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Disease-specific Complications of Chronic Lymphocytic Leukemia in Binet Stage A Patients: Analysis of Immunodeficiency, Autoimmune Constellations and Infections in the CLL1-Protocol

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This work is my mother and my late father devoted.

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1. Introduction

1.1. Chronic Lymphocytic Leukemia

1.1.1. Definition and Epidemiology

Chronic lymphocytic leukemia (CLL), the most prevalent adult form of leukemia in Western countries, is characterized by the clonal proliferation and accumulation of neoplastic B lymphocytes in the blood, bone marrow, lymph nodes, liver and spleen. The median age of patients at diagnosis is 72 years, with only 10 to 15 percent under 50 years of age (Civil Rozman et al. 1995). The risk of developing CLL increases progressively with age without plateauing and is 2.8 times higher for older men than for older women.
Since decades the clinical staging systems designed by Rai et al. and Binet et al. are the most useful methods for predicting survival in CLL (Rai et al. 1975; Binet et al. 1981).

1.1.2. Pathogenesis

CLL was considered to be a homogeneous disease of immature, immune-incompetent, minimally self-renewing B cells, which accumulate relentlessly because of faulty apoptotic mechanisms. But these views have been changed in the past decade. Now CLL is a clinically heterogeneous disease originating from B lymphocytes that may differ in activation, maturation state, or cellular subgroup. Whereas CLL in historical view is a disease derived from naive B lymphocytes, CLL in current view is a disease derived from antigen-experienced B lymphocytes that differ in the level of immunoglobulin V-gene (IgVH) mutations (Nicholas Chiorazzi et al, 2005).

The exact cause of CLL is not known. Anyway, CLL is characterized by clonal proliferation of mature, small but not-functional B lymphocytes in bone and blood. It can be assumed that genetic somatic changes play a decisive role.

Chromosomal aberrations are detected in 40 to 50% of CLL cases by conventional cytogenetics and approximately half of the patients show single abnormalities. Chromosomal aberrations in CLL are important independent predictors of disease progression and survival. The most frequent genetic abnormalities are deletions in 13q (55%) and 11q (18%), trisomy 12 (16%), and deletions in 6q (6%). Five categories are defined with a statistical model: 17p deletion, 11q deletion, trisomy 12, normal karyotype, and 13q deletion as single abnormality; the median survival times for patients in these groups were 32, 79, 114, 111 and 133 months. Deletion of 17p and an increased number of deletions at diagnosis were significantly associated with a shorter survival. And patients in the 17p- and 11q-deletion groups had more advanced disease than those in the other three groups. Patients with 17p deletions had the shortest median treatment-free interval (9 months), and those with 13q deletions had the longest (92 months). In multivariate analysis, the presence or absence of a 17p deletion, the presence or absence of an 11q deletion, age, Binet stage, the serum lactate dehydrogenase level (LDH), and the white-cell count gave significant prognostic information in advanced CLL stages (Sivia Bea et al. 2002; Hartmut Döhner et al. 2000).

1.1.3. Clinical Presentations

As with other lymphomas, there is not a reliable symptom of chronic lymphocytic leukemia. In the early stages of CLL the disease is usually asymptomatic. The most common symptom is the appearance of enlarged lymph nodes. In the advanced stage patients suffer from reduced performance state, B-symptoms and infections.

CLL is often discovered by accident during a blood test as part of the diagnosis of other diseases. The following symptoms occur during the course of the illness: lymph node swelling, liver enlargement, spleen enlargement, skin symptoms such as pale skin and mucous, pruritus, eczema, mycoses, herpes zoster, skin bleeding, knotty infiltrates, parotid, leukocytosis with lymphocytosis >10,000/ µl, high percentage in the bone marrow of mature lymphocytes, and antibody deficiency syndrome by replacing the normal B-cells.

Infections are the major cause of morbidity and mortality in patients with CLL. Predisposition to infection in CLL is mediated through various abnormalities including the immune defects inherent in the primary disease (impairment in humeral and cellular immunity) and the additional immunosuppression related to the therapeutic management of CLL. Hypogammaglobulinemia is probably the most important immune defect in terms of risk of severe bacterial infections, its frequency and severity progressing with the duration of the disease (Morra et al. 1999).

1.1.4. Disease-specific Complications

The majority of disease-specific complications in CLL are infections and autoimmune phenomena, based on the dysfunction of immunity. Both cellular and humeral immunity are impaired with qualitative and quantitative defects in B cells, T cells, NK cells, neutrophils and the monocyte/macrophage lineage. In fact, all patients have reduced immunoglobulin levels, even in early stages, and this might explain an association with an increased frequency and severity of infection (Dearden C. 2008).

1.1.4.1. Impaired Immunity

Patients with CLL show the dysregulated immune system, such as T- and B-cell dysfunction, hypogammaglobulinemia and autoimmune complications.
There is increasing evidence of T-cell dysfunction in chronic lymphocytic leukemia which may contribute to the etiology and progress of the disease. An absolute CD8+ lymphocytosis correlates with disease progression and low expression of CD4 and CD8 (as found in autoimmune disease) is seen with abnormal expression of other surface molecules. (Scrivener S. et al 2002).

<u>T-cell dysfunction</u>

Through many studies, we know that decreased T-cell responses to mitogenic and T-cell dysfunction have been described in patients with CLL (Scrivener et al 2003; Scrivener et al 2001). But the exact cellular and molecular mechanisms are still unclear (Wang et al. 2004).
In untreated CLL, absolute T-cell numbers are either normal (Briggs et al. 1990) or more usually increased (Kimby et al. 1989), but there are deficient T-helper function and increased T-suppressor activity with a reversal of the CD4/CD8 ratio and a dominant Th2 response.

The T-cell dysfunction has significant abnormalities of expression of the many antigens and ligands necessary for production of immunoglobulin in CLL.
Human CD4+CD25+ T-cells contain cells that suppress antigen-specific T-cell immune responses. In murine models, regulatory T cells (T_{reg} cells) prevent autoimmune and inflammatory disease and inhibit antitumor immune responses. Most surprisingly, in the majority of patients with CLL treated with fludarabine-containing therapy regimens the inhibitory function of T_{reg} cells was decreased or even abrogated. In addition, frequencies of T_{reg} cells were significantly decreased after therapy with fludarabine (Beyer et al. 2005).

Hypogammaglobulinemia
During the course of CLL, reduction of normal immunoglobulins (Ig) levels is observed in most patients with CLL. All classes (IgG, IgA, and IgM) are affected. Furthermore, the severity tends to increase with the duration, stage and progress of the CLL. About 70% of patients with CLL have developed hypogammaglobulinemia within 7 years from diagnosis.

1.1.4.2. Autoimmune Complications
Autoimmune complications in patients with CLL are well known. The autoimmune hemolytic anemia (AIHA) is the most common autoimmune complication, followed by the autoimmune thrombocytopenia (AITP). AIHA may occur in 10% to 25% of patients with CLL during the course of their disease, and AITP may occur in 1% to 2%. Rarely, pure red cell aplasia (PRCA) has been encountered. These manifestations may occur even when the CLL is otherwise latent (Ward JH. 2001). Indeed, CLL is known as the most common cause of all AIHA.

1.1.4.2.1. Autoimmune hemolytic anemia (AIHA)
As mentioned above, AIHA is the most common autoimmune complication and CLL itself is the most common cause of AIHA. Therefore, AIHA may occur in asymptomatic untreated patients as well. Hamblin et al reported that AIHA was less frequently observed in non-progressive Binet stage A (2.9%; $p < 0.02$) than in other stages (13.3%) (Hamblin et al. 1986). And De Rossi G et al showed that CLL patients with AIHA and AITP represent a poor prognosis (De Rossi G et al. 1988). Therefore, the National Cancer Institute (NCI)-Sponsored Working Group Guidelines for CLL included AIHA among CLL-related signs of active disease.
AIHA may be triggered by fludarabine, one of the most widely used and effective chemotherapeutic agents for CLL, and AIHA associated with Fludarabine may be difficult to treat and is often life-threatening. Beyer et al showed the influence of fludarabine-containing therapy regimens on autoimmune diseases in his study. He assessed 73 patients with B-cell chronic Lymphocytic leukemia (CLL) and 42 healthy controls. Through his study he found that in the majority of patients with CLL treated with fludarabine-containing therapy regimens the inhibitory function of T_{reg} cells was decreased. In addition, frequencies of T_{reg} cells were significantly decreased after therapy with fludarabine (Beyer et al. 2005). As we know, human CD4+CD25+ T cell contain cells that suppress antigen-specific T-cell immune responses. These naturally occurring regulatory CD4+CD25+ T cells (T_{reg} cells) play a central role in the maintenance of peripheral tolerance by suppression of autoreactive T-cell populations. Decrease of these T_{reg} cells was found in patients with autoimmune diseases (Shevach EM. 2000).

1.1.4.2.2. Autoimmune thrombocytopenia (AITP)

Autoimmune thrombocytopenia (AITP), a condition of low platelets, can occur from primary causes, often referred to as idiopathic thrombocytopenic purpura (ITP), or secondary to an underlying disease, such as an autoimmune disorder or an infection. Secondary AITP can also occur with lymphoproliferative malignancies, such as chronic lymphocytic leukemia (CLL), Hodgkin's disease (HD), and non-Hodgkin's lymphomas (NHL). AITP associated with lymphoproliferative disorders has the same mechanism of platelet destruction as in idiopathic or primary AITP. The current treatment paradigm for secondary ITP varies according to the underlying condition. Standard treatments for primary AITP, which include corticosteroids, intravenous immunoglobulin (IVIG), anti-D, and splenectomy, are often successful in secondary AITP. However, in most situations with secondary AITP, treatment should focus on resolving the underlying disorder before treating the shortage of platelets, and, in the circumstances of AITP developing in patients with lymphoproliferative disorders, responses are frequently linked to remission of the primary malignancy (Liebman HA. 2009).

1.1.4.3. Infections

Infections frequently occur during course of CLL. The pathogenesis of infections in CLL is associated with cellular and humoral immune dysfunction, like hypogammaglobulinemia and the immunosuppressive effects of chemotherapy for CLL. It is also well-known that immune dysfunction and hypogammaglobulinemia are the main complications of CLL.

Many therapies used to treat CLL, particularly purine analogues and alemtuzumab, are cytotoxic to T cells. This unintended effect of CLL therapy places patients at risk of opportunistic infections (e.g. pneumocystis carinii, cryptococcus, aspergillus) and reactivation of latent viral infection (CMV, VZV). In addition, other studies suggest that purine analogues also reduce the number and function of regulatory T cells that suppress antigen specific immune responses, illustrating the complex effects of therapy on the T cell compartment of CLL patients (Beyer et al. 2005).

1.1.5. Diagnostic

A National Cancer Institute-sponsored Working Group (NCI-WG) on CLL published guidelines for the design and conduct of clinical trials for patients with CLL in 1988, which were updated in 1996. Passing the past few decades, new prognostic markers, diagnostic parameters and new treatments have been achieved. Therefore, Hallek et al published updated guidelines for the diagnosis and treatment of CLL (Hallek et al. 2008).

To diagnose CLL correctly, it is essential to evaluate the blood count, blood smear, and the immune phenotype of the circulating lymphoid cells.

In the peripheral blood, the presence of at least 5 x 10^9 B lymphocytes/l is required for diagnosis of CLL. The clonality of the circulating B lymphocytes needs to be confirmed by flow cytometry, demonstrating a kappa or lambda light chain restriction. The immunophenotype of this lymphocyte population includes the co-expression of CD5 and CD19, as well as positivity for CD20, CD21, CD23, and CD24. Several variable immunophenotypic findings with prognostic importance include CD38 expression and intracellular expression of zeta-associated protein (ZAP70). Peripheral blood should be sent for cytogenetics and a fluorescence in situ hybridization (FISH) panel for common

chromosomal abnormalities (del [17p], del [11q22-23], del [13q14], and trisomy12) should be done in order to provide superior prognostic information (Abbott BL. 2006).

The leukemia cells found in the blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatic. These cells may be found admixed with larger or atypical cells, cleaved cells, or prolymphocytes, which may comprise up to 55% of the blood lymphocytes. Finding prolymphocytes in excess of this percentage would favor a diagnosis of prolymphocytic leukemia (B-cell PLL). Gumprecht nuclear shadows, or smudge cells, found as cell debris, are other characteristic morphologic features found in CLL (Hallek et al. 2008).

Bone marrow finding in CLL includes normal to high cellularity with a B lymphocyte population that is monoclonal for kappa or lambda light chain expression as shown by immunohistochemistry.

1.1.6. Staging

It is known that CLL has a variable course. Some patients after diagnosis survive for many years without therapy, while others might die within 1 year despite of aggressive therapy.
For the assessment of prognosis of a patient with CLL either at the time of diagnosis or during the course of the disease, it was necessary to classify CLL in different stage. In addition, the staging systems now play an important role for planning therapy.

Rai et al proposed a system for the clinical staging of CLL, which is based on Dameshek's concept (Dameshek W. 1967) and had tested its validity in predicting survival in a retrospective as well as prospective follow-up study of a large number of patients.
The following table 1 describes the staging system of CLL proposed by Rai et al. The prognostic factors are absolute lymphocytosis, bone marrow infiltration, lymphadenopathy, hepatomegaly, splenomegaly, anemia and thrombocytopenia.

Table 1: Rai Staging of CLL (Rai et al. 1975)

Stage	**Definition**	**Median Survival**
Low risk		> 10 years
0	Absolute lymphocytosis > 15.000/µl Bone marrow infiltration > 40%	
Intermediate risk		7 years
I	Absolute lymphocytosis with lymphadenopathy	
II	Absolute lymphocytosis with either hepatomegaly or splenomegaly (with or without lymphadenopathy)	
High risk		2.3 – 5 years
III	Absolute lymphocytosis and anemia (Hb < 11g/dl) (with or without lymphadenopathy, hepatomegaly, or splenomegaly)	

IV	Absolute lymphocytosis and thrombocytopenia (<100.000/ mm^3) with or without lymphadenopathy, hepatomegaly, splenomegaly, or anemia.

