Department of Medicine at Huddinge, Division of Hematology
Karolinska Institutet, Stockholm, Sweden

CLINICAL PROGNOSTIC MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Stefan Norin

Stockholm 2011

Published by Karolinska Institutet.

ISBN 978-91-7457-361-9

Printed by
REPROPRINT AB
Stockholm 2011
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

Everything has been figured out, except how to live

Jean-Paul Sartre

To My Beloved Family

ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature B lymphocytes in blood, bone marrow and lymphoid tissues. The clinical course for the individual patient is still unpredictable despite decades of research on prognostic markers and staging systems. The aim of this thesis was to review the value of existing prognostic tools in order to develop new clinical prognostic markers for CLL and to assess the impact of clonal evolution and transformation in CLL in relation to biological markers and given therapy.

Paper I: This is a long-term follow-up of the first trial of subcutaneous alemtuzumab as first-line therapy in CLL. In order to assess duration of response, infectious complications and incidence of Richter transformation, a comparison was made with historical controls. Median time to treatment failure was 28 months for the alemtuzumab-treated patients compared to 17 months for the control group (not significant). Infectious complications were not more common in the alemtuzumab-treated patients despite profound and prolonged T-cell suppression. The rate of Richter transformation was similar between the groups.

Paper II: Clinical data of 77 patients included in five phase II trials at Karolinska University Hospital were analyzed to find out whether the use of computed tomography (CT) could add prognostic information to the Rai and Binet clinical staging systems. A high nodal tumor burden evaluated by CT correlated with a shorter time to next therapy and a trend towards shorter survival. Massive splenomegaly was associated with shorter overall survival and therapy-free survival.

Paper III: The expression of the estrogen receptors (ER) α, β1 and its splice variant β2 was evaluated in peripheral blood mononuclear cells (PBMC) from CLL patients and normal controls using immunocytochemistry. The expression of ERα was generally low whereas most PBMCs expressed ERβ1 in both patients and controls. ERβ2 expression was significantly more common in CLL. Patients with high expression (> 50% of PBMC) of ERβ1 and/or ERβ2 were more likely to need therapy during follow-up.

Paper IV: Paraffin-embedded splenic tissue samples were obtained from 62 patients with CLL or SLL to assess whether chromosomal aberrations in the spleen have a prognostic impact. The cytogenetic abnormalities 11q-, 13q-, 17p- and trisomy 12 were assessed by interphase FISH and compared with samples from blood and/or bone marrow. Patients with 11q- and 17p-deletions in the spleen had significantly shorter overall and therapy-free survival. Clonal evolution seemed to occur in some cases.

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. **Norin S**, Kimby E, Lundin J. Tumor burden status evaluated by computed tomography scan is of prognostic importance in patients with chronic lymphocytic leukemia. *Med Oncol 2010;27:820-825.*

III. Yakimchuk K, **Norin S**, Kimby E, Hägglund H, Warner M, Gustafsson JÅ. Upregulated estrogen receptor β2 in chronic lymphocytic leukemia. *Submitted.*

IV. **Norin S**, Wallblom A, Sander B, Kimby E. Cytogenetic abnormalities in the spleen detected by FISH in chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) patients. *Manuscript.*

* Shared first authorship

CONTENTS

LIST OF ABBREVIATIONS

ABL	v-abl Abelson murine leukemia viral oncogene
AIG	Autoimmune granulocytopenia
AIHA	Autoimmune hemolytic anemia
AlloSCT	Allogenic stem cell transplantation
ASCT	Autologous stem cell transplantation
ATM	Ataxia telangiectasia-mutated
Bcl-2	B-cell lymphoma 2
BCR	B-cell receptor
CAP	Cyclophosphamide/doxorubicin/prednisone
CCL	Chemokine (C-C motif) ligand
CD40L	CD40 ligand
CE	Clonal evolution
CHOP	Cyclophosphamide/doxorubicin/vincristine/prednisone
CLL	Chronic lymphocytic leukemia
CLLU1	CLL up-regulated gene 1
CMV	Cytomegalovirus
CpG	Cytosine-phosphoguanine dinucleotide
CR	Complete remission
CT	Computed tomography
CXCR	C-X-C chemokine receptor
DAPK1	Death-associated protein kinase 1
DAT	Direct antiglobulin test
EBMT	European group for Blood and Marrow Transplantation
EBV	Epstein-Barr virus
EBV-LPD	EBV-driven lymhoproliferative disease
ECOG	Eastern Cooperative Oncology group
EMA	European Medicines Agency
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERIC	European Research Initiative on CLL
FC	Fludarabine/cyclophosphamide
FCA	Fludarabine/cyclophosphamide/alemtuzumab
FCR	Fludarabine/cyclophosphamide/rituximab
FDA	U.S. Food and Drug Administration
FISH	Fluorescence in situ hybridization
FOXP3	Forkhead box P3
GC	Germinal center
GELF	Groupe d'Etude des Lymphomes Folliculaires
GR	Glucocorticoid receptor
HCDR3	Heavy chain complementarity-determing region 3
HL	Hodgkin's lymphoma
Ig	Immunglobulin
IGHD	Immunglobulin heavy diversity

IGHJ	Immunglobulin heavy join
IGHV	Immunglobulin heavy variable
IGKV	Immunglobulin kappa variable
IGLV	Immunglobulin lambda variable
ITP	Immune thrombocytopenic purpura
IWCLL	International Workshop on Chronic Lymphocytic Leukemia
KCDR3	Kappa chain complementarity-determing region 3
LCDR3	Lambda chain complementarity-determing region 3
LDH	Lactate dehydrogenase
LDT	Lymphocyte doubling time
LPL	Lipoprotein lipase
MBL	Monoclonal B-cell lymphocytosis
MDR	Minimally deleted region
MGUS	Monoclonal gammopathy of undetermined significance
MLUS	Monoclonal lymphocytosis of undetermined significance
MRD	Minimal residual disease
NF-κB	Nuclear factor kappa B
NLC	Nurse-like cells
OFAR	Oxaliplatin/fludarabine/cytarabine/rituximab
PC	Proliferation center
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression-free survival
PRCA	Pure red cell anemia
PS	Performance status
R-CHOP	Rituximab/cyclophosphamide/doxorubicin/vincristine/prednisone
RS	Richter's syndrome
SHM	Somatic hypermutation
SLL	Small lymphocytic lymphoma
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TLS	Tumor lysis syndrome
TNF	Tumor necrosis factor
TP53	Tumor protein p53
T_{reg}	Regulatory T cells
TTTF	Time to treatment failure
ZAP-70	Zeta-chain-associated protein kinase 70

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 INTRODUCTION

CLL is the most common chronic leukemia in the western world and is characterized by the accumulation of small mature appearing B lymphocytes in blood, bone marrow and lymphoid tissues [1]. In Sweden around 500 patients are diagnosed with CLL each year [2]. The median age at diagnosis is 72 years.

CLL is incurable with the therapeutic regimens currently in use except for the small minority of patients who are suitable for allogenic stem cell transplantation. The clinical course in CLL is extremely variable ranging from a stable disease over decades to a rapidly progressive disease despite intensive chemoimmunotherapy.

1.2 HISTORY

The term leukemia, “weisses blut” was first coined by Virchow in 1847 [3]. During the late 19th century enhanced staining techniques made it possible to separate different types of leukemia and in 1903 criteria for the diagnosis of chronic lymphocytic leukemia (CLL) were published [4]. However due to lack of accurate immunophenotyping, CLL could not be distinguished from other leukemic lymphomas. With the recognition of the antigens CD5 and CD23 a unique CLL phenotype could be defined. [5]

1.3 ETIOLOGY AND EPIDEMIOLOGY

Predisposing factors for CLL development remains largely unknown. Known carcinogenic agents such as tobacco smoke, ionizing radiation and chemical compounds appears to have little or no role in CLL. Some rather weak associations with pesticides, farming, animal breeding and magnetic fields have been published [6-8].

CLL incidence varies considerably throughout the world and is most common in countries with large populations of European descent, whereas the lowest incidence can be found in sub-Saharan Africa and south-east Asia. Japan is the country with the lowest recorded CLL rate. In contrast to studies of breast and colon cancer incidence, Asians who have migrated to the United States do not have a higher incidence of CLL [9,10].

There is a male predominance in CLL with a ratio of 1.8:1. Women have a better prognosis independent of age and stage of disease [11,12]. Most cases are sporadic, but there is strong support for a genetic component in CLL, as relatives to CLL patients have an eightfold increased risk to develop CLL [13]. In recent years, the hypothesis that an antigen-driven process contributes to CLL development has been supported by experimental data [14].

1.4 DEFINITION OF CLL AND MONOCLONAL B CELL LYMPHOCYTOSIS

According to the iwCLL guidelines, CLL is defined as clonal B-lymphocytosis in blood (at least 5 x 10^9 lymphocytes/L) with a typical phenotype (CD5+, CD19+, CD20dim+, CD23+) [15]. In some healthy persons, small clonal B cell populations that phenotypically and cytogenetically resemble CLL can be detected [16]. This condition has been coined monoclonal B-cell lymphocytosis (MBL) or monoclonal lymphocytosis of undetermined significance (MLUS), in analogy with monoclonal gammopathy of undetermined significance (MGUS) [17]. The exact distinction between these mostly benign conditions and CLL is not biologically evident. A diagnosis of MBL requires fewer than 5 x 10^9 clonal B cells/L and no evidence of tissue involvement. MBL frequency increases with age, being virtually undetectable under 40 years of age but is present in 50-75% in people older than 90 years [18].

MBL is more common in first-degree relatives to patients with CLL [19]. A population-based prospective study using prediagnostic frozen samples shows that CLL is generally preceded by MBL [20].

Cases that present without lymphocytosis but with lymphadenopathy, splenomegaly and/or bone marrow involvement of clonal B lymphocytes with a typical CLL phenotype are classified as small lymphocytic lymphoma (SLL), a condition not distinguished from CLL in the current WHO classification guidelines [21]. In a large study, similar prognostic factors in CLL and SLL corresponded to a shorter survival [22].

1.5 BIOLOGY AND PATHOGENESIS

CLL cells resemble mature lymphocytes and have undergone rearrangement of the immunoglobulin genes. The cellular origin has not been clarified [23], but current evidence supports that CLL evolves from an antigen-experienced B cell.

The ability of the human immune system to create antibodies against a vast array of antigens is dependent on the B cell diversity created by the recombination of the V(D)J gene recombinations of the Ig loci. To further fine-tune the B cell response, somatic hypermutation (SHM) occurs during the maturation of the B cell. For many years, all CLL cases were thought to have mutated IGHV genes, but in the late 1990s it was shown that about 50% of CLL cases were unmutated [24]. Unmutated cases have IGHV genes with less than 2% somatic mutations and have a worse prognosis [25,26].

There is strong evidence that mutated CLL cells are derived from post-germinal center (GC) memory B cells which have encountered antigens [1]. Unmutated and mutated cases have a common characteristic gene expression signature [27,28], supporting the hypothesis that unmutated CLL also stems from antigen-experienced B cells. Nevertheless a distinct set of genes is differentially expressed in the two subtypes including zeta-chain-associated protein kinase 70 (ZAP-70) and lipoprotein lipase (LPL), both more expressed in unmutated cases [29,30]. In the gene expression study by Klein et al [27], most of the genes specifically expressed (or overexpressed) in CLL are involved in signal transduction pathways but Ror-1, an orphan tyrosine kinase receptor, is also highly expressed. Downregulated genes in CLL are, among others cyclin B and dihydrofolate reductase, mainly involved in cell cycle progression and metabolism, reflecting the quiescent phenotype of most CLL cells.

1.6 CHROMOSOMAL ABERRATIONS, GENE MUTATIONS AND CLONAL EVOLUTION

The first studies on chromosomal aberrations in CLL used karyotyping methods. Due to the low mitotic activity of CLL cells in blood, cytogenetic abnormalities could only be found in 40 to 50% of the cases, the most common being trisomy 12 and deletion of 13q [31]. New stimulating techniques to enhance metaphase cultivation have been developed, but are not yet in use in the routine clinical setting [32].

With interphase FISH on non-stimulated, non-dividing cells, recurrent genomic aberrations can be found in more than 80% of CLL cases [33], the most common being deletions in chromosome 13q, 11q or 17p and trisomy 12.

Deletion of 13q14 is found in about 50% of CLL cases. The minimally deleted region (MDR) involves two microRNAs (miRs); 15a and 16-1. Both these miRs have a nine base pair long nucleotide sequence that is complementary to the mRNA encoding the anti-apoptotic protein Bcl-2 [34]. This protein is upregulated in CLL and critical for tumor cell survival [35]. The expression of Bcl-2 is inhibited by interactions of miR-15a/16-1 with Bcl-2 transcripts; deletion of 13q14 can thus indirectly lead to increased expression of Bcl-2.

Deletion of the 11q22-q23 region encompasses the ATM (Ataxia telangiectasia-mutated) tumor suppressor gene; a central component of the DNA damage response pathway to double-strand breaks [36]. This deletion is present in about 20% of CLL cases.

Deletion of 17p13 affects the tumor suppressor gene TP53, which encodes the protein p53, a transcription factor critical for DNA damage repair and promotion of apoptosis after genotoxic stress [37]. This deletion is uncommon in early-stage disease, but more frequent in refractory CLL, affecting 4% and 31% of cases, respectively [1]. Cases with deletion of 17p frequently have mutations that inactivate TP53 on the other allele [38].

Trisomy 12 was the first recurrent abnormality described in CLL [39], present in 20% of CLL cases. The genes on chromosome 12 of potential importance for CLL pathogenesis are largely unknown, but CLLU1, a gene located at 12q22, has been shown to be uniquely overexpressed in CLL, even in cases without trisomy 12 [40].

Clonal evolution (CE) is defined as the accumulation or acquisition of genomic aberrations over time. Previous studies have shown that this occurs in a low frequency in CLL [41-43]. CE is more common in ZAP-70-positive and/or unmutated CLL [44].

An unusual feature of CLL is abnormally short telomeres despite the low proliferation of most CLL cells [45] and telomere length has shown to be a prognostic marker [46].

