MEDICAL UNIVERSITY VARNA
FACULTY OF MEDICINE

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PROGNOSTIC FACTORS: INTEGRATING RISK ASSESSMENT AND TIME TO TREATMENT SCALE IN NAÏVE B- CLL PATIENTS IN CLINICAL PRACTICE

ABSTRACT

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The dissertation is presented on 123 pages and is illustrated with 49 figures and 30 tables. The bibliography includes 199 sources, of which 5 in Cyrillic and 194 in Latin. All clinical trials related to the dissertation were conducted on the territory of "Dr. Georgi Stranski" University Hospital, - Pleven, the Clinic of Hematology, Central Clinical Laboratory, Medical Laboratory of Immunodiagnostics, Clinic of Imaging Diagnostics and Laboratory of Molecular Biology and Cytogenesis NSHATHZ – Sofia. The trials were based on two research projects funded by the Medical University - Pleven

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Members of a scientific jury

Chairman:
Prof. Liudmila Angelova, MD PhD- review

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Assoc. Prof. Ilina Micheva, MD PhD opinion
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Prof. Dr. Margarita Genova, MD PhD position
Assoc. Prof. Veselina Goranova, MD PhD review

The defense materials are published on the website of MU-Varna http//www.mu-varna.bg

CONTENTS

Abbreviations used:

B - CLL - B chronic lymphocytic leukemia

β-2 MG - beta 2 microglobulins

ABLC - absolute B- lymphocyte count

LDT - lymphocyte doubling time

TTFT - time to first treatment

OS - overall survival

IGVH - mutation status

Flt 3 - fms - tyrosine kinase 3

c - kit - protooncogene c- kit

IL 7 R – interleukin 7 receptor

PU .1- transcription factor PU .1

Ikaros - DNA-binding protein Ikaros

EBF 1 - early B-cell factor 1

PAX -5 - B-cell specific transcription factor

Fox 01- transcription factor Fox 01

Runx 1- connected to Runx 1 transcription factor 1

c – Myb- proto-oncogene

RAG genes - recombinant activating genes

BCR - B-cell receptor

NF - κB - nuclear factor κB

SF3B1 - splicing factor 3B1

CXCR 4 - C-C-C chemokine receptor type 4

smIg - membrane-bound immunoglobulin

SRC - protein kinase

LYN - protein kinase

SYK- splenal tyrosine kinase

PI3 K - phosphatidylinositol 3-kinase

BTK - Bruton tyrosine kinase

PKC - protein C kinase

TNF - tumor necrosis factor

VLA - 4 integrin alpha 4beta1 - very late antigen / integrin

VCAM - 1 vascular cell adhesion molecule 1

MBL - monoclonal B-cell lymphocytosis

DLBCL -diffuse B-cell lymphoma

CT- computed tomography

PET- positron emission tomography

FISH - fluorescent in situ hybridization

VEGF - vascular endothelial growth factor

Mcl- 1- antitapoptotic protein

AID - activation inducing cytidine deaminase

DNA - DNA

ATM - ataxia telangiectasia gene

NOTCH 1 - gene encoding transmembrane protein

PFS - progression-free survival

RT - PCR – Real-time polymerase chain reaction

LPL - lipoprotein lipase

ADAM 29– ADAM metallopeptidase domain 29

NAD - nicotinamide adenine dinucleotide

cADPR - cyclic ADP ribose

NAADP - nicotinamide adenine dinucleotide phosphate

MMP-9 - matrix metallopeptidase 9

TLRs - Toll - like receptors

CIT- immunochemotherapy

WBC – white blood cell count

Epidemiological data and relevance of the problem:

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in adults. The disease occurs mainly in Western countries and relatively rarely in Asian countries. The average age at diagnosis is about 70 years. The risk of disease progressively increases with age. According to the literature, more than 70% of patients are older than 65, and a significant proportion of them have more than two comorbidities (Pinilla-Ibarz et al., 2015; Strati, P et al., 2019).

For Bulgaria, the incidence among men of all age groups is 5.0 per 100,000 people at global standard levels of 3.7 and 2.9, respectively, compared to 2.1 for women as of 2014. (Valerianva Z, et al. 2014).

The clinical course of the disease is extremely diverse and variable. Although controllable over a period of time, the disease can progress from a sluggish to an aggressive form.

In 2008, the definition of B-CLL was revised and the current diagnosis of B-CLL requires the presence of 5,000 cells/μl of morphologically mature, monoclonal B-lymphocytes expressing B-cell antigenic markers CD19, CD23, low levels of sIgM and co-expression of the T-cell antigen CD5, in peripheral blood, for at least 3 months.

The critical absolute lymphocyte count (ABLC) threshold of 5 000 cells/μl was replaced with an absolute B-cell lymphocyte count. This served as the basis for introducing the term monoclonal B-cell lymphocytosis (MBL) and the re-classification of early-stage patients Rai 0 / I into MBL or small cell lymphocytic lymphoma (Marti E Gerald, 2008).

Due to the generally accepted fact that about 80-85% of newly diagnosed patients remain under observation, as well as in order to predict the evolution and control the disease, for years measurable indicators have been sought that are reliable, stable and at the same time related to the complex and not well-known pathogenetic processes occurring in neoplastic cells.

Prognostic factors are variables that are assessed prior to treatment and based on which good or poor clinical outcome can be expected, regardless of treatment choice (Simms L et al., 2013). Some of them can be considered as prognostic and predictive factors (Nalejska, E., et al. 2014).

In clinical practice, they have been introduced to identify which patients will need early treatment, to determine the time to initiate treatment, to select risk-adapted therapy (drug toxicity), and to determine a strategy for monitoring patients.

After 2000, thanks to the development and improvement of immunology, immunogenetics and molecular biology, a number of author teams have intensively developed and proposed prognostic models, indices and scales for risk stratification in patients with newly diagnosed B-CLL.

Genomic aberrations, referred to by some authors as “moving targets” that change dynamically throughout the course of the disease (Amaya-Chanaga et al., 2016), are present in most risk assessment models and in treatment choices. The mutational status of the immunoglobulin heavy chain variable region (IGVH) genes, defined by sequencing methods, is a stable indicator that does not change in the course of the disease.

Considered in a combination with the so-called "classical prognostic factors", the latter serve as a basis for prognosis and selection of targeted therapy and are part of the international prognostic index - CLL-IPI presented in 2016.

Although originally developed to predict overall survival, several clinical trials with multiple patient groups have validated its use in estimating the time to initiation of initial treatment (Parikh A S, 2018).

The last mentioned indicator, like the factors influencing it, are extremely important and require good knowledge, because, taken together they indirectly reflect the aggressiveness of the disease and are related to the survival and outcome of the disease.

In clinical practice, besides the two staging systems, the feasible methods for diagnosis, risk assessment and disease monitoring are reduced to flow cytometric analysis, fluorescent insitu hybridization, and PCR.

At present, there is no consensus on the methods of diagnosing the condition in our country. Reported studies are based on evidence related to a comprehensive assessment of available molecular genetic and classical prognostic factors, although the need for a more accurate assessment of the risk of progression is becoming increasingly important, given the introduction of new drugs for the treatment of B-CLL.

Working hypothesis

The different and variable clinical course of chronic lymphocytic leukemia in patients classified in the same clinical stage is probably related to their different molecular genetic profiles. Some of the unfavorable prognostic markers are present before the diagnosis is made, suggesting aggressive course of the disease and determining a shorter time to initiation of antileukemic therapy. They suggest the need for early identification in each patient with B - CLL. Complex monitoring of surrogate markers of mutation status, chromosomal abnormalities, molecular deviations with classical prognostic factors proven in clinical practice would contribute to a more precise risk stratification and the time to treatment prognosis in patients with newly diagnosed B-CLL. At the same time, due to the fact that some of the prognostic factors play the role of predictive markers, their determination would help choose a therapy adapted to the specific patient, effective and free of early and late side effects, thus providing a better quality of life and limiting the need for prolonged hospitalizations.

Aim of the study:

To study and analyze the impact of available clinical-laboratory and molecular, and genetic factors related to the specific characteristics of both patient and disease, and to assess their impact on the time when initiation of treatment is needed in untreated patients with B - CLL.

Tasks of the scientific investigation:

- ✓ 1. To study the demographic indicator age and its importance as a factor related to the time to treatment in patients with untreated B - CLL .
- ✓ 2. To analyze the significance of the stages of the disease determined in the diagnosis, as a prognostic factor related to TTFT .
- ✓ 3. To look for a correlation between the serum marker β-2 MG and the time to treatment.
- ✓ 4.To investigate the frequency of some clinically significant chromosomal aberrations among untreated patients with B - CLL and their distribution depending on the stage of the disease.
- ✓ 5. To determine mutation status based on the surrogate markers LPL, ADAM 29 and seek a link between molecular and genetic changes in patients with untreated B - CLL.

- ✓ 6. To make a comprehensive assessment of the prognostic factors and their importance in determining the time to first treatment .

Materials and methods:

Place and time of the study

The study was conducted on the territory of "Dr. Georgi Stranski" University Hospital - Pleven, including the following units: Hematology Outpatients Consulting Room, Clinic of Hematology, Central Clinical Laboratory, Medical Laboratory of Immunodiagnostics, Clinic of Imaging Diagnostics and Laboratory of Molecular Biology and Cytology at The National Hematology Hospital - Sofia.

The study period was within three years 2016-2019. The design includes two types of observation: prospective and retrospective. The study involved trained medical professionals from the above units. A total of 97 patients with documented B - CLL, over 18 years of age were studied.

Data from available medical documentation and laboratory tests were used. A patient questionnaire was designed, presented below as Appendix 1. Routine laboratory tests were performed, including complete blood count, differential count, and a biochemical panel. Additionally, in cases of suspected concomitant disease, autoimmune hemolytic anemia and / or thrombocytopenia, the Coombs' test, reticulocyte count, and antiplatelet antibodies test were performed. Patients with a proven autoimmune phenomenon were excluded from the study.

Screening of the patients was carried out based on the performed flow cytometric analysis of peripheral blood. To this purpose, after signing an informed consent approved by the Local Ethics Commission of MU-Pleven, three milliliters of venous blood were taken from each patient by venipuncture in a vacutainer containing EDTA. The blood samples were stored at room temperature (22-24 ° C). All samples were processed within 6-24 hours after taking. Leukocytes were analyzed using a dual laser flow cytometer FACSCalibur (Becton Dickinson, Heidelberg, Germany) and Cell Quest Pro Software (Becton Dickinson). Lymphocytes were separated by CD 45 / SSC gating. A panel of the following monoclonal antibodies was used (Immunize , Salamanca , Spain); CD45, CD5, CD19, CD20, CD22, CD23, CD 38, CD 11 a , CD 49 d , CD 29.

Patients with the so-called "atypical CLL ", i.e., cases in which the expression of CD5, CD23 was weak to absent, or those with strong expression of CD20, or a combination of the

above, were not included in the study. Patients with monoclonal B cell lymphocytosis (MBL) with absolute lymphocyte count <5.0 x 10^9/l, as well as those, meeting the criteria of small lymphocytic lymphoma, were also excluded from the study.

Staging the patients:

The Binet staging system was used to stage the disease, consisting of three stages as follows:

Stage A: less than three enlarged lymph-node areas, the absence of anemia and thrombocytopenia from laboratory examination of peripheral blood, and presence of lymphocytosis;

Stage B*:* three or more enlarged groups of lymph nodes, in the absence of anemia and thrombocytopenia from laboratory examination of peripheral blood;

Stage C*:* presence of anemia (hemoglobin below 100g/l) and / or thrombocytopenia (platelet count below $100x10^9$/ l).

Five anatomical lymph areas of involvement have been identified *(Binet, JL, et al. 1981):*

1. Head and neck, including the Waldeyer`s ring (considered a single area, even if there was more than one group of enlarged lymph nodes).
2. Axillary (whether engaged unilaterally or bilaterally, reported as one affected area).
3. Inguinal (involvement of the two inguinal groups of lymph nodes is considered as one area).
4. Palpable spleen.
5. Palpable liver

Imaging tests - X-ray of the chest, abdominal ultrasound, computer tomography were also conducted. The following sizes were accepted for deviations from normal: spleen - longitudinal size over 130 mm, transverse over 50 mm; of abdominal lymph nodes over 15 mm, established by ultrasound diagnostics *(Daksalov I. et al., 2004)*. Sizes of the lymph nodes in adults by region measured by CT were as follows: when short axis of one or more lymph nodes greater than 10 mm, this was considered lymphadenomegaly. The regional variations considered were as follows: inguinal 10 - 20 mm; pelvis - 10 mm (for ovoid lymph nodes) and 8 mm for round ones.

Serum marker for proliferative activity and tumor volume:

For investigation the serum concentration of beta 2-microglobulin, 3 ml of fresh or frozen serum was used, harvested by venipuncture transported into vacutainer with silicon particles to accelerate the clotting process. A turbidimetric method was applied to determine

the concentration. Concentrations were calculated automatically from the calibration curve. An automatic SPA Plus protein analyzer was used. Values from 0.80 to 2.35 mg / L were taken as reference limits.

Chromosomal aberrations and molecular markers

Fluorescence *in situ* hybridization (FISH) was performed according to a standard Vysis® protocol (Vysis®; Abbot Molecular Inc., Abbott Park, Illinois, USA) on interphase nuclei from peripheral blood cells. For the study of *p53, ATM and DLEU 1* genes, locus-specific deletion DNA probes were used: Vysis TP53 / CEP17 FISH Probe Kit, Vysis ATM / CEP11 FISH Probe Kit, Vysis D13S319 / 13q34 FISH Probe Kit. The target genes were marked with red fluorochrome, and the control regions of chromosomes 11 (centromere heterochromatin), 17 (centromere heterochromatin) and 13 (telomeric heterochromatin) were marked with green fluorochrome. Signal patterns on interphase nuclei were recorded and described according to ISCN (An International System for Human Cytogenetic Nomenclature), 2013. Signal readings were performed on a fluorescence microscope on at least 200 interphase nuclei from two independent analysts. The deletion of the target gene was considered clinically significant if more than 10% of the studied nuclei were affected. A digital camera and software from Olympus were used for documentation.

Expression of the LPL and ADA 29 genes was investigated by multiplex polymerase-chain reaction after reverse transcription [Reverse Transcription (RT) Polymerase Chain Reaction (PCR)]. For this purpose, from 1 μ g total RNA isolated from peripheral blood nucleated cells by Trizol Reagent, complementary DNA (coda) was synthesized in a medium containing random hexamers and MMLV reverse transcriptase with an appropriate buffer. The availability of RNA suitable for analysis and efficiency of reverse transcription were assessed by amplification of β - Actin cDNA. The expression of LPL and ADAM 29 was determined by co-application of *LPL and ADAM 29 cDNA* with primers - *GGAATGTATGAGAGTTGGGTGC / CAATGCTTCGACCAGGGGACC* [*LPL*] and TCTTATGTGGGCTGGTGGATCC / GACCTAGATGATGAGCCAC - TG [*ADAM 29*] under the following temperature conditions: 1 x 94 C ° / 5 min, 30 x [95 C ° / 30 sec ; 62 ° C / 30 sec ; 72 ° C / 30 sec.] , 1x 72 C ° / 5 min. PCR products were analyzed by electrophoresis in 2% agarose gel stained with SYBR Safe DNA gel stain and visualization after irradiation with UV rays in a photo documentation system Vilber Lourmat BLX-254. The expected size of the obtained PCR products was 445 a*nd* for *ADAM29* and 410 for *LPL,* respectively. In determining *LPL / ADAM29* status (as a surrogate marker of IGVH mutation

status), the recommendations of Oppezzo et al. (2005) were followed: [1] the presence of hypermutation in IGVH at establishing 1 band corresponding to the expression of the *ADAM29,* or 2 products corresponding to *ADAM29* and *LPL;* [2] unmutated IGVH status - when one band corresponding to of *LPL* expression was found. When visible products of amplification of *ADAM 29* and *LPL* were absent, despite the presence of *β -Actin* amplification, the respective sample was classified as of an indeterminate IGVH status.

Basic terms used for evaluation:

Time to first treatment (TTFT) was defined as the interval from diagnosis of the disease to the start of treatment, or the date of the last follow-up, or death (censored).

The indications for treatment were in accordance with the recommendations of IWCLL –2008, listed as text and Table № 1*(Hallek M et al., 2008).*

Constitutional symptoms were considered positive presence of one or more of the following associated with the disease:

- weight loss of 10% or more within the previous 6 months;
- fever over 38^0C with duration of two or more weeks, without evidence of infection;
- general fatigue, ECOG 2 (functional status), inability to perform normal physical activities;
- night sweats with duration of more than 1 month, without evidence of infection;

Table №1. Indications for treatment according to the stage of the disease (*Modified after Hallek M., 2008)*

Stage of disease	Clinical practice
Binet stage A	treatment is not recommended
Binet stage B	treatment is recommended in cases with active disease
Binet stage C	treatment is recommended

Disease progression for A stage patients is considered in the presence of at least one of the following:

- constant increase in lymphocyte count, lymphocyte doubling time less than 12 months;
- tendency to decrease hemoglobin and / or platelet counts;

- increase in the size of the lymph nodes, and / or spleen, and / or liver by 50%;
- emerging lymphadenomegaly and / or splenomegaly and / or hepatomegaly;
- symptoms: fever, night sweats, weight loss, weakness (London *Cancer CLL Guidelines 2015- 16v 1.0.*)

Statistical methods for data processing:

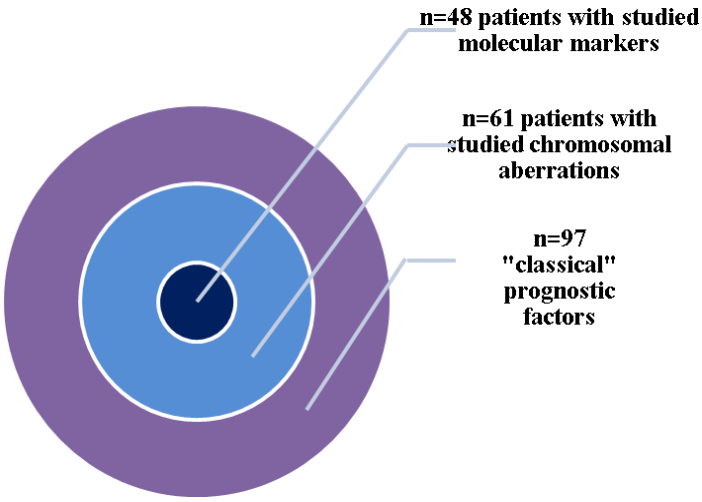
The study data was processed with a statistical software product SPSS 19. For the level of significance of the conclusions, which rejects the null hypothesis, $p < 0.05$ was chosen. The results are illustrated by tables, graphs and numerical values. Some of the data were processed with EXCEL. The methods used were description of quantitative variables, Chi-square test (χ^2) Pearson test), Kruscol - Wallistest, cross-tabulation to search for a connection between categorical features. To compare the time to initiation of initial treatment in different groups of patients depending on the indicators: age (Fig. № 4, 5, 6), sex (Fig. № 9), stages of the disease (Fig. № 11) involvement of the spleen (Fig. № 12), absolute B-cell lymphocyte count (Fig. № 14), values of serum beta-2 microglobulin (Fig. № 15), percentage of lymphocytes sow del13q (Fig. № 27) , mutational status (Fig. № 29), risk groups according to the modified CLL-IPI (Fig. № 30), the Logrank test (Kaplan-Meier) was used .