Binet et al proposed a new classification in three prognostic groups. The prognostic factors in this staging system are as follows: anemia (Hb < 10 g/dl), thrombocytopenia (platelet count <100.000/ mm^3), and the number of areas of lymphoid enlargement. In this system, thrombocytopenia and anemia appear as the most important risk factors. This three-stage classification only requires clinical examination and routine hematological analysis, and has a good prognostic value which was confirmed on the series of Montserrat and Rozman (Binet et al. 1981).
The following table 2 describes the staging system of CLL proposed by Binet et al.

Table 2: Binet Staging of CLL (Binet et al. 1981)

Stage	**Definition**	**Median Survival**
Low risk		> 10 years
A	Hb ≥10 g/dl Platelet count normal Less than three areas of lymphoid enlargement (≥ 1cm)*	
Intermediate risk		7 years
B	Hb ≥10 g/dl Platelet count normal Three or more areas of lymphoid enlargement (≥ 1cm)*	
High risk		2.3 – 5 years
C	Hb < 10g/dl and/or Platelet count < 100.000/ mm^3 Regardless of the number of areas of lymphoid enlargement (≥ 1cm)*	

* Lymphoid areas include cervical, axillary, inguinal unilateral or bilateral, liver, and spleen.

In general, Binet staging system is commonly used in Europe, and Rai staging system in North America. Through these staging systems, we can see that there is a strong relationship between the stage of CLL and the prognosis; patients in earlier stages have long-term survival. However, there is a wide range of results even in patients within a given stage, and the stage alone does not precisely predict the prognosis for an individual patient, especially in patients at the early stages (Han T et al. 1984).

1.1.7. Prognostic Factors

The individual prognosis of patients with CLL is extremely variable. The staging systems of CLL provide useful tools for predicting survival and planning therapy. Patients with low-risk disease (Rai stage 0, Binet stage A) have a median survival time of more than 10 years, those with intermediate-risk disease (Rai stage I or II, Binet stage B) have a median survival of 7 years, and those with high-risk disease (Rai stage III or IV, Binet stage C) have a median survival of 2.3 – 5 years. For assigning these staging systems, we need to require only a physical examination and a blood count. Even though the staging systems are still very useful tools for assessing prognosis in patients with CLL, they have some limitations; for example, patients who will have a rapid progressive disease and those in whom the leukemia will run an indolent course are not identified. On the other hand, the majority of patients are currently diagnosed during routine medical examinations, when still asymptomatic. As a result, up to 80% of the patients have low-risk disease at diagnosis, thus limiting the prognostic value of clinical stages as a whole. Finally, the prognosis of a patient with CLL ultimately depends on complex relationships between the characteristics of the patient (age, gender, co-morbidity, performance status), the disease (burden, kinetics, genetics and biology of the tumor), as well as sensitivity of the disease to treatment. Staging systems are only one of the parameters in this complex interaction (Montserrat E. 2006).
Montserrat showed in his study as well that a number of biological parameters, particularly serum markers, cytogenetics, IgVH mutational status, CD38 and ZAP-70 expression in leukemic cells, are important independent prognostic markers. ZAP-70 and IgVH mutations basically provide similar prognostic information and therefore they can substitute each other (Montserrat. 2006).

Lymphocyte doubling time, serum levels of β2-microglobulin, thymidine kinase (Hallek et al. 1996), soluble CD23 (Sarfati et al. 1996), as well as CD38 expression on malignant cells (Damle et al.1996) can help to predict disease activity and rapid progression, but the presence in the leukemic B cells of cytogenetic abnormalities like 11q or 17p deletions (Dohner et al. 2000), or somatic mutations in the immunoglobulin heavy chain genes (Damle et al. 1999; Hamblin et al. 1999) are better predictors of survival. A recent retrospective study from the French Cooperative Group on 146 patients for whom the Ig sequence could be obtained and with a long follow-up stressed the importance of the mutational profile of Ig genes in predicting the progression in Binet stage A patients. Stage A patients expressing mutated Ig genes have a 75% 12-year survival and a progression free survival of 156 months, as compared to a medium overall survival of 97 months and a progression free survival of 42 months for patients with unmutated Ig genes.
These results suggest that a high percentage of stage A patients requiring early treatment are included within this group. However, this study also showed that a small percentage of mutated cases (about 10%) may also require early treatment and may die from disease related causes (Vasconcellos et al. 2003). These data confirm the results of a monocentric German study which represents the greatest Binet stage A population so far with 189 patients. The estimated median overall survival time of the group with IgVH homology of 98% or greater was 79 months, whereas the median OS was 152 months for the group with IgVH homology less than 98% (Kröber et al. 2002).

A recent preliminary analysis performed on a common data base of the German and French

cooperative study groups identified short LDT and high sTK as the strongest predictors of rapid progression.

1.1.8. Treatment Options

CLL is an incurable disease. With chemotherapy, however, the prolongation of life and improvement of life's quality can be achieved.

Some patients in early stage of CLL (Rai stage 0, Binet stage A) survive for many years without therapy. Other patients in early stage need to be treated because of rapid progressive disease. A chemotherapy is generally recommended in patients with high-risk stage (Rai stage III/IV, Binet stage C) and in some patients with low-risk stage (Rai stage II, Binet stage A and B) according to the recommendation proposed by the Italian Society of Hematology (SIE) and two affiliates societies, the Italian Society of Experimental Hematology (SIES) and the Italian Group for Bone Marrow Transplantation (GITMO). They developed clinical practice guidelines for the therapy of CLL. The recommendations were developed through a systematic search of evidence and formulated according to explicit methods for consensus development. The indications for initiation of disease-specific therapy in CLL include the presence of at least one of these features: B symptoms (i.e. fever, sweats, extreme fatigue, or weight loss), progressive/obstructive lymphadenopathy or organomegaly, rapid lymphocyte doubling time, anemia or thrombocytopenia (of new onset, worsening or steroid-resistant) (Brugiatelli et al. 2006).

With conventional chemotherapy CLL is incurable, making palliation of symptoms as well as prolongation of progression free survival and overall survival the goals of therapy. For many years alkylating agents, such as chlorambucil, with or without corticosteroids, have been the mainstay of treatment in CLL resulting in response rates of 40-77% among untreated patients. New therapeutic options arose by introduction of the purine analogues. Fludarabine has been most extensively investigated and showed marked activity with response rates between 28-67% in relapsed patients and about 80% among untreated patients (Kalil et al. 2000; Rai et al. 2000). Further improvement may be achieved by combination therapies with fludarabine. In vitro studies revealed a synergistic activity of fludarabine with alkylating agents, such as cyclophosphamide (Bellosillo et al. 1999). Combination therapies with fludarabine and cyclophosphamide showed significant response rates between 80-100% (O'Brien et al. 1998; Flinn et al. 2000; Hallek et al. 2001) with CR rates between 35-50% in untreated patients. So far, the only treatment, which is able to prolong overall survival in CLL patients, is the combination of immunotherapy and chemotherapy, combining fludarabine, cyclophosphamide and rituximab. The CLL8 trial of the German CLL Study Group demonstrated a significant better 3-year overall survival from 87% in comparison to 83% for patients treated with chemotherapy alone (Hallek et al 2010).

Fludarabine (F)

Fludarabine (Fludara®) is distributed in Germany by the company Bayer Schering Pharma. It is the best studied purine analogue used for the treatment of CLL. It is available in injection vials containing 50 mg dry substance. It is a fluorinated adenine (active ingredient: fludarabine-dihydrogen phosphate) and thus by definition an antimetabolite.

Concerning the FDA approval, fludarabine is indicated for the treatment of adult patients with B-cell chronic lymphocytic leukemia (CLL) who have not responded to or whose disease has progressed during treatment with at least one standard alkylating-agent containing regimen. In Germany and more than 60 other countries fludarabine is also approved for first-line treatment of CLL.
The major side-effects are myelosuppression with neutropenia and immunosuppression and reduction of the T helper cells, at higher doses central nervous system (CNS) toxicity (rarely progressive encephalopathy (PML)), nausea, vomiting, mucositis/stomatitis, diarrhea, anorexia and elevation of transaminases (Adkins et al. 1997; Pott et al. 1997).

Cyclophosphamide (C)

Cyclophosphamide (Endoxan®, Cyclostin®) is distributed by the companies Pharmacia and Asta Medica, among others. It is available in injection vials containing 100 mg, 200 mg, 500 mg and 1000 mg dry substance. The major side-effects are myelosuppression, nausea, vomiting (often delayed), allergic reactions, hair loss, mucositis/stomatitis, anorexia, cardiotoxicity (particularly at high doses), nephrotoxicity, hemorrhagic cystitis, neurotoxicity in the form of acute encephalopathy (with high-dose therapy), dermatotoxicity and rarely a syndrome of inappropriate ADH secretion. In diabetics, acute hypoglycemia may occur rarely.

Combined Immunochemotherapy of CLL

In recent years the development of monoclonal humanized antibodies such as rituximab (anti-CD20) and alemtuzumab (anti-CD52) has significantly increased the treatment options for CLL. In vitro studies have shown additional cytotoxic activity towards CD20 positive B cells when rituximab is combined with a number of different chemotherapeutic agents including purine analogues and alkylating agents. Four phase II studies explored the combination of fludarabine with rituximab or fludarabine plus cyclophosphamide with rituximab. Wierda et al conducted a study of rituximab in combination with fludarabine and cyclophosphamide in 135 previously untreated patients with advanced disease (stage Rai III or IV). Patients received six cycles of the triple combination. For the first cycle of treatment, rituximab was given at a dose of 375 mg/m² on day 1, and doses of cyclophosphamide and fludarabine at 250 and 25 mg/m² IV respectively on days 2 to 4. For the second and subsequent cycles, rituximab was given at doses of 500 mg/m² on day 1 and cyclophosphamide and fludarabine at the above doses were given on days 1 to 3. 76% of patients completed all six treatment cycles. In 79 pts evaluable so far, the ORR was 95% (75/79), of these 66% (52/79) were in CR, 14% (11/79) were in nodular PR and 15% (12/79) were in PR. Four percent did not respond and there was 1 early death. At 24 months, 61/63 responders remained in CR or nodular PR (nPR). Molecular remission by PCR for IgVH mutations was documented in 59% (22/37) of a subset of patients studied. After a median follow up of 24 months, 93.7% (74/79) of patients survived. During the first infusion, fever and chills were experienced by nearly half the patients and infusion related symptoms (hypotension, nausea and dyspnea) occurred in 10% -18% of patients. These events were rare with subsequent infusions. Grade 4 neutropenia was seen in 20% of cycles administered, and grades 3 or 4 thrombocytopenia in 4%. Major infections (sepsis, pneumonia) were associated with 3% of cycles and minor infections (FUO, HSV, soft tissue) with 14% of cycles. Byrd et al compared in randomized phase II study fludarabine with concurrent versus sequential treatment with rituximab in symptomatic, untreated patients with CLL. The overall response rate with the concurrent regimen was 90% (47% CR) compared with 77%

(28% CR) with the sequential regimen (Byrd et al. 2003).
Recently Keating et al reported the use of FCR combining fludarabine 25 mg/m² per day for 3 days, cyclophosphamide 250 mg/m² per day for 3 days and rituximab 375 – 500 mg/m² on day 1 as initial therapy for 224 CLL patients with 70% CR, 10% nPR, 15% PR (ORR 95%, Keating et al. 2005).
In the CLL4B study of the GCLLSG (Wendtner et al. 2004), patients with advanced CLL responding to initial chemotherapy with fludarabine alone or in combination with cyclophosphamide were randomized for treatment with alemtuzumab (CAMPATH-1H) in a dose of 30 mg i.v. TIW, 12 weeks or observation. Of 21 evaluable patients, 11 were randomized to alemtuzumab. At six months after randomization, two patients in the alemtuzumab arm converted to CR, while three patients in the observation arm progressed.
After alemtuzumab treatment, five of six patients achieved a molecular remission in peripheral blood while all patients in the observation arm remained MRD positive. At 21.4 months median follow-up, patients receiving alemtuzumab showed a significant longer progression free survival. Due to severe infections in seven of 11 patients in the alemtuzumab arm which were successfully treated the study was stopped. In conclusion, a consolidation therapy with alemtuzumab is able to achieve molecular remissions and longer survival in CLL, but a safe treatment regimen needs to be determined.
The data described above show that when used in combination with a chemotherapy regimen containing fludarabine and cyclophosphamide, antibodies like alemtuzumab or rituximab are very effective agents for the treatment of CLL. The regimen of fludarabine plus cyclophosphamide (FC) and rituximab has shown so far the highest response rate in untreated patients to date, with molecular remissions in a large proportion of responding patients. The safety profile was as expected and manageable. This is in line with the superiority of rituximab plus chemotherapy combinations documented in follicular and aggressive NHL.
At present there is no further information about the immunochemotherapy with fludarabine plus cyclophosphamide (FC) and rituximab in CLL patients with early disease (Binet A or asymptomatic B or Rai 0 or 1) and a high risk for disease progression. In these patients the achieving of molecular complete remissions should be the aim of treatment in order to prolong the progression free survival or the overall survival significantly. A randomized phase III trial of the German CLL study group treats stratified patients at high risk for disease progression with FCR versus observation (CLL7 protocol).