1.7 EPIGENETICS IN CLL

Aberrant DNA methylation has been shown to have a strong role in tumorigenesis, with genome-wide hypomethylation and regional hypermethylation of tumor suppressor gene promoters [47]. One study using genome-wide methylation analysis in CLL showed that 2% to 8% of cytosine-phosphoguanine dinucleotide (CpG) islands were aberrantly methylated compared with normal controls [48]. A strong correlation between promoter methylation and transcriptional silencing has been shown for certain individual gene promoters in CLL, for example death-associated protein kinase 1 (DAPK1) and ZAP-70 [49,50]. Methylation profiles have been shown to vary between different prognostic subsets of CLL [51].

1.8 MICROENVIRONMENT

CLL cells are long-lived in vivo compared to normal B cells [23], but rapidly undergo apoptosis in vitro unless co-cultivated with monocyte-derived nurse-like cells (NLC) or bone marrow stromal cells [52,53]. The apoptotic resistance in CLL is thus dependent on external factors rather than being an intrinsic attribute [54,55]. The view that CLL consists mainly of slowly accumulating cell arrested in G0/G1 phase has been challenged by an in vivo labelling study of CLL cells [56]. The results showed a higher cell turnover than expected and patients with high cell birth rates were more likely to have active or progressive disease. A high amount of CLL cells in S-phase has been shown to be associated with a short therapy-free and overall survival [57].

Proliferation centers (PC) have mainly been recognized in spleen and lymph nodes [58-60], in which a fraction of the CLL cells divide. In the PCs, the malignant lymphocytes are in contact with CD3+ T cells (mainly CD3+, CD4+ T cells), that express the CD40 ligand (CD40L) and support the growth of CLL cells through ligation of CD40, a member of the TNF receptor super family highly expressed in CLL [61]. Data supports that NF-κB activation of CLL cells also takes place in PCs [62].

CLL cells are not just passive bystanders in the microenvironment but can actively create a suitable microenvironment by secreting chemokines such as CCL3, CCL4 and CCL22 [63,64]. In addition CLL cells express functional CXCR3, CXCR4 and CXCR5 chemokine receptors that direct neoplastic cell chemotaxis in vitro [65]. Thus the CLL microenvironment is likely created by a dynamic interplay between neoplastic and normal bystander cells [66].

1.9 ANTIGENS AND MUTATIONAL STATUS IN CLL

There is a bias toward usage of certain Ig gene segments in CLL, in particular IGHV1-69, IGHV4-34, IGHV3-7, and IGHV3-21 [24,67]. Patients with CLL cells that use IGHV3-21 have relatively aggressive disease, even when mutated [68].

Some CLL cases share B cell receptors (BCRs) of remarkably similar amino acid sequence. These "stereotyped" BCRs exhibit highly homologous HCDR3s, often encoded by identical IGHV, IGHD, and IGHJ segments. Furthermore, many stereotyped BCRs use the same IGKV or IGLV. Thus the KCDR3s/LCDR3s are very similar in protein structure. The likelihood that these similar rearrangements could have occurred by chance is extremely remote ($< 1 \times 10^{-6}$ to $< 1 \times 10^{-12}$). Stereotyped receptors can be found in approximately 30% of CLL cases, and is more common in unmutated CLL [69,70]. The antigen specificities of CLL BCRs are often skewed towards polyreactivity, which permits binding to autoantigens as well as exoantigens [14,71]. Interestingly, there is molecular evidence for a link between persistence of Epstein-Barr virus (EBV) and cytomegalovirus (CMV) and usage of a specific stereotyped IGVH 4-34 receptor [72].

These findings suggest that antigen selection may play an important role in CLL pathogenesis and may also influence outcome [73].

1.10 OTHER RECEPTORS IN CLL

Apart from BCR and CD40, several other receptors are expressed in CLL cells. Toll like receptors (TLRs) recognize molecular patterns found in microbial components, trigger an immediate innate immune response in monocytes and granulocytes after infection [74] and acts as co-stimulatory signals that induce B cell maturation, proliferation and antibody production after pathogen recognition [75]. It has been shown that TLRs are expressed by CLL cells and that bacterial lipopeptides can protect CLL cells from spontaneous apoptosis through TLR signaling [76].

Expression of steroid hormone receptors has been reported in CLL. Glucocorticoid receptors (GR) are expressed in CLL [77], while estrogen receptor (ER) expression was shown in CLL in early studies, although with variable results [78-80]. In 1995 it was demonstrated that two different ERs exist, ERα and ERβ with opposing clinical effects

[81,82]. In addition several splice isoforms of ERβ have been described [83]. The most studied splice variant is ERβ2, which lacks ligand binding ability [84], but can form heterodimers with ERα inhibiting its binding to DNA. Normal B lymphocytes express ERβ [85], but so far no data regarding ERα and ERβ expression in CLL have been published.

2 DIAGNOSIS AND PROGNOSTIC MARKERS IN CLL

2.1 DIAGNOSIS

According to iwCLL guidelines the diagnosis of CLL requires an absolute B cell lymphocytosis (≥5 x 10^9/L) with a duration of at least three months with a typical phenotype of CD5+, CD19+, CD20dim+, CD23+ and a low level of surface immunoglobulin with a light chain restriction [15]. The phenotype needs to be confirmed by immunohistochemistry or preferably by flow cytometry. A bone marrow sample is not required to establish a diagnosis of CLL.

2.2 CLINICAL STAGING AND THE USE OF IMAGING TECHNIQUES IN CLL

The extremely heterogeneous course of CLL makes it difficult to predict the clinical course for individual patients. For more than 30 years the Rai and Binet staging systems have been in clinical use [86,87]. These systems are based on the presence or absence of lymphadenopathy, splenomegaly and bone marrow failure due to infiltrating CLL cells and are thus classifying patients according to the amount of tumor burden. However, contrary to the staging procedures in other lymphoid malignancies, Rai and Binet are based on clinical examination only and do not incorporate results from imaging techniques [88]. The use of CT scans in CLL have remained controversial and are not recommended for staging and response evaluation outside clinical studies according to the latest IWCLL guidelines [15].

Positron emission tomography in combination with CT (PET/CT) is seldom used in CLL. However PET/CT can be of value to exclude transformation to a high-grade lymphoma and may be used to direct lymph node biopsies [89].

2.3 CYTOGENETICS

Cytogenetic analysis has emerged as an important prognostic tool and findings of certain aberrations can predict response to therapy. In clinical praxis, the four most common aberrations in CLL are assessed by interphase FISH. A deletion of 13q is associated with a favorable course if no other aberrations are present [33], but the prognosis might be related to the percentage of 13q deleted CLL cells [90]. In a recent study, large deletions affecting genes outside the MDR on chromosome 13 were associated with inferior outcome [91]. It has been proposed that 13q deletions can be categorized into two types; Type I deletions targeting a region involving the MDR, whereas larger Type II deletions also include the RB1 gene locus. Type II deletions are more common in patients with high Rai stage and after therapy [92].

Trisomy 12 was considered as a poor prognostic marker in early karyotyping studies [31], however this has not been confirmed in later studies using FISH [33,93] and now correlates to an intermediate prognosis.

Deletion of 11q has been associated with extensive lymphadenopathy and a negative impact on progression-free and overall survival [33,93,94]. However, data from a recent trial indicate that a deletion of 11q22-q23 is not an adverse prognostic factor for patients receiving immunochemotherapy with FCR [95].

Deletion of 17p implies a dismal prognosis with poor response to chemotherapy including rituximab-containing regimens [33,95]. However, alemtuzumab can be effective in 17p-deleted cases [96].

Most patients with a 17p deletion have a TP53 mutation of the other allele [38]. Recent data suggest that the clinical behavior of CLL with a monoallelic TP53 inactivation due to mutation is similar to cases with 17p deletion [38,97]. However, a small subgroup of patients with TP53 abnormalities has a more indolent course [97,98]. Mutations in the ATM gene without corresponding deletions are also associated with impaired response to therapy and survival [99].

2.4 MUTATIONAL STATUS

IGHV gene mutational status is a prognostic factor in CLL and patients with unmutated CLL have an inferior outcome [25,26]. Usage of specific IGHV genes can also influence outcome. CLL cases with IGHV 3-21 usage have an inferior prognosis regardless of mutational status [68]. Cases with IGHV 4-34 usage generally have an indolent course [72]. Interestingly, IGHV 4-39 expression is associated with an increased risk for transformation to diffuse large B-cell lymphoma (DLBCL) [100].

Analysis of mutational status is technically challenging for clinical routine use. Therefore possible surrogate markers have been evaluated with CD38 and ZAP-70 being the most studied.

2.5 CD38

CD38 is a cell surface molecule that is expressed in approximately one-third of CLL cases, mainly in patients with unmutated IGHV genes and is correlated to a worse outcome [25]. The expression of CD38 is regulated by the tumor microenvironment and can be considered as an activation marker [101] and the expression level can change over time [102]. There is currently no consensus about which cut-off value to use, which limits the clinical value of CD38 analysis in CLL.

2.6 ZAP-70 AND LPL

Microarray studies of mutated and unmutated cases have shown that the gene expression pattern is similar [27]. However some genes are differentially expressed. ZAP-70, a SYK-family protein tyrosine kinase with a key role in signalling via the T-cell receptor [103], is generally more expressed in unmutated cases and associated with an impaired prognosis [29]. Discordance between mutational status and ZAP-70 status occurs in up to 25% of cases [104]. ZAP-70 can be analyzed by flow cytometry or immunohistochemistry, but lack of standardization has hampered its routine clinical use. However within the settings of the ERIC group, a consensual technique has recently been described [105].

High expression of LPL, is also more common in unmutated cases and predicts for a poor response to chemotherapy [106] and has also been proposed as a surrogate marker for mutational status.

2.7 OTHER PROGNOSTIC MARKERS

Several other markers have shown to be of prognostic relevance in CLL. Elevated thymidine kinase and β2-microglobulin in serum are associated with a worse prognosis [107]. Lymphocyte doubling time (LDT) has also shown to be prognostically important [108] and a LDT of less than six months is a criterion of active disease requiring therapy according to the latest iwCLL guidelines [15].

3 CLINICAL MANIFESTATIONS AND TREATMENT OF CLL

3.1 CLINICAL MANIFESTATIONS

Most CLL patients are asymptomatic with a low tumor burden at diagnosis [109]. The most common symptom is fatigue [110]. Enlarged lymph nodes and increased susceptibility to infections are also common. Splenomegaly may occur, but massive symptomatic splenomegaly is more common later in the course of the disease. Bone marrow failure due to CLL infiltration with anemia, neutropenia and thrombocytopenia is a common reason for institution of therapy [15]. So-called B-symptoms; weight loss, night sweats and unexplained fever, can be experienced by patients with advanced disease. Immunologic hematologic complications such as autoimmune hemolytic anemia (AIHA) and immune thrombocytopenic purpura (ITP) are more common in CLL than in other lymphoid malignancies and can be the first symptoms of disease [111,112].

3.2 INDICATIONS FOR THERAPY

Historically the therapeutic intention for CLL patients has been palliative, focusing on reduction of disease-related symptoms. With the emergence of more effective therapeutic regimens, and evidence that improved remissions are associated with prolonged survival [113], the goal has shifted towards obtaining CRs or even eradication of minimal residual disease (MRD). MRD is generally assessed by PCR or flow cytometry in blood or bone marrow [114].

However more intensive therapy is often accompanied by higher toxicity and performance status (PS) and comorbidity must be carefully considered before therapy is instituted.

Several studies have shown that alkylator-based therapies in asymptomatic CLL do not prolong survival [115,116]. Therefore observation (“wait and watch”) is generally recommended. The criteria for active disease in the iwCLL guidelines [15] are used as recommendations for initiation of therapy:

- Evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia
- Massive (i.e., at least 6 cm below the left costal margin) or progressive or symptomatic splenomegaly.
- Massive nodes (i.e., at least 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) of less than 6 months. In patients with initial blood lymphocyte counts of less than $30*10^9$/L LDT should not be used as a single parameter to define a treatment indication.
- Autoimmune anemia and/or thrombocytopenia that is poorly responsive to corticosteroids or other standard therapy.
- Constitutional symptoms, defined as any one or more of the following disease-related symptoms or signs:
  - Unintentional weight loss of 10% or more within the previous 6 months;
  - significant fatigue (i.e., Eastern Cooperative Oncology Group (ECOG) PS 2 or worse; inability to work or perform usual activities);
  - fever higher than 38.0°C for two or more weeks without other evidence of infection; or
  - night sweats for more than 1 month without evidence of infection.

3.3 CHEMOTHERAPY

3.3.1 Chlorambucil

Chlorambucil is an alkylating agent used for treatment of CLL since the 1950s and has until recently been a cornerstone in CLL therapy [117]. However, the complete remission (CR) rate for chlorambucil is low, generally below 5% [118]. Therefore, chlorambucil is now generally reserved for elderly patients with severe comorbidity.

3.3.2 Purine analogs

Purine analogs (fludarabine, cladribine and pentostatin) were introduced in the 1990s.

With these agents some patients achieved CR, which previously were seldom seen in CLL. Moreover, phase III trials comparing purine analogs and chlorambucil could demonstrate a longer progression-free survival (PFS) in the purine analog arms, but no improvement in overall survival [118,119]. However, in a later trial with patients aged more than 65 years, no benefit in PFS was seen and there was a non-significant trend towards shorter OS [120]. Bendamustine, a purine analog/alkylator hybrid agent, has also been tested against chlorambucil in a phase III trial with a higher CR rate, longer PFS but without an improvement in OS [121].

3.3.3 Chemotherapy combinations

An early study compared CAP and ChOP ("French CHOP", with a lower doxorubicin dose than in standard CHOP) with single-agent fludarabine. ChOP and fludarabine had similar response rates and showed better results than CAP, but there were no differences in overall survival [122]. In vitro data suggested that additional cytotoxicity could be obtained by combining fludarabine with the alkylating agent cyclophosphamide (FC) [123], since DNA repair mechanisms induced by cyclophosphamide, are inhibited by fludarabine [124]. In a large non-randomized trial with FC, a high response rate and long PFS were demonstrated [125].

Three randomized studies have compared FC with fludarabine, all demonstrating an improved CR rate and increased PFS, but no benefit in overall survival [93,126,127]. No phase III studies comparing single-agent fludarabine and cladribine have been published, but in a randomized trial comparing FC with cladribine and cyclophosphamide, the two combinations had similar remission rates and toxicity [128].