RESULTS:

Depending on the number of the investigations carried out, the 97 patients were divided into three groups as follows (Fig. №1)**:**

- **Group 1** included 97 patients in whom standard laboratory tests, flowcytometric analysis of peripheral mononuclear cells, beta 2-microglobulin level and staging procedures were performed at diagnosing the disease.
- **Group 2** included 61 randomly selected patients from group 1. On these patients, fluorescent *in situ* hybridization was performed.
- **Group 3** included 48 patients selected at random from group 2. These patients underwent additional PCR analysis

Figure №1. Distribution of patients by groups depending on the performed laboratory tests

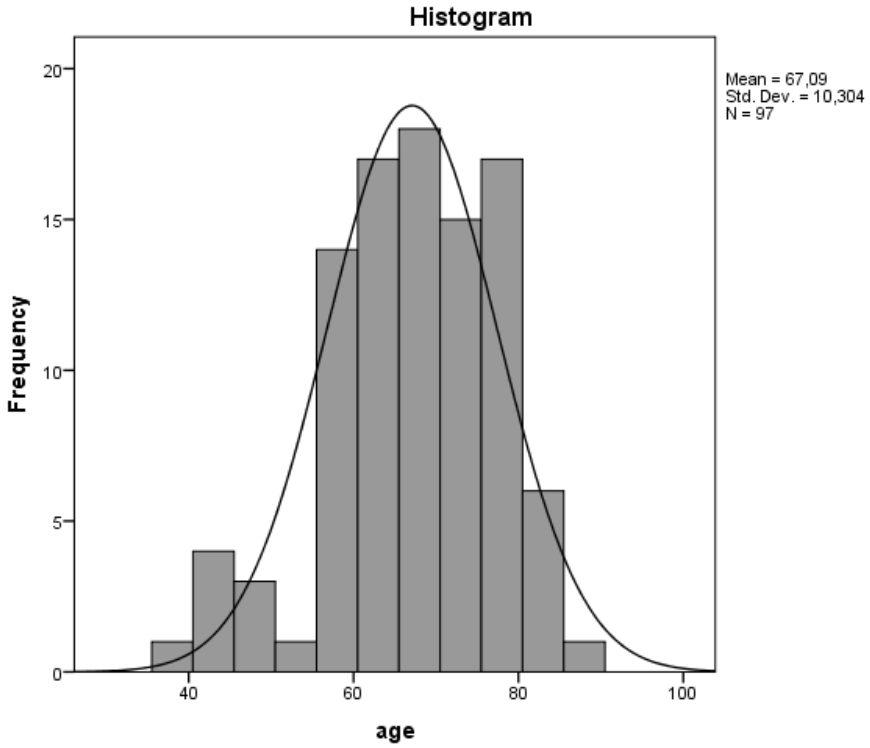


Genetic disorders and clinically significant chromosomal aberrations: 17 p-, 11q-, 13 q - were studied once over a three-year period in 61 patients. From this randomized group, PCR analysis was performed in 48 patients, the method was used as an alternative method for determination of mutational status.

Demographic factors and TTFT

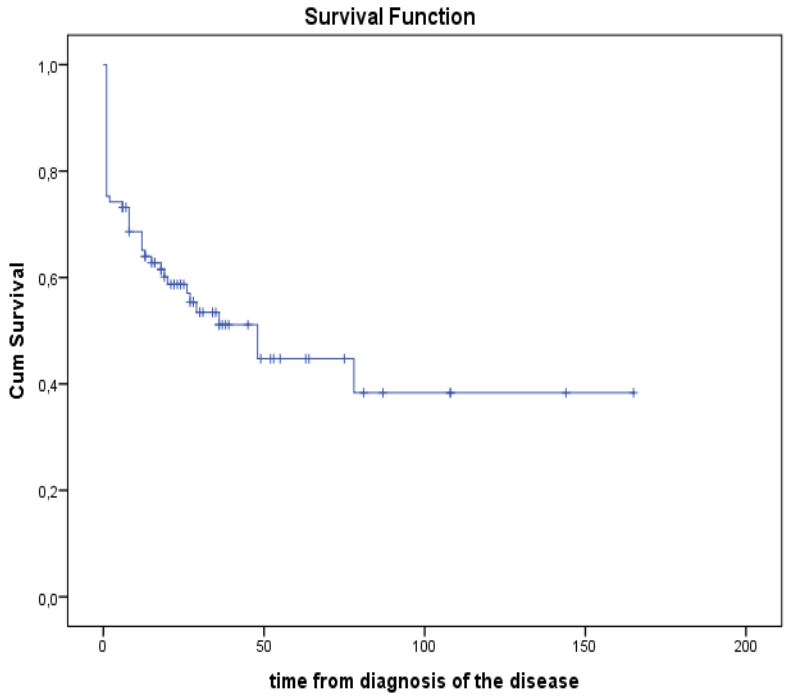
The distribution of the study group of 97 patients by age was parametric and is presented as a bell-shaped (Gaussian) curve (Fig. №2). The average age of the patients was 67 ± 10 years, ranging from 38 to 89 years.

Figure №2. Distribution of the patients by age



Because the data for the TTFT factor were not parametric, we determined the median time to treatment, which was 48 months in the group of 97 patients (Fig . № 3).

Figure №3. Median time from diagnosis to initiation of treatment, or last follow-up check

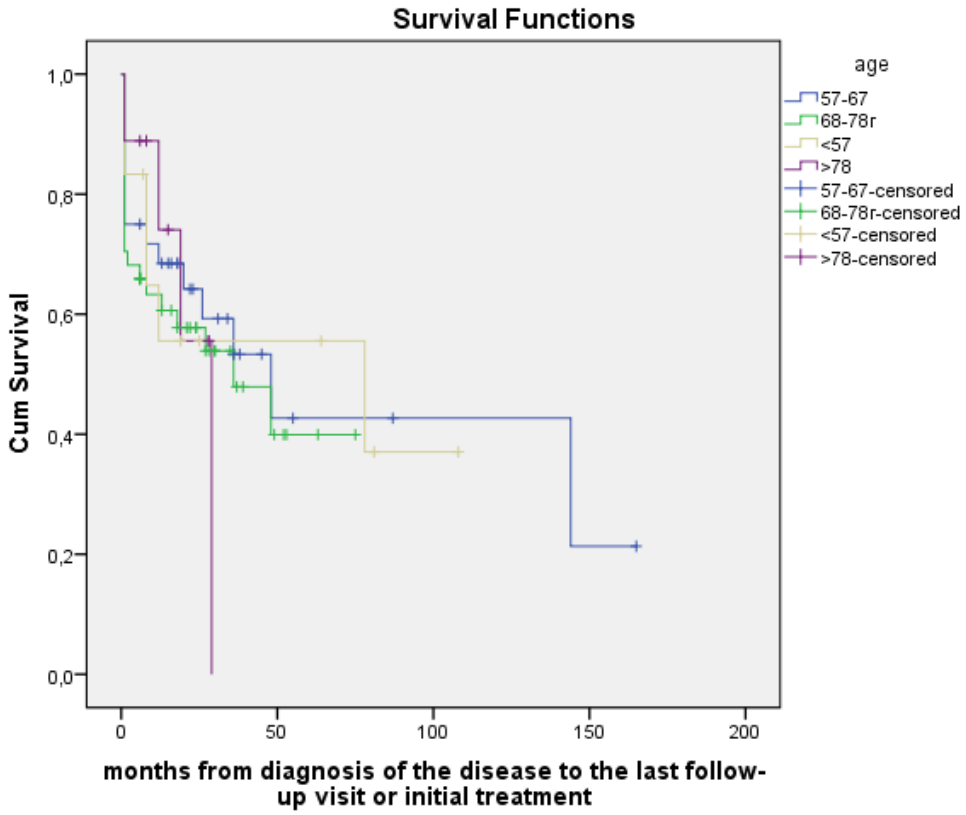


To investigate the relationship between TTFT and the age factor, we transformed age from an interval to a nominal variable. Because the mean age of the patients was 67 years, the patients were initially divided into four groups as follows: 1- age group 57-67 years, 2-age group 68-78 years, 3-age group under 57 years, 4-age group over 78 years, to looked for a difference in time to treatment for the individual groups through comparing the Kaplan - Meier curves (Table № 2 , Fig. № 4)

Table № 2. Distribution of patients by age groups and TTFT

	Time to treatment depending on the demographic factor age			
			interval	
group/age	months	standard error	lower bound	upper bound
1/ 57-67	76.216	16.084	44.691	107.741
2/ 68-78	38.523	5.578	27.589	49.456
3/< 57	57.204	14.426	28.928	85.479
4/> 78	21.519	4.044	13.592	29.445
total	69.519	9.830	50.253	88.785

Figure №4. TTFT for a patient in the four age groups (p = 0.915)



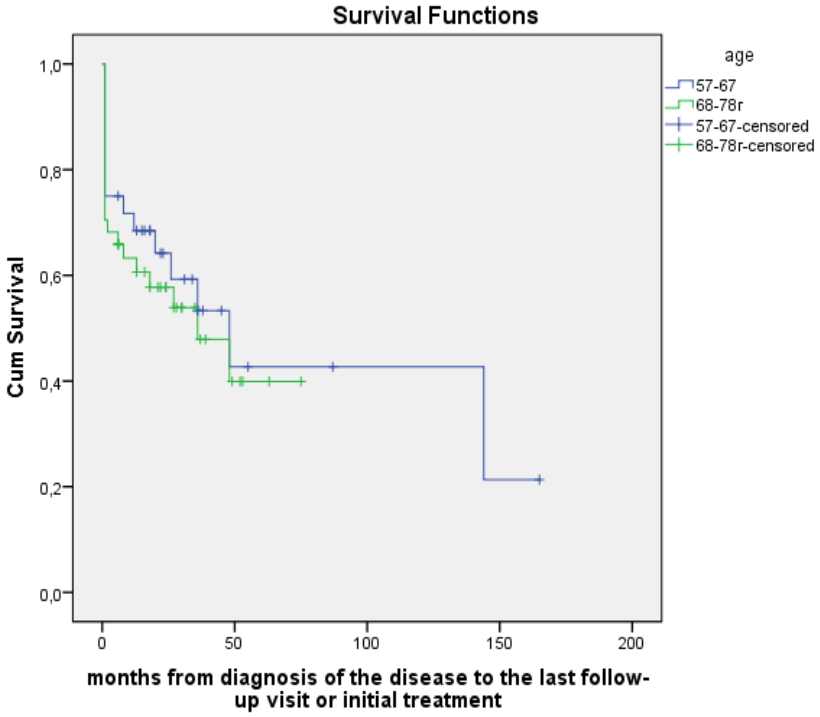
In the 4 age groups thus formed, no significant difference was found in the time to the first treatment (Kaplan-Meier , p = 0. 915) (Fig. №.4).

Due to the small number of patients in the two end groups (3 and 4) the groups with the largest number of patients was analyzed, respectively, 1 and 2 (Table. No 3 Fig. №5). Since the distribution of patients was almost balanced in each group (patients in treatment and untreated), a difference in time to treatment for individuals in these two groups was sought.

Table №3.TTFT for a patient in age groups 1 and 2

Group / age	Age groups and TTFT			
	months	standard error	interval	
			lower bound	upper bound
1/ 57-67	76.216	16.084	44.691	107.741
2/ 68-78	38.523	5.578	27.589	49.456
total	72.837	10.703	51.858	93.815

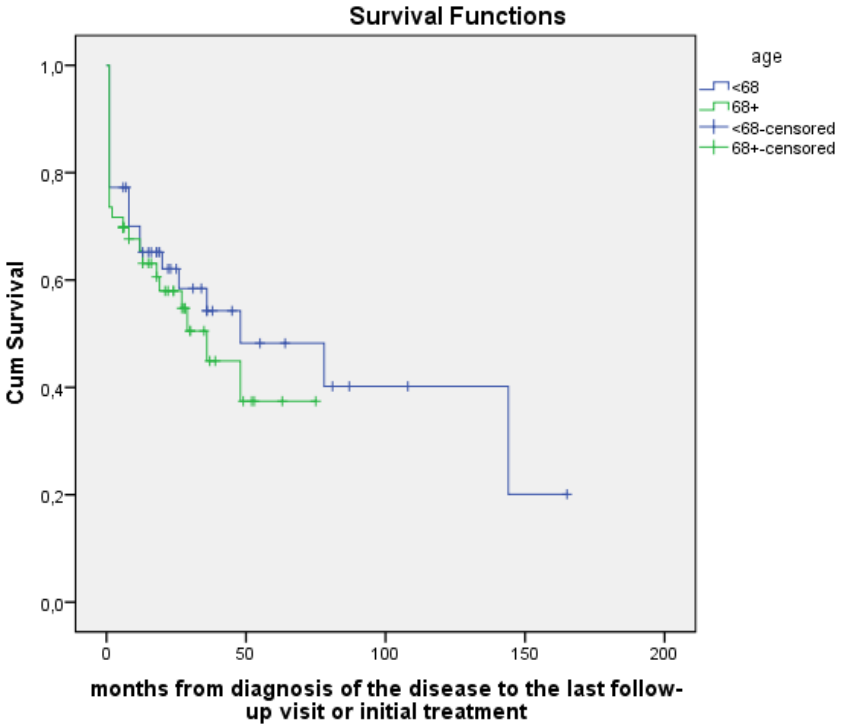
Figure № 5. Time to treatment for the age groups 57-67 and 68-78.



No significant difference was found in the TTFT between the two age groups. The results of the study did not confirm the influence of the age as a significant factor for TTFT (Kaplan - Meier, p = 0.596) (Fig. №.5). Even when assigning young patients (group 3) to the

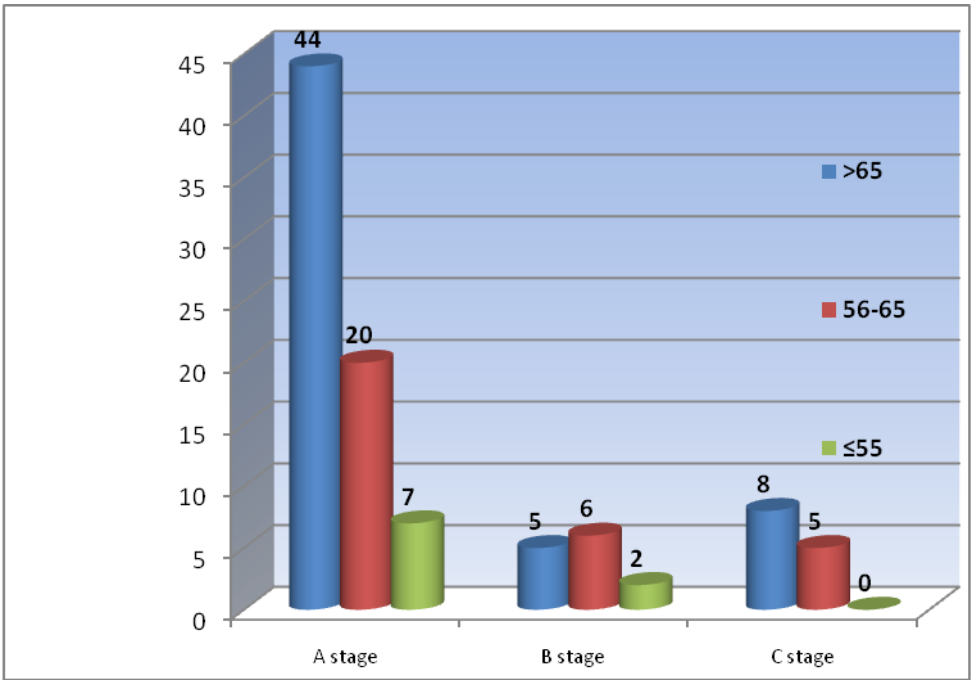
first group and the oldest (group 4) to the second group (Fig. № 6), age proved to be an insignificant factor in terms of TTFT (Kaplan - Meier, p = 0.47).