However, despite the high remission rates achieved by fludarabine-based regimens, relapse of CLL after remission durations of months to several years is the rule. Therefore, additional treatment strategies aiming at eradication of the disease are currently being investigated. Hallek et al showed recently the better efficiency and good safety of the combination of FC with rituximab (FCR) in their study. They analyzed 817 first-line patients with CLL: 408 patients were assigned to fludarabine, cyclophosphamide, and rituximab (chemoimmunotherapy group) and 409 to fludarabine and cyclophosphamide (chemotherapy group). At 3 years after randomization, 65% of patients in the chemoimmunotherapy group were free of progression compared with 45% in the chemotherapy group (hazard ratio 0·56 [95% CI 0·46—0·69], p<0·0001); 87% were alive versus 83%, respectively (0·67 [0·48—0·92]; p=0·01). Chemoimmunotherapy was more frequently associated with grade 3 and 4 neutropenia (136 [34%] of 404 *vs.* 83 [21%] of 396; p<0·0001) and leucocytopenia (97 [24%] *vs.* 48 [12%]; p<0·0001). Other side-effects, including severe infections, were not increased. There were eight (2%) treatment-related

deaths in the chemoimmunotherapy group compared with ten (3%) in the chemotherapy group (Hallek et al 2010). Therefore, the combination of FC with rituximab (FCR) is at present a new and promising treatment gold standard.

1.2. Rationale and Study Purpose of the CLL1-Protocol

1.2.1. Rationale

Two long-term French trials and a meta-analysis of most randomized trials demonstrated that therapy with chlorambucil, an oral alkylating agent and the standard treatment of CLL so far, could be deferred for Binet stage A patients. Moreover, deferring therapy until forced by disease progression does not compromise survival (CLL Trialists' Collaborative Group 1999, Dighiero et al. 1998).
However, over 25 percent of these indolent cases, die of causes related to chronic lymphocytic leukemia, 40 percent progress to advanced stages, and 50 percent ultimately require treatment.
The analysis of treatment requirement in this trial demonstrated that one third of patients included in the abstention arm required treatment before a 3-year follow-up (Dighiero et al. 2002).
Preliminary data of the CLL1 protocol of the GCLLSG on 182 patients showed that the cytogenetic abnormalities 17p-deletion and 11q-deletion, elevated serum thymidine kinase and a short lymphocyte doubling time (below 12 months) are independent prognostic factors with regard to progression free survival. Subsequently the next generation CLL7 trial used a combination of four prognostic factors (cytogenetics, s-TK, LDT, IgVH) to define patients at high risk for disease progression within Binet stage A patients.

Although, presently available evidence favors the view that deferral of treatment for stage A patients, does not compromise their survival, these studies were conducted with alkylator-based regimens (Dighiero et al. 1998). This recommendation may change, provided that new drugs demonstrated an advantage in CLL. In addition, these long term studies have shown that about one third of Rai stage 0 and Binet stage A patients die of CLL related causes. It is presently unclear whether young patients with Binet stage A whose leukemic B cells express unmutated V genes or deleterious chromosomal abnormalities like 11q deletions or alterations in the p53 protein or even mutated patients with a short doubling time or elevated levels of other prognostic indicators would benefit from early treatment. This possibility should therefore be tested in a prospective clinical trial.

Until now there are no data available about the early use of purine analogues as single agents or in combination with alkylating agents like cyclophosphamide (FC) or antibodies (rituximab) as first-line therapy in Binet stage A patients.

1.2.2. Purpose of the Study

CLL is now increasingly diagnosed in younger patients and in the early stages, who represent a very inhomogeneous group regarding its illness and progression risk. It agrees that patient with smoldering CLL will not be treated. By using new prognostic parameters, a risk group among the remaining patients of stage A can be identified. Patients in a risk-group, in contrast to those in a low-risk group, tend to progress in higher stage (Binet stage B or C) within 12 months (Hallek et al. 1997).

Based on the hypothesis that a good complete remission may result in prolongation of

disease-free interval and overall survival, it is objective of this study to examine whether an early treatment with fludarabine, currently the most effective therapeutic substance in CLL, would prevent or at least delay the progression of CLL in early stage.

Through stratification, the initiation of the chemotherapy can be determined. Namely, the stratification can prevent the needless chemotherapy for patients with indolent CLL and make sure that only patients, who should receive chemotherapy within 2 years, would be treated (Hallek et al. 1997).

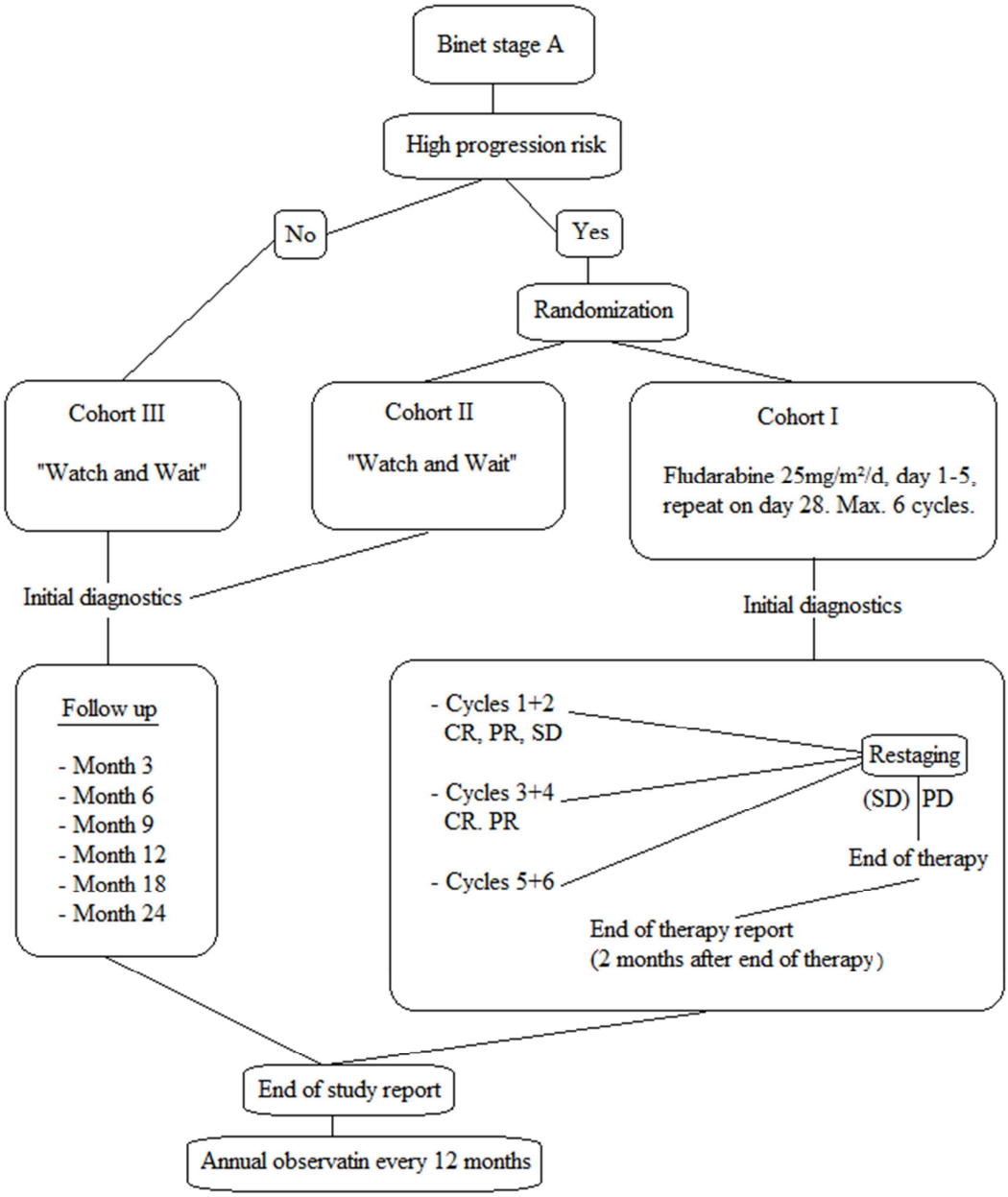
After randomized allocation, patients with high risk of progression are divided in two groups: the experimental arm "Early treatment with Fludarabine" and the standard arm "Watch and Wait". As endpoints, the quality of response, progression-free survival, overall survival and quality of life are evaluated.

Through this study, we aim to analyze the influence of fludarabine in infections, impaired immunity and autoimmune disease by comparing the incidence of infections, hypogammaglobulinemia, AIHA and AITP in all three study arms.

2. Materials and Methods

2.1. CLL-1 Protocol: Study Design

Fig. 1: CLL-1 study flow sheet



It is a multi-center, risk-stratified, randomized phase III study on therapy with fludarabine of CLL in Binet stage A. The study has three different groups: The cohort I and II include patients with high risk of progression. Patients at high risk of progression are randomized between treatment (cohort I) and watch and wait (cohort II) according to a phase III protocol. In these two cohorts, the efficacy of treatment with fludarabine-chemotherapy versus watch and wait will be compared.

The cohort III is an observation arm (watch and wait) in which all patients have low risk of progression.

It is very important to answer the following question:

- Can the progression-free survival and the overall survival of CLL patients in Binet stage A by early, risk adapted therapy with fludarabine be extended?
- What values have new prognostic factors in CLL patients in Binet stage A?

2.1.1. Study on risk-adapted therapy of CLL in Binet stage A

The risk of progression in patients in Binet stage A is assessed on the basis of bone marrow histology (infiltration type: non-nodular versus nodular), lymphocyte doubling time (LDT), serum thymidine kinase, and serum ß2-microglobulin.
High risk of disease progression is defined as follows:

Not-nodular bone marrow infiltration *or* LDT < 12 months

and

Serum thymidine kinase > 7.0 U/l *or* serum-$ß_2$- microglobulin > 3.5 mg/l

Through the definition of high risk of disease progression, patients in Binet stage A can be divided in two groups: patients with high risk of progression and those without high risk of progression. In our study cohort I and cohort II belong to the group with high risk of progression, and cohort III belongs to the group with low risk of progression.
As already mentioned, patients with high risk of progression are randomized between early treatment with fludarabine (cohort I) and "watch and wait" (cohort II). Therefore, only patients in cohort I receive the therapy with fludarabine. And patients in other two groups (cohort II and cohort III) are only observed and are treated only in case of progression according to the NCI-WG guidelines.

2.1.1.1. Endpoints of the study.

Primary endpoint

- Progression-free survival

Secondary endpoints

- Overall survival
- Therapy efficacy (CR, PR, SD, Progression, remission duration)
- Occurrence of severe side effects caused by therapy with fludarabine.
- Occurrence of infections
- Quality of life

2.1.2. Assessment of the significance of new prognosis factors for CLL-patients in Binet stage A.

For the analysis of prognostic factors, the following parameters are recorded in all patients at inclusion in the study:

- Age
- Gender
- Binet stage
- Rai stage
- Lymphocyte count in peripheral blood
- Hemoglobin
- Platelet count
- Bone marrow histology and cytology
- Lymph node histology, when lymph node enlargement with at least 2cm diameter exists and aspiration with acceptable risk is possible (including assessment of the histological subtype of B-CLL and B-CLL with plasmacellular differentiation) (Harris et al. 1994)
- Lymphocyte doubling time
- Serum immunoglobulin level (IgA, IgM, IgG)
- Serum-ß2-microglobulin
- Serum thymidine kinase
- Serum lactate dehydrogenase (LDH)
- Serum albumin
- Determination of the number of enlarged lymph nodes through clinical examination plus chest X-ray plus abdominal sonography
- Splenomegaly (determined by sonography)
- Hepatomegaly (determined by sonography)
- Molecular cytogenetic using FISH (including detection of p53 alterations, 11q-deletion)
- B-symptoms
- ECOG performance status

2.1.2.1. Endpoints for the analysis of prognostic factors

Primary endpoint

- Progression-free survival

Secondary endpoint

- Overall survival

2.2. Study Population

2.2.1. Study Population

Previously untreated male or female patients with Binet stage A CLL, as defined by NCI criteria (Cheson et al. 1996).

2.2.2. Inclusion Criteria (Cheson et al. 1996)

1. Established diagnosis of B-CLL in Binet stage A.

   The diagnostic criteria for B-CLL are:

   - Persistent (> 3 months) increase in absolute lymphocyte counts in the blood (> 5000/µl)
   - More than 30% mature lymphocytes in the bone marrow of normal or increased cellularity
   - Immunophenotype confirmation of the diagnosis according to the following criteria: Low expression of surface immunoglobulin, CD5+, CD19+, CD20+, CD23+, and the double labeling of CD5/CD19 (Matutes et al.1994, Rozman et al.1995)

2. First diagnosis within 3 years before inclusion in study
3. No prior treatment
4. Age between 18 and 75 years old
5. ECOG performance status 0-2
6. No insufficiency of important organ functions
7. Written informed consent for study participation
8. Existence of the parameters for risk stratification
9. Willingness to accept contraception if randomized to the treatment arm (cohort I) for the duration of therapy

2.2.3. Exclusion Criteria (Cheson et al. 1996)

1. Age less than 18 years old and over 75 years old
2. ECOG performance status >2
3. Clinically apparent immune hemolysis
4. Positive Coombs test
5. Clinically apparent immune thrombocytopenia
6. Active secondary malignancy
7. Simultaneous presence of other neoplasia, and previous radiotherapy or chemotherapy for any neoplastic diseases.

8. HIV-infection
9. Pregnancy and Lactation
10. Participation in another clinical trial before and during the study
11. The following concomitant diseases:
    - Clinical apparent heart insufficiency
    - Cardiomyopathy
    - Myocardial infarction within the past 6 months prior to the study
    - Severe chronic obstructive lung disease with hypoxemia
    - Severe diabetes mellitus
    - Hypertension difficult to control
    - Infection difficult to control
    - Impaired liver function with serum bilirubin > 2mg/dl and/or transaminase over 3 times of the normal
    - Impaired renal function with creatinine > 3mg/dl
    - Clinically apparent cerebral dysfunction
    - Serious psychiatric or neurological diseases that would preclude participation in the required study procedures

2.3. Treatment Schedule

2.3.1. Procedures of risk stratification and randomization

The first step following after the registration is the assessment of lymphocyte doubling time (LDT) by the study center; at least 4 blood lymphocyte counts are therefore needed. Bone marrow histology is also important to evaluate the infiltration. These two parameters, together with serum thymidine kinase and serum-ß2-microglobulin, would be used to perform risk stratification in order to determine whether the patients have high risk of progression or not. Patients stratified to the high risk group will furthermore be randomized between the treatment arm (cohort I) and the watch and wait arm (cohort II). (see chapter 2.1.1.)