3.4 MONOCLONAL ANTIBODIES

3.4.1 Alemtuzumab

Alemtuzumab is a humanized monoclonal antibody directed against the antigen CD52, which is highly expressed on normal as well as malignant B and T lymphocytes [129]. The approved route of administration is by intravenous infusion. Most alemtuzumab-treated patients will be affected by a cytokine release syndrome [130]. These symptoms are less pronounced if alemtuzumab is given by subcutaneous injections [131-133]. Alemtuzumab has been tested against chlorambucil in a phase III trial and showed higher OR and CR rates as well as a longer PFS, but did not improve OS [130]. Alemtuzumab has also been shown to be effective in patients with 17p deletion or mutated TP53 [96] and recent guidelines recommend that alemtuzumab should be considered as first-line therapy in patients with 17p deletions [134].

3.4.2 Rituximab and ofatumumab

Rituximab is a chimeric monoclonal antibody directed against the CD20 antigen expressed by mature B lymphocytes [135]. Compared to normal B lymphocytes and other types of indolent B cell lymphoma, expression of CD20 is lower in CLL [136]. In non-randomized trials low response rates was demonstrated [137]. Dose-escalation of rituximab led to better OR but a still a very low CR rate [138]. No randomized studies comparing single-agent rituximab with other agents have been reported.

Ofatumumab is an antibody targeting a different epitope of CD20 than rituximab and is approved for treatment of refractory CLL by both EMA and FDA. Studies in previously untreated patients are ongoing [139].

3.5 CHEMOIMMUNOTHERAPY

The FC + rituximab (FCR) regimen was initially evaluated in a large phase II study at the MDACC showing a CR rate of 70%, the highest CR rate recorded in published CLL trials at that time [140]. The German CLL8 phase III randomized trial between FC and FCR confirmed the high response rate and showed a longer PFS in the FCR arm. Moreover, FCR improved overall survival for the first time in a randomized CLL trial [95]. The patient cohort in this study consisted of relatively young physically fit patients with a median age of 61 years and 57% of the patients had an ECOG performance status of 0.

A study comparing FCR with FC + alemtuzumab (FCA) had to be stopped due to increased toxicity in the FCA arm. Moreover, no significant differences in response rate were recorded, but a trend towards a lower CR and OR in the FCA arm [141]. A phase III study comparing FC and FCA with a low dose of alemtuzumab in untreated high-risk patients (HOVON 68) has finished recruitment, but no results have yet been published. Several phase III studies comparing single-agent chlorambucil with addition of rituximab or ofatumumab are ongoing [139].

3.6 THERAPY AFTER RELAPSE

All CLL patients will eventually relapse after therapy, and many patients will require multiple courses of chemotherapy during the course of the disease. Patients with relapsed CLL are very heterogeneous regarding age, performance status, first-line therapy and duration of response after that therapy. This makes it difficult to conduct randomized trials and to transfer the results into the general CLL population. In one phase III trial that compared FCR with FC in previously treated patients, FCR improved OR, CR and PFS but not overall survival [142].

Ofatumumab has shown to be active in advanced CLL. In a study with patients refractory to both fludarabine and alemtuzumab or fludarabine-refractory with bulky lymphadenopathy OR were 58% and 47% respectively [143], which led to approval of ofatumumab for patients refractory to fludarabine and alemtuzumab.

3.7 STEM CELL TRANSPLANTATION

Allogenic stem cell (alloSCT) transplantation is currently the only potentially curative therapeutic option in CLL. However, due to advanced age and comorbidities in the general CLL population, only a minority of patients are eligible for alloSCT. The European group for Blood and Marrow Transplantation (EBMT) has published a consensus document stating that alloSCT is a procedure with evidence-based efficacy in poor-risk CLL and proposed indications for alloSCT in CLL [144]. Criteria for alloSCT in biologically fit patients are:

- Non-response or relapse < 1 year after purine analog therapy
- Relapse < 2 years after purine analog-containing combination therapy
- p53 abnormalities in patients with treatment indication

Today, reduced-intensity conditioning regimens are mostly used, with less toxicity and lower non-relapse mortality. Long-term follow-up has shown that sustained PFS can be obtained in about 40% of cases [145,146].

3.8 OTHER THERAPEUTIC MODALITIES

Splenectomy is mainly used in CLL complicated by refractory immune cytopenias, but is also a therapeutic option in patients with anemia/thrombocytopenia due to hypersplenism or symptomatic splenomegaly not responding to other therapy [147]. No prospective, randomized trials have evaluated the role of splenectomy in CLL, but retrospective single-center case series have shown that durable responses can be obtained [148].

Radiation therapy can be considered in patients with local symptoms from lymph nodes refractory to other therapy [149].

3.9 EMERGING THERAPIES

Despite the recent advances in therapy, CLL remains incurable in the majority of patients and the outlook is poor for patients with refractory disease or early relapse after purine analogue-containing regimens [150,151]. Moreover, patients who relapse after several lines of therapies mostly have a deteriorated immune function and a high risk of infections. However, several new compounds have shown promising results in early phase I-II trials.

Oblimersen is an antisense oligonucleotide that can downregulate the antiapoptotic Bcl-2 protein. In a phase III trial with patients who had relapsed after a fludarabine-containing regimen were randomized between FC with or without oblimersen. The rate of CR/nodular PR was higher in the FC+oblimersen arm, but no differences in PFS were recorded [152].

Flavopiridol is a broad cyclin-dependent kinase inhibitor that induces apoptosis in CLL cells by a p53-independent pathway [153]. Early studies were disappointing in CLL [154]. However, with a modified administration schedule a PR rate of 45% was noted in a study of refractory CLL patients, including response in 5 of 12 patients with 17p deletions [155]. Tumor lysis syndrome (TLS) sometimes requiring hemodialysis was the main dose-limiting toxicity.

Tyrosine kinase inhibitors (TKIs) have shown impressive effects in several malignancies [156-158]. In CLL, dasatinib, a drug approved for therapy of chronic myeloid leukemia, has so far been the most studied TKI. In addition to its inhibition of Abl, dasatinib binds to several other tyrosine kinases of which Lyn, a member of the Src family, is considered to the most important in CLL [159]. In a recent phase II trial in heavily pretreated patients, the response rate (all PRs) was 20% [160]. Fostamatinib disodium, a clinically available oral Syk inhibitor, has shown an objective response in 6 of 11 chemotherapy-resistant SLL/CLL patients [161].

Lenalidomide is a derivative of thalidomide and belongs to the class of immune modulating drugs. It has multiple effects, such as inhibition of TNF-α synthesis, immune cell modulation, angiogenesis inhibition and direct antineoplastic effects [162], however the exact mechanism of action in CLL remains unknown. Two phase II trials have evaluated lenalidomide as a single-agent in patients with refractory or relapsed CLL. [163,164]. Reported OR rates were 47% and 32% with 9% and 7% CRs, respectively. Tumor flare reactions and TLS were among the main side-effects. Therefore, the optimal dosing of lenalidomide for CLL has not yet been established.

4 IMMUNE DEFECTS AND INFECTIOUS COMPLICATIONS IN CLL

4.1 INTRODUCTION

Infectious complications are a main cause of morbidity in CLL and the primary cause of death in 50-60% of the patients [165]. The cause of the infectious susceptibility is multifactorial and involves secondary hypogammaglobulinemia, T cell defects, neutropenia and defects in the complement pathway. Respiratory tract bacterial infections are the most common, but also infections of the urinary tract and the skin can occur [165]. The most common bacterial infectious agents are Staphylococcus aureus, Streptococcus pneumoniae, Hemophilus influenzae, Klebsiella pneumoniae and Escherichia coli.

Purine analog-containing regimens and alemtuzumab have a pronounced T cell immunosuppressive effect. Therefore, with the emerging use of these agents, opportunistic infections like pneumocystis jiroveci, mycobacterial infections, CMV reactivation and fungal infections have become more common [166].

4.2 B CELL DEFECTS

Immunoglobulin synthesis is decreased in many CLL patients. The mechanisms are not entirely clear, but CLL cells can induce apoptosis in immunoglobulin-secreting plasma cells through CD95/CD95L interaction [167]. Hypogammaglobulinemia was present in 47% of untreated patients in one study [168], but in more advanced cases virtually all patients have decreased immunoglobulin levels [169]. IgG, IgA and IgM levels are all affected [170].

In addition, CLL patients respond poorly to vaccines, especially polysaccharide vaccines [171]. However, at least in patients with early stage disease, a conjugate pneumococcal vaccine could provide antibody responses in 40% of the patients [172].

4.3 T CELL DEFECTS

The absolute numbers of T-cells are increased in CLL, especially CD8+ cells, which lead to a reverse CD4/CD8 ratio and a skewed T-cell repertoire [173]. The number of CMV-specific CD4+ and CD8+ cells are markedly expanded and are particularly high in patients who receive chemotherapy, comprising up to 46% of all CD4+ cells [174,175].

T regulatory cells (T_{reg}) (CD4+ CD25+, FOXP3+), a subset with a main role in maintaining self-tolerance by suppressing autoreactive T-cells, are also increased in CLL [176]. Fludarabine therapy reduces the number of T_{reg}, which may explain the increased risk for severe autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia (ITP) after therapy with purine analogs [177].

5 AUTOIMMUNE CYTOPENIAS

5.1 INCIDENCE AND DIAGNOSTIC CHALLENGES

Compared with other lymphoid malignancies, CLL is frequently associated with autoimmune cytopenias affecting up to 25% of CLL patients at some time during their disease course [177], particularly AIHA, but also ITP, PRCA and very rarely autoimmune granulocytopenia (AIG) [112,177,178]. AIHA is the most common autoimmune complication affecting 5-10% of CLL cases and CLL is the most common cause of AIHA [179]. An incidence of ITP in 1-2% of CLL has been reported, whereas less than 1% of CLL patients develop PRCA.

There are no strict diagnostic criteria for any of the CLL-related cytopenias. A diagnosis of AIHA is usually based on findings of anemia, reticulocytosis, increased lactate dehydrogenase (LDH) and unconjugated bilirubin levels and a positive direct antiglobulin test (DAT). However, the interpretation of the results is often difficult, since many of the findings can be disease or therapy-related. To further complicate the issue, at least in patients receiving chemoimmunotherapy DAT-negative AIHA is not uncommon [180].

There are two main types of AIHA. In warm AIHA antibodies attach to the erythrocytes at 37°C, whereas in cold AIHA the antibodies directed against red blood cells are only active in lower temperatures. In CLL warm-antibody AIHA accounts for 90% of the cases, in contrast to other lymphoid malignancies [181]. Warm AIHA IgG antibodies are polyclonal whereas cold AIHA is mediated by monoclonal IgM antibodies produced by the CLL cells. [178].

For ITP the diagnostic criteria are even less clear, since autoantibody platelet tests lack both specificity and sensitivity [182]. A deep and unexpected drop in thrombocyte count despite normal or increased numbers of megakaryocytes in bone marrow and the absence of hypersplenism is generally considered as ITP. In PRCA, there is an absence of erythropoietic precursors in the bone marrow [170]. A diagnosis of AIG should be considered in cases with decreased neutrophil production with other possible causes ruled out [178].

5.2 RISK FACTORS AND PROGNOSTIC IMPORTANCE

Risk factors associated with autoimmune cytopenia are advanced disease, older age, ZAP-70 positivity and unmutated IGHV genes [182,183].

The association of CLL therapy and AIHA/ITP is well-known. Several reports of severe immune cytopenia after fludarabine monotherapy have been published [177,184]. Fludarabine in combination with cyclophosphamide was associated with a lower incidence of AIHA than fludarabine monotherapy in two studies (2.8 and 5% compared with 7.7% and 11%, respectively) [126,185]. A similar incidence of AIHA (6.5%) has been reported after FCR therapy [180]. A positive DAT before initiation of chemotherapy is a strong predictor for later development of AIHA, but also an independent negative prognostic factor in a recent clinical trial regardless of subsequent AIHA development [185]. Neither the Rai nor the Binet staging systems consider the origin of cytopenia when assigning the clinical stage for a given patient. However, in recent study patients with Binet C due to autoimmune cytopenias had an overall survival of 7.4 years compared with 3.7 years for patients with advanced stage due to an infiltrated bone marrow [186].

5.3 THERAPY FOR AUTOIMMUNE CYTOPENIAS

Therapy for AIHA or ITP in CLL has not been subject for randomized trials. Therefore therapeutic recommendations are largely based on expert opinions or consensus in national guidelines [147,187,188]. In general, first-line therapy for autoimmune cytopenia in patients with non-progressive CLL is steroids, in Sweden prednisolone 1mg/kg [147], which can induce responses in the majority of patients. However, durable responses are only seen in about a third of the cases [178]. Splenectomy can induce long-term responses in cases refractory to steroids [148]. However, splenectomy carries a small risk of mortality and morbidity, especially in elderly patients. Laparoscopic surgery is feasible in many cases [189].

Rituximab monotherapy has provided good results in AIHA unrelated to CLL in several small uncontrolled retrospective studies [188], and promising results have also been reported in CLL-related AIHA [190]. Rituximab is usually considered as a therapeutic alternative in patients too frail to undergo splenectomy or in relapses after removal of the spleen. A recent study combining rituximab, dexamethasone and cyclophosphamide showed a response rate of 89.5% in mostly pretreated patients [191]. Cyclosporin A and alemtuzumab are further options in refractory AIHA/ITP [177,192].

In patients with progressive CLL and concomitant AIHA/ITP data are more limited. Monotherapy with purine analogues, known to increase the risk of autoimmune cytopenia should be avoided [185]. Although combination therapy with FC or FCR carries a lower risk, there are currently no data to support their use in active autoimmune cytopenia. Therefore alternative regimens have been explored [182]. In a small study with R-CVP in 20 patients with autoimmune cytopenia and progressive CLL, the cytopenia responded in 95% of cases and the progressive CLL in 85%, but the duration of response was short [193].

