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ZAP-70, THYMIDIN KINASE, CD38, BETA 2 MICROGLOBULINE AND LAKTATDEHYDROMINAZE CHANGES FOR PATIENTS WITH CHRONIC LYMPHOID LEUKEMIA AND ITS EFFECT TO DISORDER PROCESS AND PROGNOSIS

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Code of Ethics

The research is authorized by Central Medical Ethics Committee on 22nd March 2007, A-9 (decision No.9) and the agreement was received from all the patients.

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ABBREVIATIONS USED

β_2 MG	-	β_2 mikroglobuline
CD	-	lymphocytes receptor disegnation (engl. lang. *claster of differentiation*)
EDTA	-	ethylenediaminetetraacetate
EGL	-	E.Gulbja laboratory
ELISA	-	enzyme-linked immunosorbent assay
FITC	-	fluorescein isothiocyanate
HLA -		human leukocyte antigene
HLA-DR	-	stem cell, activated T cell, monocytes, macrophages B cell marker
CLL	-	chronic lymphoid leukosis
LDH	-	laktatdehidrogenaze
NCI	-	National Cancer Institute
NHL	-	Ne Hodgkin's lymhoma
NK	-	natural killers
p	-	probability, the significance level, p value
PE	-	fikoeritrine
RECUH	-	Riga Eastern clinical university hospital
RSU	-	Riga Stradins university
T HLL	-	T cell chronic lympholeucozis
TK	-	thymidine kinase
WHO	-	World Health organisations
ZAP-70	-	zeta associated protein

1. GENERAL DESCRIPTION OF THE STUDY AND IMPORTANCE OF THE PROBLEM

CLL is one of the most common leucosis variant in modern hematology. A lot of researches have been conducted for prognostic marker discovery, such as ZAP-70 and CD154. The goal of the research is as follow: to discover prognostic marker; to predict disorder course; to choose the right individual therapy. This therapy must be the most effective and the least aggressive for the general state of the health. Nowdays standard diagnostic criteria are used (lymphocytes number, atypical cell number and bone marrow infiltrate) which are accepted by USA National Cancer institute and CLL international workgroup. For CLL patient control and diagnostics, different laboratory indicators are used in treatment process. The most common are: LDH, beta 2 (β_2 MG), CD19, CD5, CD23 phonotypical markers. Today's clinical institutions widely use 70-kDa zeta associated protein (ZAP-70) for diagnostics. Different researchers recommend to use thymidine kinase (TK), but this parameter until now has not been widely used. Considering modern discovery options and time conditions, for CLL problem research in Latvia, we choose such diagnostic parameters as ZAP-70, differential cluster 38 (CD38), TK, LDH, β_2 MG. It is important, that all chosen methods are non-invasive and patient friendly. For the first time in the world and in Latvia, ZAP-70 and TK have been used CLL diagnostic.

ZAP-70 protein is determined to detect IgVH gene mutations and may be a successful determination parameter of the mutative status in laboratories. In recent years researches have shown that CLL varied manifestations are related and they reflect TK level. It is determined that in any case of tumor growth, TK level raise, as this marker reflect tumor cell mitotic divide activation stage. Researchers notes, TK level rise in CLL patient blood serum

typical for patients with rapidly progressive disorder variant. High TK level often determined for primary patients in advanced disorder stages. For the same patient group, TK level slowly decreases or remain high, despite treatment. It is important to determine relation between TK level raise and CD38, which is immunological prognostic factor. Many scholars have analyzed two of these parameters, however no publications have been made. As majority of authors analyzed two of these parameters independently one of each other, there are no publications which indicates TK and CD38 dependency. Many authors consider that CD38 is a basic indicator which describes CLL progressive variant. The obtained data, that tumor clone activity raise, related to CD38 marker level raise on cell surface and disease variant metastatic process acceleration. From all the above follows, that its necessary ZAP-70, TK and CD38 expression comprehensive analysis, prognostic parameter connectivity determination with such classic prognostic parameters as leucocytes level and absolute lymphocytosis, LDH and β_2 MG. It is vitally important to determine prognostic parameter level for CLL patient to choose upon a proper and successful treatment.