Figure №6. Dependence between the factor age and time to treatment, in the groups (1 + 3) and (2 + 4) (p = 0.47)



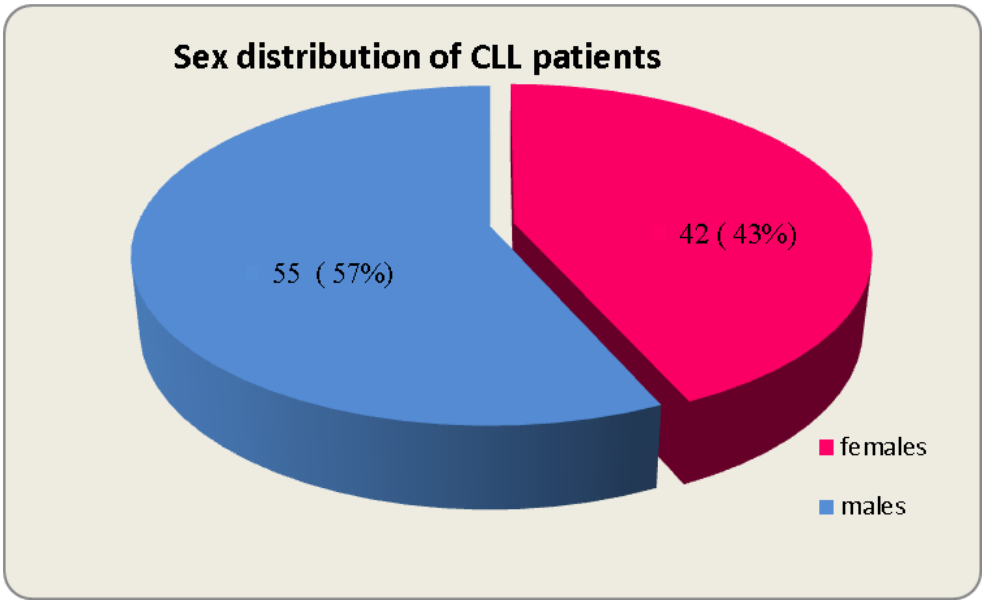
The distribution of patients by age according to the stage of the disease was also examined (Fig. №7). Of all the subjects included in the study, the number of patients in stage A (Binet) aged >65 was the largest (n = 44/97), and the lowest number of patients was that in the group under 55 years of age in stage B (n = 2/97). There were no patients under 55 in the advanced (C) clinical stage in the cohort we studied.

Figure №7 Distribution of patients by stages and age structure



The structure and distribution of patients according to the demographic factor "sex" were as follows: 57% (55) males, 43% (42) females, respectively in a ratio of 1.3: 1, with a predominance of males (Fig. № 8).

Figure №8.Characteristics of the analyzed group by sex



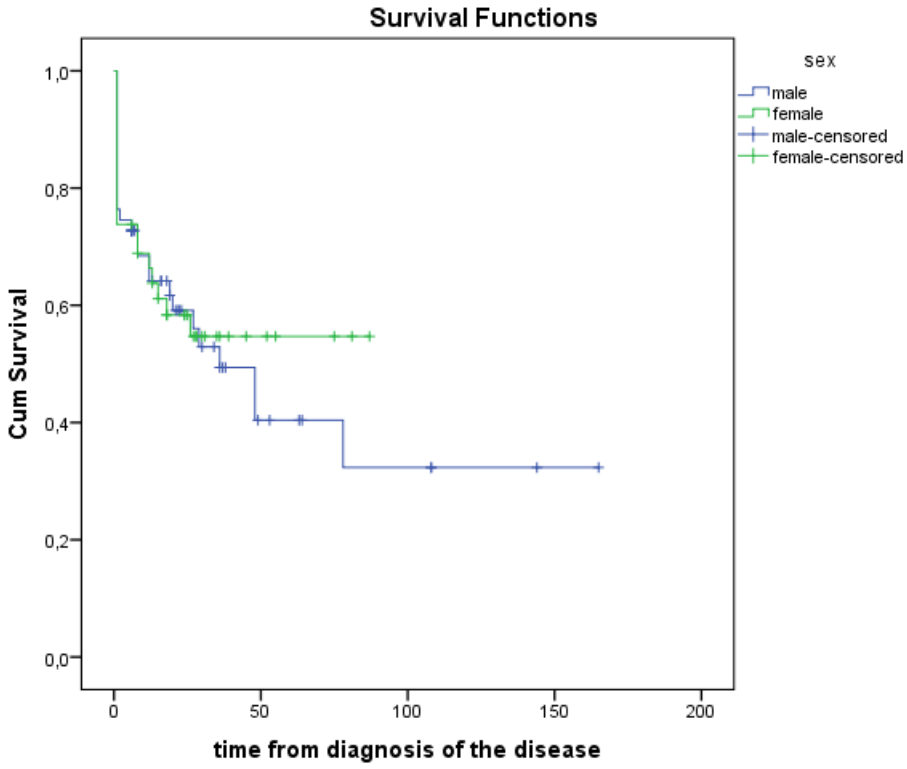
The distribution of patients according to the sex and stages of the disease is also presented in table. № 4.

Table № 4. Distribution of patients by demographic factor sex

Binet stage	**Number of patients male (n =)**	**Number of patients female (n =)**
A- stage	36(65%)	35 (83%)
B- stage	10 (18%)	3 (7%)
C- stage	9 (16%)	4 (10%)
total number	55 (100%)	42 (100%)

The data from the analysis did not show a statistically significant difference (p> 0.05) in the time to first treatment for the two groups of patients (Fig. №9) The summarized results of our study did not establish the influence of demographic characteristics age and sex on the time from diagnosis of the disease to its initial treatment.

Figure №9. Relationship between TTFT and gender demographics in untreated CLL patients

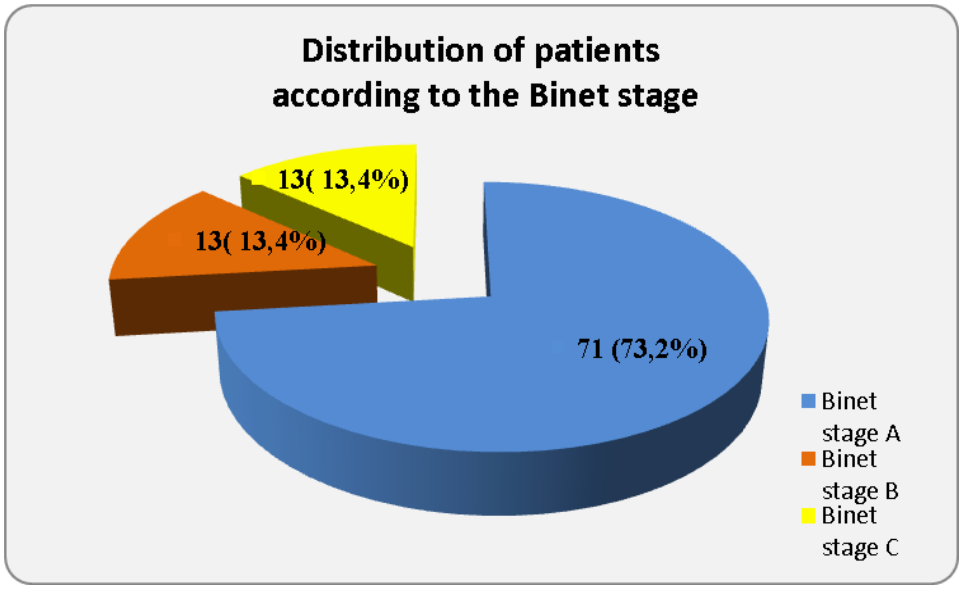


Clinical and laboratory parameters, determining the stage of disease and their effect on TTFT

Clinical indicators:

Given that at the time of diagnosis the disease does not always manifest with abnormal objective findings, staging of patients was based on comprehensive evaluation of physical status, imaging (abdominal ultrasound, CT scan, X-ray of the chest), and routine laboratory tests. The results summarizing the data from the conducted studies and the distribution of the patients depending on the stage of the disease are presented in Fig . № 10.

Figure №10. Characteristics of patients by the prognostic factor stage of the disease

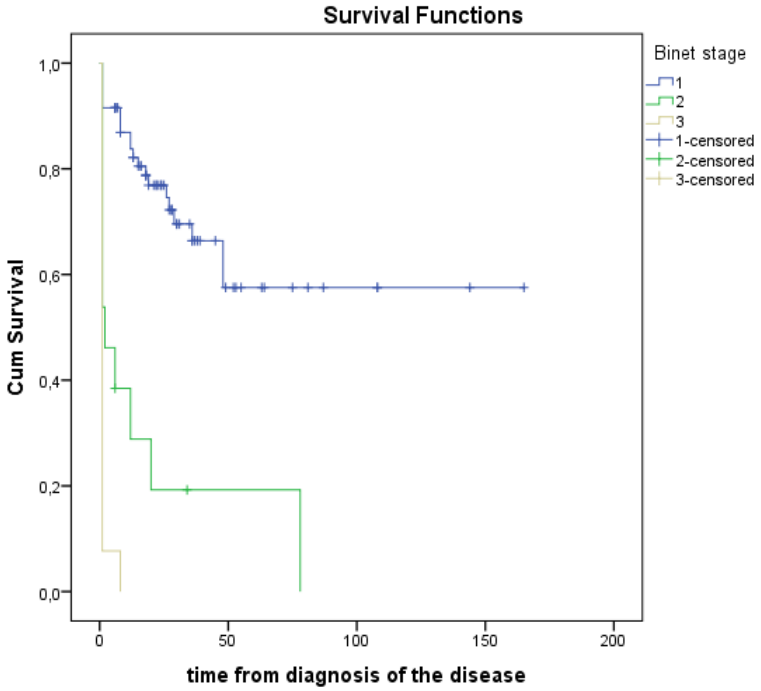


In the present study, it was found that approximately three-fourths (73.2%) the of patients with B - CLL at the time of diagnosis of the disease were in the early A stage, and the remaining one-fourth (26.8%) were balanced between stages B and C (13. 4%) . The time to treatment for the three groups of patients is presented in table № 5 (Kaplan-Meier analysis). There was a statistically significant difference in time to treatment between groups of patients (log-rank test, p <0.001, Fig. №11), and it was the shortest for patients in advanced stage C and the largest - in patients from stage A.

Table №5. Time to treatment for patients with untreated CLL

Binet stage	Time to treatment (months)			
	estimate	standard error	interval	
			lower bound	upper bound
A	104.294	11.142	82.455	126.133
B	19.154	8.976	1.561	36.747
C	1.538	.538	.483	2.594
overall	75.820	10.011	56.199	95.442

Figure № 11. Kaplan-Meier curves displaying the TTFT of the patients from the different Binet stages(1- A stage, 2- B stage, 3- C stage according to Binet)



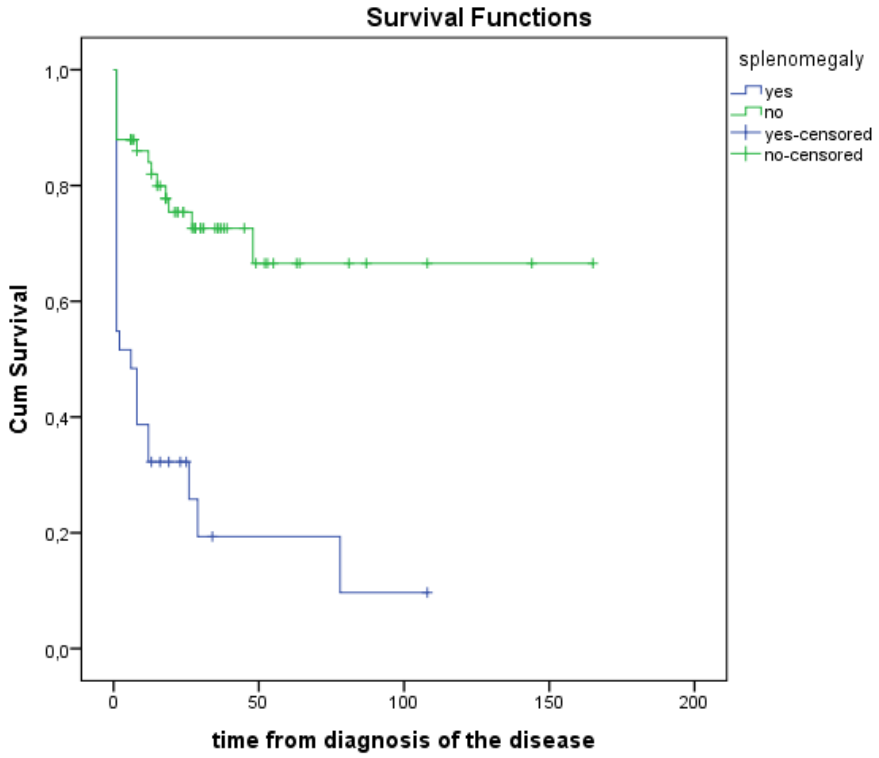
Our results confirmed the importance of the clinical stage as a significant factor, related to the TTFT. We sought a connection between the involvement of the spleen (as defined at the time of diagnosis by physical examination and imaging procedures) and TTFT. To this purpose, we divided the patients into two groups, which are presented in Table. №6. Comparing the two groups according to the TTFT (Fig. № 12), we found a statistically significant difference between the mean time to treatment, which was 23.8 ± 7 months,

respectively, for patients with proven splenomegaly and 115.4 ± 11 months for group a in which there was no evidence of splenic involvement (p <0.001).

Table №6. Patients distribution depending on presence of splenomegaly

Splenomegaly	*Number of patients (N=)*	*%*
yes	31	32.0
no	58	59.8
missing data	8	8.2
total number	97	100

Figure № 12. Kaplan-Meier comparison of TTFT between patients with or without splenomegaly

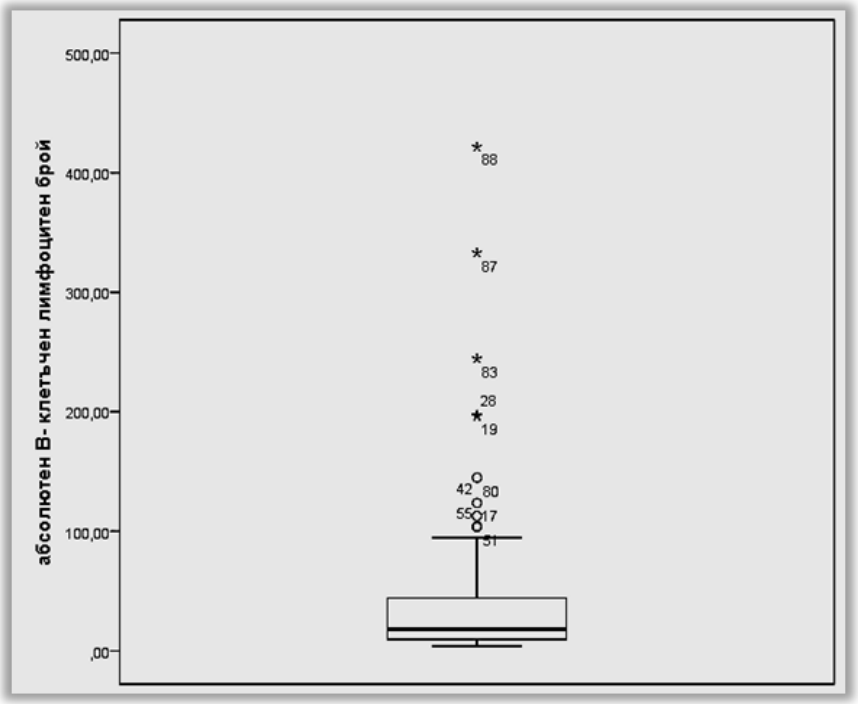


Laboratory indicators related to tumor load

As part of the diagnostic process, all patients underwent a panel of standard tests, including complete blood count, differential count, and absolute lymphocyte counts. Flow

cytometric analysis of peripheral mononuclear cells was performed in patients with absolute lymphocytosis. Absolute B-cell lymphocyte count was calculated by multiplying the percentage of CD19 / CD5 positive lymphocytes by total leukocyte count. In the group studied, this laboratory indicator ranged from 5.2 x 10^{9}/ l to 421.5x 10^{9}/ l . We looked at the distribution of patients according to ABLC (Fig. №13) and looked for a relationship between ABLC and the time to treatment.

Figure №13.Box & Whisker plot distribution diagram of absolute lymphocyte count values



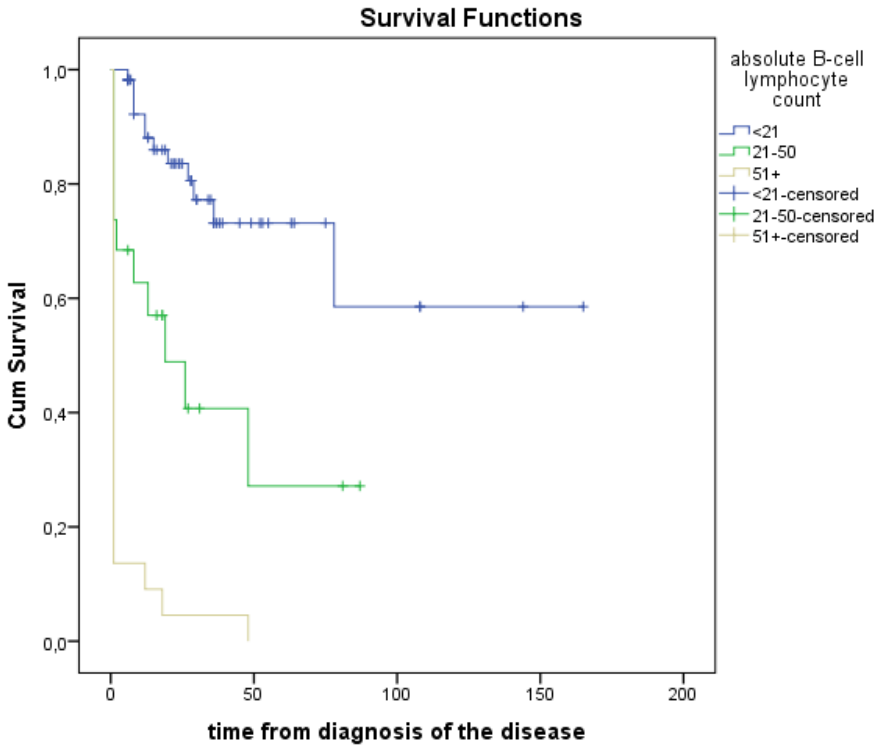
The patients were divided into 4 groups, presented in Table № 7. The largest number of patients who started treatment were these with ABLC count above 50 (n = 21), followed by the group with count of 20-49 x10^{9} / l. The largest number of patients without treatment were those with ABLC count of 5-9 x 10^{9} / l and 10-19 x 10^{9} / l. The ratio of the number of patients in the last two groups was 1:1. Studying the association between ABLC and time to treatment, a positive correlation was found with a Spearmen coefficient of 0.647 between the two factors in the different patient groups.