6 RICHTER'S SYNDROME

Transformation of CLL to a more aggressive disease is called Richter's syndrome (RS) after Maurice N Richter who published the first case in 1928 [194]. Although initially limited to transformation to DLBCL, the term RS has been expanded to include other diagnoses such as prolymphocytic leukemia, Hodgkin's lymphoma and multiple myeloma [195]. The incidence of RS varies significantly between studies with 2-15% being reported, reflecting both heterogeneity of studied CLL populations and different biopsy policies [196]. Median time between CLL diagnosis and RS is 23-48 months [100,197]. Transformation should be suspected in cases with discordant rapid growth of lymph nodes, an unexpected rise in LDH without evidence of AIHA or the emergence of B-symptoms [198]. A diagnosis of RS should be made on biopsy material [15], since cytology cannot distinguish between CLL with numerous proliferation centers and transformation to DLBCL [196]. PET/CT can identify local transformation sites and might be useful to guide biopsies [89,199]. EBV-driven lymhoproliferative disease (EBV-LPD) can occur in CLL, especially after alemtuzumab-based therapy [200,201]. EBV-LPD can mimic RS and should be ruled out before a diagnosis of RS is set.

Stereotyped BCR usage is a risk factor for RS development [202], in particular the IGHV 4-39 subset [100]. Additional independent risk factors in this study were lymph nodes ≥ 3cm in diameter, CD38 expression and absence of 13q- aberrations. However, in another study no such correlations were found [203]. In one study of 401 patients, short telomere length was shown to be a risk factor for RS [45].

The profound immunosuppression caused by treatment with purine analogs and alemtuzumab has raised concern about an increased risk for RS. In a retrospective observational study from the Mayo clinic, there were an increased number of RS cases after therapy with purine analogs, 5.2%, compared to 1.8% in other patients [203]. However, in the large LRF CLL4 trial, there were no differences in RS incidence between patients in the chlorambucil, F and FC arms [93]. In the phase III trial comparing alemtuzumab and chlorambucil there were no recorded RS cases at a median follow-up of 24.6 months [130]. The phase II trial of FCR at MD Anderson had a RS incidence of 2.5% at a follow-up of 6 years [204].

Studies of clonal relationship between the developing DLBCL and CLL in RS have shown that two types of RS may exist, clonally related to CLL or unrelated. The former accounts for the majority of RS cases [205,206], and have a worse prognosis than unrelated DLBCL cases [207]. The outcome in RS is generally worse than in *de novo* DLBCL [198,208], with a median survival of only 8 months in a study from MDACC including 148 RS patients over a 30-year period [209]. The CR rate was only 12%, but a small number of patients had long-lasting remissions for up to 15 years.

No randomized trials regarding RS treatment has been made, therefore there is no consensus on the best therapeutic approach in RS [196]. R-CHOP, which can be considered a standard therapy for *de novo* DLBCL is often used in RS, however the results are far from satisfactory [209,210]. A regimen with oxaliplatin, fludarabine, cytarabine and rituximab, OFAR, has been tested in a phase I-II trial with increasing doses of oxaliplatin in patients with RS or refractory CLL. Twenty RS cases were enrolled with a median age of 66 years. Response rate was 50% with 20% being CRs. However, 6-month survival rate was only 60% [211]. Patients with CLL that has transformed to Hodgkin's lymphoma (HL) usually respond to standard HL therapy [195].

In follicular lymphoma transformed to DLBCL autologous stem cell transplantation (ASCT) is an established therapeutic alternative [212], however very little data is published regarding ASCT for RS [213]. AlloSCT can be a therapeutic option in selected patients. In the study from MDACC, seven patients who underwent alloSCT after obtaining at least PR after previous therapy, there was a 3-year estimated cumulative survival of 75% [209].

7 AIMS OF THE THESIS

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

II. To review the clinical benefit of CT scans in CLL in assessment of response. An additional aim was to asses if nodal and splenic tumor burden status detected by CT before therapy had any impact on therapy-free and overall survival.

III. To analyze the expression of the estrogen receptors ERα, ERβ1 and ERβ2 in blood from CLL patients and normal donors.

IV. To detect the chromosomal aberrations by interphase FISH; deletion of 13q14, 11q22-q23, 17p13 and trisomy 12 on formalin fixed, paraffin embedded material from splenic tissue in patients with CLL/SLL and to relate the findings to abnormalities in bone marrow or blood. Additional aims were to analyze if cytogenetic aberrations in the spleen had prognostic relevance and to assess clonal evolution.

8 MATERIALS AND METHODS

Paper I: This work is a long-term follow-up study of the previously published phase II trial of alemtuzumab given subcutaneously to previously untreated patients [132]. The Swedish Cancer Registry was used to identify historical control patients diagnosed with CLL during the period 1990-1997, before the aforementioned study started. 351 patients with CLL at four hospitals in Stockholm were identified. Patients (n=75), who matched the inclusion criteria for the study but received other first-line therapies and were not treated with alemtuzumab during the course of the disease were included as controls. Data were collected through case reviews. The two-sided Chi-square exact test was used to test differences in response rate and infections between study patients and historical controls. The Wilcoxon-Gehan exact test was used to test differences between curves representing time to treatment failure (TTTF) and time to Richter's transformation.

Paper II: Seventy-seven CLL patients included in five phase II trials, all using CT scans both before initiation of therapy and for response assessment [132,214-217], were included in the study. Assessment of response to therapy was based on information from the charts of the patients using the 1996 National Cancer Institute (NCI) Working Group criteria for CLL [218]. Since no established criteria for interpretation of CT scans in CLL has been published, International Working Group NHL criteria were used to define responses [219]. To assess the amount of nodal tumor burden, GELF criteria developed for follicular lymphoma were used [220]. No established criteria for grading of splenic enlargement in CLL exists, therefore the following system was used: no splenic enlargement, no palpable spleen but enlargement on CT (≥ 11cm in longest axis), and palpable spleen < 6 cm or > 6 cm under left costal margin respectively, the latter being a criterion for active disease (treatment indication) according to the NCI criteria [218]. The Wilcoxon-Gehan univariate test was used to test the possible correlation between nodal tumor burden status and overall and therapy-free survival. Multivariate analysis was performed using Cox regression.

Paper III: After informed consent, peripheral blood samples were obtained from 26 CLL patients and 30 healthy donors. The diagnosis of CLL was reviewed according to the updated CLL guidelines [15]. PBMCs were collected from the blood samples.

Expression of ERα, ERβ1 and ERβ2 were analyzed by immunocytochemistry. Clinical data were collected by case reviews. For comparison between groups, the two-sided Chi-square test was used for nominal variables and Mann-Whitney U test for ordinal and continuous measurements. Log-rank test was used to test for differences in therapy-free and overall survival.

Paper IV: Using existing registries at the Pathology department, Karolinska University Hospital, 62 patients with CLL (n=57) or SLL (n=5) who underwent splenectomy between 1989-2010 were identified. Patient data were obtained by chart review. Paraffin-embedded sections from spleens were obtained from the Pathology department, Karolinska University Hospital. Interphase FISH was used to evaluate chromosomal aberrations in splenic tissue and the results were compared with aberrations in blood and/or bone marrow before and after splenectomy, if available. Analyzed aberrations were deletion of 11q22-q23, 13q14, 17p13 and trisomy 12. To avoid false-positive results in paraffin-embedded sections due to cutting of the tissue, loss of the centromere in chromosome 12, an abnormality rarely occurring in CLL, was used as a marker to detect the amount of incomplete nuclei in the tissue sections. Flow cytometry data was used, or if not available, tumor involvement was assessed by immunohistochemistry. Spearman's rank correlation coefficient was used to correlate FISH results from splenic tissue with blood and bone marrow. The log-rank test was used to test differences between groups in time to next therapy and overall survival.

9 RESULTS, DISCUSSION AND CONCLUSIONS

9.1 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*, Norin* et al, Leukemia 2006;20:2204-07) (Shared first authorship)

This long-term follow-up of the phase II trial studied the TTTF, the frequency of infectious complications and the incidence of RS in 38 patients who received alemtuzumab by subcutaneous injections as first-line treatment. The results were compared with 75 consecutive matched historical controls. Median follow-up from initiation of therapy to last follow-up were 64 (13-102) months and 61 (4-132) months in the two groups, respectively. Median TTTF for alemtuzumab-treated patients were 28 months compared with 17 months for the controls (p=0.07). Responders to alemtuzumab therapy (n=33) had a median TTTF of 32 months, whereas 7 patients reaching CR had a median TTTF of 77 months.

No grade 4 infectious complications were observed in the alemtuzumab group during therapy. Grade 3 infections were observed in four patients (10%) and consisted of cytomegalovirus (CMV) reactivation that caused fever without pneumonitis (n=3) and Pneumocystis jiroveci pneumonia (n=1, in a patient without 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 infectious complications was not statistically significant.

During long-term unmaintained follow-up, 7/38 alemtuzumab-treated patients (18%) experienced 10 episodes of reversible grade 3 infections, including one symptomatic EBV-reactivation. No grade 4 or fatal infections were observed. In the control group with a shorter TTTF, the observed incidence was 8/75 (11%) including two fatal cases. The difference was not statistically significant.

In the alemtuzumab group, 6/38 (16%) developed RS compared with 9/75 (12%) in the other group. Median time from CLL diagnosis to transformation was 44 (20-75) months and 41 (14-94) months, respectively. Median time from start of first-line therapy to RS was 16 (3-32) months for the alemtuzumab-treated patients and 36 months (1-84) months in the matched historical control group (not significant).

The current work represents the first long-term follow-up of patients who have received first-line therapy with alemtuzumab for CLL. In the absence of randomized trials, retrospective historic comparisons may provide meaningful preliminary information. A significantly higher OR rate was observed in the alemtuzumab group than in the historical controls (p=0.01). The response rates and PFS and TTF in the alemtuzumab group were all comparable with the results from the phase III trial comparing alemtuzumab intravenously and chlorambucil [130]. In that study CMV PCR was performed weekly during therapy. Asymptomatic CMV reactivation was found in 52.4% whereas symptomatic infection was recorded 15.6% of the patients. However, only 6 (4.1%) patients had a grade 3 CMV activation compared with 3 (8%) in this study where CMV-PCR was taken only if early symptomatic CMV was suspected. It therefore seems reasonable not to monitor CMV outside clinical studies, and this has been implemented in the Swedish national guidelines for CLL [147].

In our study, transformation occurred in 16% of alemtuzumab-treated patients and in 12% of the patients in the historical control group. The difference was not statistically significant although the incidence of RS in this study is higher than in most other studies [196,198]. A possible explanation might be that all cases in this study had an active disease requiring therapy and the relatively long follow-up period. Many of the patients in the alemtuzumab had advanced disease, known to increase the risk for RS. However it cannot be ruled out that the profound immunosuppression caused by alemtuzumab can be a risk factor for development of RS. In the phase III trial comparing alemtuzumab and chlorambucil no RS cases were observed, but the follow-up time is still relatively short [130].

In conclusion, this long-term follow up study shows that despite the long-lasting immunosuppression, alemtuzumab appears to be effective and safe as first-line therapy with no apparent increase in serious infectious complications or RS compared to the historical control group.

9.2 PAPER II

Tumor burden status evaluated by computed tomography scan is of prognostic importance in patients with chronic lymphocytic leukemia.

(Norin et al, Med Oncol 2010;27:820-25)

In this study 77 patients, previously included in five phase II trials, all using CT for response assessment, were evaluated regarding response rate with and without CT.

Before therapy, enlarged lymph nodes and/or an enlarged spleen was noted by clinical examination in 60 (78%) and 32 (42%) patients, respectively and with CT in 64 (83%) and 54 (70%), respectively. Massive splenomegaly (≥6 cm below left costal margin) was noted in 11 patients. The GELF criteria for high nodal tumor burden assessed by CT were fulfilled in 19 patients, with three or more nodes ≥3 cm (n = 8), a single node ≥7 cm (n = 2), or both (n = 9). In 11 patients (58%), the bulky lymph nodes and/or high nodal tumor burden were not noted by clinical examination.

A retrospective evaluation of the response was possible for 69 of the 77 patients in the study. The CR rate using NCI criteria was 22% (n=15) and PR rate was 62% (n=43). Thus, the overall response rate (OR) was 84%. If data from the CT scans were taken into account, the response rates (CR, PR, and OR) dropped to 17% (12 patients), 59% (41 patients) and 76%, respectively. In eight patients, evaluation of response was not possible due to the lack of a CT evaluation in four patients, or early withdrawal from the study in four cases.

The time from initiation of first-line treatment to start of next therapy (TTT) was 29 months (1-156+) and overall survival was 70 (3-172+) months. Patients with a high lymphadenopathy tumor burden according to the GELF criteria had a significantly shorter time to next therapy, 12 months (1–78) compared to 35 months (1-156+) for patients with less advanced lymphadenopathy (p=0.002). In a multivariate analysis the two factors that had an impact on therapy-free survival were type of chemotherapy regimen and the degree of lymph node enlargement. There was also a non-significant trend for a shorter overall survival in the high nodal tumor burden group: 58 (9-172+) months compared to 75 (3-159+) months (p = 0.098).

In 23 patients without splenomegaly, the time to next therapy was 36 (5-156+)

months and overall survival was 82 months. In 22 patients with splenomegaly on CT scan only, the time to next therapy was 26 (1-133) months and overall survival 70 (9-159) months. The time to next therapy and overall survival for the 21 patients with palpable splenomegaly ≤ 6 cm below costal margin were 29 (1-123) and 63 (13-172+) months and in the 11 patients with palpable splenomegaly ≥6 cm below costal margin 16 (2-93) and 58 (3-117) months. There was a significant trend for an impaired therapy-free (p=0.037) and overall survival (p=0.017) depending on the grade of splenomegaly.

In recent years, the clinical approach to patients with CLL has changed remarkably. The quality of the remission obtained has shown to be of importance for survival [113]. Detection of minimal residual disease in blood and/or bone marrow has therefore gained a lot of attention [221]. In contrast, there has been relatively little interest in how tumor burden status prior to therapy affects response and survival in CLL. Eichhorst et al. reported that the CR rate was reduced by almost one-third when patients were routinely scanned with CT [126], similar to this study, where the CR rate was reduced by one-fifth. In a meta-analysis of three phase III trials results from CT scans did have a prognostic impact after administration of conventional chemotherapy, but not after chemoimmunotherapy [222], but it should be noted that CT were not mandatory and were performed in a minority of patients in the study. Therefore, a selection bias cannot be excluded. In contrast, in a study of clinical Rai stage 0 patients the presence of CT-verified abdominal lymphadenopathy correlated with a shorter time to progression [223].