2. THE RESEARCH GOAL

To research proteins ZAP-70, CD38, TK, LDH, β_2 MG and other indicators in cases of chronic lymphoid leukemia and their effect to disorder process and therapy efficiency.

3. THE RESEARCH OBJECTIVES

1. To determine ZAP-70 marker for chronic lymphoid leukemia patients at disease diagnosis moment and the connection with TK, CD38, LDH, β_2 MG parameters.

2. To determine ZAP-70 marker changes dynamics in chronic lymphoid leukemia therapy in the period after 6 and 12 months.

3. To determine TK for chronic lymphoid leukemia patients at diagnosis moment and in connection with other parameters.

4. To determine TK marker changes in chronic lymphoid leukemia therapy in the period after 6 and 12 months and its connection with other parameters.

5. To determine CD38 for chronic lymphoid leukemia patients at diagnosis moment and in connection with other parameters.

6. To determine CD38 marker changes in chronic lymphoid leukemia therapy in the period after 6 and 12 months and its connection with other parameters.

7. To determine LDH for chronic lymphoid leukemia patients at diagnosis moment and in connection with other parameters.

8. To determine LDH changes dynamics in chronic lymphoid leukemia therapy in the period after 6 and 12 months and in connection with other parameters.

9. To determine β_2 MG for chronic lymphoid leukemia patients at diagnosis moment and in connection with other parameters.

10. To determine β_2 MG marker changes dynamics in chronic lymphoid leukemia therapy in the period after 6 and 12 months and its connection with other parameters.

4. THEORETICAL AND PRACTICAL IMPORTANCE

New prognostic criteria for CLL clinical process disease temper determination have appeared. Individual prognostic factor – ZAP-70 is determined for every disorder process variant. CD38, TK level, β_2 MG, LDH and other disorder parameters have been determined, depending on the leucosis process dynamics. The obtained results are taken as the basis for differentiated treatment therapy determination of CLL. It is important for therapeutical tactic determination for CLL patients at early disease stages.

5. HYPOTHESIS

1. CLL progress speed depends on ZAP-70 expression. Patients with this marker positive expression are different from disorder progressive process, in ZAP-70 negative expression group. ZAP-70 negative subgroup has positive prognosis.
2. For patients with II, III and IV stage and progressive course variant TK level is more elevated than for patients with 0 and I stage and positive course variant.
3. There is correlation between ZAP-70 positive expression and elevated CD38, TK and other prognostic marker.
4. For CLL patients with positive ZAP-70 expression and progressive disorder variant, it is possible to raise significantly the level of CD38 and TK, which manifest by fast progressing disorder variant.
5. For CLL patients with positive ZAP-70 expression and progressive disorder variant, it is possible to raise significantly the level of CD38 and TK,

which manifest by lack of remission after 6 and 12 month chemotherapy course.

6. ZAP-70 expression, high CD38 and TK content, for CLL patients in a diagnosis moment, can be considered as additional criteria of an adverse forecast: the prognosis is unfavorable if there is ZAP-70 positive expression, high TK and CD38 levels.

6. THE RESEARCH NOVELTY

The received data are based on the selected prognostic factors. There is individual prognostic factor – ZAP-70 mutative status surrogate marker which selects disorder determination and allows to predict disorder course variant. TK level analysis is managed the first time for these patients. TK is analyzed for patients depending on stage and disorder course variant and other prognosis factors, considering disorder course variant. ZAP-70 and TK prognostic factors are determined as prognostic factors importance and notability clinical developments in the CLL case.

7. OUTLINE OF THE DISSERTATION

The Doctoral Thesis is written in Latvian language. It consits of 15 chapters: Introduction, Importance of the Problem, Objective of the Work and Terms of Reference, Hypotheses of the Work, Review of Publications, Materials and Methods, Results, Discussion, Conclussions, Practical References, List of Publications. It has 5 annexes. Total amount of work is 133

pages, including 53 tables and 42 bar charts. In research are included 199 references.