Table №7. Distribution of patients by groups according to the determined ABLC

Absolute B-cell lymphocyte count $x10^9$ / l	Number of patients		
	without therapy	on therapy	total
5-9	21	2	23
10-19	21	8	29
20-49	8	15	23
50 +	1	21	22
Total	51	46	97

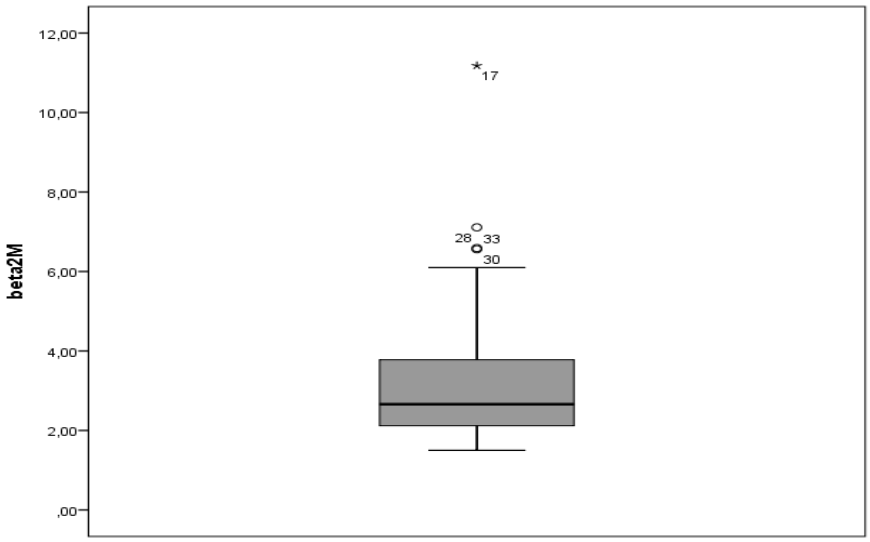
Due to the small number of patients from groups with ABLC 5-9 and 10-19x109/ l and who had started treatment, the last two groups were combined into one, and the three groups formed were as follows: ABLC ≤20x10^9 / l, 21-50x10^9 / l, and ≥ 51x10^9. Further examination of the impact of ALBC on the time to treatment revealed a statistically significant difference between the three groups (p <0.001). This period was for the group of patients with ABLC ≥51 × 10^9 / l (4.4 months ± 2,2), as compared with the group with ABLC < 20 x10 ^{9} / l, in which a median time to treatment was 113 months ± 14.6 (Fig. No 14).

Figure № 14. Kaplan-Meier curves for TTFT and ABLC



A relationship was sought between the serum marker в2-M, as an indicator indirectly reflecting the proliferative activity and tumor burden, and whether the factor had a significant effect on TTFT. The indicator was studied in 93 patients (Fig. 15), and in 35 (37.6%) the marker had values below the upper reference limit. The remaining 58 (62.4%) had values above 2.35 mg / l (normal range from 0.80 to 2.35mg / l)

Figure № 15. Box diagram - distribution of patients according to the levels of serum β2-MG.

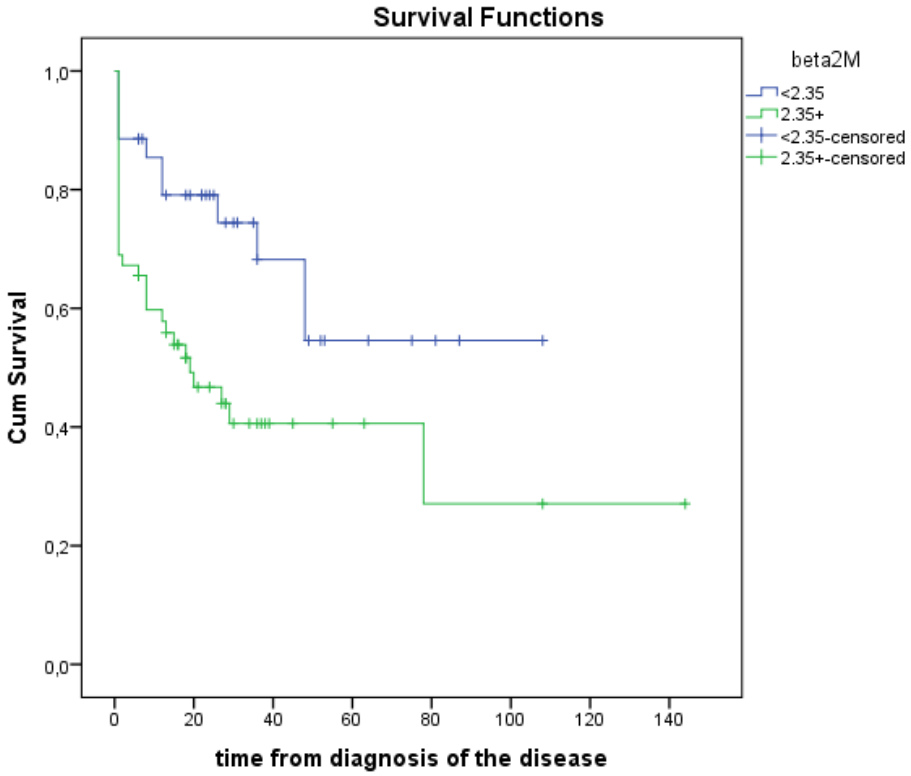


Considering β2-MG as a single prognostic factor related to the time to first treatment, a statistically significant difference was found between the groups with normal value or values exceeding 2.35 mg / l. The results are presented in (Table 8 and Fig. 16).

Table №8. Serum level of β2-MG and time to first treatment

	Time to first treatment			
		Standard	Confidence interval 95%	
β2-MG	Months	error	lower bound	upper bound
<2.35 mg/l	70.067	9.022	52.384	87.751
>2.35+ mg/l	54.300	10.934	32.869	75.732
total	67.052	8.740	49.921	84.183

Figure №16. Mean time to treatment for patients with normal or high level of serum β2-MG values (p=0.010)



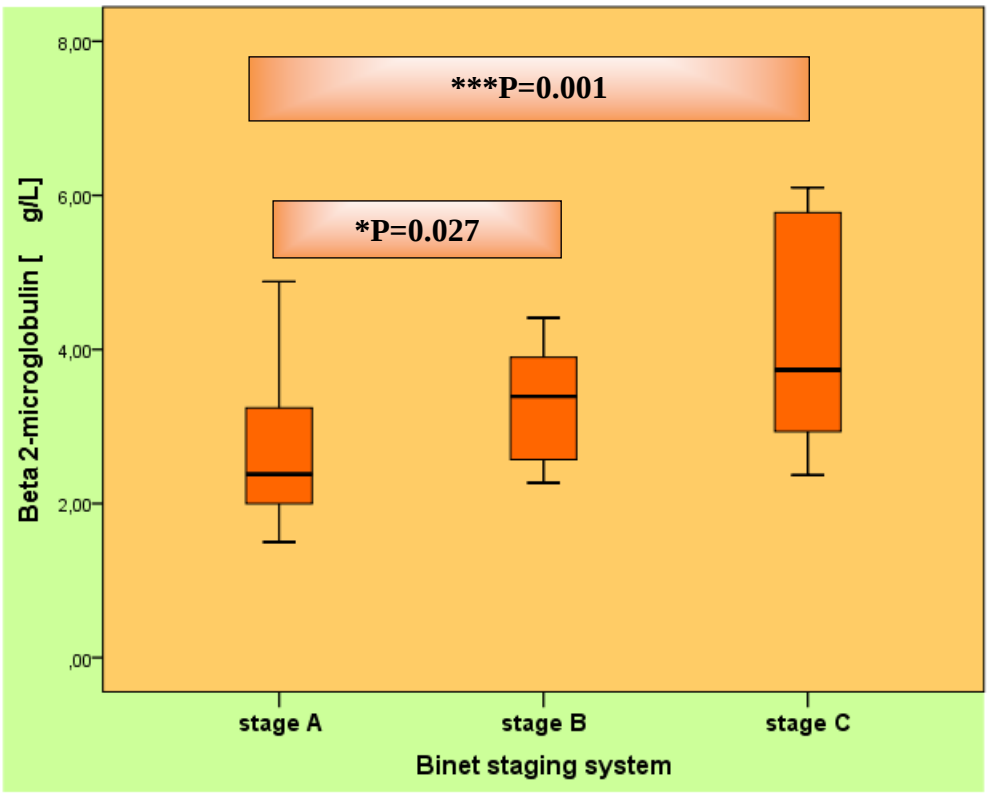
The TTFT of patients with values of the indicator above the reference limit was 54.3 ± 10 months. In four of the patients, β2-MG values were significantly elevated above the upper limit of normal. The clinical and laboratory characteristics of these patients are presented in Table № 9.

Table № 9. Clinical and laboratory characteristics of patients with pathological levels of β2-MG

Patient №	***Binet stage***	***Absolute B- cell lymphocyte count***	***β2-MG level***	***Therapy***
17	C	112G/l	11,8	+
28	A	197G/l	6.56	+
30	B	8.4 G/l	6.59	-
33	A	5.25 G/l	7.11	-

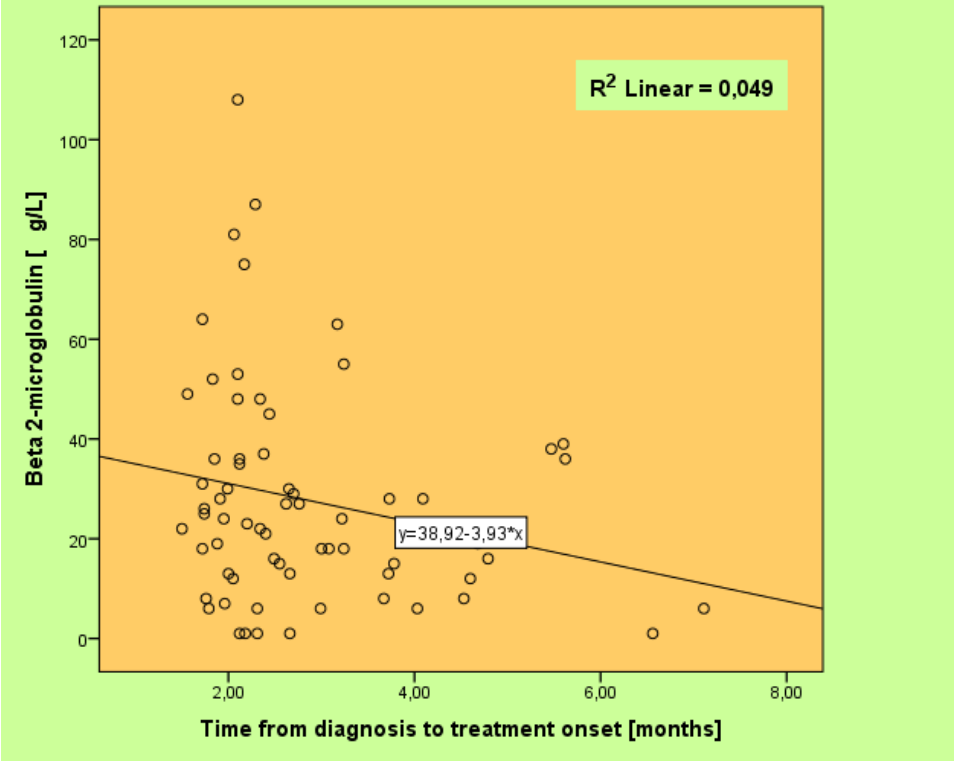
The relationship between disease stage and β2-MG values as two different indicators indirectly reflecting tumor burden was also examined. To this purpose, the patients were divided into three groups depending on the stage (1st group - patients from A clinical stages; 2nd group - patients from B clinical stages; 3rd group - patients staged in advanced C clinical stages). The results of the analysis showed a difference in time to treatment in different groups (Fig. 17).

Figure №17.Dependence between TTFT and serum concentration of β2-MG



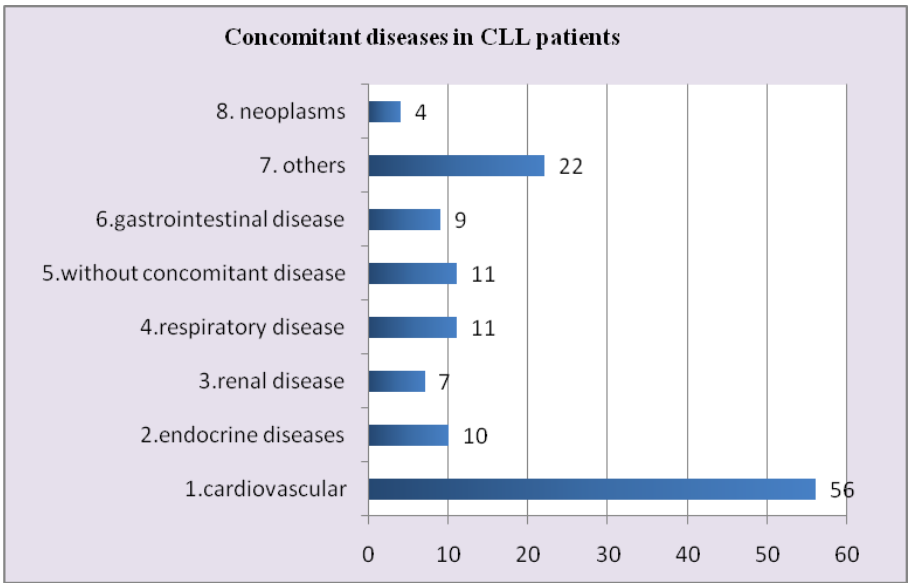
There was a tendency for a weak negative relationship between the determined concentration of beta 2-microglobulin and the time from diagnosis to treatment for patients in stage A (Rs = -0.213, p = 0.086). In other words, the higher the concentration of the marker, the shorter the expected time from diagnosis to the start of treatment (Fig. №18).

Figure № 18. Correlation between β2-MG and TTFT for stage A patients



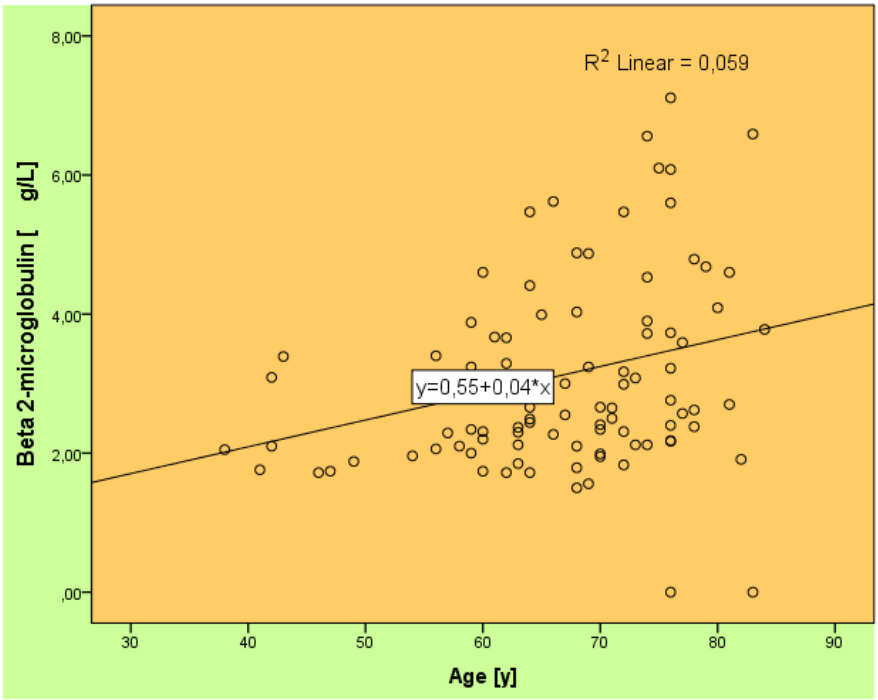
We further studied the comorbidities of the patients, given the need to recalculate the indicator depending on renal function. (Fig. №19). Twenty-three (23.7%) of a total of 97 patients had two or more diseases. Only 11 of the patients were without a history and an objective finding for concomitant pathology. Documented grade I-II chronic renal failure was found in two of the patients.

Figure №19. Concomitant diseases in CLL patients. In the group of other disease are included psoriasis, Parkinson's disease, thrombophlebitis, rheumatoid arthritis, GIT (gastrointestinal diseases)



A relationship between age and serum beta 2 microglobulin values was sought. There was a moderate, significant correlation between its concentration and the age of the patients [Rs = 0.304, p = 0.003] (Fig. № 20).