In conclusion, our study shows that nodal tumor burden as well as splenomegaly assessed by CT before initiation of therapy predict the duration of response. Moreover, splenomegaly fulfilling the Cheson criteria for active disease had a negative impact on overall survival. The widely accepted GELF criteria for follicular lymphoma appear to be of value for assessment of nodal involvement in CLL and should be validated in a larger prospective study.

9.3 PAPER III

Upregulated estrogen receptor β2 in chronic lymphocytic leukemia

(Yakimchuk et al, submitted)

In this study, estrogen receptor expression was analyzed in 26 CLL patients and compared with 30 healthy controls. Nuclear expression of ERα in PBMCs was found in a minority of both CLL patients and normal controls (in 31% and 27% respectively), while ERβ1 was expressed in the majority in both groups (65% and 83%) with no significant differences between the two groups. Moreover, ERβ1 was expressed in more than 50% of the cells in 11 of 17 positive CLL patients.

A positive nuclear staining for ERβ2 was found in 18 out of 26 (69%) of CLL patients. In contrast, just 5 out of 30 (17%) normal controls expressed ERβ2 ($p<0.05$).

Immunofluorescence double staining of PBMCs from several CLL patients showed expression of ERβ2 in CD19-positive B-lymphocytes but not in CD3-positive T-cells. ERβ2 expression was also found in the cytoplasm of CD14+ and CD68+ cells in several CLL patients. Large CD14+ ERβ2+ cells were surrounded by smaller ERβ2+ cells. In ERβ2-positive normal controls the nuclear staining for ERβ2 was observed in the majority of cells of different morphology representing both lymphoid and myeloid cells in 4 out of 5 samples.

There were more previously treated CLL cases with low or no expression of ERβ2 ($p<0.05$), but for ERβ1 no such relation was noted. A trend towards a longer time between diagnosis and sampling was noted for the ERβ2-low cases, 86 months compared to 15 months for cases expressing ERβ2 in >50% of cells ($p=0.05$).

At last follow-up 14 CLL patients had required therapy after sampling. Patients with ERβ2 expression in more 50% of mononuclear cells (n=13) were more likely to need therapy ($p<0.05$), while patients expressing ERβ1 (n=11) in the majority of mononuclear cells had a significantly shorter time to therapy requirement than patients with less or no ERβ1 positive cells) (median 22 vs. 31 months, $p<0.05$). No differences regarding overall survival were seen between any ER expression groups.

This study shows for the first time that the expression of nuclear ERβ2 is much more common in CLL cells and that ERβ2 expression is associated with co-expression of

ERβ1 in both CLL and normal lymphocytes. Thus, most ERβ2-negative CLL patients lacked ERβ1 expression and the majority were also ERα−negative.

The importance of the ERs and estrogen hormone in the immune system has been demonstrated in animal models [224,225]. These results indicate an important role for ERβ in regulating the differentiation of pluripotent hematopoietic progenitor cells.

Expression of ERβ splice variants have been described in human immune system with expression of ERβ2 and ERβ5 in thymus and spleen. [226]. This is in line with our results in which 17% of healthy controls that expressed ERβ2.

In 50% of the CLL patients more than 50% of PBMCs were ERβ2-positive. The clinical characteristics of CLL patients in this study indicate that ERβ2 expression may correlate with a more indolent disease. Most of the ERβ2-positive CLL patients (83.3%) had not received any treatment, while 50 % of the ERβ2-negative CLL patients were treated. Interestingly, in CLL cases with ERβ2 expression in the majority of mononuclear cells only one of 13 patients had been treated compared with 6/13 (46 %) with less or no expression of ERβ2, but patients with high expression of ERβ1 and/or ERβ2 were more likely to require therapy during follow-up.

In addition to the findings in CLL tumor cells we also detected expression of ERβ2 in the cytoplasm of CD68+ and CD14+ cells in three CLL patients. These cells may be precursors of nurse-like cells (NLC) [52]. The finding of ERβ2 expression in these cells indicates that ERβ2 may also play a role in the microenvironment around CLL cells.

In conclusion, this study shows that ERβ1 is expressed in both patients and healthy controls, whereas ERβ2 is significantly more common in CLL. Based on our findings of ERβ expression in CLL cells, further investigation of the effect of estrogen and selective ERβ agonists might give rise to new therapeutic modalities in the future.

9.4 PAPER IV

Cytogenetic abnormalities in the spleen detected by FISH in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma

(Norin et al, manuscript)

In this study, cytogenetic abnormalities in splenic tissue were analyzed by FISH in 62 patients who have underwent splenectomy for AIHA/ITP (n=31) or symptomatic splenomegaly (n=30) (data missing in one patient). Median age at splenectomy was 68 (44-83) years. Chemo- and/or immunotherapy were administered to 51 of the 61 (records missing in one patient) patients before splenectomy. Median lymphocyte count at splenectomy was 16.4*10^9/L (0.1-265.3).

In all but two samples, FISH results from the splenic sections could be obtained. Cytogenetic aberrations were detected in 43 of 57 patients (75%); in 31 cases as a single abnormality and in 12 cases as multiple aberrations. The most common aberration was 13q-, detected in 60% of cases (a homozygous deletion was found in 16%) followed by 11q- in 21%, trisomy 12 in 9% and 17q- in 7%. Spleens from patients with autoimmune cytopenias more commonly had more than one cytogenetic aberration, in 8 out of 28 cases compared to 4 out of 28 cases in the splenomegaly group.

In 15 cases FISH samples from blood or bone marrow were available from time points both pre- and post- splenectomy, 6 additional cases had FISH data before splenectomy and 12 cases only after splenectomy. All patients with FISH abnormalities affecting >20% CLL cells in pre-splenectomy blood or bone marrow (n=19) were also found in the spleen, except in three cases.

All aberrations found in splenic tissue could be detected in later blood samples, when available. However, in the patient who had an accessory spleen removed 8 months after splenectomy, an 11q- clone present in splenic tissue was not detected in the accessory spleen. There was a significant correlation between the amount of cells with 13q and 11q deletions in spleen and blood/bone marrow ($p<0.01$ respectively). In 4 of 27 cases with follow-up samples after splenectomy, new abnormalities were detected in blood/bone marrow.

After a median follow-up of 43 months after splenectomy, 23 patients are alive and 39 have died. Only one patient died within 30 days after splenectomy. In total, 33 patients have required further therapy. Median time to next therapy was 9 (range 0-

255+) months. Patients with high-risk cytogenetic abnormalities in the spleen ((11q-) and/or (17p-)) (n=16) had a significantly shorter overall survival, 25 months compared to 58 months for patients with 13q-, trisomy 12 or a normal karyotype (n=21) ($p<0.05$). Time to next therapy was also shorter for the patients with high-risk cytogenetic abnormalities: 7 months compared to 19 months ($p<0.01$). Patients with 13q as a single aberration (n=23) had a significantly longer median overall survival of 85 months compared with 36 months for all other cases (n=34) ($p<0.05$), but there were no significant difference regarding time to next therapy (14 months and 9 months respectively, p=0.35). When combining these results it was possible to discern three groups with different overall survival ($p<0.05$). Patients with more than one chromosomal aberration had a tendency towards a shorter overall survival (27 months compared to 53 months for patients with one or no aberration (p=0.17) and shorter time to next therapy: 7 months and 14 months respectively (p=0.08).

Patients splenectomized due to AIHA or ITP were older, more heavily pretreated and had a shorter overall survival after splenectomy compared with patients who underwent splenectomy due to splenomegaly. There were no significant differences in time to next therapy. However, during a follow-up of 3 years or longer, 10 out of 30 patients who had splenomegaly as indication for surgery have not required any further therapy and 4 patients are alive with stable indolent disease ≥ 10 years after splenectomy. In the AIHA/ITP group only 5 out of 31 patients did not receive any additional chemotherapy for at least 3 years.

There are few studies regarding cytogenetic abnormalities in CLL in other tissues than blood and bone marrow. To our knowledge there is no published FISH data on tissue sections in CLL/SLL. However a study on fine needle aspirations from lymph nodes in CLL and SLL has been published [227] and demonstrated a short overall survival associated with deletion of 11q or 17p.

By analyzing cytogenetic aberrations in the spleen in patients with CLL/SLL we can now define three groups with different overall survival: deletion of 13q as a single aberration had a relatively favorable course, followed by patients with trisomy 12 or normal karyotype, whereas patients with a high-risk karyotype had a dismal prognosis.

In general, FISH results on splenic tissue correlated well to previous blood/bone marrow samples and CE was a relatively rare event compared to previous studies [43,44]. However, it should be noted that by setting the cut-off to 40%, necessary to avoid false-positive results due to tissue cutting and incomplete nuclei, smaller clones of 11q- and 17p- will not be detected, and this might therefore lead to an underestimation of CE in this study.

In our material most of the patients were in Binet C stage. Neither the Rai nor the Binet staging systems consider the origin of anemia or thrombocytopenia when assigning the stage. In a recent study, patients with Binet C stage due to autoimmune cytopenia had a longer survival than patients with advanced stage due to bone marrow infiltration [186]. Although not confirmed, it seems reasonable to suggest that cytopenia due to consumption in an enlarged spleen might also be a favorable subgroup of Binet stage C patients which might explain the apparently long therapy-free survival in some patients.

In conclusion, cytogenetic abnormalities in splenic CLL tissue are prognostically important. In our study of splenectomized patients, aberrations detected by FISH on paraffin-embedded spleen sections have an impact on overall survival and therapy-free survival. Clonal evolution was only detected in a few patients during long-term follow-up. In selected patients splenectomy can induce very long remissions.

10 FUTURE PERSPECTIVES

During the past decade there has been a significant increase in our knowledge of CLL. We have begun to understand the underlying mechanisms in the development of CLL and its predecessor MBL. Many new prognostic markers have been developed and can to a certain extent predict response to therapy. New treatment regimens have led to improved responses and long-term remissions are now common.

However despite the recent advancements, there are still many issues to be solved in the management of CLL. Standard CLL therapy is still not curative and most patients will eventually succumb to the disease. Several new phase III studies have shown impressive remission rates and for the first time a prolonged survival was demonstrated in one trial [95]. Long-term follow-up results are needed to confirm the results. The management of advanced disease has been far less studied, and with the exception of alemtuzumab, there are no good approved therapy alternatives for patients with 17p deletions [96]. In the first paper of this thesis we did a long-term follow up of the results from the first study with subcutaneous alemtuzumab as primary therapy confirming the good results [132], but also presented data regarding infectious complications and Richter's syndrome.

RS is a well-recognized complication to CLL, but data presented are mainly in the form of case series from single-centers [209]. With the emergence of national and/or European registries for CLL, it should be possible to present multi-center results for frequently used therapies such as R-CHOP-14 and also to finally answer the question whether modern immunosuppressive chemo- and/or immunotherapy can cause an increase in RS incidence.

Therapy for AIHA and ITP in CLL patients has not been investigated in any formal trial, but proper use of registry data will add a lot of information. Since both alemtuzumab and rituximab have provided good results in cases series [190,192], phase I/II trials with rituximab and/or alemtuzumab for autoimmune cytopenias can hopefully be initiated.

The new prognostic markers should be tested in clinical trials and it is also important to continuously evaluate the role of existing prognostic markers. In our second paper in this thesis we provided data regarding the use of CT for evaluation of remission and assessment of tumor burden in CLL. Several studies have now presented data regarding the use of CT, but with conflicting results [222,223,228], probably due to differences in patient material, given therapy and selection bias. Hopefully this outstanding question can be answered by examining results from phase III trials in which CT scans are mandatory.

FISH has emerged as the most used prognostic marker in CLL. However, the study that established FISH as a prognostic tool is largely based on results from untreated patients [33]. Ideally, the results should be reproduced by studying the incidence and relevance of cytogenetic aberrations in more advanced disease. Given the emerging knowledge about the importance of proliferation centers in CLL [58], comparing the interphase FISH from blood and bone marrow with lymphoid tissues might provide interesting results regarding clonal selection and evolution in different compartments. In our last study in the thesis, we showed that detection of common CLL aberrations in paraffin-embedded material is feasible and can provide prognostic information.

Despite the recent therapeutic advances in CLL new drugs are needed. Our findings that ERβ1 is expressed and ERβ2 is upregulated in CLL might provide a new target for therapy. ERβ agonists might be potential agents in the treatment of CLL, but monitoring for immunosuppressive effects might be necessary given the high rate of ERβ1 expression in normal lymphocytes. Genistein is one of the main isoflavones derived from soybeans with high binding affinity for ERβ1. Several studies have shown a suppressive effect of genistein on CLL and lymphoma cells in vitro alone or in combination with fludarabine [229,230]. Based on our findings of ERβ expression in CLL cells, studies of the effect of estrogen and selective ERβ agonists in CLL might give rise to new therapeutic modalities in the future.

11 ACKNOWLEDGEMENTS

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

Eva Kimby, my main supervisor for introducing me to the field of CLL. You have always been available and enthusiastic about my work and believing in my capacity. I would also like to thank you for spending endless hours improving our papers and sharing your enormous network. I would also like to thank you for your interest in the life of me and my family outside the clinical work and for welcoming us into your own.

Birgitta Sander, my co-supervisor for always giving me good advice. Your ability to catch sight of possible areas for research and to pose the right scientific questions to improve papers will always amaze me.

Hans Hägglund, my co-supervisor. You were the first person to lead me into the field of science and have guided my steps towards scientific maturation.

Eva Hellström-Lindberg and **Jan Bolinder** for creating excellent conditions for research at the Department of Medicine Huddinge, Karolinska Institutet.

Per Ljungman and **Eva Löfvenberg** for providing excellent conditions to combine clinical work and research at Hematology Center, Karolinska University Hospital.

All co-authors for your help with the manuscripts, especially:

Claes Karlsson, for great discussions and work together in our common paper. I look forward to further cooperation in the future.

Jeanette Lundin, for being an excellent co-author and for your skills in designing a study.