8. MATERIALS AND METHODES

8.1. Patients, Clinical Description

120 first-time patients with chronic B-cell lymphoid leucosis (CLL) took part in the present research. Riga Eastern clinical university hospital (RECUH), National Hematology center (now Chemotherapy and hematology clinics) is base for research.

The age of researched CLL patients ranged from 35 till 81 years, average age was 66.87 ± 1.007. Among patients, 60 patients (50%) were men and 60 (50%) women. Average men age 68.51 ± 8.72 years, women – 65.42 ± 1.56. 97 patient fully finished research, 3 patients died during research – death cause – progressing B CLL, 20 patients withdrew from research – cause unknown.

For all patients, CLL diagnosed according to World Health Organization (WHO) recommendations that includes: integrated blood test, milligram data, flow cytometry analyze of peripheral blood and bone marrow lymphoid element.

8.2. Division of Patients Depending on Disorder Stage

During research all patients were divided in stages after Rai classification. The stage found at the diagnosis time. There are the greatest

number of patients in I and II stages, respectively 38 and 40 patients.0 stage – 7 patients; III stage – 19 pacients; IV stage – 16 patients.

8.3. Division of Patients Depending on Variant

According to publications, considering progressing risk, as described in T.Seiler 2006-th year work, patients depending on disorder course variant were divided into 3 groups – early (Rai 0, Binet I), average (Rai I, Binet B), progressive (Rai III, IV, Binet C). For each group representative special disorder course variant. Average by disorder course variant in this research made considering clinical features. The CLL patients were divided into 3 groups depending on clinical course features. In first group were included 7 patients with 0 stage, in second group combined 78 patients with I and II stage, from those for 38 patients set I stage, but for 40 people – II stage. In third group combined 35 patients with III and IV stage. From those for 19 people set III stage, but for 16 patients – IV stage. Patients dividing after stages are presented on table 2. In the first patient group were 7 people (6%) with benign disorder variant. In the second patients group were 78 people (65%), disorder had slowly progressive character. In the third group were 35 patients (29%), disorder had fast progressive character.

8.4. Diagnostics of Chronic Lymphoid Leukemia

All standard laboratory investigations made RECUH. TK level determination made in RSU August Kirhensteins Microbiology and virusology institute.

ZAP-70 and CD38 expression made in Riga Hematology centers hemapatology laboratory base (Head of the laboratory A.Rivkina).

Ultrasonoscopic investigation made in RECUH clinic „Linezers" in National Hematology center.

Trepanbiopsy and lymph node biopsies made in Riga Hematology center.

8.5. Summary Diagnostically Amount

To determine chronic B cell lymphoid leukemia, for studied patients, various standard analysis and research methods have been used during the research.(Table 8.1 and 8.2) The total quantity of research after 6 months are: quantity of patient – 109. Eleven patients are withdrawn during 6 months period from research. Two patient's death due to progressing disorder, for 9 – unknown reason. The total quantity of research after 12 months are: patients – 97. Twelve patients are withdrawn from research during 12 months period, comparing with after 6 months examined patients number. One patient death cause due to progressing disorder, 11 – unknown reason.

Table 8.1

Standard examination volume

No.	Studied parameter	Researches made at the diagnosis moment	Researches made at the diagnosis moment	Researches made at the diagnosis moment
1.	Clinical examination	120	109	97
2.	General blood test	120	109	97
3.	LDH	120	109	97
4.	β_2 mikroglobuline	120	109	97

Table 8.1extension

No.	Studied parameter	Researches made at the diagnosis moment	Researches made after 6 month	Researches made after 12 month
5.	Myelogram	120	109	97
6.	USI	120	109	97

Table 8.2

Total volume of parameters set out

No	Studied parameter	Researches made at the diagnosis moment	Researches made after 6 month	Researches made after 12 month
1.	Peripheral blood cells immunophenotyping	120	109	97
2.	ZAP-70 expression level	120	109	97
3.	CD38 expression level	120	109	97
4.	TK level in blood serum	120	109	97

8.6. Patients Clinical Status Assessment

Certified doctors - hematologists performed the first inspection in RECUH Chemotherapy and hematology clinic. The present research had no affect on the patients' treatment.