2.3.2. Regimen

The chemotherapy regimen of patients at high risk of disease progression, who were randomized to the therapy arm (Cohort I), is as follows:

Fludarabine 25mg/ m^2/day as a 30-minutes i.v. infusion from day 1-5 Cycle repeat on day 28.

Study medication will be given in at least 4 cycles, but not more than 6 cycles. The restaging is scheduled after 2 and 4 cycles of therapy. The number of treatment cycles follows the following guidelines:

- Complete Remission (CR) after 2 cycles: Administration of 2 additional (total 4) cycles of fludarabine
- Complete Remission (CR) first after 4 cycles: Administration of 2 additional (total 6) cycles of fludarabine.
- Partial Remission (PR) after 4 cycles: Administration of additional (total 6) cycles of fludarabine
- Progression after 2 or more cycles: Therapy stop
- No change after 2 cycles: Administration of 2 additional cycles of fludarabine
- No change after 4 cycles: Therapy stop

2.3.3. Dose Modification

At the following side effects, the dose of fludarabine in the next cycle should be reduced:

- Occurrence of severe general infection after administration of chemotherapy in the phase of neutropenia: 75%
- Severe neutropenia (if no severe neutropenia existed before the therapy):
  - Neutrophil nadir under 500/µl: 50%.
  - Neutrophil nadir under 1000/µl: 75%.
- Severe thrombocytopenia (if no severe thrombocytopenia existed before the therapy):
  - Platelet nadir under 20.000/µl: 50%
  - Simultaneous occurrence of thrombocytopenia and bleeding complications: 50%

The following table shows the detailed plan for dose reduction in the presence of hematological toxicity in CLL (Cheson et al. 1996).

Table 3: Plan for dose reduction

Drop of thrombocyte* or of Hb#(nadir) compared to values before therapy.	Toxicity grad◦	Absolute neutrophil count$	Recommended dose reduction
No change	0		No
10%	1	= 2.000	No
11% - 24%	2	=1.500 und <2.000	No
25% - 49%	3	=1.000 und <1.5000	No
50% - 74%	4	= 500 und <1.000	75%
= 75%	5	<500	50%

*****Every drop of thrombocyte under 20.000/µl is considered as grade 4-toxicity.

Baseline and subsequent Hb values must be determined before administration of packed red blood cells.

◦ Degrees: 1 = mild, 2 = moderate, 3 = severe, 4 = life threatening, 5 = fatal.

$ If the absolute neutrophil counts reach less than 1.000/µl, a toxicity grade 3 is adopted. Changes in white blood cells or lymphocytes are not considered, because the decrease in white blood cell count is a therapeutic target. If the neutrophil count is less than 1.000/µl before therapy, the toxicity is not evaluable.

2.3.4. Criteria for therapy termination

The following criteria lead to discontinuation of fludarabine chemotherapy:

- Lack of response or disease progression after 2 cycles
- Occurrence of severe adverse effects, especially life threatening complications

After discontinuation of therapy, no further chemotherapy is performed until the occurrence of an indication for therapy.

2.3.5. Procedures in the observation arm

Patients in cohort II and III belong to the observation arm. They are not treated with fludarabine, but only observed. They would be only observed until the occurrence of therapy indications.

2.3.6. Therapy for progression or relapse

It is scheduled to include patients with progression or relapse in the CLL-4 study (fludarabine vs. combination of fludarabine-cyclophosphamide) if they are younger than 65 years old. Patients over 65 years old would be included in the CLL-5 study (fludarabine vs. chlorambucil).
If patients in cohort I show progression or relapse after fludarabine therapy, they should be further treated according to the CLL-6 protocol (therapy combination consisting of fludarabine, cyclophosphamide, and mitoxantrone).

If patients reject the above mentioned therapy in different studies, they would be treated with fludarabine, unless they have history of no-response under fludarabine therapy.

According to the consensus recommendations of the German Study Group, indications of therapy for progression or relapse are defined as follows:

- Progression to Binet stage C or symptomatic Binet stage B
- Progression with continuous increase of absolute lymphocyte count ≥ 100% and / or increase of leukocyte count > 300.000/ µl
- Development of severe B-symptoms.

2.4. Evaluation

2.4.1. Definition of treatment success and disease progression

The Assessment of treatment success and disease progression is based on the new criteria of "National Cancer Institute-Sponsored Working Group" (Cheson et al. 1996; Cheson et al. 1988). The results of treatment are compared with initial status at study inclusion, not at the beginning of treatment.

Complete Remission (CR)
Following criteria must for at least 2 months be met:

- Lymph nodes < 1 cm (previous lymph nodes enlargement no more detectable), evaluated by clinical examination, chest x-ray, abdomen ultrasound and in case of doubt CT-scan
- Normal liver and spleen sizes
- Absence of disease related symptoms
- Peripheral blood lymphocytes <4.000/µl
- Peripheral blood neutrophils ≥ 1.500/µl
- Platelets ≥ 100.000/µl
- Hemoglobin > 11g/dl (untransfused)
- Lymphocytes <30% in bone marrow

Patients with CR, who still have a focal infiltration in the histology of the bone marrow, are represented as nodular partial remissions (nPR). The aim is to check the quality of remission

through using molecular methods.

Partial Remission (PR)
The definition of a partial remission requires ***all*** of the following features (if abnormal prior to therapy) for at least 2 months:

- ≥ 50% decrease in peripheral blood lymphocyte count from the pretherapeutic staging
- ≥ 50% reduction in lymphadenopathy
- ≥ 50% reduction of the size of the enlargement of liver and/or spleen.

As well as ***one or more*** of the remaining features:

- ANC ≥ 1500/µl or 50% improvement over baseline
- Platelets > 100.000/ µl or 50% improvement over baseline
- Hemoglobin > 11.0 g/dl or 50% improvement over baseline without transfusions

Progression (PD)
Progression occurs when at least ***one*** of the following criteria is met:

- Progression to symptomatic Binet stage B or C
- Apparent, otherwise unexplained lymphadenopathy ≥ 100% on two consecutive tests at intervals of at least two weeks.* One of the lymph nodes should be given a minimum diameter of 2 cm.
- Appearance of new, not otherwise explicable lymphadenopathy (at least 1 cm in diameter) on two consecutive tests at intervals of at least two weeks.
- Sonographically measured increase (≥ 25%) in size of liver and / or spleen (at least one diameter). occurrence of an apparent, previously not detectable hepatomegaly or splenomegaly (confirmed by sonography).
- Long-lasting increase of absolute lymphocyte counts ≥100% (= doubling).
- Transformation into a highly malignant NHL (Richter's syndrome) or into a prolymphocytic leukemia (> 55% prolymphocytes).

Stable Disease (SD)
Stable Disease (SD) is defined as the remission status if the criteria for Complete Remission (CR), Partial Remission (PR), and Progression (PD) are not met.

2.5. Statistic Analysis

2.5.1. Progression Free Survival (PFS) and Overall Survival (OS)

The analysis of the progression free survival and overall survival is based on the method of Kaplan and Meier. Survival curves are compared using the Log-Rank-Test. The level of significance (one-sided test) is at 5%.

2.5.2. Efficacy of the Fludarabine Therapy

Patients in both groups (cohort I and II) are at high risk of disease progression. But only

patients of cohort I are treated with fludarabine. And patients of cohort II are only observed. If the results of both groups, therefore, are compared, the efficacy of fludarabine could be good evaluated. The analysis of the response rate (occurrence of CR, PR, PD, and Stable Disease) is descriptive.

2.5.3. Impaired Immunity

The frequency of AIHA and AITP are evaluated in each study arm. Especially, the influence of fludarabine in impaired immunity could be evaluated by comparing the result of two groups (Cohort I and II). The analysis of the result is descriptive.

2.5.4. Infection

The frequency, duration and spectrum of infections are evaluated in each study arm. The results in three different arms are compared. In addition, prognostic factors for infection are analyzed. The influence of fludarabine in infections could be showed through this analysis.

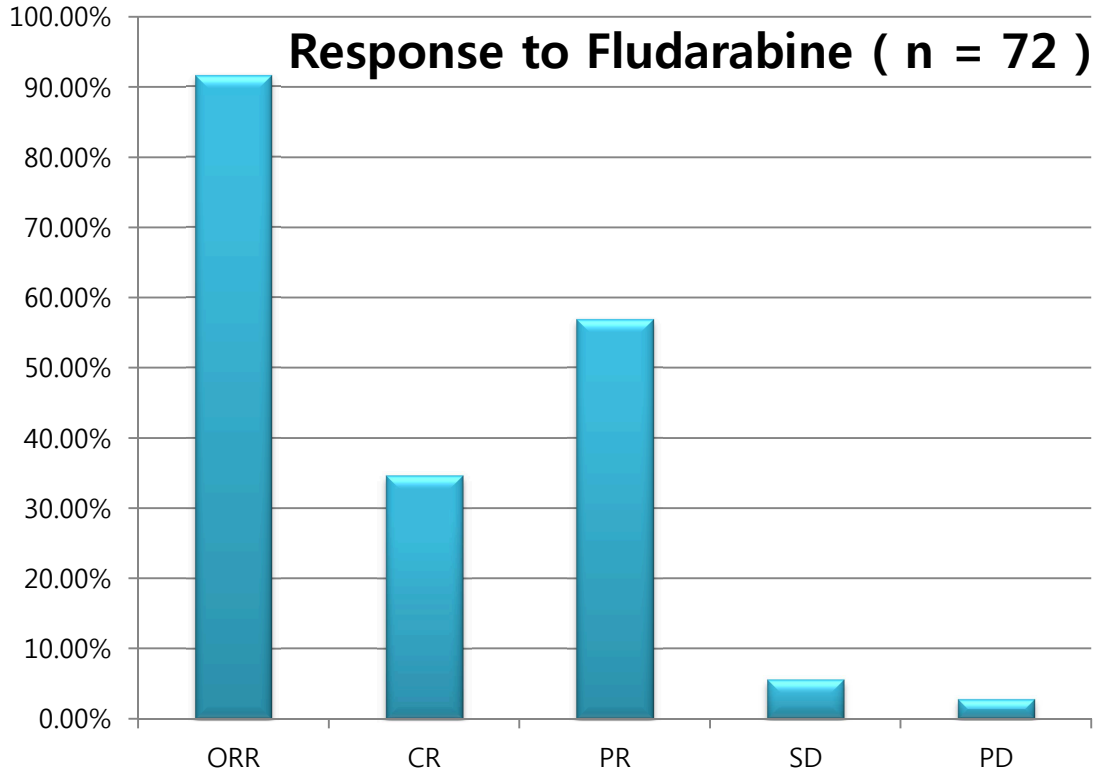
3.Results

3.1. Response to treatment with Fludarabine.

Table 4: Response to treatment with fludarabine.

Response to treatment with fludarabine (n = 72)				
ORR	CR	PR	SD	PD
91,6% 66 pts	34,7% 25 pts	56,9% 41 pts	5,6% 4 pts	2,8% 2 pts

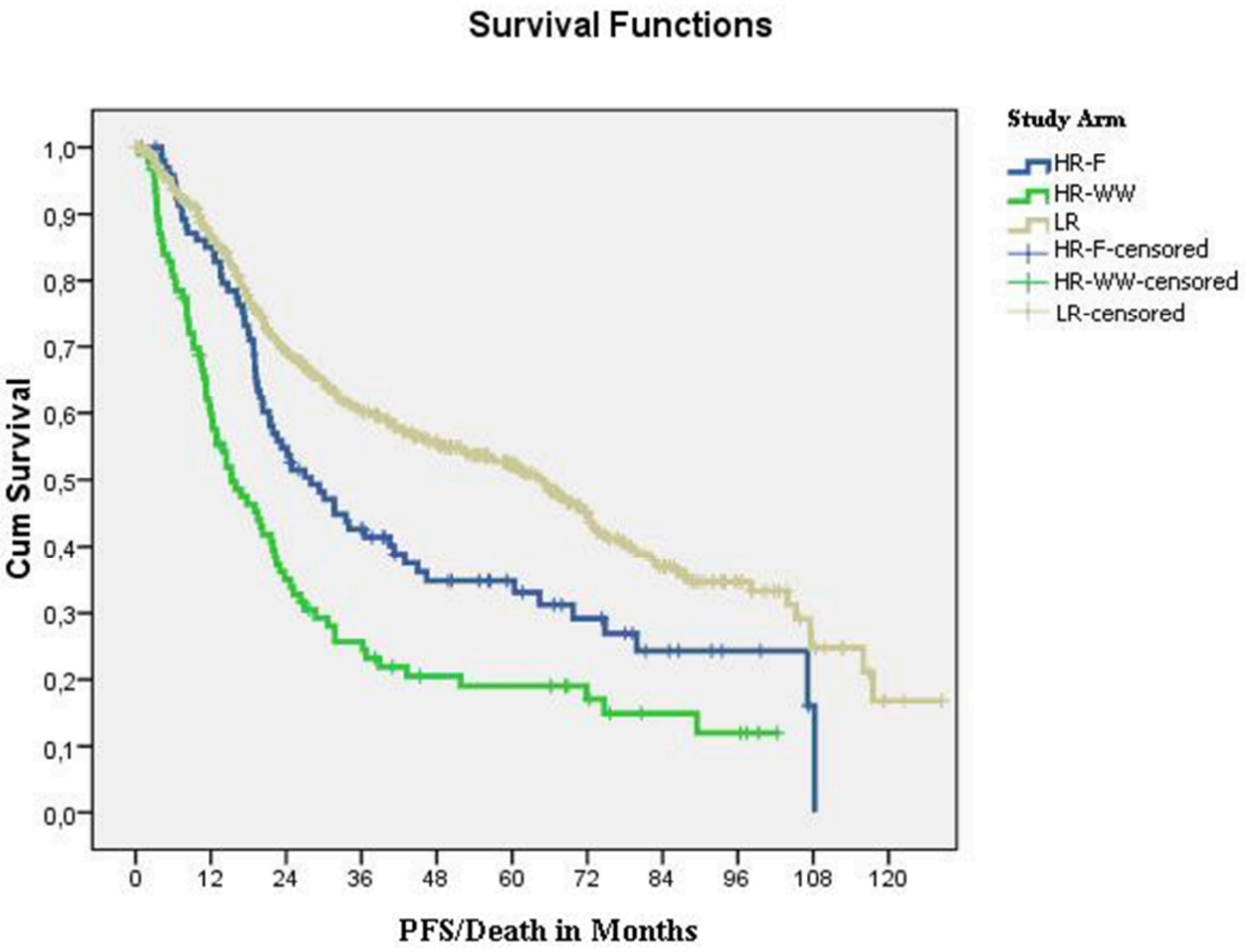
Diagram 1: Response to treatment with fludarabine