Anders Österborg, for creating resources for and encouraging our work in the first paper in this thesis.

Konstantin Yakimchuk, for being an excellent co-worker and co-author and for sharing your laboratory skills.

Jan-Åke Gustafsson for sharing your expertise on estrogen receptors.

Ann Wallblom, for great laboratory work and endless patience with my questions regarding FISH methods.

Hareth Nahi and **Björn Wahlin** for great discussions about hematology, life in general and much more during our lunch breaks.

Jan Palmblad, for being a scientific role-model with your broad knowledge of not just hematology, but science in general. You have taught me to look for and enjoy the most obscure articles in Nature and Science.

Christer Paul, for always standing up for your beliefs and for your great stories. I would also like to thank you and **Robert Hast** for your last-minute feedback on the thesis-kappa.

Eva Eriksson, Helena Pettersson, Ulrika Dahlin and **Hannele Kleemola** among all the nurses and other staff, for excellent help in taking care of the patients.

All other past and present colleagues at the Hematology Center – The "M64 team", among others **Stefan Deneberg, Daniel Tesfa, Martin Jädersten, Kalle Malmberg, Andreas Björklund, Johanna Ungerstedt, Johannes Admasie, Richard Lerner, Mats Merup, Lars Möllgård, Ragnhild Lindquist, Sören Lehmann, Maciej Machaczka, Bo Björkstrand** and **Per-Anders Broliden** for creating such a nice atmosphere not only promoting discussions about hematology and general science, but also about other things in life, especially during our Friday afternoon sessions.

All other friends and colleagues in the lymphoma team Huddinge/Solna not previously mentioned.

All members of the Swedish CLL Group for inspiration and encouragement.

All CLL patients for participating in our studies and reminding me why science is important.

Storkyrkans kör and Gustaf Sjökvist for keeping Monday evenings free from science and for letting me share great musical moments.

To all my friends outside the clinical and scientific world.

My parents**, Klas** and **Birgitta** for your life-long support and encouragement, always trusting in my capability.

My brothers **Karl, Martin** and my sister-in-law **Maria** for your support, and friendly competitions at the golf course.

My father- and mother-in-law **Lars** and **Birgitta** and all other family members for your support.

To my fantastic family, Kristina, Emma and **Erik**. Without your support and encouragement, this thesis would never have been completed. You really have showed me what is important in life.

This work has been supported by the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Insitutet, and by grants from ILEX Corporation, the Swedish Hematology Society's Roche grant for lymphoproliferative diseases, Swedish Cancer Fund, The Cancer Society in Stockholm, Robert A Welch foundation and the memorial fund of Dagmar Ferb.

12 REFERENCES

1. Zenz T, Mertens D, Kuppers R, Dohner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer. 2010;10:37-50.
2. Svenska Lymfomregistret. Rapport för 2000-2006. http://www.ocsyd.se/VP-verksamhet/Kvalitetsreg/SvenskalymfomregistretRapport2000-2006.pdf.
3. Virchow R. Weisses blut und milztumoren. Medicinische Zeitung 1847;16:9-15.
4. Türk W. Ein system der lymphomatosen. Wien Klinische Wochenschrift 1903;16:1073-85.
5. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia. 1994;8:1640-5.
6. Nanni O, Amadori D, Lugaresi C, et al. Chronic lymphocytic leukaemias and non-Hodgkin's lymphomas by histological type in farming-animal breeding workers: a population case-control study based on a priori exposure matrices. Occup Environ Med. 1996;53:652-7.
7. Amadori D, Nanni O, Falcini F, et al. Chronic lymphocytic leukaemias and non-Hodgkin's lymphomas by histological type in farming-animal breeding workers: a population case-control study based on job titles. Occup Environ Med. 1995;52:374-9.
8. Feychting M, Forssen U, Floderus B. Occupational and residential magnetic field exposure and leukemia and central nervous system tumors. Epidemiology. 1997;8:384-9.
9. Gale RP, Cozen W, Goodman MT, Wang FF, Bernstein L. Decreased chronic lymphocytic leukemia incidence in Asians in Los Angeles County. Leuk Res. 2000;24:665-9.
10. Haenszel W, Kurihara M. Studies of Japanese migrants. I. Mortality from cancer and other diseases among Japanese in the United States. J Natl Cancer Inst. 1968;40:43-68.
11. Oscier DG, Gardiner AC, Mould SJ, et al. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. Blood. 2002;100:1177-84.
12. Molica S. Sex differences in incidence and outcome of chronic lymphocytic leukemia patients. Leuk Lymphoma. 2006;47:1477-80.
13. Goldin LR, Bjorkholm M, Kristinsson SY, Turesson I, Landgren O. Elevated risk of chronic lymphocytic leukemia and other indolent non-Hodgkin's lymphomas among relatives of patients with chronic lymphocytic leukemia. Haematologica. 2009;94:647-53.
14. Lanemo Myhrinder A, Hellqvist E, Sidorova E, et al. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood. 2008;111:3838-48.
15. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008;111:5446-56.
16. Rawstron AC, Bennett FL, O'Connor SJ, et al. Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia. N Engl J Med. 2008;359:575-83.
17. Kimby E, Mellstedt H, Nilsson B, Bjorkholm M, Holm G. Differences in blood T and NK cell populations between chronic lymphocytic leukemia of B cell type (B-CLL) and monoclonal B-lymphocytosis of undetermined significance (B-MLUS). Leukemia. 1989;3:501-4.
18. Scarfo L, Dagklis A, Scielzo C, Fazi C, Ghia P. CLL-like monoclonal B-cell lymphocytosis: are we all bound to have it? Semin Cancer Biol. 2010;20:384-90.
19. de Tute R, Yuille M, Catovsky D, et al. Monoclonal B-cell lymphocytosis (MBL) in CLL families: substantial increase in relative risk for young adults. Leukemia. 2006;20:728-9.

20. Landgren O, Albitar M, Ma W, et al. B-cell clones as early markers for chronic lymphocytic leukemia. N Engl J Med. 2009;360:659-67.
21. Swerdlow S, Campo E, Harris N, et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC; 2008.
22. Tsimberidou AM, Wen S, O'Brien S, et al. Assessment of chronic lymphocytic leukemia and small lymphocytic lymphoma by absolute lymphocyte counts in 2,126 patients: 20 years of experience at the University of Texas M.D. Anderson Cancer Center. J Clin Oncol. 2007;25:4648-56.
23. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005;352:804-15.
24. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998;102:1515-25.
25. Damle RN, Fais F, Ghiotto F, et al. Chronic lymphocytic leukemia: a proliferation of B cells at two distinct stages of differentiation. Curr Top Microbiol Immunol. 2000;252:285-92.
26. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-54.
27. Klein U, Tu Y, Stolovitzky GA, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001;194:1625-38.
28. Rosenwald A, Alizadeh AA, Widhopf G, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med. 2001;194:1639-47.
29. Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood. 2003;101:4944-51.
30. Oppezzo P, Vasconcelos Y, Settegrana C, et al. The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. Blood. 2005;106:650-7.
31. Juliusson G, Oscier DG, Fitchett M, et al. Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. N Engl J Med. 1990;323:720-4.
32. Haferlach C, Bacher U. Cytogenetic methods in chronic lymphocytic leukemia. Methods Mol Biol. 2011;730:119-30.
33. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000;343:1910-6.
34. Cimmino A, Calin GA, Fabbri M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci U S A. 2005;102:13944-9.
35. Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. Blood. 1993;82:1820-8.
36. Bassing CH, Alt FW. The cellular response to general and programmed DNA double strand breaks. DNA Repair (Amst). 2004;3:781-96.
37. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell. 1997;88:323-31.
38. Zenz T, Krober A, Scherer K, et al. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. Blood. 2008;112:3322-9.
39. Gahrton G, Robert KH, Friberg K, et al. Cytogenetic mapping of the duplicated segment of chromosome 12 in lymphoproliferative disorders. Nature. 1982;297:513-4.
40. Buhl AM, Jurlander J, Jorgensen FS, et al. Identification of a gene on chromosome 12q22 uniquely overexpressed in chronic lymphocytic leukemia. Blood. 2006;107:2904-11.
41. Oscier D, Fitchett M, Herbert T, Lambert R. Karyotypic evolution in B-cell chronic lymphocytic leukaemia. Genes Chromosomes Cancer. 1991;3:16-20.

42. Shanafelt TD, Witzig TE, Fink SR, et al. Prospective evaluation of clonal evolution during long-term follow-up of patients with untreated early-stage chronic lymphocytic leukemia. J Clin Oncol. 2006;24:4634-41.
43. Berkova A, Zemanova Z, Trneny M, et al. Clonal evolution in chronic lymphocytic leukemia studied by interphase fluorescence in-situ hybridization. Neoplasma. 2009;56:455-8.
44. Stilgenbauer S, Sander S, Bullinger L, et al. Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival. Haematologica. 2007;92:1242-5.
45. Rossi D, Lobetti Bodoni C, Genuardi E, et al. Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia. Leukemia. 2009;23:1062-72.
46. Roos G, Krober A, Grabowski P, et al. Short telomeres are associated with genetic complexity, high-risk genomic aberrations, and short survival in chronic lymphocytic leukemia. Blood. 2008;111:2246-52.
47. Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science. 2003;300:455.
48. Rush LJ, Raval A, Funchain P, et al. Epigenetic profiling in chronic lymphocytic leukemia reveals novel methylation targets. Cancer Res. 2004;64:2424-33.
49. Raval A, Tanner SM, Byrd JC, et al. Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia. Cell. 2007;129:879-90.
50. Corcoran M, Parker A, Orchard J, et al. ZAP-70 methylation status is associated with ZAP-70 expression status in chronic lymphocytic leukemia. Haematologica. 2005;90:1078-88.
51. Kanduri M, Cahill N, Goransson H, et al. Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia. Blood. 2010;115:296-305.
52. Burger JA, Tsukada N, Burger M, et al. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood. 2000;96:2655-63.
53. Lagneaux L, Delforge A, Bron D, De Bruyn C, Stryckmans P. Chronic lymphocytic leukemic B cells but not normal B cells are rescued from apoptosis by contact with normal bone marrow stromal cells. Blood. 1998;91:2387-96.
54. Burger JA, Kipps TJ. Chemokine receptors and stromal cells in the homing and homeostasis of chronic lymphocytic leukemia B cells. Leuk Lymphoma. 2002;43:461-6.
55. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol. 2003;123:380-8.
56. Messmer BT, Messmer D, Allen SL, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J Clin Invest. 2005;115:755-64.
57. Kimby E, Mellstedt H, Nilsson B, et al. S-phase lymphocytes in chronic lymphocytic leukemia (CLL) in relation to immunoglobulin isotypes on the leukemic clone and to disease activity. Leukemia. 1987;1:432-6.
58. Gine E, Martinez A, Villamor N, et al. Expanded and highly active proliferation centers identify a histological subtype of chronic lymphocytic leukemia ("accelerated" chronic lymphocytic leukemia) with aggressive clinical behavior. Haematologica. 2010;95:1526-33.
59. Schmid C, Isaacson PG. Proliferation centres in B-cell malignant lymphoma, lymphocytic (B-CLL): an immunophenotypic study. Histopathology. 1994;24:445-51.
60. Lampert IA, Wotherspoon A, Van Noorden S, Hasserjian RP. High expression of CD23 in the proliferation centers of chronic lymphocytic leukemia in lymph nodes and spleen. Hum Pathol. 1999;30:648-54.
61. Wang D, Freeman GJ, Levine H, Ritz J, Robertson MJ. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. Br J Haematol. 1997;97:409-17.

62. Herreros B, Rodriguez-Pinilla SM, Pajares R, et al. Proliferation centers in chronic lymphocytic leukemia: the niche where NF-kappaB activation takes place. Leukemia. 2010;24:872-6.
63. Burger JA, Quiroga MP, Hartmann E, et al. High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. Blood. 2009;113:3050-8.
64. Ghia P, Strola G, Granziero L, et al. Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. Eur J Immunol. 2002;32:1403-13.
65. Lopez-Giral S, Quintana NE, Cabrerizo M, et al. Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination. J Leukoc Biol. 2004;76:462-71.
66. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood. 2009;114:3367-75.
67. Mauerer K, Zahrieh D, Gorgun G, et al. Immunoglobulin gene segment usage, location and immunogenicity in mutated and unmutated chronic lymphocytic leukaemia. Br J Haematol. 2005;129:499-510.
68. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood. 2002;99:2262-4.
69. Tobin G, Thunberg U, Karlsson K, et al. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood. 2004;104:2879-85.
70. Stamatopoulos K, Belessi C, Moreno C, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood. 2007;109:259-70.
71. Broker BM, Klajman A, Youinou P, et al. Chronic lymphocytic leukemic (CLL) cells secrete multispecific autoantibodies. J Autoimmun. 1988;1:469-81.
72. Kostareli E, Hadzidimitriou A, Stavroyianni N, et al. Molecular evidence for EBV and CMV persistence in a subset of patients with chronic lymphocytic leukemia expressing stereotyped IGHV4-34 B-cell receptors. Leukemia. 2009;23:919-24.
73. Rosen A, Murray F, Evaldsson C, Rosenquist R. Antigens in chronic lymphocytic leukemia--implications for cell origin and leukemogenesis. Semin Cancer Biol. 2010;20:400-9.
74. Muzio M, Mantovani A. Toll-like receptors (TLRs) signalling and expression pattern. J Endotoxin Res. 2001;7:297-300.
75. Lanzavecchia A, Sallusto F. Toll-like receptors and innate immunity in B-cell activation and antibody responses. Curr Opin Immunol. 2007;19:268-74.
76. Muzio M, Scielzo C, Bertilaccio MT, et al. Expression and function of toll like receptors in chronic lymphocytic leukaemia cells. Br J Haematol. 2009;144:507-16.
77. Simonsson B, Terenius L, Nilsson K. Glucocorticoid receptors, clinical characteristics, and implications for prognosis in chronic lymphocytic leukemia. Cancer. 1982;49:2493-6.
78. Rosen ST, Maciorowski Z, Wittlin F, et al. Estrogen receptor analysis in chronic lymphocytic leukemia. Blood. 1983;62:996-9.
79. Zaniboni A, Di Lorenzo D, Simoncini E, et al. Estrogen and progesterone receptor guideline for tamoxifen therapy in chronic lymphocytic leukemia: a pilot study. Acta Haematol. 1986;75:92-5.
80. Melo N, Hobday C, Dowsett M, et al. Oestrogen receptor (ER) analysis in B-cell chronic lymphocytic leukemia: correlation of biochemical and immunocytochemical methods. Leuk Res. 1990;14:949-52.
81. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A. 1996;93:5925-30.
82. Matthews J, Gustafsson JA. Estrogen signaling: a subtle balance between ER alpha and ER beta. Mol Interv. 2003;3:281-92.