8.7. TESTS

8.7.1. Blood Test Determination

Blood scene determination has been done for each patient at his first doctor's visit and then every next visit. E. Gulbja Laboratory (EGL) uses these blood scene determination methods.

8.7.2. β_2 mikroglobuline Determination

EGL use standard method for β_2 microglobuline determination – immunochemical luminescence. Refferental values are 1.01–1.73 mg/L.

8.7.3. Laktatdehydrogenaza Determination

EGL uses standard method for LDH determination.Refferental values are 200–480 U/L.

8.7.4. Immunophenotyping

The lymphoid element superficial markers have been used antibody panel, which included CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD22, CD23, and CD38. The immunophenotyping of peripheral blood are used EDTA containing containers. Into the blood was added 10 μl fluorescence conjugated monoclonal antibody: anti- CD5, -CD 10, -CD 19, -CD20, -CD22, -CD23, -

CD38 (company *Beckman Coulter Immunocytometry systems, Miami, Florida*, USA), incubated 30 minutes at the room temperature, in the dark. After that cells were double washed, suspended with 500 µl in buffered saline solution, analyzed samples on flow cytofluometer Epics XL, *Beckman Coulter*, Miami, Florida.

8.7.5. Determination of ZAP-70 Expression

The brefeldine A in10 µg/ml concentration and ionomicine – 1 µg/ml were added in tubes and incubated 4 hours under 37° C temperature in CO_2 incubator with 7.5% humidity. After incubation period completion, cells were mixed and transferred by 100 µl into tubes. Further, blood 100 µl of were added 20 µl of monoclonal antibodies anti-CD5, -CD19 to superficial antigens and incubated 20 minutes at the room temperature, in the dark. In each tube were added 0.5 ml *Optilyse* solution and were incubated 15 minutes at the room temperature for erythrocyte lyses. Further the cell fixation was done with adding 250 µl solutions *Cytofix/Cytopenn* (*Beckman Coulter*, Miami, Florida) and 20 minutes incubation at the room temperature, in the dark. Cells washed twice (PBS+1% BSA, 0.1% NaN_3) environment. Into the washing cells were added permabilisation solution *Perm/Wash*. Last stage cells were conjugated with fluoriscine monoclonal antibody 20 µl intracellular anti-ZAP-70 (*Beckman Coulter*, Miami, Florida), staining. The sample analysis made by flow cytofluorimeter.

8.7.6. Determination of TK Level in Blood Serum

Thymidine kinase (TK) is one of important enzymes which show cells activity. Increased cells quantity in division process associated with malignant disorder and active tumor process. Similar process is CLL. Thymidine kinase level in blood serum is low and corresponds to the number of malignant cell division. In work used ELISA method, which includes these actions. The blood serum of healthy persons, TK level must be not less than 50 ng/l, but this TK minimal discovery method (sensitivity) was 100 ng/l.

8.8. Statistical Analysis Methods of Results

Distribution of patients testing made with D'Agostino-Pearson test. In case of normal distribution dissonance in groups were compared using repeated measures ANOVA test, but in normal distribution dissonance case with Kruskal-Wallis test with further Dunns aftertest. If results between groups were statistically reliable, then after Kruskal- Wallis test did Mann-Whitbey U test. In normal distribution dissonance case, correlations were rated with Spearman test, but, if data was inconsistent with normal distribution, then made Pearson test.