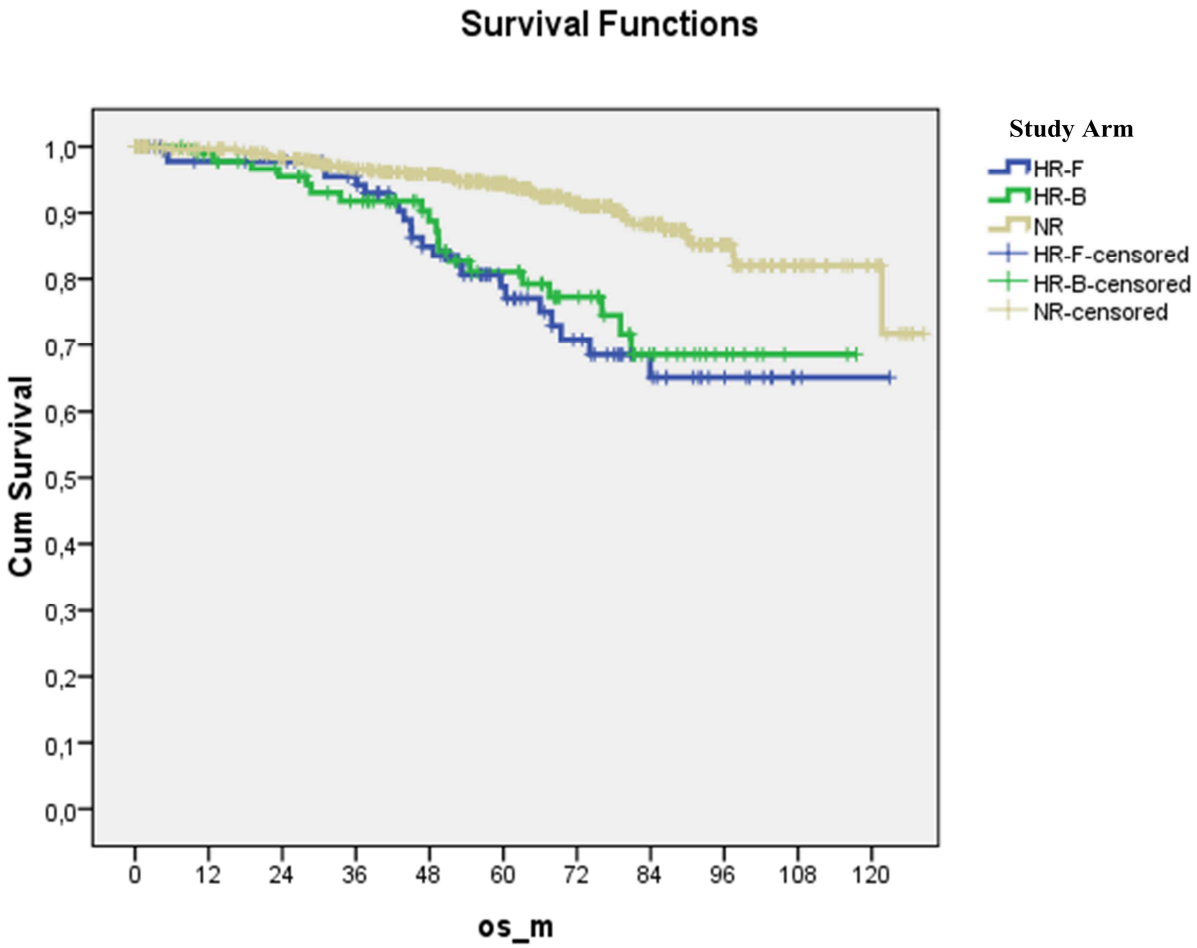
The table 4 and diagram 1 show the response to treatment with fludarabine. As we see, patients treated with fludarabine showed good results as many studies have already showed. 91.6% of patients reached the overall response (ORR). The partial response rate (PR, 56.9%) was higher than the complete response rate (34.7%). Progressive disease showed only 2.8%.

The following diagram 2 shows the overall survival rate and progression free survival rate among all three study arms.

Diagram 2: PFS and OS: HR-F vs. HR-WW vs. LR



PFS:
HR-F(cohort I) vs. HR-WW(cohort II) vs. LR(cohort III)
27.9 months vs. 15.3 months vs. 64.8 months.



OS :
HR-F vs. HR-WW vs. LR
Median not yet reached

Patients in cohort III showed the best overall survival (OS) rate and progression free survival (PFS) rate. Although, patients in cohort I showed higher PFS rate than it in cohort II, when compared the overall survival rate between patients in cohort I and in cohort II, there was no significant difference.

3.2. Impaired Immunity in Patients with CLL

3.2.1. Hypogammaglobulinemia

The majority of patients with CLL show hypogammaglobulinemia during the course of their disease. Hypogammaglobulinemia is a type of immune disorder characterized by a quantitative reduction of all types of gamma globulins. In general, hypogammaglobulinemia increases the susceptibility to infections in patients with CLL. Therefore, many patients with hypogammaglobuliemia frequently suffer from bacterial and viral infections. These infections

are the most common cause of death of patients with CLL.

IgA, IgG, and IgM are analyzed in our study. As cut-off values the normal serum values have been used.

Table 5: Low levels of immunoglobulin and infection

Immunogloulin	Patients(n)	Patients with Infections (%) (Yes/No)	P-value
IgA (mg/dl) ≥70	441 (88.7%)	34,0% (150/291)	ns
<70	56 (11.3%)	37,5% (21/35)	
IgG (mg/dl) ≥600	456 (91.6%)	33,8% (154/302)	ns
< 600	42 (8.4%)	38,1% (16/26)	
IgM (mg/dl) ≥30	421 (84.7%)	33,7% (142/279)	ns
<30	76 (15.3%)	36,8% (28/48)	

Through the table 5, we observed that most of patient with CLL in Binet stage A had still normal levels of immunoglobulins. Only 11.3% in IgA, 8.4% in IgM and 15.3% in IgM showed reduced levels of immunoglobulins. As known well, the severity tends to increase with the duration and stage of the CLL.

We compared the frequency of infections in normal or high levels of immunoglobulin and in low levels of immunoglobulin. As we see in the table 5, the frequency of infections in low levels of immunoglobulin was higher than in normal or high levels of immunoglobulin. Namely, difference of the frequency of infections in IgA was 3.5%, 4.3% in IgG, and 3.1% in IgM. Even though the difference is not clearly significant, we could observe a correlation between low levels of IgA, IgG and IgM and the frequency of infections. In fact, if we consider the stage and duration of CLL, we could see the tendency of patients with low levels of immunoglobulin to have more frequently infections.

3.2.2. Infections

Infection is one of the most common complications of CLL. Infection can be occurred by immune dysfunction, and hypogammaglobulinemia, and the toxic effect of chemotherapy like fludarabine. Moreover, infection is well known as the most common cause of death of patients with CLL.

We evaluated the difference of the incidence, the spectrum and the duration of infection in all 3 study arms.

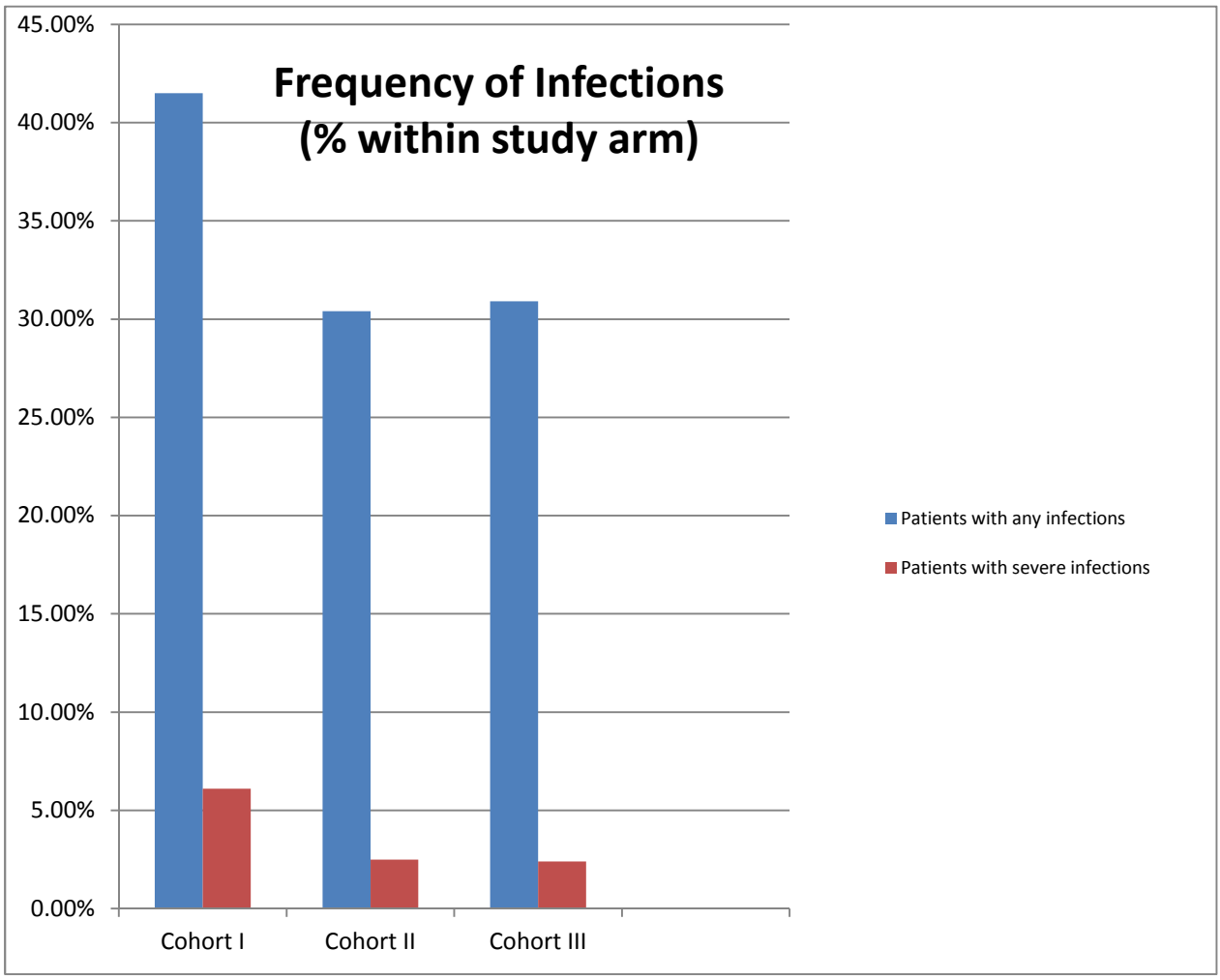
3.2.2.1. Frequency of infections in different study arms.

Table 6: Frequency of infections according to treatment arm.

	Total (n =617)	Cohort I (n = 82)	Cohort II (n = 79)	Cohort III (n = 456)
Number of patients with any infections	200 (200/617 =32.4%)	34 (34/82 =41.5%)	24 (24/79 =30.4%)	142 (142/456 =30.9%)
Number of patients with severe infections*	18 (18/617 =2.9%)	5 (5/82 =6.1%)	2 (2/79 = 2.5%)	11 (11/456 = 2.4%)

*Severe infection: defined as Common Toxicity Criteria (CTC) grades 3 and 4 infections (infection requiring intravenous antibiotic, antifungal or antiviral treatment or life threatening infections)

Diagram 3: Frequency of infection



Through this study, we aimed to find out whether fludarabine therapy could increase the infection rate or dysregulate the immune system. We could observe different infection rates among in cohort I, cohort II and cohort III. As we see in the table 6 and Diagram 3, the

incidence of infections in cohort I was 41.5%, in cohort II 30.4% and in cohort III 30.9%. That is, the incidence of infections in patients treated with fludarabine was about 10% higher than in patients without early treatment. The incidence of severe infections showed the same tendency although we could relatively seldom observe severe infections.