83. Zhao C, Dahlman-Wright K, Gustafsson JA. Estrogen receptor beta: an overview and update. Nucl Recept Signal. 2008;6:e003.
84. Ogawa S, Inoue S, Watanabe T, et al. Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor ofestrogen action in human. Nucleic Acids Res. 1998;26:3505-12.
85. Shim GJ, Gherman D, Kim HJ, et al. Differential expression of oestrogen receptors in human secondary lymphoid tissues. J Pathol. 2006;208:408-14.
86. Rai KR, Sawitsky A, Cronkite EP, et al. Clinical staging of chronic lymphocytic leukemia. Blood. 1975;46:219-34.
87. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer. 1981;48:198-206.
88. Cheson BD, Pfistner B, Juweid ME, et al. Revised response criteria for malignant lymphoma. J Clin Oncol. 2007;25:579-86.
89. Bruzzi JF, Macapinlac H, Tsimberidou AM, et al. Detection of Richter's transformation of chronic lymphocytic leukemia by PET/CT. J Nucl Med. 2006;47:1267-73.
90. Van Dyke DL, Shanafelt TD, Call TG, et al. A comprehensive evaluation of the prognostic significance of 13q deletions in patients with B-chronic lymphocytic leukaemia. Br J Haematol. 2010;148:544-50.
91. Parker H, Rose-Zerilli MJ, Parker A, et al. 13q deletion anatomy and disease progression in patients with chronic lymphocytic leukemia. Leukemia. 2011;25:489-97.
92. Ouillette P, Erba H, Kujawski L, et al. Integrated genomic profiling of chronic lymphocytic leukemia identifies subtypes of deletion 13q14. Cancer Res. 2008;68:1012-21.
93. Catovsky D, Richards S, Matutes E, et al. Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. Lancet. 2007;370:230-9.
94. Dohner H, Stilgenbauer S, James MR, et al. 11q deletions identify a new subset of B-cell chronic lymphocytic leukemia characterized by extensive nodal involvement and inferior prognosis. Blood. 1997;89:2516-22.
95. Hallek M, Fischer K, Fingerle-Rowson G, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376:1164-74.
96. Lozanski G, Heerema NA, Flinn IW, et al. Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions. Blood. 2004;103:3278-81.
97. Gonzalez D, Martinez P, Wade R, et al. Mutational Status of the TP53 Gene As a Predictor of Response and Survival in Patients With Chronic Lymphocytic Leukemia: Results From the LRF CLL4 Trial. J Clin Oncol. 2011. [Epub ahead of print]
98. Tam CS, Shanafelt TD, Wierda WG, et al. De novo deletion 17p13.1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the M. D. Anderson and Mayo Clinic experience. Blood. 2009;114:957-64.
99. Austen B, Powell JE, Alvi A, et al. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. Blood. 2005;106:3175-82.
100. Rossi D, Cerri M, Capello D, et al. Biological and clinical risk factors of chronic lymphocytic leukaemia transformation to Richter syndrome. Br J Haematol. 2008;142:202-15.
101. Patten PE, Buggins AG, Richards J, et al. CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. Blood. 2008;111:5173-81.
102. Hamblin TJ, Orchard JA, Ibbotson RE, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood. 2002;99:1023-9.
103. Chu DH, Morita CT, Weiss A. The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol Rev. 1998;165:167-80.

104. Krober A, Bloehdorn J, Hafner S, et al. Additional genetic high-risk features such as 11q deletion, 17p deletion, and V3-21 usage characterize discordance of ZAP-70 and VH mutation status in chronic lymphocytic leukemia. J Clin Oncol. 2006;24:969-75.
105. European research initiative on CLL. Standardization & harmonization of cytometric analysis of ZAP70 and CD38. http://www.ericll.org/projects/ZAP70_CD38_harmonization.php.
106. Maloum K, Settegrana C, Chapiro E, et al. IGHV gene mutational status and LPL/ADAM29 gene expression as clinical outcome predictors in CLL patients in remission following treatment with oral fludarabine plus cyclophosphamide. Ann Hematol. 2009;88:1215-21.
107. Hallek M, Wanders L, Ostwald M, et al. Serum beta(2)-microglobulin and serum thymidine kinase are independent predictors of progression-free survival in chronic lymphocytic leukemia and immunocytoma. Leuk Lymphoma. 1996;22:439-47.
108. Montserrat E, Sanchez-Bisono J, Vinolas N, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. Br J Haematol. 1986;62:567-75.
109. Rozman C, Bosch F, Montserrat E. Chronic lymphocytic leukemia: a changing natural history? Leukemia. 1997;11:775-8.
110. Else M, Smith AG, Cocks K, et al. Patients' experience of chronic lymphocytic leukaemia: baseline health-related quality of life results from the LRF CLL4 trial. Br J Haematol. 2008;143:690-7.
111. D'Arena G, Cascavilla N. Chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. Leuk Lymphoma. 2007;48:1072-80.
112. Ward JH. Autoimmunity in chronic lymphocytic leukemia. Curr Treat Options Oncol. 2001;2:253-7.
113. Moreton P, Kennedy B, Lucas G, et al. Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. J Clin Oncol. 2005;23:2971-9.
114. Moreno C, Ritgen M, Rawstron A. Is MRD eradication a desirable goal in CLL? Best Pract Res Clin Haematol. 2010;23:97-107.
115. Dighiero G, Maloum K, Desablens B, et al. Chlorambucil in indolent chronic lymphocytic leukemia. French Cooperative Group on Chronic Lymphocytic Leukemia. N Engl J Med. 1998;338:1506-14.
116. Shustik C, Mick R, Silver R, et al. Treatment of early chronic lymphocytic leukemia: intermittent chlorambucil versus observation. Hematol Oncol. 1988;6:7-12.
117. Galton DA, Israels LG, Nabarro JD, Till M. Clinical trials of p-(di-2-chloroethylamino)-phenylbutyric acid (CB 1348) in malignant lymphoma. Br Med J. 1955;2:1172-6.
118. Rai KR, Peterson BL, Appelbaum FR, et al. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. N Engl J Med. 2000;343:1750-7.
119. Robak T, Blonski JZ, Kasznicki M, et al. Cladribine with prednisone versus chlorambucil with prednisone as first-line therapy in chronic lymphocytic leukemia: report of a prospective, randomized, multicenter trial. Blood. 2000;96:2723-9.
120. Eichhorst BF, Busch R, Stilgenbauer S, et al. First-line therapy with fludarabine compared with chlorambucil does not result in a major benefit for elderly patients with advanced chronic lymphocytic leukemia. Blood. 2009;114:3382-91.
121. Knauf WU, Lissichkov T, Aldaoud A, et al. Phase III randomized study of bendamustine compared with chlorambucil in previously untreated patients with chronic lymphocytic leukemia. J Clin Oncol. 2009;27:4378-84.
122. Leporrier M, Chevret S, Cazin B, et al. Randomized comparison of fludarabine, CAP, and ChOP in 938 previously untreated stage B and C chronic lymphocytic leukemia patients. Blood. 2001;98:2319-25.
123. Bellosillo B, Villamor N, Colomer D, et al. In vitro evaluation of fludarabine in combination with cyclophosphamide and/or mitoxantrone in B-cell chronic lymphocytic leukemia. Blood. 1999;94:2836-43.

124. Yamauchi T, Nowak BJ, Keating MJ, Plunkett W. DNA repair initiated in chronic lymphocytic leukemia lymphocytes by 4-hydroperoxycyclophosphamide is inhibited by fludarabine and clofarabine. Clin Cancer Res. 2001;7:3580-9.
125. O'Brien SM, Kantarjian HM, Cortes J, et al. Results of the fludarabine and cyclophosphamide combination regimen in chronic lymphocytic leukemia. J Clin Oncol. 2001;19:1414-20.
126. Eichhorst BF, Busch R, Hopfinger G, et al. Fludarabine plus cyclophosphamide versus fludarabine alone in first-line therapy of younger patients with chronic lymphocytic leukemia. Blood. 2006;107:885-91.
127. Flinn IW, Neuberg DS, Grever MR, et al. Phase III trial of fludarabine plus cyclophosphamide compared with fludarabine for patients with previously untreated chronic lymphocytic leukemia: US Intergroup Trial E2997. J Clin Oncol. 2007;25:793-8.
128. Robak T, Jamroziak K, Gora-Tybor J, et al. Comparison of cladribine plus cyclophosphamide with fludarabine plus cyclophosphamide as first-line therapy for chronic lymphocytic leukemia: a phase III randomized study by the Polish Adult Leukemia Group (PALG-CLL3 Study). J Clin Oncol. 2010;28:1863-9.
129. Treumann A, Lifely MR, Schneider P, Ferguson MA. Primary structure of CD52. J Biol Chem. 1995;270:6088-99.
130. Hillmen P, Skotnicki AB, Robak T, et al. Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia. J Clin Oncol. 2007;25:5616-23.
131. Stilgenbauer S, Zenz T, Winkler D, et al. Subcutaneous alemtuzumab in fludarabine-refractory chronic lymphocytic leukemia: clinical results and prognostic marker analyses from the CLL2H study of the German Chronic Lymphocytic Leukemia Study Group. J Clin Oncol. 2009;27:3994-4001.
132. Lundin J, Kimby E, Bjorkholm M, et al. Phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). Blood. 2002;100:768-73.
133. Karlsson C, Lundin J, Kimby E, et al. Phase II study of subcutaneous alemtuzumab without dose escalation in patients with advanced-stage, relapsed chronic lymphocytic leukaemia. Br J Haematol. 2009;144:78-85.
134. Osterborg A, Foa R, Bezares RF, et al. Management guidelines for the use of alemtuzumab in chronic lymphocytic leukemia. Leukemia. 2009;23:1980-8.
135. Rose AL, Smith BE, Maloney DG. Glucocorticoids and rituximab in vitro: synergistic direct antiproliferative and apoptotic effects. Blood. 2002;100:1765-73.
136. Almasri NM, Duque RE, Iturraspe J, Everett E, Braylan RC. Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukemia. Am J Hematol. 1992;40:259-63.
137. Huhn D, von Schilling C, Wilhelm M, et al. Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. Blood. 2001;98:1326-31.
138. O'Brien SM, Kantarjian H, Thomas DA, et al. Rituximab dose-escalation trial in chronic lymphocytic leukemia. J Clin Oncol. 2001;19:2165-70.
139. http://www.clinicaltrials.gov.
140. Keating MJ, O'Brien S, Albitar M, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. J Clin Oncol. 2005;23:4079-88.
141. Lepretre S, Aurran T, Mahé B, et al. Immunochemotherapy with fludarabine (F) cyclophosphamide (C) and rituximab (R) (FCR) versus fludarabine (F) cyclophosphamide (C) and mabcampath (CAM) (FCCAM) in previously untreated patients (pts) with advanced B-chonic lymphocytic leukemia. (B-CLL): experience on safety and efficacy with a randomised multicenter phase III trial of the French cooperative group in CLL and WM (FCGCLL/MW) and the “Groupe ouest-est d'etudes des leucemés aigues et autres maladies du sang” (GOELAMS): CLL2007FMP (for fit medically patients). Haematologica. 2009;94:S67.
142. Robak T, Dmoszynska A, Solal-Celigny P, et al. Rituximab plus fludarabine and cyclophosphamide prolongs progression-free survival compared with fludarabine

and cyclophosphamide alone in previously treated chronic lymphocytic leukemia. J Clin Oncol. 2010;28:1756-65.
143. Wierda WG, Kipps TJ, Mayer J, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol. 2010;28:1749-55.
144. Dreger P, Corradini P, Kimby E, et al. Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus. Leukemia. 2007;21:12-7.
145. Sorror ML, Storer BE, Sandmaier BM, et al. Five-year follow-up of patients with advanced chronic lymphocytic leukemia treated with allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. J Clin Oncol. 2008;26:4912-20.
146. Dreger P, Dohner H, Ritgen M, et al. Allogeneic stem cell transplantation provides durable disease control in poor-risk chronic lymphocytic leukemia: long-term clinical and MRD results of the German CLL Study Group CLL3X trial. Blood. 2010;116:2438-47.
147. Swedish CLL Group. CLL National Guidelines, updated version 2010-12-13. http://www.swecll.org/Nationella-riktlinjer.
148. Neal TF, Jr., Tefferi A, Witzig TE, et al. Splenectomy in advanced chronic lymphocytic leukemia: a single institution experience with 50 patients. Am J Med. 1992;93:435-40.
149. Chan EK, Fung S, Gospodarowicz M, et al. Palliation by Low-dose Local Radiation Therapy for Indolent Non-Hodgkin Lymphoma. Int J Radiat Oncol Biol Phys. 2010 [Epub ahead of print].
150. Keating MJ, O'Brien S, Kontoyiannis D, et al. Results of first salvage therapy for patients refractory to a fludarabine regimen in chronic lymphocytic leukemia. Leuk Lymphoma. 2002;43:1755-62.
151. Tam CS, O'Brien S, Lerner S, et al. The natural history of fludarabine-refractory chronic lymphocytic leukemia patients who fail alemtuzumab or have bulky lymphadenopathy. Leuk Lymphoma. 2007;48:1931-9.
152. O'Brien S, Moore JO, Boyd TE, et al. Randomized phase III trial of fludarabine plus cyclophosphamide with or without oblimersen sodium (Bcl-2 antisense) in patients with relapsed or refractory chronic lymphocytic leukemia. J Clin Oncol. 2007;25:1114-20.
153. Byrd JC, Shinn C, Waselenko JK, et al. Flavopiridol induces apoptosis in chronic lymphocytic leukemia cells via activation of caspase-3 without evidence of bcl-2 modulation or dependence on functional p53. Blood. 1998;92:3804-16.
154. Byrd JC, Peterson BL, Gabrilove J, et al. Treatment of relapsed chronic lymphocytic leukemia by 72-hour continuous infusion or 1-hour bolus infusion of flavopiridol: results from Cancer and Leukemia Group B study 19805. Clin Cancer Res. 2005;11:4176-81.
155. Byrd JC, Lin TS, Dalton JT, et al. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. Blood. 2007;109:399-404.
156. O'Brien SG, Guilhot F, Larson RA, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003;348:994-1004.
157. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med. 2005;353:123-32.
158. Geyer CE, Forster J, Lindquist D, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med. 2006;355:2733-43.
159. Contri A, Brunati AM, Trentin L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. J Clin Invest. 2005;115:369-78.
160. Amrein PC, Attar EC, Takvorian RW, et al. Phase II Study of Dasatinib in Relapsed or Refractory Chronic Lymphocytic Leukemia. Clin Cancer Res. 2011 [Epub ahead of print].