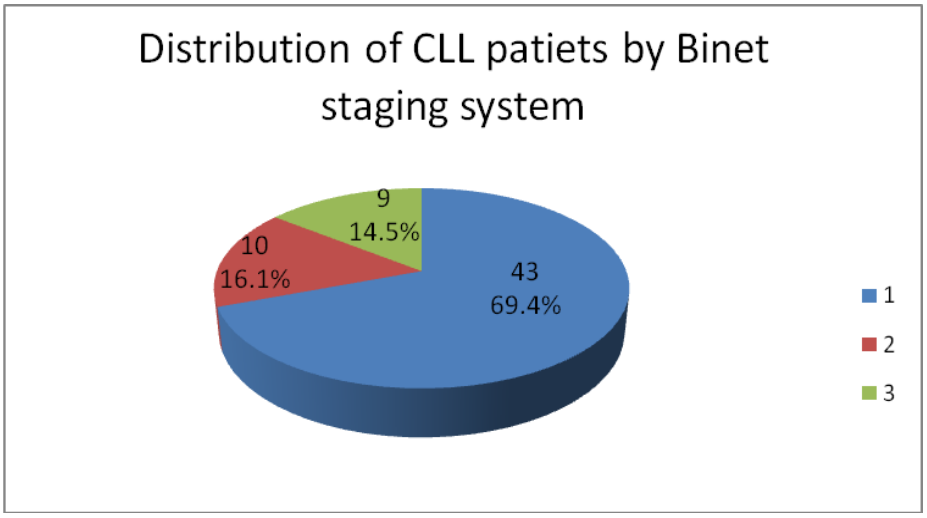
Figure №20. Correlation between the factor age and β2-MG levels



Molecular and genetic markers

The relative proportion of patients with some of the most common, clinically significant chromosomal aberrations identified by locus-specific probes for del13, del11, del17p / p53 was analysed. To this purpose, 62 of 97 patients underwent FISH analysis on the interphase nuclei of mononuclear cells isolated from peripheral blood. The distribution of patients from this group according to the stage of the disease is presented on Fig. № 21.

Figure №21. Distribution of patients by stage of the disease :1 – Binet stage A, 2 – Binet stage B, 3 – Binet stage C



Based on the results of the FISH test, the patients were divided into 5 subgroups as follows: group A - with isolated del13q, B - with isolated del11q, C - with del17p / p53, D - with more than one chromosomal aberration, and E - none of the chromosomal abnormalities searched for were identified. One of the results was found inappropriate for interpretation. The distribution of individuals according to the observed karyotype aberrations determined by FISH analysis is presented in Fig. №22. Among the group of 62 patients, the most common chromosomal abnormality was del13q (n = 26) - at 42.6%. In 22 (36.1%) patients no chromosomal abnormalities were found. The number of patients with del17p / p53- 6 was significantly lower (9.8%). The result of the FISH analysis of the above mentioned chromosomal aberration is shown on Fig. №23.

Figure №22. Distribution of chromosomal aberrations in the group of 61 untreated CLL patients based on FISH analysis

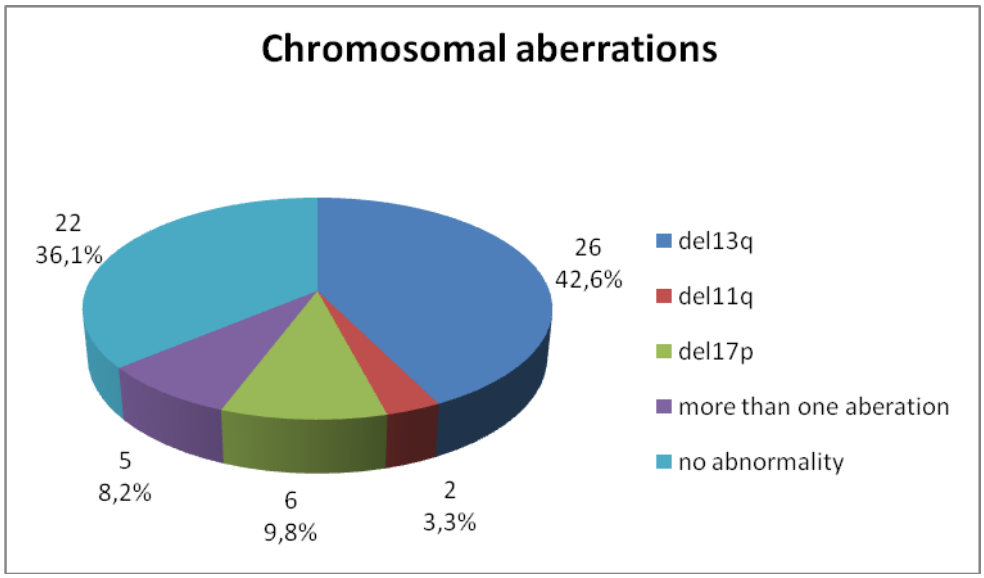
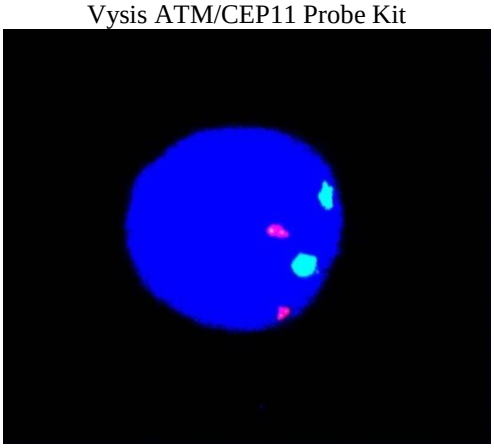
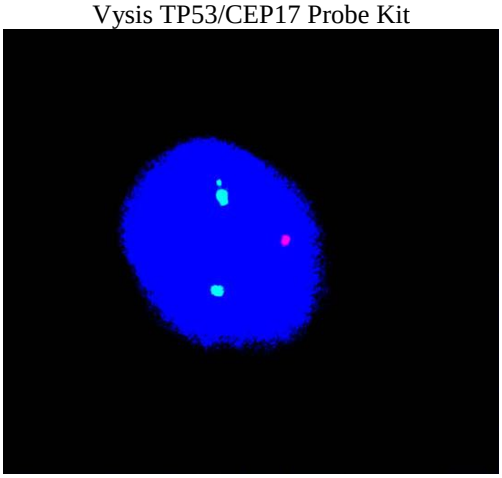
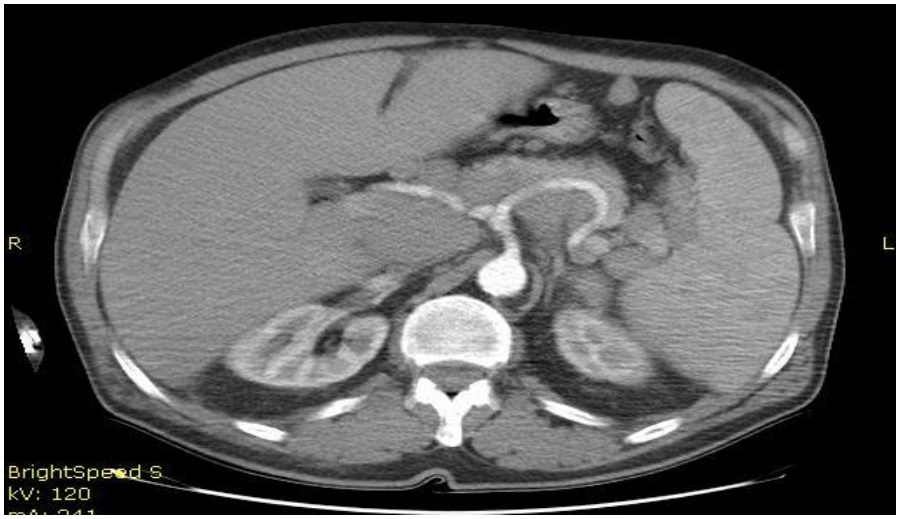


Figure №23. In 94% of the interphase nuclei, one of the alleles of the p53 gene is missing: in 76% of the nuclei the chromosomal aberration is in the form of del (17) (p13), and in 18% it is presented as a monosomy.

Interphase nuclei marked with probes:	
Vysis ATM/CEP11 Probe Kit	Vysis TP53/CEP17 Probe Kit
	
The signal pattern (2G2R) corresponds to 2 intact ATM genes.	The signal pattern (2G1R) indicates the loss of one allele of the p53 gene

Isolated del11q was found in two of the patients (3.3%). Fig. 24 shows a CT image of a patient with the aforementioned chromosomal aberration.

Figure №24 Result of a CT scan proving a systemic lymphoproliferative process. Spleen with overall increased axial dimensions up to 176/102 mm



In four of the patients, del (11)(q 22) was combined with del (13)(q14) or del (17) (p13). The results of the FISH analysis, laboratory tests and main clinical characteristics of the patients are presented in Table 10 Fig. № 25.

Figure №25. In 13% of the studied interphase nuclei there is a loss of one allele of the p53 gene, and in 20% - a loss of the ATM gene.

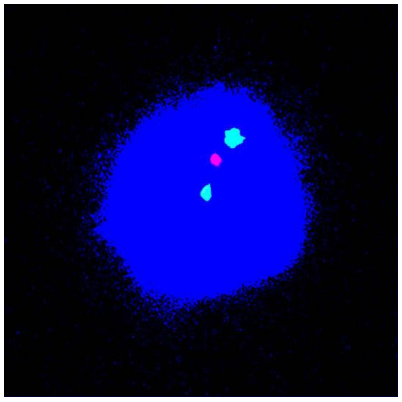
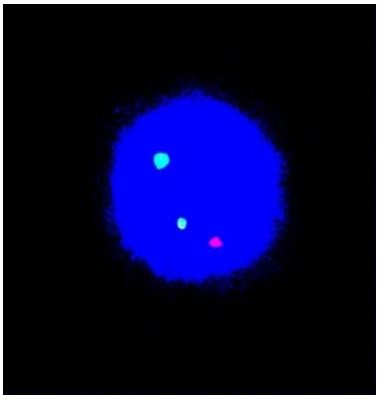
Interphase nuclei marked with probes:	
Vysis ATM/CEP11 Probe Kit 	Vysis TP53/CEP17 Probe Kit 
The signal pattern (2G1R) indicates the loss of one allele of the ATM gene.	The signal pattern (2G1R) indicates the loss of one allele of the p53 gene

Table № 10. Clinical and laboratory characteristics of patients with more than one chromosomal aberration

Patient №	Clinical presentation	Absolute – B-lymphocyte count	Cytogenetic abnormalities
№ 13	peripheral lymphadenomegaly	53.99 $X10^9$/ l	del(11)(q22) del(13)(q14)
№ 19	splenomegaly and abdominal LAM	196.1 $X10^9$/ l	del(13)(q14) del(11)(q22)
№ 36	splenomegaly and abdominal LAM	21.42 $X10^9$/ l	del(17)(p13) del(11)(q22)
№ 44	peripheral lymphadenomegaly	94.66 $X10^9$/ l	del(13)(q14) del(11)(q22)
№ 51	splenomegaly	104.37 $X10^9$/ l	del(17)(p13) del(13)(q14)

Del (17 p) was found in 4.8% (2/42) of patients in stages A, in 20, 0 % (2/10) in stage B and in 22, 2 % (2/10) in the stage C (Table . № 11 and fig № 26)

Table №11. Distribution of chromosomal aberrations among patients from different stages of the disease

chromosomal aberrations	Binet stages						Binet stages			
	A		B		C		early (1 & 2)		advanced(3)	
	n	%	n	%	n	%	n	%	n	%
del13	20	47.6%	5	50.0%	1	11.1%	25	48.1%	1	11.1%
del11	1	2.4%	1	10.0%	0	0.0%	2	3.8%	0	0.0%
del17p	2	4.8%	2	20.0%	2	22.2%	4	7.7%	2	22.2%
more than one	2	4.8%	1	10.0%	2	22.2%	3	5.8%	2	22.2%
without chromosomal aberrations	17	40.5%	1	10.0%	4	44.4%	18	34.6%	4	44.4%

In the form of loss of one allele of 53 gene, the chromosomal abnormality varied widely from 10 to 95%.

Figure №26.Distribution of chromosomal aberrations depending on the stage of the disease. Early A and intermediate B stages according to Binet (1 and 2), advanced stage C (3)

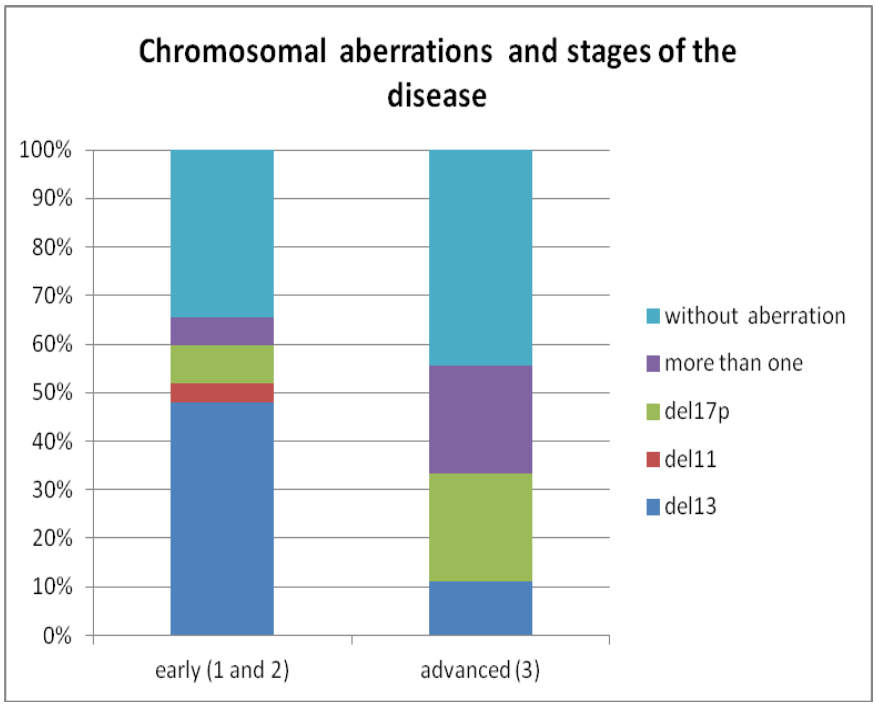
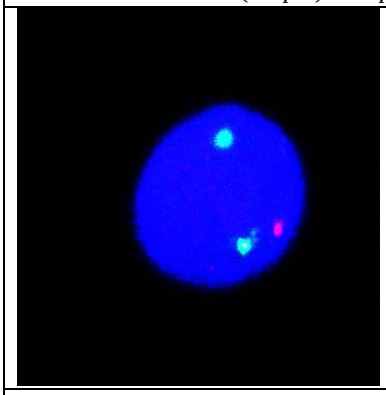
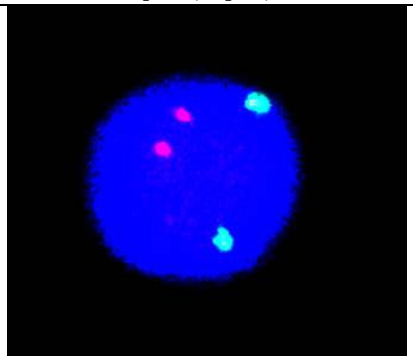


Figure № 27. A Poseidon™DLEU1 (13q14) / 13qter Control probe was used to identify the DLEU1 gene in the long arm of chromosome 13. Loss of the DLEU1 gene (2G1R) was observed in 85% of the studied interphase nuclei

Interphase nuclei marked with probes:	
Poseidon ™ DLEU (13q14)&13qter Control probe	Poseidon ™ p53 (17p13)&SE 17 Control probe
	
The signal model (2G1R) demonstrates the absence of the DLEU1 gene.	The signal pattern (2G2R) corresponds to two intact p53 genes.

Additionally, a relationship between the percentage of cells with del13q and the time to start treatment was searched for. The data for this percentage was converted from interval to nominal and the patients with del13q were divided into two subgroups (≥ 70% cells with aberration - code 1 and with <70% - code 0). Based on the Kaplan-Meier analysis , there was

no statistically significant difference in time to treatment between patients in the two groups (p > 0.05)(Fig. № 28). Table №12 shows the distribution of patients with isolated del (13q) depending on the percentage of cells, in which this chromosomal aberration was observed.

Figure № 28. Dependence between the percentage of cells with 13q- and TTFT

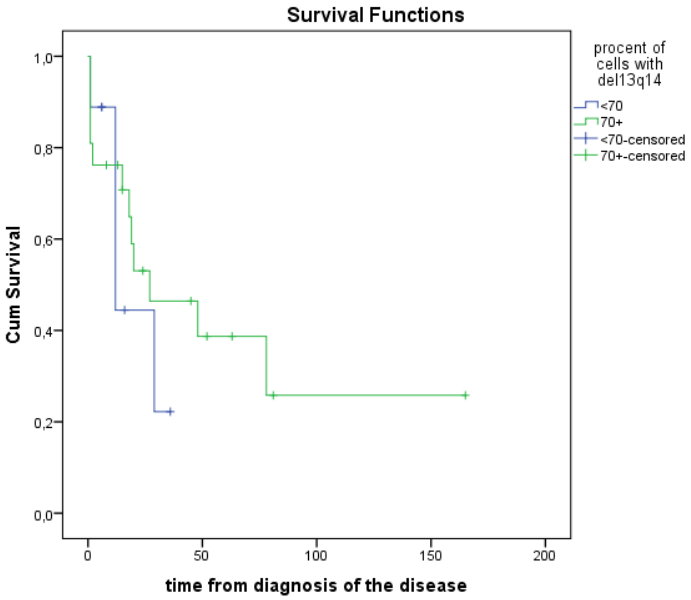


Table №12. Distribution of patients according to the percentage of cells with del (13q) and treatment started

CLL cells with isolated del13q	*N patients of treatment*	*N patients without treatment*	*Total count*
≥ 70%	**10**	**8**	**18**
<70%	**4**	**4**	**8**
Total count	**14**	**12**	**26**

Mutational status

In a subgroup analysis of 48 randomly selected patients, with documented data from staged procedures and FISH analysis, multiplex PCR was additionally performed to determine the mutational status. Based on the alternative variant used to determine IGVH status and on the obtained results, patients were divided into two groups as follows: group A,

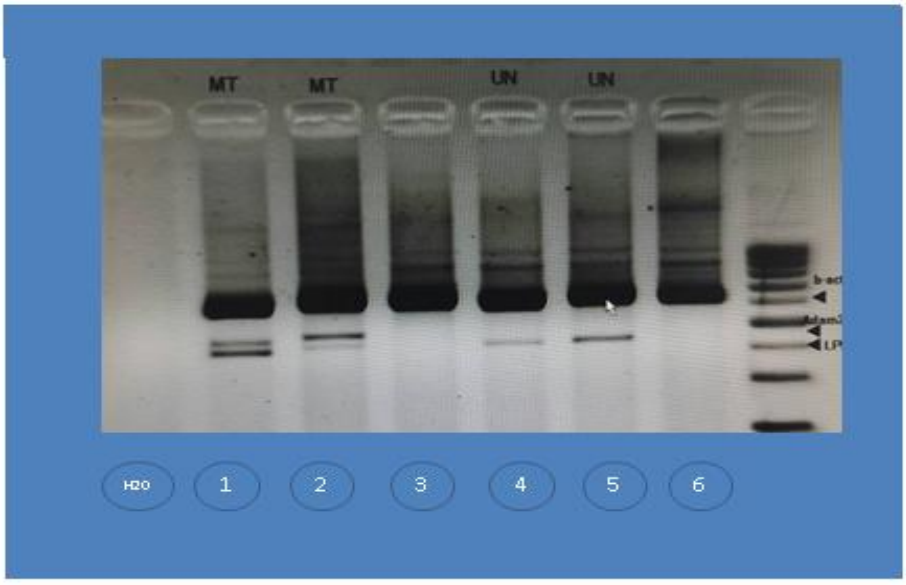
comprising 32 patients (66.6%) with mutated IGVH status and group B, consisting of 16 patients (33 , 3 %) with UM status. A sample of the results of the PCR analysis is presented on Fig. № 29.