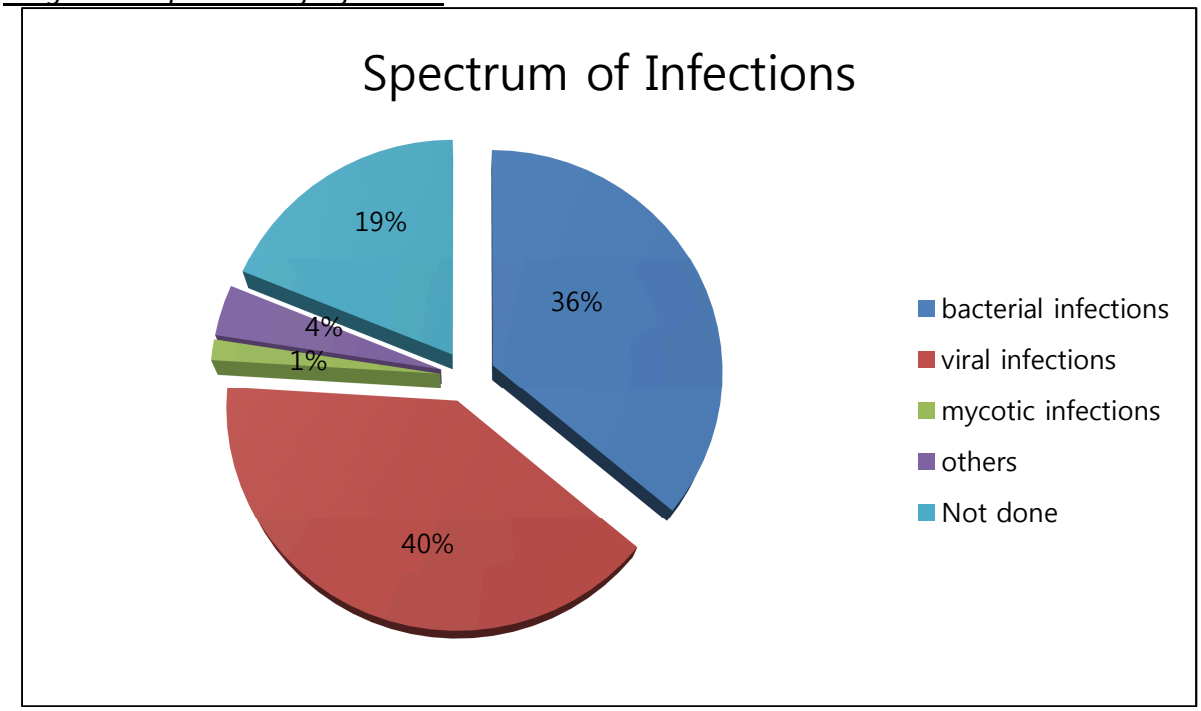
3.2.2.2. Spectrum and Duration of Infections

• Spectrum of Infections.

Table 7: Spectrum of infections (with causative organisms) according to treatment arm

	Total	Cohort I	Cohort II	Cohort III	P-value
Number of infections	324 (100%)	66 (100%)	40 (100%)	218 (100%)	
Bacterial infections	117 (36.1%)	25 (37.9%)	21 (52.5%)	71 (32.6%)	0.19
Viral infections	129 (39.8%)	24 (36.4%)	11 (27.5%)	94 (43.1%)	0.23
Mycotic infections	5 (1.5%)	1 (1.5%)	0 (0%)	4 (1.8%)	0.10
Others	12 (3.7%)	5 (7.6%)	1 (2.5%)	6 (2.8%)	0.10
Not applicable	61 (18.8%)	11 (16.7%)	7 (17.5%)	43 (19.7%)	0.15

Diagram 4: Spectrum of infections



The table 7 and diagram 4 show the spectrum of infections. 263 infections of total 324 infections were analyzed. The viral (39.8%) and bacterial (36.1%) infections were the most common spectrums. The viral infections were more frequently observed than the bacterial infection. However, it showed the slight difference between both spectrums. When we see

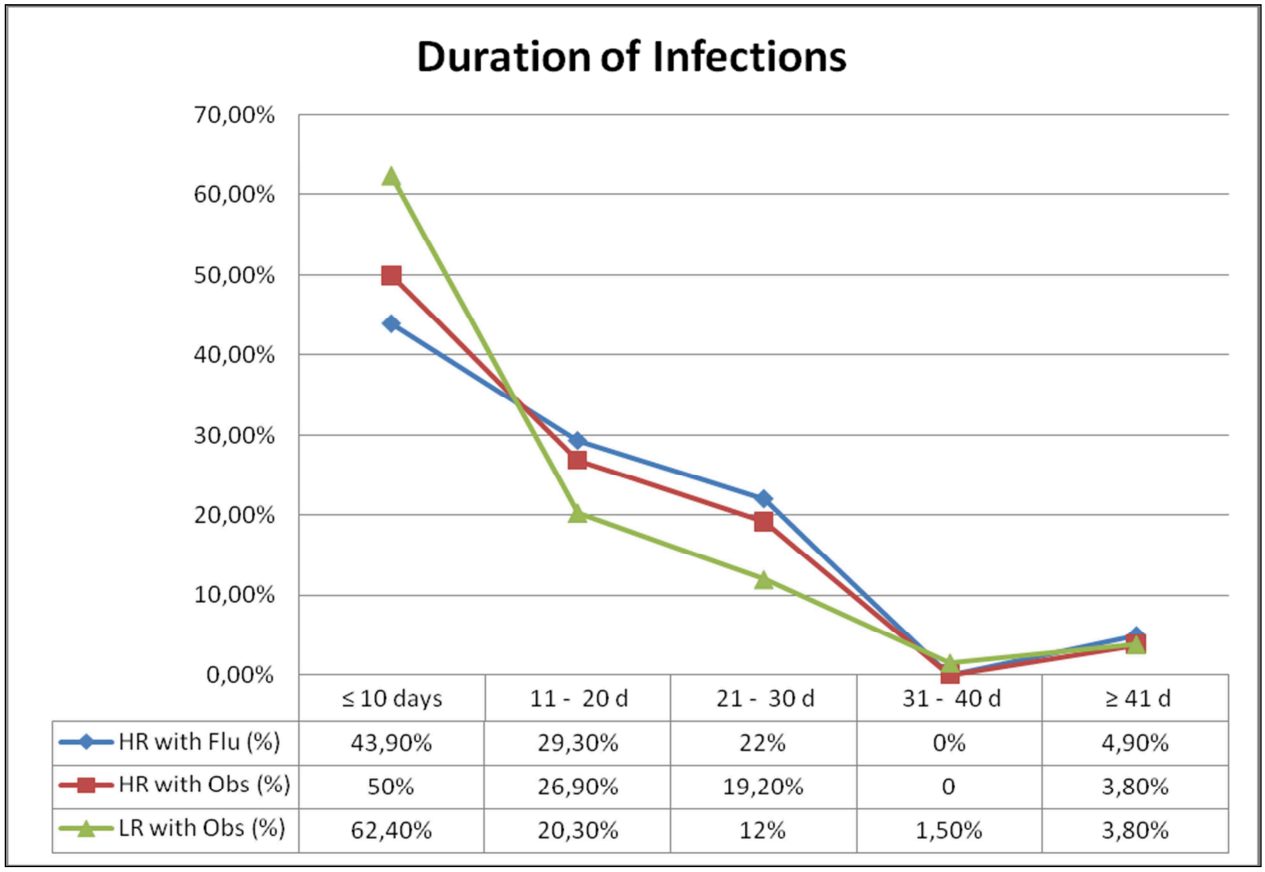
the spectrum of infection in 3 study arms, in contrast to in cohort III, the bacterial infection rates in cohort I and cohort II was higher than the viral infection rate. We could seldom observe the mycotic infections (1.5%).
Unfortunately, we could not analyze 18.8% of infections because causative organisms were not documented.

• Duration of Infections.

Table 8: Duration of Infections

	Total (%)	Cohort I (%)	Cohort II (%)	Cohort III (%)	P-value (%)
Number of infections	324 (100%)	66 (100%)	40 (100%)	218 (100%)	
Duration of infections					
≤ 10 days	114 (35.2%)	18 (27.0%)	13 (32.5%)	83 (38.1%)	0.19
11 - 20 d	46 (14.2%)	12 (18.2%)	7 (17.5%)	27 (12.4%)	0.13
21 - 30 d	30 (9.3%)	9 (13.6%)	5 (12.5%)	16 (7.3%)	0.12
31 - 40 d	2 (0.6%)	0 (0.0%)	0 (0.0%)	2 (0.9%)	0.09
≥ 41 d	8 (2.5%)	2 (3.0%)	1 (2.5%)	5 (2.3%)	0.10
Not applicable	124 (38.3%)	25 (37.9%)	14 (35%)	85 (39.0%)	0.21

Diagram 5: Duration of infections



114 of total 200 infections (57%) ended within 10 days. With increasing duration of infections, the incidence of infections was reduced. However, 124 of total 324 infections (38.3%) could

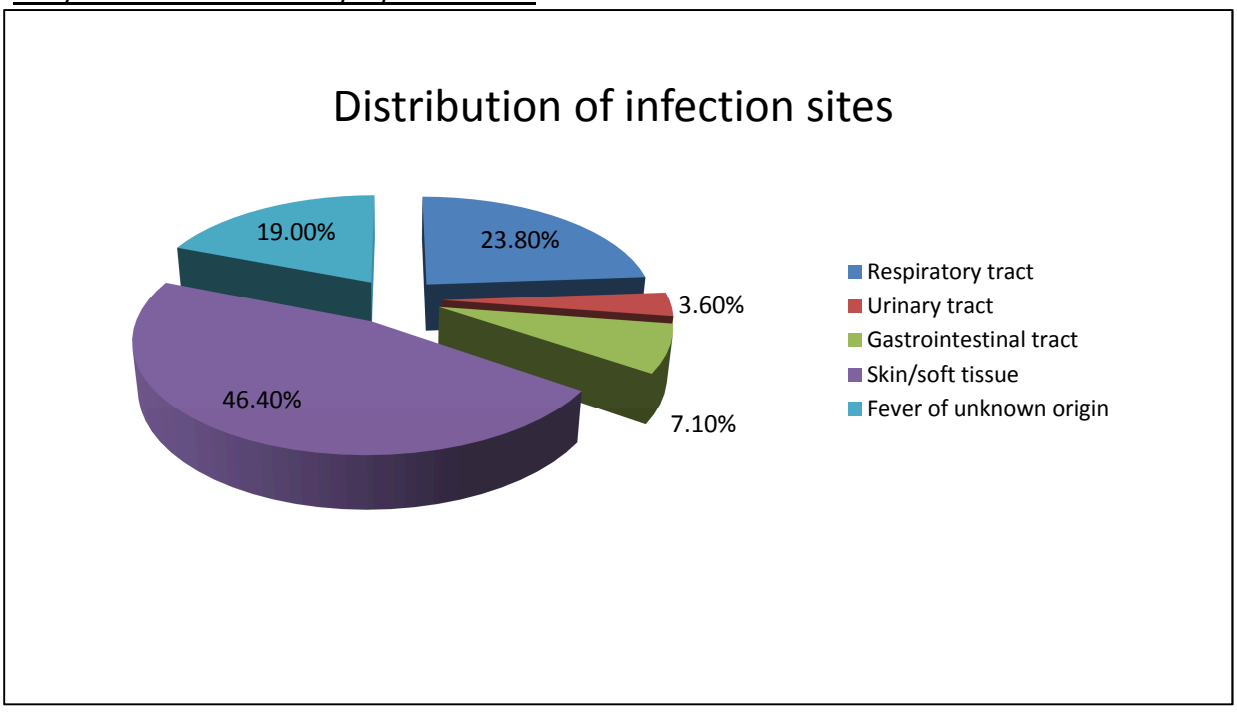
not be analyzed. Because many cases (124 of total 324 infections (38.3%)) of infections had to be excluded from the statistics we could not show the result completely. But we could see the tendency. When we compared results of each arm, infections having long duration were more frequently observed in cohort I than in cohort II or in cohort III. But there was a slight difference. Maybe, we can hypothesize through the table 8 and diagram 5 that more patients with infection in cohort I had longer infection than in cohort II and cohort III.

3.2.2.3. Distribution of infection sites according to treatment arm

Table 9: Distribution of infection sites according to treatment arm

	All infections					Severe infections				
	Total	**C. I**	**C. II**	**C. III**	**P-value**	**Total**	**C. I**	**C. II**	**C. III**	**P-value**
Respiratory tract	20 (23.8%)	6	4	10	0.001	2	2	0	0	0.116
Urinary tract	3 (3.6%)	1	2	0	0.026	0	0	0	0	
Gastro-intestinal tract	6 (7.1%)	0	1	5	0.058	0	0	0	0	
Skin/soft tissue	39 (46.4%)	11	3	25	0.156	7	5	0	2	0.033
Fever of unknown origin	16 (19.0%)	0	3	13	0.053	0	0	0	0	
Not applicable	241	48	27	166	0.021	12	3	1	8	0.018

Diagram 6: Distribution of infection sites



Skin/soft tissue (46.4%) was the most common infection site. Respiratory tract followed with 23.8%. In addition to these infection sites, we could observe infections in urinary tract and gastrointestinal tract. 19% of infections showed fever of unknown origin.

3.2.2.4. Infection related Mortality

Table 10: Patients with infections who died due to any reason

	Infection	Severe Infection	death	cause of death	comment
Cohort III	Yes	unknown	Yes	Unknown	No information
Cohort III	Yes	Yes	Yes	Unknown	No information
Cohort II	Yes	No	Yes	Second disease	Lung cancer
Cohort II	Yes	No	Yes	Second disease	Inoperable central lung cancer

During and after therapy 4 patients with the report of infections died. Two patients died of incurable lung cancer as secondary disease. Other two patients died of unknown cause and one of both had severe infection.
Through only this table 10, we could not see the manifest relation between infection and mortality, even though all dead patients had at least one report of infections.

3.2.2.5. Prognostic factors of Infections

Table 11: Analysis of risk factors for infection across 3 treatment arms.

