, such as 17p-), as well as, later-line-setting (i.e. fludarabine-refractory disease). The results presented in this thesis have provided additional information on: long-term follow-up, efficacy in severe multi-agent refractory complex AIHA, simplified SC route of administration and in-depth analysis of immunological effects and its clinical consequences. However, several issues remain to be elucidated.

In pharmacokinetic studies, high alemtuzumab concentrations have been correlated to improved clinical responses [221-223] and it has been hypothesized that optimized treatment could be better achieved if alemtuzumab administration was based on serum levels [223]. This approach could possibly help to individualize alemtuzumab therapy and needs to be further investigated, in particular in relation to safety when the tumor burden is low.

Several strategies to achieve minimal residual disease (MRD)-free status by incorporating alemtuzumab into the approach have been evaluated, hypothesizing that this may translate into longer remission duration and survival [214, 222-225]. However, a major concern for the use of alemtuzumab as consolidation therapy is infections [214, 215]. To decrease toxicity and increase efficacy, new strategies for alemtuzumab consolidation therapy have to be developed.

Alemtuzumab still lacks an effective and safe "partner" for optimal combination therapy. In combination with FC, alemtuzumab have been associated with increased toxicity and such therapy is not recommended outside clinical studies [112]. In an ongoing first-line phase III study for biological high-risk CLL, patients are randomized between FC +/- alemtuzumab therapy (HOVON 68). The use of a relatively low alemtuzumab dose and selection of high-risk patients performed in this trial may hopefully result in a better outcome than what was seen in a previous study which was prematurely stopped because of increased toxicity in the FC plus alemtuzumab arm [121]. Interestingly, fludarabine plus alemtuzumab (in the absence of cyclophosphamide) was equally safe as fludarabine mono-therapy in a phase III trial [127].

In one phase II trial, combination therapy with alemtuzumab and rituximab has been tested as first-line CLL treatment [226]. Early data on 24 patients demonstrated a high ORR and CR rate, 92% and 79%, respectively. The promising data on the new anti-CD20 antibody, ofatumumab, makes this antibody, potentially, an even better combination candidate [130]. Theoretically, the alemtuzumab and ofatumumab combination could be a suitable alternative for CLL patients with severe BM failure, concomitant autoimmune cytopenia or 17p deletion, i.e. patients, in whom current chemotherapy-based combination treatments are either too toxic or ineffective. This will be explored in a up-coming study.

In a small series of patients with p53 defects and bulky lymphadenopathy, high-dose methylprednisolone in combination with alemtuzumab has shown promising results. This will be evaluated more thoroughly within the NCRI UKCLL06 trial [227]. The

immunomodulatory agent lenalidomide is another potential partner. Its effects on the immune system may result in synergistic effects when combined with alemtuzumab. Further, both lenalidomide and alemtuzumab have shown efficacy in CLL with 17p deletion making the combination especially interesting for this subgroup of patients. Our research group has currently a phase I/II study on the combination of alemtuzumab and lenalidomide in chemorefractory CLL patients.

Kinases have become effective targets for cancer treatment, especially in chronic myelogenous leukemia (CML). In recent years, kinases have been identified that are potential targets in CLL treatment as well, e.g. spleen tyrosine kinase (SYK) and phosphatidylinositol 3-kinase-δ (PI3Kδ) [228]. Ror-1 is another potential target as the receptor contains a tyrosine kinase domain [229]. New in vitro data on CAL-101, a selective PI3Kδ inhibitor, indicate that this might be a suitable drug to combine with alemtuzumab [230]. CAL-101 promoted apoptosis in CLL cells, independently of common prognostic markers (including deletion 17p). Further, it was demonstrated that this agent greatly decreases the production of several inflammatory cytokines and it was speculated that this effect might diminish the antibody related infusion toxicity (cytokine release syndrome). It was also shown that CAL-101 does not interfere with alemtuzumab-mediated ADCC.

CXC chemokine receptor type 4 (CXCR4) antagonists may disrupt adhesive interactions between bone marrow stromal cells and CLL cells and thereby reduce growth and drug-resistance signals to the malignant cells (reviewed in [231]). In vitro studies have demonstrated that the addition of CXCR4 antagonists may abrogate the protective effect of stroma on CLL cells and thereby increase the sensitivity to alemtuzumab [232]. Thus, the addition of CXCR4 antagonists to alemtuzumab therapy could potentially lead to improved efficacy by mobilization of less therapy resistant CLL cells from protective niches in bone marrow and secondary lymphoid tissues.

In conclusion, potential candidates for a alemtuzumab "partnership" are not lacking. The number of possible clinical trials is constantly increasing.

The role of alemtuzumab in treatment of autoimmune cytopenia is poorly investigated. We have demonstrated efficacy in a small series of patients with progressive CLL and AIHA refractory to conventional therapy (Paper II). However, larger series of patients are needed as well as formal consensus on the definitions of autoimmune cytopenia treatment evaluations [161].

The long-term risks of alemtuzumab immunosuppressive effects need to be evaluated in a larger series of patients, such as the patients participating in the CAM307 study [82]. This would improve the knowledge regarding the risks for development of secondary infections and malignancies. We have, in relation to CLL therapy, identified significant decreases of specific IgG content in serum (Paper V). Some of these patients might need revaccination/vaccination. However, CLL patients have weak antibody responses to vaccination [145] and vaccination with live attenuated organisms should, because of risk for side effects, be avoided [137]. Thus, development of improved vaccination strategies is needed. Notably, it has recently been shown that lenalidomide may augment both cellular and humoral responses to pneumococcal vaccination in relapsed myeloma patients [233].

This needs to be investigated also in CLL patients and our research grpup has started to explore lenalidomide as an adjuvant in an ongoing CLL antitumor vaccination program.

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