Figure No 29. Multiplex PCR was used for simultaneous detection of the products of ADAM29 and LPL genes. For internal control β-Actin was used.

(a) In cases of mutated IGVH status (MT) 2 products were observed corresponding to ADAM29 and LPL, or 1 band, corresponding to the expression of the ADAM29 (columns 1,2)

(b) Unmutated IGVH status (UM): the presence of a single-band corresponding to LPL was established (columns 4,5)

(c) In the absence of ADAM 29 and LPL amplification products, regardless of the presence of β-Actin amplification product, the relevant sample was classified as indeterminate IGVH status (columns 3,6).



Based on the results of the FISH analysis, we looked for a relationship between mutation status and the studied chromosomal abnormalities (Table 13). The largest number of patients were those with del 13q - 22 out of a total of 48 patients . In the group of patients in whom the mutational status was accepted as mutated, chromosomal aberration was found in 50% (16/32) of patients. Data analysis revealed the presence of a significant association between mutational status and the karyotype aberrations investigated (p = 0.035).

Table №13.Distribution of chromosomal aberrations among groups with different mutation status (A = MT- mutated IGVH status and B = UM– non-mutated IGVH status)

Studied chromosomal aberrations								
		del13q	del11q	del17p	More than one aberration	No abnormality	unrepresentative	Total
IGVH	Group A-MT	16	0	2	2	12	0	32
status	група B-UM	6	2	2	3	2	1	16
N	total	22	2	4	5	14	1	48

Del17p, whose presence is associated with an unfavorable prognosis, was observed in 12.5% (2/16) in the group with UM status and in 6.25% (2/32) in patients with MT status. We additionally examined patients with isolated del13q and certain mutational status. Out of a total of 22 patients, six had unmutated VH status and started treatment within 20 months of diagnosis on average. We also analyzed the influence of mutational status on the TTFT factor. The distribution of patients according to these indicators is presented in Table №14.

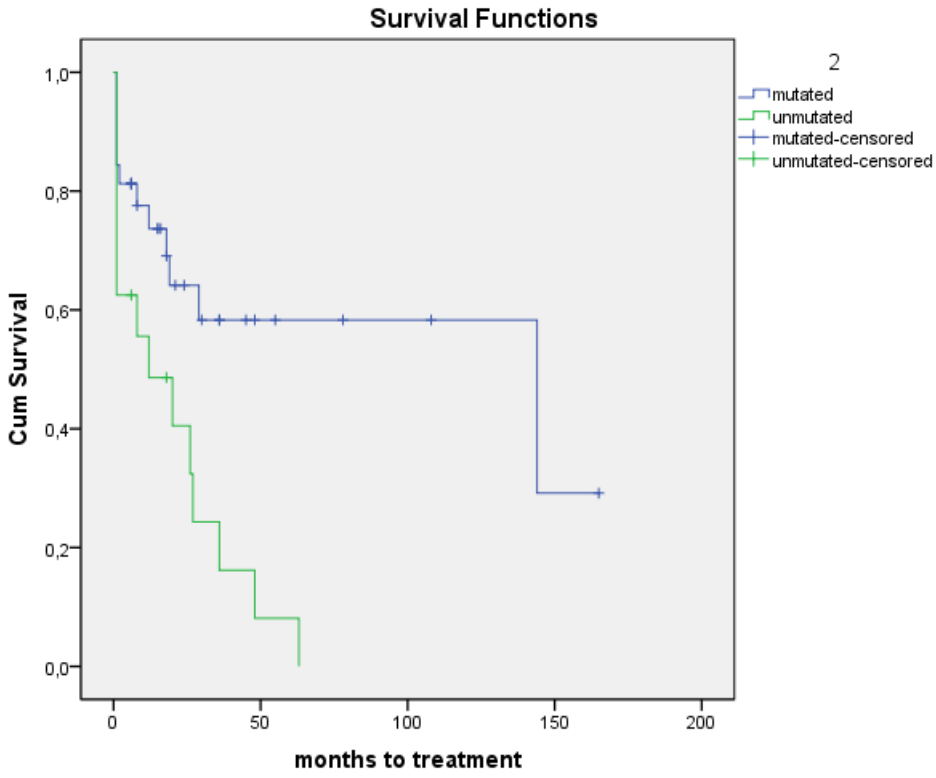
Table №14. Distribution of patients according to mutational status and TTFT

Mutational status	Total (N=)	Treated	Without treatment
MT	32	12 (37.5%)	20 (62.5%)
UMT	16	14(87.5%)	2 (12.5%)
total	48	26(54.2%)	22 (45.8%)

The differences in time to treatment for the two groups were determined by comparing the Kaplan-Meier curves (Fig. №30). The mean time to treatment for 50% of patients with

UM status was 19 ± 5 months (confidence interval 9-30 months), as opposed to the mean time to treatment for patients with mutated status, which was 94.5 ± 15.8 months.

Figure № 30.TTFT for the groups with mutated and non - mutated status (p = 0.003)



The distribution of patients by risk category and time to treatment was analyzed based on the risk assessment scale proposed in CLL-IPI, using the alternative to determine the mutation status. The distribution of patients according to the prognostic factors set in CLL-IPI is presented in table. № 15.

Table №15. Distribution of the number of patients according to the prognostic factors in CLL-IPI

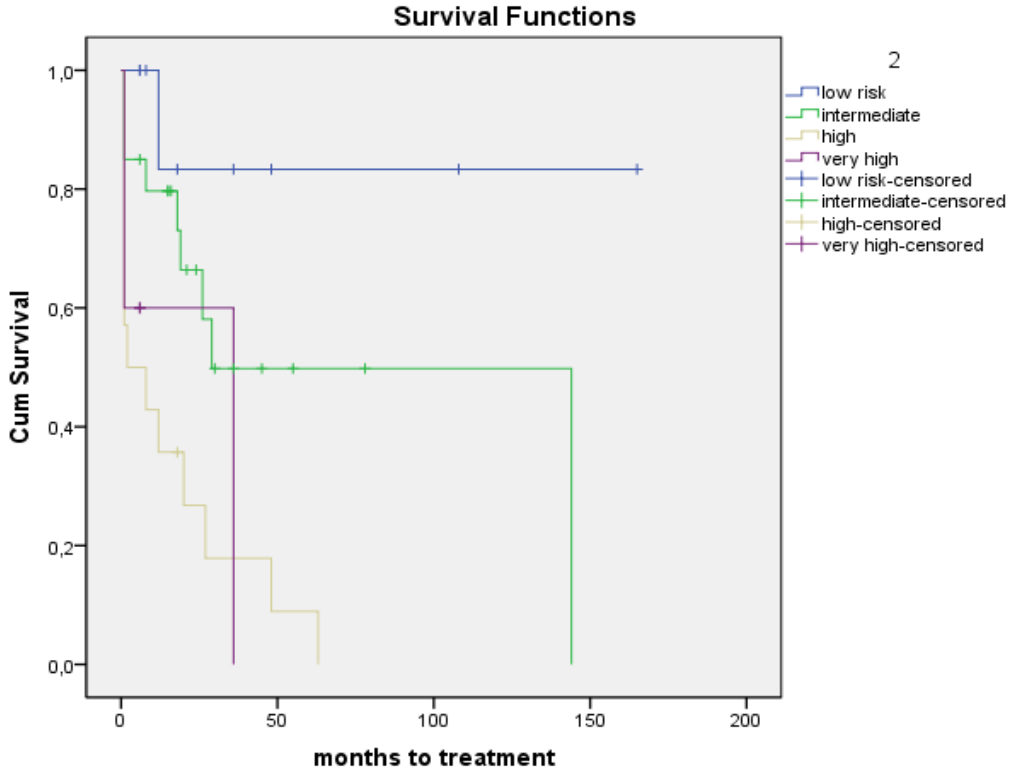
Factor	Number of patients
Age	
>65 years	28
<65 years	20
Binet stage	
A	32
B, C	16
del17p	
+	6
-	42
Mutational status	
UM	16
MT	32
β2 microglobulin	
<2.35 mg/l	12
>2.35mg/l	36

When determining the risk groups, recording of the prognostic factors was performed, as presented in the original model for risk stratification. After collecting the obtained points and depending on the established results, the patients were divided into four risk groups, presented in Table. № 16 and Fig. № 31. The detected by FISH analysis del17p was used as the only marker to determine TP53.

Table № 16. Distribution of patients by risk group status

Risk group	Total number N=	Number of treated patients N=	Censored	
			Untreated	Percentage
Low risk	9	1	8	88.9%
Intermediate	20	9	11	55.0%
High risk	14	13	1	7.1%
Very high risk	5	3	2	40.0%
Total	48	26	22	45.8%

Figure № 32.TTFT for the different risk groups, assessed as follows: low risk group 0-1 points, intermediate risk 2-3, high risk 4-6, very high risk 7-10 points



The stratification of patients by risk groups based on prognostic factors age, β2 microglobulin values, disease stages, mutational status and cytogenetic aberrations is presented in Table. № 17.

Table №17. Risk groups of patients and TFT.

	Risk stratification							
		Standard Error	95% confidence interval				95% confidence interval	
Risk group	months		Lower bound	Upper bound	months	Stand Error	Lower bound	Upper bound
Low risk	139.500	23.278	93.875	185.125	.	.	.	.
Intermediate	79.316	17.891	44.251	114.382	29.000	27.012	.000	81.943
High	16.107	5.854	4.632	27.582	2.000	6.548	.000	14.834
Very high	22.000	9.391	3.593	40.407	36.000	.000	.	.
total	59.514	12.120	35.759	83.269	27.000	9.241	8.888	5.112

Out of a total of 48 patients, 9 (19%) were assessed as low-risk, of which only one had started treatment. The time to treatment for patients in this group was 139.5 ± 23.2 months. In the groups with the highest number of patients, treatment was not required in 55% of cases with an intermediate risk of progression and in 7.1% of patients, rated as high-risk. In the group of 20 patients at intermediate risk, we observed an almost balanced distribution of patients on treatment (9/20) and without therapy (11/20). The median time to treatment for this group was 79.3 ± 17.8 months. In the high and very high risk groups, almost all patients started treatment. The mean time to treatment for high-risk subjects was 16.1 ± 5.8 months, and 22 ± 9.3 months in the very high-risk group, respectively. The close time-to-treatment outcomes for the latter two groups could be due to the small number of very high-risk cases (5/48). Of a total of 32 patients staged in early A stages, 8 (25%) were identified with UM status, and a 17p- mutation was found in four (12%).

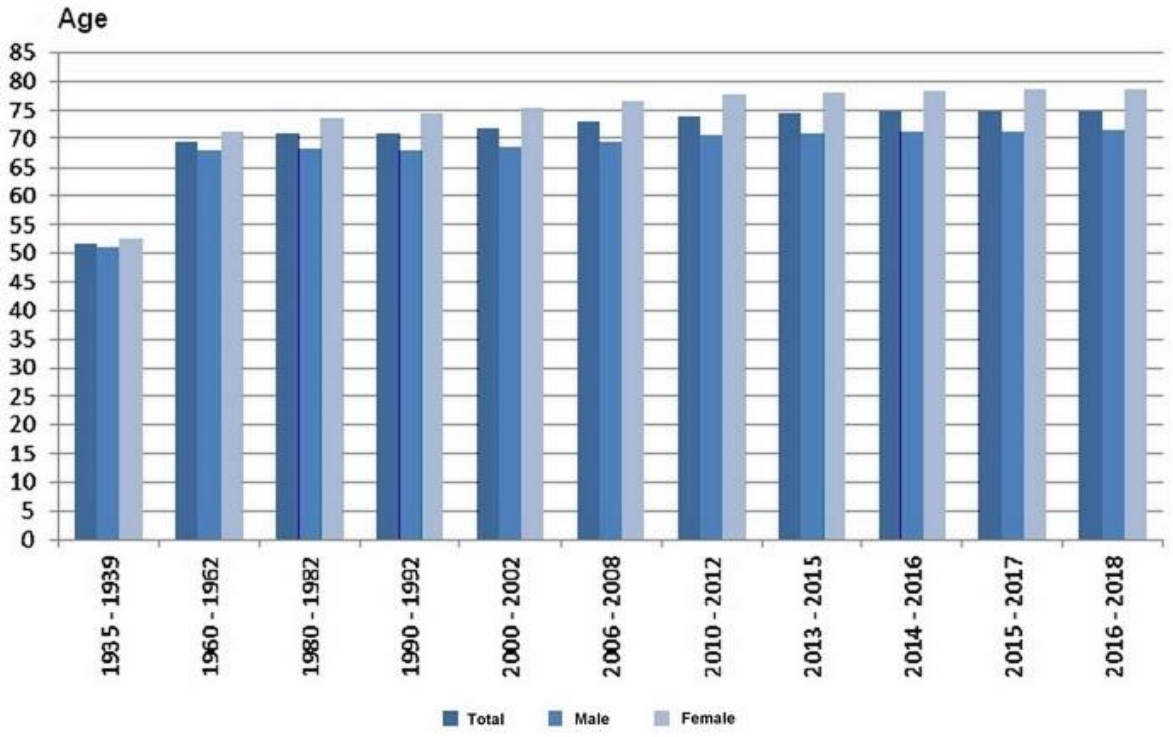
Discussion

Referring to the fact that CLL is a disease of elderly patients, and age is one of the main factors that is taken into account when choosing therapy, this indicator is included in most prognostic models for risk stratification. In a study of Parikh et al., in which young patients (aged ≤ 55 years) were screened, the authors analyzed the influence of age on time to treatment. The results of the study showed a significantly shorter time to treatment for patients in the young age group *(Parikh S et al., 2014.* Another research group, whose main target were patients ≥75 years of age, suggested that the usefulness of many of the clinical and biological prognostic markers used to determine survival and time of first treatment varied with age, considering the higher mortality from comorbidities among more elderly patients (*Shanafelt T et al 2010*). The mean age of the patients in our study was 67 and was close to that cited in most scientific reports. In 2017, Paolo Strati et al. on the basis of a prospective study, comprising a huge cohort of 1143 patients, reported an average age group of 63 years *(Strati P et al. 2017).* In 2018, Michael Hallek et al. determined an average age of patients at diagnosis - 67-72 years. Our results did not confirm the influence of demographic factors age and gender on TTFT. Taking as a limit even the proposed by CLL - IPI age of 65 years, the analysis of the group of the 97 patients we studied did not establish a statistically significant difference in time to treatment among those aged over 65 year and those under 65 years. We assume**,** that issues related to the age factor to a degree are close to those related to the proliferative processes and aging. In this respect, the studies of two research groups are interesting. In a report by Pietro Bulian et al. have suggested that demographic factors are important for determining overall survival in patients under 70 years, but these factors have virtually no prognostic value in determining the time to treatment *(Bulian P et al., 2012).* A study carried out by Davide Rossi et al. whose purpose was to monitor the clonal evolution of the disease trough a comprehensive approach, including FISH and molecular analysis has established a link between aging and instability of the of leukemia clone. The authors presume that these processes are based on a deterioration of the mechanisms responsible for maintaining the genome in older patients, or a greater tendency of such patients to select positively and expand the clones carrying the high-risk genetic disorders *(Rossi D et al., 2013).*

To specify the characteristics of patients by indicators of age and stages of the disease to the diagnosis, similar to the prognostic index proposed by William G. Wierda et al. (2007), we divided the patients into three age groups as follows: under 50 years, 50-65 years and over

65 years. What made an impression on the distribution of patients was the predominant number of people over the age of 65 who were in the early. i.e., A clinical stage. In practice, if we take into consideration the average life expectancy for this group, which is equal to or greater than 10 years, then the life expectancy for such patients people would be close to the average life expectancy for disease-free people (Fig. №33). On the other hand, over time in a significant part of patients, diagnosed at an early stage, the disease will progress and this would reduce their estimated median survival. The independent analysis of the age factor in our study did not validated the prognostic value of this indicator regarding the time treatment would be needed.

Figure №33. Average life expectancy in Bulgaria: 71.4 - 78.4 years (according to data from the National Statistical Institute as of 2018)



All this made us add indicators reflecting characteristics of the disease, namely the proliferative activity of neoplastic cells (by examining the level of β2-MG) and the degree of disease spread (stages).