161. Friedberg JW, Sharman J, Sweetenham J, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. Blood. 2010;115:2578-85.
162. Bartlett JB, Dredge K, Dalgleish AG. The evolution of thalidomide and its IMiD derivatives as anticancer agents. Nat Rev Cancer. 2004;4:314-22.
163. Chanan-Khan A, Miller KC, Musial L, et al. Clinical efficacy of lenalidomide in patients with relapsed or refractory chronic lymphocytic leukemia: results of a phase II study. J Clin Oncol. 2006;24:5343-9.
164. Ferrajoli A, Lee BN, Schlette EJ, et al. Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood. 2008;111:5291-7.
165. Wadhwa PD, Morrison VA. Infectious complications of chronic lymphocytic leukemia. Semin Oncol. 2006;33:240-9.
166. Morrison VA. Infectious complications of chronic lymphocytic leukaemia: pathogenesis, spectrum of infection, preventive approaches. Best Pract Res Clin Haematol. 2010;23:145-53.
167. Sampalo A, Brieva JA. Humoral immunodeficiency in chronic lymphocytic leukemia: role of CD95/CD95L in tumoral damage and escape. Leuk Lymphoma. 2002;43:881-4.
168. Rozman C, Montserrat E, Vinolas N. Serum immunoglobulins in B-chronic lymphocytic leukemia. Natural history and prognostic significance. Cancer. 1988;61:279-83.
169. Hansen DA, Robbins BA, Bylund DJ, et al. Identification of monoclonal immunoglobulins and quantitative immunoglobulin abnormalities in hairy cell leukemia and chronic lymphocytic leukemia. Am J Clin Pathol. 1994;102:580-5.
170. Dearden C. Disease-specific complications of chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program. 2008:450-6.
171. Sinisalo M, Aittoniemi J, Oivanen P, et al. Response to vaccination against different types of antigens in patients with chronic lymphocytic leukaemia. Br J Haematol. 2001;114:107-10.
172. Sinisalo M, Vilpo J, Itala M, et al. Antibody response to 7-valent conjugated pneumococcal vaccine in patients with chronic lymphocytic leukaemia. Vaccine. 2007;26:82-7.
173. Scrivener S, Goddard RV, Kaminski ER, Prentice AG. Abnormal T-cell function in B-cell chronic lymphocytic leukaemia. Leuk Lymphoma. 2003;44:383-9.
174. Pourgheysari B, Bruton R, Parry H, et al. The number of cytomegalovirus-specific CD4+ T cells is markedly expanded in patients with B-cell chronic lymphocytic leukemia and determines the total CD4+ T-cell repertoire. Blood. 2010;116:2968-74.
175. Mackus WJ, Frakking FN, Grummels A, et al. Expansion of CMV-specific CD8+CD45RA+CD27- T cells in B-cell chronic lymphocytic leukemia. Blood. 2003;102:1057-63.
176. Beyer M, Kochanek M, Darabi K, et al. Reduced frequencies and suppressive function of CD4+CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. Blood. 2005;106:2018-25.
177. Hamblin TJ. Autoimmune complications of chronic lymphocytic leukemia. Semin Oncol. 2006;33:230-9.
178. Zent CS, Kay NE. Autoimmune complications in chronic lymphocytic leukaemia (CLL). Best Pract Res Clin Haematol. 2010;23:47-59.
179. Engelfriet CP, Overbeeke MA, von dem Borne AE. Autoimmune hemolytic anemia. Semin Hematol. 1992;29:3-12.
180. Borthakur G, O'Brien S, Wierda WG, et al. Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab--incidence and predictors. Br J Haematol. 2007;136:800-5.
181. Hauswirth AW, Skrabs C, Schutzinger C, et al. Autoimmune hemolytic anemias, Evans' syndromes, and pure red cell aplasia in non-Hodgkin lymphomas. Leuk Lymphoma. 2007;48:1139-49.
182. Hodgson K, Ferrer G, Montserrat E, Moreno C. Chronic lymphocytic leukemia and autoimmunity: a systematic review. Haematologica. 2011 [Epub ahead of print].

183. Visco C, Novella E, Peotta E, et al. Autoimmune hemolytic anemia in patients with chronic lymphocytic leukemia is associated with IgVH status. Haematologica. 2010;95:1230-2.
184. Bastion Y, Coiffier B, Dumontet C, Espinouse D, Bryon PA. Severe autoimmune hemolytic anemia in two patients treated with fludarabine for chronic lymphocytic leukemia. Ann Oncol. 1992;3:171-2.
185. Dearden C, Wade R, Else M, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. Blood. 2008;111:1820-6.
186. Moreno C, Hodgson K, Ferrer G, et al. Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance. Blood. 2010;116:4771-6.
187. Gribben JG. How I treat CLL up front. Blood. 2010;115:187-97.
188. Lechner K, Jager U. How I treat autoimmune hemolytic anemias in adults. Blood. 2010;116:1831-8.
189. Hill J, Walsh RM, McHam S, Brody F, Kalaycio M. Laparoscopic splenectomy for autoimmune hemolytic anemia in patients with chronic lymphocytic leukemia: a case series and review of the literature. Am J Hematol. 2004;75:134-8.
190. D'Arena G, Laurenti L, Capalbo S, et al. Rituximab therapy for chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. Am J Hematol. 2006;81:598-602.
191. Rossignol J, Michallet AS, Oberic L, et al. Rituximab-cyclophosphamide-dexamethasone combination in the management of autoimmune cytopenias associated with chronic lymphocytic leukemia. Leukemia. 2011;25:473-8.
192. 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;21:511-4.
193. Bowen DA, Call TG, Shanafelt TD, et al. Treatment of autoimmune cytopenia complicating progressive chronic lymphocytic leukemia/small lymphocytic lymphoma with rituximab, cyclophosphamide, vincristine, and prednisone. Leuk Lymphoma. 2010;51:620-7.
194. Richter MN. Generalized Reticular Cell Sarcoma of Lymph Nodes Associated with Lymphatic Leukemia. Am J Pathol. 1928;4:285-92 7.
195. Tsimberidou AM, Keating MJ. Richter's transformation in chronic lymphocytic leukemia. Semin Oncol. 2006;33:250-6.
196. Molica S. A systematic review on Richter syndrome: what is the published evidence? Leuk Lymphoma. 2010;51:415-21.
197. Seymour J, Campbell J. Richter's syndrome. In: Cheson BD, ed. Chronic Lymphoid Leukemias 2nd ed. Basel, Switzerland: Marcel Dekker; 2001:459-83.
198. Tsimberidou AM, Keating MJ. Richter syndrome: biology, incidence, and therapeutic strategies. Cancer. 2005;103:216-28.
199. Bodet-Milin C, Kraeber-Bodere F, Moreau P, et al. Investigation of FDG-PET/CT imaging to guide biopsies in the detection of histological transformation of indolent lymphoma. Haematologica. 2008;93:471-2.
200. Ghobrial IM, Otteman LA, White WL. An EBV-positive lymphoproliferative disorder after therapy with alemtuzumab. N Engl J Med. 2003;349:2570-2; discussion 70-2.
201. O'Brien SM, Kantarjian HM, Thomas DA, et al. Alemtuzumab as treatment for residual disease after chemotherapy in patients with chronic lymphocytic leukemia. Cancer. 2003;98:2657-63.
202. Rossi D, Spina V, Cerri M, et al. Stereotyped B-cell receptor is an independent risk factor of chronic lymphocytic leukemia transformation to Richter syndrome. Clin Cancer Res. 2009;15:4415-22.
203. Maddocks-Christianson K, Slager SL, Zent CS, et al. Risk factors for development of a second lymphoid malignancy in patients with chronic lymphocytic leukaemia. Br J Haematol. 2007;139:398-404.

204. Tam CS, Otero-Palacios J, Abruzzo LV, et al. Chronic lymphocytic leukaemia CD20 expression is dependent on the genetic subtype: a study of quantitative flow cytometry and fluorescent in-situ hybridization in 510 patients. Br J Haematol. 2008;141:36-40.
205. Cherepakhin V, Baird SM, Meisenholder GW, Kipps TJ. Common clonal origin of chronic lymphocytic leukemia and high-grade lymphoma of Richter's syndrome. Blood. 1993;82:3141-7.
206. Timar B, Fulop Z, Csernus B, et al. Relationship between the mutational status of VH genes and pathogenesis of diffuse large B-cell lymphoma in Richter's syndrome. Leukemia. 2004;18:326-30.
207. Rossi D, Spina V, Deambrogi C, et al. The genetics of Richter syndrome reveals disease heterogeneity and predicts survival after transformation. Blood. 2011;117:3391-401.
208. Rossi D, Gaidano G. Richter syndrome: molecular insights and clinical perspectives. Hematol Oncol. 2009;27:1-10.
209. Tsimberidou AM, O'Brien S, Khouri I, et al. Clinical outcomes and prognostic factors in patients with Richter's syndrome treated with chemotherapy or chemoimmunotherapy with or without stem-cell transplantation. J Clin Oncol. 2006;24:2343-51.
210. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. N Engl J Med. 2002;346:235-42.
211. Tsimberidou AM, Wierda WG, Plunkett W, et al. Phase I-II study of oxaliplatin, fludarabine, cytarabine, and rituximab combination therapy in patients with Richter's syndrome or fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol. 2008;26:196-203.
212. Bernstein SH, Burack WR. The incidence, natural history, biology, and treatment of transformed lymphomas. Hematology Am Soc Hematol Educ Program. 2009:532-41.
213. Friedberg JW, Neuberg D, Gribben JG, et al. Autologous bone marrow transplantation after histologic transformation of indolent B cell malignancies. Biol Blood Marrow Transplant. 1999;5:262-8.
214. Karlsson K, Stromberg M, Liliemark J, et al. Oral cladribine for B-cell chronic lymphocytic leukaemia: report of a phase II trial with a 3-d, 3-weekly schedule in untreated and pretreated patients, and a long-term follow-up of 126 previously untreated patients. Br J Haematol. 2002;116:538-48.
215. Juliusson G, Liliemark J. Long-term survival following cladribine (2-chlorodeoxyadenosine) therapy in previously treated patients with chronic lymphocytic leukemia. Ann Oncol. 1996;7:373-9.
216. Osterborg A, Dyer MJ, Bunjes D, et al. Phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. European Study Group of CAMPATH-1H Treatment in Chronic Lymphocytic Leukemia. J Clin Oncol. 1997;15:1567-74.
217. Juliusson G, Christiansen I, Hansen MM, et al. Oral cladribine as primary therapy for patients with B-cell chronic lymphocytic leukemia. J Clin Oncol. 1996;14:2160-6.
218. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood. 1996;87:4990-7.
219. Cheson BD, Horning SJ, Coiffier B, et al. Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. J Clin Oncol. 1999;17:1244.
220. Solal-Celigny P, Lepage E, Brousse N, et al. Recombinant interferon alfa-2b combined with a regimen containing doxorubicin in patients with advanced follicular lymphoma. Groupe d'Etude des Lymphomes de l'Adulte. N Engl J Med. 1993;329:1608-14.
221. Rawstron AC, Villamor N, Ritgen M, et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. Leukemia. 2007;21:956-64.

222. Eichhorst BF, Fischer K, Fink AM, et al. Limited clinical relevance of imaging techniques in the follow-up of patients with advanced chronic lymphocytic leukemia: results of a meta-analysis. Blood. 2011;117:1817-21.
223. Muntanola A, Bosch F, Arguis P, et al. Abdominal computed tomography predicts progression in patients with Rai stage 0 chronic lymphocytic leukemia. J Clin Oncol. 2007;25:1576-80.
224. Shim GJ, Kis LL, Warner M, Gustafsson JA. Autoimmune glomerulonephritis with spontaneous formation of splenic germinal centers in mice lacking the estrogen receptor alpha gene. Proc Natl Acad Sci U S A. 2004;101:1720-4.
225. Shim GJ, Wang L, Andersson S, et al. Disruption of the estrogen receptor beta gene in mice causes myeloproliferative disease resembling chronic myeloid leukemia with lymphoid blast crisis. Proc Natl Acad Sci U S A. 2003;100:6694-9.
226. Girault I, Andrieu C, Tozlu S, et al. Altered expression pattern of alternatively spliced estrogen receptor beta transcripts in breast carcinoma. Cancer Lett. 2004;215:101-12.
227. Caraway NP, Thomas E, Khanna A, et al. Chromosomal abnormalities detected by multicolor fluorescence in situ hybridization in fine-needle aspirates from patients with small lymphocytic lymphoma are useful for predicting survival. Cancer. 2008;114:315-22.
228. Blum KA, Young D, Broering S, et al. Computed tomography scans do not improve the predictive power of 1996 national cancer institute sponsored working group chronic lymphocytic leukemia response criteria. J Clin Oncol. 2007;25:5624-9.
229. Mansour A, Chang VT, Srinivas S, Harrison J, Raveche E. Correlation of ZAP-70 expression in B cell leukemias to the ex vivo response to a combination of fludarabine/genistein. Cancer Immunol Immunother. 2007;56:501-14.
230. McCall JL, Burich RA, Mack PC. GCP, a genistein-rich compound, inhibits proliferation and induces apoptosis in lymphoma cell lines. Leuk Res. 2010;34:69-76.