Risk factors	Patients(n)	Patients with infections (%) (Yes/No)	P-value
Age (year)			
<60	290	32,1% (93/197)	0.863
≥60	327	32,7% (107/220)	
Sex			
Male	365	34,5% (126/239)	0.187
Female	248	29,4% (73/175)	
IgA (mg/dl)			
≥70	441	34,0% (150/291)	0.61
<70	56	37,5% (21/35)	
IgG (mg/dl)			
≥600	456	33,8% (154/302)	0.57
< 600	42	38,1% (16/26)	
IgM (mg/dl)			
≥30	421	33,7% (142/279)	0.60
		36,8%	

<30	76	(28/48)	
Hemoglobin (g/dl)			
≥13	509	33,0% (168/341)	0.64
<13	98	30,6% (30/68)	
Serum creatinine (mg/dl)			
≥1.5	10	90,0% (9/1)	<0.01
<1.5	553	31,8% (176/377)	
Platelet count			
≥150,000	493	31,2% (154/339)	0.18
<150,000	111	37,8% (42/69)	
Monocyte (G/l)			
≥ 1.0	113	35,4% (40/73)	0.57
< 1.0	430	32,6% (140/290)	
Initial Neutrophil Count (G/l)			
≥2	513	32,7% (168/345)	0.574
<2	40	35,0% (14/26)	
LDH (U/l)			
≥250	88	27,3% (24/64)	0.16
<250	464	34,9% (162/302)	
Splenomegaly (Sono/Clinic)			
Yes	39	43,6% (17/22)	0.14
No	542	32,1% (174/368)	

We analyzed the risk factors of infections. Increased serum creatinine can be regarded as the best risk factors of infection in our study. Only 10 patients with CLL had more than 1.5 mg/dl serum creatinine. And 9 of these 10 patients had infections. That means, 90% of patients having more than 1.5mg/dl serum creatinine had infections, although the most patients (98%) had less than 1.5 mg/dl serum creatinine. The difference of infection rate between patients with ≥1.5 mg/dl serum creatinine and in patients with <1.5mg/dl serum creatinine is 58.2%.

When we analyzed IgA, IgG, and IgM, we could observe that hypogammaglobulinemia was a good risk factor of infections. We can see in table 11 that infection rates in normal or high immunoglobulins (IgA, IgG, IgM) accounts were lower than in lower immunoglobulins accounts. Here we only see a statistical trend. In addition, other factors, for example splenomegaly, low LDH and low platelet count can be regarded as proper risk factor of infection.

3.3. Autoimmune Complications of CLL

Patients with chronic lymphocytic leukemia (CLL) are at increased risk for autoimmune complications. Autoimmune hemolytic anemia (AIHA) and autoimmune thrombocytopenia (AITP) are observed in approximately 20% to 35% and 2% of patients, respectively.

3.3.1. The incidence of AIHA

AIHA (Autoimmune hemolytic anemia) is the most common complications of B-CLL and occurs in 10 – 20% of the case (Rozman et al. 1995). It is also reported that the use of fludarabine may trigger the occurrence of AIHA and this complication may be life-threatening (Tertian et al. 1996; Bastion et al. 1992; Maclean et al. 1996; Tosti et al. 1992).

Hamblin et al reported that a positive direct antiglobulin test in non-progressive Binet stage A is significantly less likely to occur (2.9%; $p<0.02$) than in the other stages (13.3%). Therefore, the occurrence of AIHA in non-progressive stage A is lower than in other stages.

The positive Coombs test is the exclusion criteria. Even though there are some reports of Coombs negative AIHA, it is known that up to 98 % of patients with AIHA have a positive direct Coombs test. Therefore, we have postulated that all patients in our study have no AIHA at enrollment of study.

We analyzed the incidence of AIHA in three different study arms.
We show three tables as follows: the incidence of AIHA during therapy, after therapy, and the total incidence of AIHA during and after therapy.

The incidence of AIHA during therapy

Table 12: Incidence of AIHA during therapy

			Study Arm			Total
			Cohort I	**Cohort II**	**Cohort III**	
AIHA during Therapy	No	Count of patients	85	83	487	655
		% within AIHA during therapy	13.0%	12.7%	74.4%	100.0%
		% within study arm	96.6%	100.0%	100.0%	99.5%
	Yes	Count	**3**	**0**	**0**	**3**
		% within AIHA during therapy	100.0%	0.0%	0.0%	100.0%
		% within study arm	**3.4%**	**0.0%**	**0.0%**	**0.5%**
Total		Count of patients	88	83	487	658
		% within AIHA during therapy	13.4%	12.6%	74.0%	100.0%
		% within study arm	100.0%	100.0%	100.0%	100.0%

We analyzed the incidence of AIHA during therapy (***Criteria: hemoglobin <11 g/dl and ≥ 3.5 g/dl drop from baseline***).

As we see in the table 12, we found 3 patients with AIHA only in the cohort I arm. In other groups patient with AIHA was not found. AIHA occurred only 0.5% of total patients, and 3.4% of patients in cohort I group.

It can be confirmed through this result that fludarabine may trigger AIHA in patients with CLL.

The incidence of AIHA after therapy

Table 13: Incidence of AIHA after therapy

			Study Arm			Total
			Cohort I	**Cohort II**	**Cohort III**	
AIHA after Therapy	No	Count of patients	70	73	441	584
		% within AIHA after therapy	12.0%	12.5%	75.5%	100.0%
		% within study arm	95.9%	94.8%	98.0%	97.3%
	Yes	Count of patients	**3**	**4**	**9**	**16**
		% within AIHA after therapy	18.8%	25.0%	56.3%	100.0%
		% within study arm	**4.1%**	**5.2%**	**2.0%**	**2.7%**
Total		Count	73	77	450	600
		% within AIHA after therapy	12.2%	12.8%	75.0%	100.0%
		% within study arm	100.0%	100.0%	100.0%	100.0%

In the table 13, we could find patients with AIHA in all study arms: 4.1% in cohort I, 5.2% in cohort II, and 2.0% in cohort III. CLL is well known as the most common cause of AIHA. We could see slightly the difference of the incidence of AIHA between in cohort I and in cohort II.

The incidence of AIHA during & after therapy

Table 14: Incidence of AIHA during and after therapy

			Study Arm			Total
			Cohort I	**Cohort II**	**Cohort III**	
AIHA during/after Therapy	No	Count of patients	82	79	478	639
		% within AIHA during/after therapy	12.8%	12.4%	74.8%	100.0%
		% within study arm	93.2%	95.2%	98.2%	97.1%
	Yes	Count of patients	**6**	**4**	**9**	**19**
		% within AIHA during/ after	31.6%	21.1%	47.4%	100.0%

		therapy				
		% within study arm	**6.8%**	**4.8%**	**1.8%**	**2.9%**
Total		Count	88	83	487	658
		% within AIHA during/after therapy	13.4%	12.6%	74.0%	100.0%
		% within study arm	100.0%	100.0%	100.0%	100.0%

We could see a slightly higher incidence of AIHA during and after therapy in cohort I than cohort II and III: 6.8% in cohort I, 4.8% in cohort II, and 1.8% in cohort III. And total 2.9% of patients in study have developed AIHA.

3.3.2. The incidence of AITP

AITP occurs in 2-3% of CLL patients and it occurs in early stage disease and may be a presenting manifestation. Initial therapy for AITP should consist of prednisone. Seventy percent of patients respond. Splenectomy is a reasonable second-line treatment. Autoimmune phenomena, largely related to blood cells, are based in the immune dysregulation of CLL. Longer survivals in CLL patients, more treatment regimens per patient, and more immunosuppression with modern treatments, allow us to predict an increasing incidence of autoimmune blood cell diseases in CLL (Diehl LF, Ketchum LH. 1998).

The cause of autoimmune phenomena in B-CLL is unclear, although it is thought to be associated with disturbances in T cell subsets secondary to the B cell proliferation (Catovsky D. 1984). In most cases the cause of AITP is bone marrow infiltration with leukemia. Because of the unsatisfactory nature of platelet antibody tests, the true prevalence of AITP in CLL is unknown.

Fludarabine has been reported to be causative in the onset of autoimmune thrombocytopenia with CLL. Montillo et al first reported relapse of CLL-associated AITP after treatment with fludarabine (Montillo et al. 1994). And then Hamblin TJ reported that a total of 25 cases of fludarabine-associated AITP have been reported (Hamblin TJ, 2001). Contrary to the reports of Montillo et al and Hamblin, Dearden reported that the development of AITP occurred predominantly in patients who were not receiving therapy, 25% of the cases occurring at, or shortly after, CLL diagnosis. There was no association with use of fludarabine although other cases have been reported in the literature (Dearden C, 2008). Actually, there is no consensus about standard criteria of AITP and the association with treatment with fludarabine.

We analyzed the incidence of AITP (***Criteria: platelet count: < 100.000/µl and ≥ 50.000/µl drop from baseline***).

The incidence of AITP during therapy

Table 15: Incidence of AITP during therapy

			Study Arm			Total
			Cohort I	Cohort II	Cohort III	
AITP during Therapy	No	Count of patients	69	83	485	637
		% within AITP during therapy	10.8%	13.0%	76.1%	100.0%
		% within study arm	79.3%	100.0%	100.0%	97.3%
	Yes	Count of patients	**18**	**0**	**0**	**18**
		% within AITP during therapy	100.0%	.0%	.0%	100.0%
		% within study arm	**20.7%**	**.0%**	**.0%**	**2.7%**
Total		Count of patients	87	83	485	655
		% within AITP during therapy	13.3%	12.7%	74.0%	100.0%
		% within study arm	100.0%	100.0%	100.0%	100.0%

In the table 15, the occurrence of AITP during therapy was observed in 18 patients (20.7%) only in cohort I.

The incidence of AITP after Therapy

Table 16: Incidence of AITP after therapy

			Study Arm			Total
			Cohort I	Cohort II	Cohort III	
AITP after Therapy	No	Count of patients	68	74	433	575
		% within AITP after therapy	11.8%	12.9%	75.3%	100.0%
		% within study arm	94.4%	96.1%	96.7%	96.3%
	Yes	Count of patients	**4**	**3**	**15**	**22**
		% within AITP after therapy	18.2%	13.6%	68.2%	100.0%
		% within study arm	**5.6%**	**3.9%**	**3.3%**	**3.7%**
		Count of patients	72	77	448	597

Total	% within AITP after therapy	12.1%	12.9%	75.0%	100.0%
	% within study arm	100.0%	100.0%	100.0%	100.0%

After therapy we found more occurrences of AITP in all study arms. The incidence of AITP in cohort I was higher than in other groups: 5.6% in cohort I, 3.9% in cohort II, and 3.3% in cohort III.

The incidence of AITP during & after Therapy

Table 17: Incidence of AITP during & after therapy

			Study Arm			Total
			Cohort I	Cohort II	Cohort III	
AITP during/after Therapy	No	Count of patients	68	80	470	618
		% within AITP during/after therapy	11.0%	12.9%	76.1%	100.0%
		% within study arm	78.2%	96.4%	96.9%	94.4%
	Yes	Count of patients	**19**	**3**	**15**	**37**
		% within AITP during/ after therapy	51.4%	8.1%	40.5%	100.0%
		% within study arm	**21.8%**	**3.6%**	**3.1%**	**5.6%**
Total		Count of patients	87	83	485	655
		% within AITP during/after therapy	13.3%	12.7%	74.0%	100.0%
		% within study arm	100.0%	100.0%	100.0%	100.0%

The total incidence of AITP during and after therapy is showed in above table.
We could observe 19 patients in cohort I (21.8%), 3 patients in cohort II (3.6%), and 15 patients in cohort III(3.1%) who have developed AITP.
The incidence of ITP is significantly higher in cohort I than in cohort II and III.

4. Discussion

Before the appearance of purine analogs, such as fludarabine, cladribine, and pentostatin (Nipent), Chlorambucil was the standard first-line therapy for treatment of patients with CLL. But it has been suggested that chlorambucil might increase long-term toxicity in patients treated with it, when compared to patients without treatment with it.
Comparative, randomized and non-randomized studies showed that higher chlorambucil doses induce a higher response rate and a longer overall survival. However, higher chlorambucil doses showed, as expected, higher hematological toxicity than standard doses (Maura et al. 2006).

The appearance of purine analogs was a big step for the treatment of patients with CLL, because it was proved that fludarabine as single agent induced higher complete response rates than chlorambucil and improved patients' quality of life. (Rai et al. 2000; Eichhorst et al. 2003). The purine analogs now used are following: fludarabine, cladribine, and pentostatin (Nipent). Fludarabine of these has been most widely tested and used for treatment of patients with CLL.

In addition, fludarabine plus cyclophosphamide combination chemotherapy resulted in significantly higher complete remission rate (24%) and overall response rate (94%) compared with fludarabine alone (7% and 83%; P<.001 and P=.001). Fludarabine plus cyclophosphamide treatment also resulted in longer median progression-free survival (48 vs 20 months; P=.001) and longer treatment-free survival (37 vs 25 months; P<.001). But so far, no difference in median overall survival has been observed.
Fludarabine plus cyclophosphamide combination therapy showed that this therapy caused significantly more thrombocytopenia and leukocytopenia compared with fludarabine alone but did not increase the number of severe infections (Eichhorst et al 2006).

4.1. Response Rates

We tried to evaluate the efficacy of fludarabine as a single agent in early CLL Binet stage A through the study. The table 4 shows that 91.6% of patients reached the overall response (ORR). The partial response (PR) rate was 56.9% and the complete response (CR) rate 34.7%. And the progression disease (PD) showed only 2.8%. When compared PFS-rate and overall survival rate in three study arms, patients in cohort III showed the best overall survival rate and progression free survival (PFS) rate. There was no difference in the overall survival rate between patients in cohort I and in cohort II, although patients in cohort I showed higher PFS rate than it in cohort II.