Easily feasible staging systems are a major part of almost all risk stratification models for CLL patients. They were created more than 30 years ago and are widely used, but also repeatedly modified. Thus, initially consisting of five stages, the Rai staging system was revised in 1987 (Rai-Sawitsky). This system divides patients into three main risk categories, combining some of the stages as follows: Rai 0 - low risk, Rai I and II -intermediate risk, and

Rai III and IV -high risk *(Rai K R et al., 1975).* Both the Rai and Binet staging systems, which determine the number of involved lymph zones, are based on the method of palpation, but not all pathologically altered lymph nodes are located in areas, accessible for surface palpation. On the other hand, discrete splenomegaly may be missed on physical examination. Unlike lymphomas, in chronic lymphocytic leukemia, the lymph nodes are not always significant in size. Bilateral cervical and axillary.lymphadenopathy is found in 80% of the cases. Splenomegaly is mild and moderate, and occurs in approximately 50% of the cases *(Rodrigues AC et al., 2016).* Albeit limited, in the literature there is evidence that the risk of progression is greater for patients with enlarged lymph nodes, measuring over 1.5cm in diameter (*Strati, P et al., 2019*). In the absence of a pathological process involving the lymph nodes, the latter are not visualized by ultrasound due to their small size of less than 1 cm. In practice, ultrasound diagnostics can objectify lymph nodes larger than 15 mm *(Daskalov I et al., 2004),* i.e., deviations from these dimensions should be considered pathological. The number of affected lymphatic regions is important for staging the patients. This number can be established by ultrasound and X-ray examination of the respective anatomical areas (below/above the diaphragm). Precise measurement of the involved lymphatic structures by CT is necessary before starting treatment, when the tumor volume has to be determined, and appropriate target areas to be selected for monitoring and evaluating the therapeutic response. Based on this, in order to diagnose the disease and specify the stage and subsequent follow-up for progression in asymptomatic patients, in addition to routine laboratory tests and physical examination, we conducted abdominal ultrasound and chest X-ray. A CT examination was used as part of the diagnostic process to determine the size of the tumor mass before starting treatment. Abdominal lymphadenopathy was also found in the majority of patients with splenomegaly, and the time to treatment was relatively shorter for these patients than in patients without splenomegaly. Currently, there is no consensus whether performing CT to diagnose asymptomatic patients will have more benefits or negatives, although established occult lymphadenopathy has been reported in early-stage individuals *(Muntanola A et al., 2007*). In practice, adequate staging at diagnosis is important and it is important for the follow-up of asymptomatic patients, as the transition from one clinical stage to another is considered progressive. There are some difficulties using the staging systems in processing and reporting of patient data, related to aligning the two systems and stratifying the risks.

In the International Prognostic Index, proposed in 2016, patients in an early stage A (Binet) or O (Rai) are not assessed as at risk. However all other groups - B/C (Binet) or I-IV

(Rai), are considered as being at risk. In some scientific reports, the authors form the patient groups by combining stages Rai 0, Rai I and II (low and intermediate risk), placing them in the early stage A group as defined by Binet. If patients are to be grouped on the basis of stage and risk, then the ones in the early stage of the disease with low and intermediate risk, those corresponding to stages A, B according to Binet or 0-II according to Rai should be placed together. The group in the advanced stage should include patients in stage C (Binet) or stage III-IV (Rai). (*Cramer P et al., 2011)* The importance of stages of the disease for TTFT was confirmed: the results of our study on a group of 97 patients proved the influence of this prognostic factor on TTFT. For the group of patients subject to monitoring before initiation of treatment, as well as for those in need of treatment, we further investigated and sought a link between factors that more closely reflected the tumor burden and TTFT.

Due to the fact that the criteria for the diagnosis of B-CLL were revised more than 10 years ago, and these indicators were based on three indicators: ABLC, involvement of lymphatic structures and the presence of constitutional symptoms, we looked for a link between ABLC as a more reliable tumor load index and TTFT. In the cohort of untreated patients we monitored, extremely elevated ABLC values - above $200x10^9$ / l were found in individuals over 65 and in an advanced stage of the disease. The period to the start of treatment was significantly longer for patients with ABLC below $20x10^9$ / l. Reviewing of the literature on the problem did not find a fixed rate of lymphocyte count as a limit related to the time to treatment. In the past, WBC values > $35x10^9$ / l were considered an unfavorable prognostic factor (Simms L et al., 2013). In a retrospective study by Stefano Molica et al., covering 818 patients with CLL and stage 0 Rai, the authors proposed threshold values of ABLC $\geq 10x10^9$/l or absolute lymphocyte count $\geq 11.5x10^9$/l. These values distinguished between patients with stable disease from patients in whom the disease would progress (Molica S et al., 2011). In our study, ABLC above 50G/ l was associated with a shorter time to treatment, and with an intermediate and advanced stage of the disease. Small in size, malignant lymphocytes have the ability to infiltrate tissues and organs. Cases of pleural effusion have sometimes been reported, even at low leukocyte counts *(Berkman N et al., 1993).*

Cases of ascites with a proven CLL etiology in the presence of small paraaortic and splenic lymph nodes were first reported in 1965, and later in 2014 (*Muntanola A et al., 2007*). Such extramedullary/extranodal manifestations with or without systemic CLL most commonly affect the skin (33%), the central nervous system (27%), the kidneys, and though less frequently, the pericardium (*Hallek M, Eichhorst B et al., 2019, Ratterman M et al. 2013,*

Ho N et al., 2018). These phenomena, although not so common in clinical practice, are probably related to the expression of various molecules on the cell surface. The existence of a complex network of antiapoptotic, adhesion, proinflammatory and proto-oncogenic molecules supports the ability of cells to infiltrate and survive in tissues (*Vladimirova R et al. 2015*). Homing receptors located on the surface of lymphocytes may explain the clinical difference between CLL and SLL (*Tsimberidou A M et al., 2006*). To avoid compromising the results and the low incidence of SLL, patients with small-cell lymphocytic lymphoma were not included in our study.

Another prognostic factor associated with lymphocyte count is lymphocyte doubling time (LDT). Defined as the time for which the absolute lymphocyte count will double and as a reflection of the disease activity, this indicator is considered a reliable prognostic factor for patients at an early stage (*Cramer P et al., 2011*). Unfortunately, in practice this marker cannot be determined at the time of diagnosis (*Hallek M, 2008*). The assessment and use of the factor as a dynamic indicator related to proliferative activity has some limitations in cases, in which ALC is below 30G / l at the time of diagnosis. In such cases, double lymphocyte counts is not recommended to serve as the sole and justifiable factor for initiating treatment. The changes in the definition of B-CLL pose the question which of the two indices is to be monitored, based on the fact that at the time of dignosis ABLC is determined and is thought to indirectly reflect the tumor burden or ALC, the latter beinbg likely to change in clinical conditions not associated with progression of the disease.

β2- MG is synthesized by all nucleated cells and presents part of the light chain of human leukocyte antigen (HLA -A, -B, -C). It is present as a prognostic marker in most models for the stratification of risk in lymphoid neoplasia. It is assumed that the marker correlates with a disease's stage and the tumor burden (*Wierda W et al., 2018*). Depending on the established values of the serum index (above and below the upper reference limit), we divided the studied cohort into two groups. There was a difference in the time to initiation of treatment for the two groups of patients. This period was significantly shorter in the group with deviations above the reference value. We conducted a comparative analysis, dividing the patients into groups depending on three factors: the stage of disease, serum marker, and time to treatment. The analysis proved the existence of a significant difference in TTFT for individual subgroups. In a study of Del Giudice I et al**.,** involving patients under 65 in early A clinical stage, the goal was to determine the significance of some predictive markers and time without therapy. The authors have questioned the usefulness of β2- MG as a factor that can predict disease progression (*Del Giudice et al., 2010*). Analyzing the indicator

in persons in early A stage in our study, we found a negative correlation between the two indicators. Conducting the analysis, given the fact that the majority of patients had more than one comorbidity at the time of diagnosis, we further investigated comorbidity in the covered group, with the idea of specifying the extent to which the result of the serum β2-MG study might be compromised by concomitant pathology. In practice, only two of all patients had proven chronic renal failure, which is thought to lead to deviations from the reference range. In the group of 97 patients, the most common were the socially significant cardiovascular diseases, of which arterial hypertension was the leading one, followed by acquired valve defects and chronic congestive heart failure. Only 11 patients were without comorbidities. In a report by Paolo Strati et al., which examined the relationship between comorbidities present at diagnosis and causes of death in patients with B – CLL, the largest proportion was that of patients with rheumatic diseases, dyslipidemia and hypertension *(Strati P . et al., 2017).* We reviewed the literature on the most frequently mentioned diseases in which deviations in serum β2- MG values could be observed, excluding lymphoid neoplasms. Serum marker levels have been shown to be influenced by various extrarenal determinants, such as systolic blood pressure, sex, total cholesterol, and smoking (*Qun , Set al., 2019*). On Table №18 are presented authors and years of publication of their reports.

Table №18.Diseases associated with elevated serum beta 2 microglobulin

Diseases accompanied by elevated levels of serum beta2 microglobulin	Author / Year of publication
Solid tumors	
- Ovarian cancer	Hogdall et al.2010
- Bladder	Sun J et al. 2015
- prostate	Zhang Y et al. 2013
- Kidney	Lucarelli G et al.2014
- Breast	Klein T et al. 1996
- Thyroid gland	Buket I et al. 2016
Chronic congestive heart failure NYHA functional class II / III	Kawai K et al.2009
Type 2 diabetes mellitus	Kim M et al. 2014
Kidney diseases	Kevin T Barton et al.2018
Autoimmune diseases	Amarante G B D et al.2014

As can be seen from the brief overview, deviations in the values of the indicator could be related to concomitant diseases, which often appear in the medical history of patients over 60 years. Referring to a report by Matokovi C D et al. , presenting data from a study of the influence of age on the values of β 2 - microglobulin in individuals of different age groups, without a history of concomitant cancer, we analyzed the two indicators in the group of 97 patients. We found the existence of albeit weak relationship between β2-MG and age of patients. Due to the small diameter of the lymphocytes, their increased number should not be a cause of obstruction in different parts of the circulation. However, there are reports in the literature on the development of venous thrombosis in patients with B-CLL, involving a different mechanism. In a study by Inger Lise Gade et al. on factors related to the development of venous thromboembolism, authors report greater incidence of complication in patients with values of β2- MG above the normal range. According to them, the activated CLL -cells secrete larger amounts of β2- MG and procoagulant microvesicles, which in turn increases the risk of venous trombembolism (*GadeI et al., 2018*) and, accordingly, changes the prediction. Cytogenetic disorders underlie the pathogenetic processes of CLL. They render a different biological profile of the disease, and are one of the most important prognostic factors. About 40-50% of CLL patients have chromosomal abnormalities that can be demonstrated by classical band cytogenetics *(Hoffman Ret al., 2013; Muthusamy N et al, 2010).* As early as the 1990s, reports were published analyzing the relationship between the complex karyotype (the presence of more than 3 structural and / or numerous chromosomal aberrations) with time to treatment and overall survival. The complex karyotype was reported for 14-34% of untreated patients and up to 25-35% of patients with refractory CLL. Though it is a heterogeneous category, thevcomplex karyotype is in most cases associated with an unfavorable prognosis. Baliakas et al. found an association between a complex karyotype, unmutated VH genes, increased expression of CD 38, and a high frequency of 17 p- and del (11 q) in a group of 156 patients with CLL (*Baliakas P et al., 2014*) .Conventional cytogenetics allows tracking of the clonal dynamics and evolution of the disease, but the need for additional stimulation of cell cultures is the reason for some limitations in the use of this method . And if about 40% of clonal disorders can be detected by classical cytogenetic, this percentage increases to 80 when using fluorescent in situ hybridization on interphase nuclei *(Hoffman Ret al., 2013*). The method is applicable to non-dividing cells and allows visualization of certain genomic sequences.

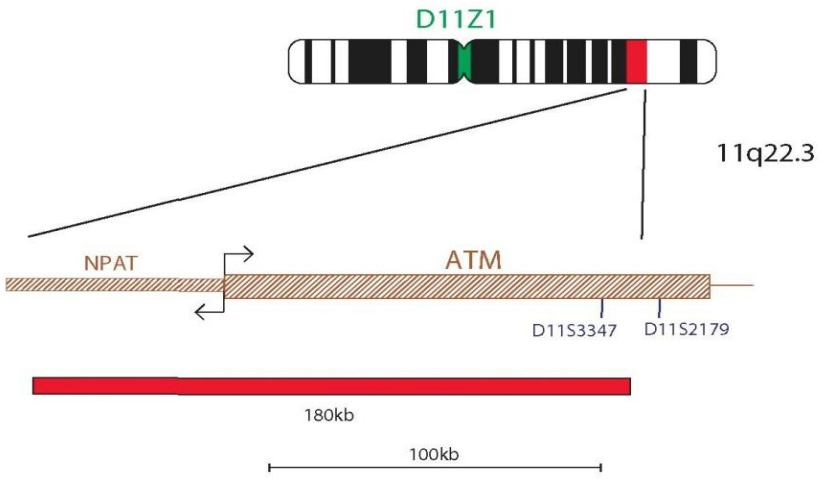
As a rule, del (17p) is found only in part of the leukemic cells. It is assumed that it is a subclonal abnormality, which in the course of the disease or after CIT or clonal selection, may become dominant *(Savov A, et al. 2017).* Approximately in about 80% of patients with del (17 p) a mutation is detected that affects TP53 *(Rossi D, 2009).* On the other hand, only 60- 70% of patients with mutation TP53 have del (17 p), detected by FISH *(Malcikova J et al., 2018).* The frequency of chromosomal aberration varies, depending on what stage of the disease the analysis it is carried out, as well as on the chosen therapeutic regimen. Unfortunately, most authors analyze this chromosome abnormality in studies involving patients at different stages of the disease and for a limited period of time.

Del 17p is observed in 3-8% of patients with untreated CLL (*Puiggros A et al., 2014)*. This percentage progressively increases to 30% in patients with relapse and reaches 50% for treatment- resistant patients (*Eichhorst B et al., 2016*). FISH analysis in the group of 62 patients proved del (17p) in six (9.8%) of them. The del17p thus established was used as the sole marker to determine the TTP53 status. In our study, loss of one allele of the p53 gene ranged in values from 10 to 95% of the interphase nuclei. In three patients it presented as monosomy 17. A review of the literature revealed conflicting opinions about the prognostic significance of mono- and biallelic disorders. Similar fluctuations exist concerning the size of the malignant clone, determined by FISH analysis as the percentage of cells with the corresponding cytogenetic abnormality (*Savov A, et al., 2017*). Van Dyke et al. considered a clinically significant threshold (related to TTFT and OS) the presence of more than 20% 17p CLL cells (*Van Dyke et al., 2016*). In one of the patients we studied, the clinical presentation of the disease was associated with extremely elevated levels of WBC and "bulky disease" although at diagnosis a loss of p53 in only 10% of the interphase nuclei (7% of them in the form of a del (17) (p 13) and in 3% as monosomy 17). Two of the patients , who were diagnosed at an advanced stage and presenting with the symptoms associated with the disease had begun treatment promptly after they were diagnosed. One patient was diagnosed at an early A clinical stage. Treatment was postponed because of lack of indications, and 48 months after the diagnosis the disease progressed and a second neoplasia was detected. Of all the 6 patients with del (17p), only 2 (one in stage A and one in stage B according to Binet) had not started treatment for six months of follow-up. Although the presence of del (17p13) / TP53 mutations alone is not considered sufficient to justify the initiation of treatment (*Savov A. et al., 2017*), underlying mutations, affecting TP53 is thought to have an adverse prognostic

factor in each stage of the development of CLL, from diagnosis, during treatment, at relapse *(Rossi D et al., 2012;Garff -Tavernier Le M et al., 2013).*

Del 11 q 22-23 always includes the ATM gene and is found in about 10% of newly diagnosed patients, and progressively increases to 20% for patients who need to start treatment (*Rossi D, Gaidano G, 2016*). In our group, del11q was found in 6 patients (9.6%): in three of them it was combined with del 13 q, and only in one it was combined with 17p- . In a group of 2184 patients with data from a FISH analysis, Patricia Greipp et al. observed what is known as "double hit" (11q- / 17p-) defect in 1% of the cases (*Greipp P T et al., 2013*). 11q - was found as an isolated chromosomal abnormality in 3.3% (2/62) of patients at an early stage (Binet, A and B). The clinical presentation of patients with del11q (alone or in a combination with another chromosomal anomaly) was associated with the presence of lymphadenomegaly, splenomegaly and values of absolute B-cell lymphocyte count **>**20G / l. Only in one of the cases within 18 months of follow-up did not need treatment, unlike the other patients in whom therapy was started at different intervals. Chromosomal aberration is present in the risk stratification models of Wiereda et al., 2011, Rossi et al., 2013, Pflug et al., 2014, but is was not considered as a prognostic factor in CLL - IPI. According to the literature, this chromosomal abnormality is associated with a unfavorable prognosis in most patients (*Guarini A et al., 2012*). Depending on the size of the affected region, two variants of 11 q have been described: "classical aberrations", including ATM loci and atypical ones - located near the centromere, without specific clinical significance (Fig. № 34) (*Sami Malek 2013).*

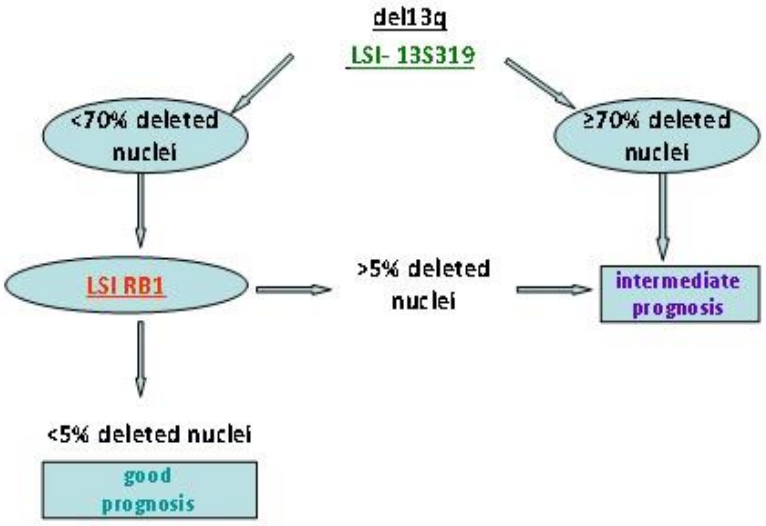
Figure №34. Affected regions in cases of 11q- in patients with CLL (Product Catalog 2017-2018 - Oxford Gene Technology)



In most scientific reports, the two high-risk chromosomal aberrations (17p- and 11q-) have been described in patients with advanced clinical stage (Zenz T et al., 2012). In 12% (8/62) of the patients we followed up, 17p- and 11q- were found, and 10% (6/62) of the carriers of these chromosomal abnormalities were in the early A and B clinical stages according to Binet.