Results were evaluated as statistically reliable, if $p< 0.05$. On bar chart data expressed as average ± average 95% confidential interval. All data are statistically processed, using statistic programs *GraphPadPrism* 5 version (*San Diego*, California, USA). All results in tables and in drawings presented as arithmetic average and its standart error (M ± t). Thereby, for 120 CLL patients done blood lymphoid cell and bone marrow complexes, morphologic,

immunologic and immunohistochemical researches with obtained statistic data processing.

9. RESULTS

9.1. Blood Test Results

9.1.1. Leukocytes Number

Results obtained during research shows. Six and twelve months therapy reduces leukocytes number in blood for chronic lymphoid leukemia patients, comparing with pre-therapy data – 20 x 10^9/l ± 3 x 10^9/l against 43 x 10^9/l ± 7x10^9/l, (p = 0.001) after 6 months, 21 x 10^9/l ± 3 x 10^9/l against 43 x 10^9/l ± 7 x10^9/l, (p = 0.001) after 12 months All patients were analyzing together before therapy, obtained statistically reliable correlation between leukocytes number (WBC) and stage (r = +0.33; p = 0.0002) and between leukocytes number and spleen size (r = +0.19; p = 0.045).

9.1.2. Lymphocyte Number

Results received during research show. Six months therapy reduces lymphocytes absolute number in blood for CLL patients, comparing with pre-therapy data (15.44 ± 1.88 to 25.68 ± 2.16 x10^9/l, p = 0.01). But 12 months therapy even with greater statistical reliability reduces lymphocyte number (14.08 ± 2.29 to 25.68 ± 1.88 x10^9/l, p = 0.001). All patients together were analyzing by stages, there were statistically significant positive correlation between stage and lymphocyte number (r = +0.32; p = 0.0003). All patients

together were analyzing, there were positive correlation between lymphocytes absolute number and spleen size. ($r = +0.20$; $p = 0.03$).

9.1.3. CD38 Quantity

Results received during research shows. Six months therapy increased CD38% number for chronic lymphoid leukemia patients, comparing with pre-therapy data – 16.01% ± 1.474% against 13.66% ± 1.526% $p = 0.01$. But 12 months therapy even with greater statistical reliability increase CD38% quantity – and comparing with pre-therapy data and with 6 months therapy results (respectively, 21.99% ± 2.008% against 13.66% ± 1.526%, $p = 0.0001$ and 21.99% ± 2.008% against 16.01% ± 1.474%, $p = 0.03$). There was no statistically significant correlation found between CD38 and stage, spleen size and lymph node diameter.

9.1.4. TK Quantity

Results received during research shows. Six months therapy reduces TK quantity for CLL patients, comparing with pre-therapy data – 1557.0 ng/l ± 213.70 ng/l against 2821.0 ng/l ± 374.70 ng/l $p = 0.0001$. But twelve months therapy also significantly reduces TK quantity (1775.0 ng/l ± 312.40 ng/l against 2821.0 ng/l ± 374.70 ng/l $p = 0.02$). All patients together were analyzing by stages, there were positive correlation between TK quantity and stage ($r = +0.36$; $p = 0.0001$), between TK quantity and spleen size ($r = +0.23$; $p = 0.015$) and between TK quantity and maximal lymph node diameter ($r = +0.30$; $p = 0.03$).

9.1.5. β_2 microglobuline Quantity

Results obtained during research shows. Six months therapy reduces β_2 MG quantity for chronic lymphoid leukemia patients, comparing with pre-therapy data – 2.84 mg/ml ± 0.22 mg/ml against 3.55 mg/ml ± 0.32 mg/ml, p = 0.0018. Twelve-month therapy reduces β_2 MG quantity, but with smaller reliability then six months therapy (3.055 mg/ml ± 0.27 mg/ml against 3.55 mg/ml ± 0.32 mg/ml, p = 0.02). There were no statistically reliable differences between 6 and 12 months therapy and β_2 MG quantity, although 6 months therapy has tendency to reduce β_2 MG quantity, comparing with 12 months therapy. All patients together were analyzing, there were positive correlation between β_2 MG quantity and stage (r = +0.23; p = 0.009), between β_2 MG quantity and maximal lymph node diameter (r = +0.39); (p = 0.0035) and correlation between β_2 MG quantity and spleen size (r = +0.17); (p = 0.05).