The study of Rai et al showed that the response rate was significantly higher for fludarabine monotherapy than for chlorambucil. According to the study, among 170 patients treated with fludarbine, 20% had a complete remission, and 43% had a partial remission. Therefore, ORR was 63%.
The corresponding values for 181 patients treated with chlorambucil were 4% CR and 33% PR (P< .001 for both comparisons). And ORR was only 37%. The median duration of remission and the median progression-free survival in the fludarabine group were 25 months and 20

months, respectively, whereas both values were 14 months in the chlorambucil group (P< .001 for both comparisons). The median overall survival among patients treated with fludarabine was 66 months, and 56 months among patients treated with chlorambucil. The difference between two groups was not significant. And severe infections and neutropenia was more frequently observed among patients treated with fludarabine than with chlorambucil.
This study has tried to assess the efficacy of the fludarabine plus chlorambucil combination therapy. But they had to stop investigating the efficacy of combined treatment because of excessive rates of life-threatening toxic effects (Rai et al. 2000).

The study of Eichhorst et al showed that fludarabine plus cyclophosphamide combination therapy in first-line treatment of younger patients with CLL resulted in higher response rates (complete remission rate: 24%, overall response rate: 94%) and the treatment-free survival (37 months) than fludarabine monotherapy (complete remission rate: 7%, overall response rate: 94%, treatment-free survival: 25 months). The patients were younger than 66 years and had predominantly advanced CLL (Eichhorst et al.2006).

Our results showed higher response rates (ORR: 91.6%, CR: 34.7%), when compared our study with studies of Rai et al (ORR: 63%, CR: 20%) and Eichhorst et al (ORR: 83%, CR: 7%). We analyzed patients in Binet stage A. Therefore, patients in our study were in early stage of CLL. But patients in study of Rai et al were in advanced stage of CLL. Therefore, we had a higher response rates than both studies.

Thomas et al investigated the activity of rituximab in untreated high risk, early-stage CLL. According to this study, the overall response rate was 90% (complete response: 19%, nodular partial response: 19% and partial response: 48%) (Thomas et al. 2002).
These results showed that rituximab has significant efficacy for treatment of patient with early-stage CLL. But the median follow-up was only 8 months (range: 2-16 months). To investigate the efficacy of rituximab as a single agent more accurately, this study requires longer follow-up.

4.2. Impaired Immunity and Infections.

Patients with CLL show a dysregulated immune system, such as T- and B-cell dysfunction, hypogammaglobulinemia and autoimmune complications.
The impaired immunity is well-known as a typical complication for patients with CLL and is caused by quantitative and qualitative defects in B- and T-cells. Therefore, a typical complication of impaired immunity is infection because of dysfunction of immune system.
We analyzed the incidence of infections in different study arms. Through this study we aimed to show influence of fludarabine therapy in impaired immunity by analyzing hypogammaglobulinemia and infection.
Firstly, we analyzed incidence of hypogammaglobuliemia. As we see in table 5, we could observe the reduction of immunoglobulin: 11.3% of patients showed low levels of IgA. 8.4% in IgM and 15.3% in IgM showed low levels of immunoglobulin. The most patients with Binet stage A presented with normal quantitative immunoglobulins and absolute neutrophils values. It is well-known that the severity tends to increase with the duration and stage of CLL.

Through the table 5, we could compare incidence of infections between the patients with normal or higher levels of immunoglobulin and patients with low levels of immunoglobulin, and patients with low levels of immunoglobulin showed slightly higher incidence of infection.

Next, we observed infections of patients. As we know, infection is well known as the most common cause of death of patients with CLL. Through the table 6, we can see the incidence of infection in 3 study arms: 41.5% of patients in cohort I, 30.4% of patients in cohort II and 30.9% of patients in cohort III. When compared the results of three study arms, we could see apparently higher incidence of infection in cohort I. And the incidence of infection between in cohort II and in cohort III almost showed no difference. If we compare the difference of incidence between in cohort I and in cohort II, we can better observe the influence of fludarabine therapy in incidence of infection. The incidence of infection showed 11.1% higher in cohort I than in cohort II, which means that fludarbine therapy increased the incidence of infection. When we observed the incidence of severe infections in three study arms, we could see the same results. We could see higher incidence of severe infections in cohort I than other two study arms, even though we could rarely observe the incidence of severe infections.
We also analyzed the duration of infections, infection-related mortality and prognostic factors of infections. When observed the duration of infections, it was difficult to find the difference of results in three study arms. However, we could see through the diagram 5 that infections with long duration were relative frequently observed in patients of cohort I than in patients of other two study arms.
In the group of patients with documented infections we observed 4 dead patients, which were all patients with high risk of progression stratified and randomized in cohort I. Infection is well-known as the main cause of death in patients with CLL. Therefore, we aimed to show association of death of patient with infections. One of them had a documentation of severe infection. However, when we observed the cause of death, lung cancer was the cause of death of two patients. And the other patients died of unknown cause. Therefore our study results can not demonstrate that the main reason for death is infection. Patients in our study had CLL in early Binet stage A. That means that most patients were still healthy and the immune system of them was not significantly weakened.
Through analyzing the risk factors of infections (see table 11), we could find that increased serum creatinine can be regarded as the best risk factor of infection. There were 10 patients with CLL having more than 1.5 mg/dl serum creatinine. And 9 of these 10 patients had infections. That means, 90% of patients having more than 1.5mg/dl serum creatinine had infections, although the most patients (98%) had less than 1.5 mg/dl serum creatinine. The difference of infection rate between in patients with ≥1.5 mg/dl serum creatinine and in patients with <1.5mg/dl serum creatinine is 58.2%.
In addition, hypogammaglobulinemia, splenomegaly, low LDH and low platelet count can be regarded as proper risk factors of infection.

4.3. Autoimmune Complications of CLL

CLL is associated with an acquired immune defect that can cause autoimmune complications, including autoimmune hemolytic anemia (AIHA) and autoimmune thrombocytopenia (AITP).
We analyzed the incidence of AIHA and AITP in 3 arms. Unfortunately, there are no fixed

standard criteria for diagnosis of AIHA and AITP now. So we have set the criteria for diagnosis of AIHA and AITP as follows [AIHA: hemoglobin < 11mg/dl and ≥ 3.4 mg/dl drop from baseline, AITP: platelet count: < 100.000/$\mu\ell$ and ≥ 50.000/$\mu\ell$ drop from baseline].

AIHA

AIHA is well-known as the most common complications of CLL. And it is repeatedly reported that AIHA can be triggered by fludarabine. Therefore, we analyzed the incidence of AIHA in 3 study arms. Especially, we compared the incidence of AIHA between in cohort I and in cohort II. Both study arms include patients with high risk of progression. But only the patients in cohort I receive treatment with fludarabine. Therefore, by comparing the incidence of AIHA in cohort I with it in cohort II, we aimed to confirm the influence of fludarabine on the occurrence of AIHA.

During therapy, we could observe 3 patients only in cohort I who developed AIHA. In other words, AIHA occurred 3.4% of patients in cohort I, and 0% of patients in cohort II and III. Through this result, it can be confirmed that fludarabine may increase the incidence of AIHA in patients with CLL.

After therapy, we analyzed again the incidence of AIHA in a observational period of 24 months. We could find 16 patients with AIHA in all 3 study arms. And the incidence of AIHA in cohort II (5.2%) was higher than in cohort I (4.1%) and III (2.0%). It is already well-known that progressive CLL is the most common cause of AIHA.

The total incidence of AIHA during and after therapy is shown on the table 14.

The total incidence of AIHA in 3 study arms is as follows: 6.8% in cohort I, 4.8% in cohort II and 1.8% in cohort III.

As expected, we could observe more incidence of AIHA in cohort I than cohort II and III. And more occurrences of AIHA were observed in cohort I and II than in cohort III.

AITP

Approximately 2% of patients with CLL develop clinically significant autoimmune thrombocytopenia (AITP). There are no clear criteria for diagnosis and the platelet antibody tests lack sensitivity and specificity. Nevertheless, the occurrence of a rapid unexplained fall in platelets, in the absence of evidence of bone marrow (BM) failure or hyposplenism, suggest an immune origin for the thrombocytopenia.

Early onset of ITP and refractoriness to treatment were associated with the poorest outcomes (Dearden. 2008)

We analyzed the incidence of AITP in 3 study arms.

During therapy we could find 18 patients with AIHA only in cohort I. After therapy we found more occurrences of AITP in all study arms. The incidence of AITP in cohort I was higher than in other two study arms: 5.6% in cohort I, 3.9% in cohort II, and 3.3% in cohort III. Totally, we could observe 19 patients in cohort I (21.8%), 3 patients in cohort II (3.6%), and 15 patients in cohort III (3.1%) who have developed AITP. The incidence of ITP is significantly higher in cohort I than in cohort II and III.

5. Conclusion

Until now, with conventional therapies CLL cannot be cured. There is an increasing trend toward no treatment at the time of initial diagnosis. Therefore, the standard option to treat patients with high risk of progression in CLL Binet stage A is the “watch and wait” strategy. Our CLL-1 study intended to answer the question, whether administration of early and risk-adapted fludarabine as a monotherapy in CLL Binet stage A patients with high risk of progression would bring an advantage for the patients. In addition, we tried to analyze the side-effect of the treatment with fludarabine.
Fludarabine is the best studied purine analogue used for the treatment of CLL. The major side-effects are myelosuppression with neutropenia and immunosuppression and reduction of the T helper cells, at higher doses CNS toxicity (rarely progressive encephalopathy), nausea, vomiting, mucositis/stomatitis, diarrhea, anorexia and elevation of transaminases. We analyzed the incidence of infection, impaired immunity and autoimmune complications (AIHA, AITP) in all three different study arms.

We could observe that the treatment with fludarabine could not prolong the overall survival rate in patients with high risk progression in CLL Binet stage A, although patients treated with fludarabine showed higher PFS rate.

The incidence of infection was higher in cohort I than in cohort II & III (41.5% vs. 30.4% & 30.9%). Furthermore, the incidence of severe infection was also higher in cohort I than other two arms (6.1% vs. 2.5% & 2.4%), even though we could rarely observe the incidence of severe infection. It was observed that the incidence of infection showed no difference between patients in the observation arms regardless of risk of progression (cohort II: 30.4%, cohort III: 30.9%). The most common spectrums were viral (39.8%) and bacterial (36.1%) infections, mycotic infections were rare (1.5%). Soft tissue infections, respiratory tract infections and FUO were the most common sites of infections. Soft tissue infections were significantly more observed in the cohort I arm. Among the severe infections significantly more respiratory tract and soft tissue infections were observed in the cohort I arm. No fatal infection was observed. In the analysis of risk factors of infection, serum creatinine can be regarded as the best risk factor in our study. In addition, IgA, IgG, IgM, splenomegaly, low LDH and low platelet count can be regarded as proper risk factor of infection. Our data show a trend for the hypothesis that the risk for infections is higher in patients treated with fludarabine.

The autoimmune complications were observed in three study arms. The occurrence of AIHA during therapy was observed in 3 patients (3.4%) of only the cohort I arm. After therapy, patients developed AIHA were observed in all study arms; cohort I: 3 patients (4.1%), cohort II: 4 patients (5.2%), cohort III: 9 patients (2.0%). Total incidence of AIHA in all study arms were as follows; cohort I: 6 patients (6.8%), cohort II: 4 patients (4.8%), cohort III: 9 patients (2.0%). We could observe that patients treated with fludarabine (cohort I) showed slightly higher incidence of AIHA than other study arms.
The occurrence of AITP during therapy was observed in 18 patients (20.7%) in only the cohort I arm. After therapy we could observe the occurrence of AIHA in all study arms; cohort I: 4 patients (5.6%), cohort II: 3 patients (3.9%), cohort III: 15 patients (3.3%). Total incidence of AITP was as follows: cohort I: 19 patients (21.8%), cohort II: 3 patients (3.6%),

cohort III: 15 patients (3.1%). Through this study we could observe that patients received fludarabine therapy (cohort I) showed significantly higher incidence of AITP.
Our study shows a trend for the hypothesis that the risk of autoimmune complications is associated with the treatment with fludarabine.

6. References

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8. Notes

9. Appendix

Table 18: ECOG Performance Status Scale

ECOG Performance Status	
Score	Description
0	Asymptomatic (Fully active, able to carry on all pre-disease activities without any restriction)
1	Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature. For example, light housework, office work)
2	Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to carry out any work activities. Up and about more than 50%
3	Symptomatic, >50% in bed, but not bedbound (Capable of only limited selfcare, confined to bed or chair 50% or more of waking hours)
4	Bedbound (Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair)
5	Death

“ECOG” represents the “performance status” of the patients according to the criteria used by ECOG (Eastern Cooperative Oncology Group) (Oken et al 1982).

The performance statuses were analyzed prior to the therapy in every patient. Patients with an ECOG-status of more than 2 would not be able to meet the inclusion criteria and therefore would be excluded from the study.

10. **Glossary of Abbreviation**

ADH	Antidiuretic Hormone
AIHA	Autoimmune Hemolytic Anemia
AITP	Autoimmune Thrombocytopenia
CLL	Chronic Lymphocytic Leukemia
CNS	Central Nervous System
CR	Complete Remission
CTC	Common Toxicity Criteria
ECOG	Eastern Cooperative Oncology Group
EORTC	European Organization for Research and Treatment of Cancer
FACS	Fluorescence Activated Cell Sorting
GCLLSG	German CLL Study Group
GITMO	Italian Group for Bone Marrow Transplantation
HR-F	High Risk-Fludarabine
HR-WW	High Risk-Watch and Wait
LDH	Lactate Dehydrogenase
LDT	Lymphocyte Doubling Time
LR	Low Risk
NCI	National Cancer Institute
NCI-WG	National Cancer Institute-sponsored Working Group
nCR	Nodular Complete Remission
NHL	Non Hodgkin Lymphoma
OS	Overall Survival
PR	Partial Response
PD	Progressive Disease
PFS	Progression Free Survival
SD	Stable Disease
SIE	Intalian Society of Hematology
SIES	Italian Society of Experimental Hematology
sTK	Serum Thymidine Kinase
W&W	Watch and Wait
WHO	World Health Organization

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