Conventional cytogenetic examination of abnormalities affecting the 13^{th} chromosome revealed a frequency of this chromosomal anomaly s 6 times lower in comparison with the results, obtained by applying the FISH method. This in turn is related to the possibility of detecting smaller or larger deletions affecting the region q 14.3 through locus-specific probes (*Hoffman R et al., 2013*).The detection of an isolated del 13q has been associated with a favorable clinical course of the disease *(Smonskey T et al, 2012; Bagacean , C et al., 2017*). However, reports have been published in recent years, calling this conclusion into question against the background of the expanding possibilities of molecular biology. The interest in the chromosomal anomaly is associated with genes DLEU 1 , DLEU 2, the gene , including the first exon of the MIR 15 A / MIR 16-1 cluster and the RB 1 gene located on the long arm of chromosome 13. These factors have focused the attention of researchers on the influence of the size of the affected chromosome region, the percentage of 13 q - CLL cells, and the different clinical course. Van Dyke et al. reported a shorter time to first treatment in patients with > 85% CLL 13 q - cells *(Van Dyke et al., 2016*). Esther Orlandi and colleagues have reported surprisingly shorter survival without treatment (TFS 22 months) in patients with 70 % CLL 13 q - cells (*Orlandi E et al., 2013*) . Nedeva et al. did not find significant differences in TTFT, at a threshold of CLL 13 q - cells of 80%, but found a difference in PFS (*Nedeva A et al., 2018).* According to some authors, the limits of prognostic significance for 13q - can vary from 65 to 90% (*Puiggros A et al., 2014*). Michele Dal Bo et al. even offered to more accurate predictive assessment particular sequence in the stages of conducting FISH analysis which are presented on Fig. № 35

Figure № 35. Prognostic chart in patients with proven del13q (modified after Michele Dal Bo https://doi.org/10.1002/gcc.20885)



In the group of 26 patients we studied, in which 13q- were observed in a different percentage of the nuclei, we did not find a significant difference in the mean time to treatment for patients with >70% or <70% of cells with said chromosomal aberration. In contrast, in subjects in whom the mutational status was defined as UM and 13 q - was detected, the mean time to treatment was 20 months. Similar data are presented in the study of Jose D . Sandoval et al., which included 273 patients and whose purpose was to determine the significance of the mutation status of patients they so-called favorable genetic aberrations. The time to treatment for persons with isolated 13q- and UM - IGHV status was 2.9 years (*Sandoval D.J et al. 2018*). This raises the question to what extent and in what direction studies should continue to conduct screening and assessment of risk for untreated CLL patients.Viewed as an isolated anomaly, this cytogenetic damage may have a clinical presentation that is different from a presentation in which it is combined with other chromosomal aberration or a molecular marker. In 2011, Gladstone et al. published a report based on data from a study, involving 94 patients with del13q, 41 of which were with UM – IGHV. The results of the study demonstrated that it was the mutational status that predetermined the clinical course of patients with del13q (*Gladstone DE et al., 2011*). In 6% (4/62) of our patients, 13q- was found in the presence of 11q- or 17p- with a phenotypic expression, associated with ABCL> 50G / l.

Cytogenetic disorders cannot fully explain the genetic basis and clinical diversity of the disease. The information obtained when determining the molecular correlates of CLL is more detailed *(Foà Ret al., 2013*). DNA sequencing methods are not routinely used in clinical practice. That is why, over the years various research groups have explored the possibilities of PCR as the method, allowing for the determination of various surrogate markers. The expression of LPL, ZAP -70, ADAM 29, SEPT10 genes is cited in the literature as one closest to the predictive value of IGVH status. In 2005, Pablo Oppezzo et al. suggested using the LPL / ADAM 29 ratio as a more accurate prognostic factor. The authors referred to the fact, that in physiological conditions lipoprotein lipase is not expressed by B-lymphocytes. The enzyme is detected in adipocytes, myocytes and is produced in significantly lower amounts of macrophages and hormone-producing cells of the suprarenal and ovarian glands (*Heintel , D. et al., 2005*). Low or absent activity of LPL in peripheral blood mononuclear cells allows this unusual expression in lymphocytes to be determined by PCR analysis (*Hartman ML et al, 2012).* Although the reason for the aberrant expression of LPL by the lymphocytes it is not quite clear, currently it is assumed, that this expression can support and amplify the transmission of signals, related to proliferation and survival on CLL cells (*Pietro D et al., 2017*). The main function of the protein is associated with participation it its lipid metabolism (*Moreno, P et al.,2012*). However, due to its ability to make bridge-like connections between the surrounding molecules, it is reasonable to assume that is also a part of this process of interaction with dendritic and stromal cells. Proliferation and cell survival of CLL depend to a great extent on the interaction with the cells of the surrounding structures, which has also been confirmed by the clinical effect of tyrosine kinase inhibitors that lead to redistribution of lymphocytes from lymphatic organs in the bloodstream (*Yosifov D et al., 2020*) . Studies on the cellular metabolism and the sources supplying ATP in CLL have established elevated levels of reactive oxygen species (board ROS) and elevated antioxidant capacity compared to normal B-lymphocytes (*Galicia - Vázquez G et al., 2018*). In practice, there is no clear evidence how CLL cells, like other neoplastic cells, use effect of Warburg, associated with an increased rate of absorption of glucose and predominently the production of lactate in the presence of oxygen and adaptive mechanism for maintaining the biosynthetic needs of leukemia cells and uncontrolled proliferation (*Liberti M V et al., 2015*). In a study of Regina Jitschin et al. on mitochondrial metabolism, the authors discovered increased oxidative phosphorylation in CLL cells as one of the key sources of generating energy *(Jitschin R et al., 2014*). Different cellular metabolism is thought to be the cause of PET/CT negative outcomes in cases of uneventful course of the disease and to be positive in cases of transformation

to DLBCL. Although the metabolic pathways that neoplastic cells use to adapt and survive, they are not fully understood and studied, different teams of researchers have focused their clinical study on the prognostic value of LPL and the search of surrogate markers of a mutation status. In 2008 Dirk Kienle et al. reported results from a clinical study, involving a group of 151 patients on whom they performed FISH analysis, VH sequencing, and PCR analysis. Based on the results from the Cox regression analysis, the authors identified surrogate marker LPL as having the strongest predicative factor, associated with overall survival of the patients, while the expression of ADAM29 was an independent prognostic factor, associated with TTFT *(Kienle , D et al., 2008*).

Referring to Pablo Oppezzo's alternative method for determining mutational status among a group of 48 patients, based on the results of multiplex PCR, we found twice as many patients with UM status as those whose status was defined as MT. Our estimated mean time to initiation of treatment in the UM status group was significantly shorter than in the MT status group. Once we determined the mutation status and based on the results of the conducted FISH analysis, we looked for an association between cytogenetic disorders and mutational status. An association between chromosomal aberrations in the study group and mutational status was observed. However, the small number of patients was a limiting factor and did not allow for drawing definite conclusions. Similar results have been presented in reports on studies including significantly larger patient groups.Thorsten Zenz et al. found a higher frequency of chromosomal aberrations associated with an unfavorable prognosis (11q -,17 q -) among individuals with UM - IGHV *(Zenz T, Dohner H, 2011).*

In 2012 Emili Montserrat analyzed the importance of prognostic and predictive markers and presented an algorithm in newly diagnosed patients with CLL, applicable in clinical trials. The author proposes for asymptomatic patients to study the prognostic markers: stage of the disease, LDT, IGHV, ZAP -70, CD38 and on the basis of which to assess the risk of progression and to determine the follow-up period. For patients who need to start treatment, the choice of therapy should be based on the result of testing for 17p-/TP53, 11q-, IGHV, ZAP-70, CD38 *(Emili Monserrat 2012).*

In the same year, Chris Pepper et al. reported the results of a study of 1,154 untreated stage A patients, proposing that this group be limited to mutation status, CD38 and LDT monitoring within the first 12 months. In cases of disease progression and need of treatment choice, further investigation of 17p- was recommended *(Pepper C et al., 2010).* According to Dirk Kienle, molecular markers allow screening of patients, but cannot completely replace

genetic factors. That is why it is suggested that they remain as basic in risk stratification approaches *(Kienle, D et al., 2008).*

Sameer Parikh published a systematic review of the literature in 2018 and proposed that all patients at diagnosis be stratified in the relevant risk categories on the basis of CLL - IPI. This is due to the fact that in about 40-50% of untreated patients unfavorable prognostic factors such as: del17p , del11q , UM - IGHV status can be found *(Parikh S, 2018).* Although CLL - IPI was designed to predict overall survival, several research teams have confirmed its use as a prognostic index related to the time to first treatment. Patients with overt symptoms, associated with the disease have begun therapy regardless of their risk category. We tried to validate CLL - IPI in 48 patients, based on the results of surveys and referring to the assumption that the prognostic model can be used to determine the time to therapy. We found a difference in the time to start treatment for the different subgroups, although for patients at high and very high risk this difference was not significant. Our results could be due to the limited number of patients in the very high risk group. On the other hand, in the group studied, the distribution of patients by disease stages was not balanced for the individual subgroups. In addition, in some patients the laboratory parameters were determined at diagnosis and staging (beta-2-microglobulin, ABLC). Molecular markers and chromosomal abnormalities were studied at different stages between diagnosis and initiation of treatment. Also, in the group of 48 patients, patients with advanced stage C and usually indicated for treatment were included. Out of a total of 32 patients staged in early A clinical stage, unfavorable prognostic factors were observed in 37.5%: del 17p and UM status, thus proving the importance of complex assessment of patients in early A clinical stage.

In a multicenter prospective study of the German CLL study group, which included patients in the early A clinical stage, the researchers confirmed the unfavorable prognostic value of the factors: age over 60 years, LDT less than 12 months, elevated β 2- MG , del 11q , del17p , and UM status. By adding two independent prognostic factors (del11q and LDT) the authors upgraded CLL - IPI and offered CLL 1- PM as an opportunity to assess TTFT and OS in patients with untreated CLL at an early stage (*Hoechstetter M A et al., 2020*). The use of mutational status as a factor that does not change in the course of the disease. It has the value as a prognostic and predictive marker *(Moia R et al., 2020*), and combined with the other progostic factors mentioned above, the mutational status provides a good opportunity for a more accurate assessment of the risk of progression among untreated patients. Each of

the studied prognostic factors carries valuable information and complements the information which the others contain.

Constantly increasing possibilities of modern targeted therapy in CLL will require more precise stratification of patient groups in view of selecting the most appropriate time to start appropriate treatment, contributing to possibly prolonging survival of patients with B-CLL as much as possible .

CONCLUSION

B - CLL is known with extremely variable and unpredictable clinical course and have been a challenge in developing different risk assessment models for years. Factors related to the time to first treatment, reflect indirectly the aggressiveness of the disease. Age is a factor, whose prognostic value in relation to the time to treatment we did not confirm. In our opinion, it would be important in determining the survival of patients with B - CLL. Carrying out staging procedures at the time of diagnosis is important because there is a need to follow up asymptomatic patients, who account for the major part of newly diagnosed individuals. An increase in absolute lymphocyte count is not always associated with disease progression, and vice versa. The involvement of lymphatic areas outside the areas available for palpation would change staging, which is based solely on physical status data. Serum β- 2 MG is an indicator whose levels are influenced by many factors related not only to the processes of lymphocyte activation, but also to a number of concomitant conditions. Dynamically changing values of the indicator can be increased in cases of chronic congestive heart failure, concomitant infection, as well as in cases of impaired, although not manifest renal dysfunction – all being, common diseases in persons over 60 years of age. Currently, the development of a prognosis for the clinical course of the disease and the corresponding determination of the clinical approach in patients with untreated CLL are based on our knowledge of the so-called " classical " prognostic factors. The addition some of the many molecular and genetic markers, indirectly reflecting the complex and dynamic processes associated with autonomous mechanisms of proliferation and apoptosis typical of neoplastic cells, provides an opportunity to better assess the risk of progression, as well as select an adequate therapy for a patient in need of treatment. It is noteworthy that some of the unfavorable prognostic factors can be found in a large number of patients with untreated B - CLL, we found in the group studied. Using RNA- based surrogate markers as an alternative for determining mutational status at this stage may serve to assess the risk of early disease progression more accurately. Considered as a complex, all the mentioned prognostic factors determined at the diagnosis of the disease can be applied in clinical practice to assess the time to treatment and the risk of progression. Notwithstanding the small group of patients**,** we tried to validate CLL – IPI using a surrogate marker of mutation status. Based on the data from the analysis, in our opinion, molecular and genetic disorders indisputably provide valuable information for clinical practice. Playing the dual role of prognostic and predictive markers, mutational status and chromosomal aberrations should be assessed as soon as the disease is

diagnosed, in order to determine the risk of early progression and more precise choice of therapy.

CONCLUSIONS:

Based on the results found and the analysis of the known prognostic factors related to TTFT in treatment-naïve patients with B-CLL, we could conclude that:

- ✓ The median age of the newly diagnosed patients with B-CLL in our study was 67 years. Age was not a factor that influenced the time to first treatment.
- ✓ The patients in early stages of the disease were the largest part of patients with B-CLL, who visited the Hematologic Outpatient Consulting Room and the Clinic of Hematology.
- ✓ The clinical stage of the disease was a significant factor associated with TTFT.
- ✓ High serum levels of β-2 MG correlated with shorter time to treatment. In patients at the early stage, a negative correlation between the values of the indicator and TTFT was established.
- ✓ The presence of del11q and del17p, or a combination of more than one chromosome aberrations, including in combination with del13q, was associated with a shorter TTFT.
- ✓ The PCR analysis allows to identify high-risk patients with a UM status, in which the mean time to initiation of treatment is significantly shorter than TTFT in subjects with MT status.
- ✓ Del13q was observed more frequently in patients with mutated status and was associated with a favorable prognosis, unlike the structural aberrations 11q– and 17p-, which were detected in subjects with unmutated status and correlated with short-term TTFT. The prognostic value of 13q- was different in cases in which it was combined with unmutated status.
- ✓ In more than one-third of the patients with an early stage of disease, adverse prognostic factors were observed: del17p and UM status. This finding proves the need to determine them at the time the diagnosis is made.
- ✓ The complex assessment, based on the molecular-genetic markers available for research in patients with untreated B-CLL provides a better opportunity to stratify the risk of progression, determine the time to treatment, the period for follow- up and, accordingly, the choice of the most adequate therapy.

CONTRIBUTIONS TO THE DISSERTATION

Contributions of original character

✓ For the first time in our country, the relationship between adverse molecular and genetic factors in patients with untreated B-CLL and their influence on the time to treatment is studied, which in turn allows the choice of the most adequate modern therapy.

✓Stratification of patients with untreated B-CLL was performed, based on the integration of a surrogate marker to classical prognostic factors and chromosomal aberrations.

Contributions of a confirmatory nature

- ✓ The importance of stage of the disease and its determining influence on the time to treatment has been confirmed.
- ✓ The presence of a correlation between the values of serum beta - 2 microglobulin and the time to treatment was confirmed .
- ✓ The need to evaluate the molecular and genetic factors, associated with the risk of disease progression and hence with a risk-adapted therapy was confirmed.

Contributions of an applied nature

- ✓A questionnaire was prepared containing information about the patient and the disease.
- ✓An approach was developed for screening high-risk patients with untreated B-CLL.

Application 1

QUESTIONNAIRE

for a patient with untreated B- chronic lymphocytic leukemia

1. First name Last name ……

2. age …….

3. Address .... phone to contact .....

4. Date and year of diagnosis :
…….
(In case, that person is established or newly diagnosed B- chronic lymphocytic leukemia)

5. Risk factors - (circle the correct one)
   - alcohol - yes / no
   - smoking - yes / no
   - work with pesticides yes / no
   - family history of cancer - yes / no

6. Concomitant diseases: ....

7. ECOG - physical capacity
(enclose the correct one) - 0 , 1 , 2, 3, 4

8. Constitutional symptoms :
   - fever over 37.5 - yes / no
   - night sweats - yes / no
   - weight loss- yes / no

Stages of the disease at the diagnosis, determined by physical examination , X-ray, ultrasound of abdominal organs , if necessary CT - (enclose the correct)

Stages Binet	**Number of lymph nodes**	**Hemoglobin g / l**	**Platelet count X 10 ^{9} / l**
A	<3	> 100	> 100
B	> 3	> 100	> 100
C		<100	<100

9. Autoimmune phenomena :
a / autoimmune hemolytic anemia - yes / no
b / autoimmune thrombocytopenia - yes / no

10. On the baseline:

Leukocyte count total …… G / l lymphocytes …… .. % ALB .....;
ABCL ……
- 6 months after diagnosis:
Leukocyte count total …… G / l lymphocytes …… .. % ALB .....;
- 12 months after diagnosis:
Leukocyte count total …… G / lymphocytes …… .. % ALB .....;

11 . Flow cytometric markers : at the diagnosis (indicate correct)
a / CD 38- positive b / CD 49 d - positive
- negative - negative

12. Serum ß2 - microglobulin - values :

13. Molecular genetic research:
chromosomal aberrations: (describe) ....
del 17p ……
del11q …….
del13q …….
LPL / ADAM29- (circle the correct one):
UM- unmutated MT - mutated

Date of completion the form: Doctor who completed:

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