9.1.6. Laktatdehydrogenaza Quantity

LDH analysis contains data, that 6 and 12 month therapy does not affect LDH level for chronic lymphoid leukemia patients.All patients together were analyzing after stages, we found statistically reliable positive correlation between LDH level and stage (r = +0.25; p = 0.006), LDH level and spleen size (r = +0.22; p = 0.014) and correlation between lymph node diameter and LDH (r = +0.27; p = 0.056).

9.2. ZAP-70 Quantity

There were no statistically reliable differences found in ZAP-70 parameters before therapy, 6 and 12 month after therapy.We found positive correlation between stage and ZAP-70 ($r = +0.18$, $p = 0.05$).

There was no correlation found between spleen size, lymph node diameter and ZAP-70.

9.2.1. ZAP-70 Expression and Other Prognostic Factor Analysis in CLL Case

ZAP-70 expression has been chosen limit 20%. This level as restrictive was suggested by *M. Ertault-Daneshpouy with co-authors* in 2008. The prognostic factors were analyzed and compared with ZAP-70 level: ZAP-70 >20% or ZAP-70+ positive variant and ZAP-70 <20% or ZAP-70– negative variant. This apportionment is important for patient groups with fast progressing and slowly progressing disorder course variant determination.

9.2.2. ZAP-70 Level Expression with CD19+/CD5+ Cells for CLL Patients with Different Prognosis Variants

We were determined ZAP-70 in tumor clone cells CD19+/CD5+. Depending on disorder course variant and stage, patients divided into groups: ZAP-70– and ZAP-70+. The first group included 7 patients with benign course variant. The second group combined 78 patients with slowly progressing disorder variant. Group with fast progressing variant included 35 patients. The

obtaining data shows, that ZAP-70 level does not change, despite time period and received treatment. This parameter can be used as basis for researched group. According to received data all patient groups can be divided into ZAP-70 negative and ZAP-70 positive patients and evaluate the course of disorder, according to the distribution. This protein level was found these regularities during the research (table 9.1).

Group divided into subgroups, one subgroup consists of 61 ZAP-70+ patient and second subgroup of 59 ZAP-70 – patients. Data presented in the table, and showed that 11% patients from group with progressing course characterized by ZAP-70+ expression. In the benign of CLL course variant group patient number after ZAP-70+ marker was 72% of total patient's number selection. The slowly progressing variant group 67% is common ZAP-70+ expression and 33% patients are negative after studied marker (ZAP-70-).

Table 9.1

ZAP-70 level expression with CD19+/CD5+ cells for CLL patients with different disorder course variants

ZAP-70 expression	**L** (n – 7)		**LP** (n – 78)		**ĀP** (n – 35)	
	number	**%**	**number**	**%**	**number**	**%**
ZAP-70–	2	28	26	33	31	89
ZAP-70+	5	72	52	67	4	11

Table 9.2

ZAP-70 level expression with CD19+/CD5+ cells for CLL patients with different disorder course variants after 6 months

ZAP-70 expression	**L** (n – 6)		**LP** (n – 70)		**ĀP** (n – 33)	
	number	**%**	**number**	**%**	**number**	**%**
ZAP-70–	2	33	22	31	30	91
ZAP-70+	4	67	48	69	3	9

ZAP-70+ subgroup were 55 patients and in ZAP-70- subgroup were 54 patients after 6 months. From data presented in table, there are 9% of patients from group with progressing course ZAP-70+ expression subgroup and 91% progressing course ZAP-70- expression sub group. In the benign group, patients number ZAP-70+ marker were 67%, from total patient number selection and in ZAP-70- subgroup were 33%. In the slowly progressing variant group 69% ZAP-70+ expression and 31% of patients were negative (ZAP-70-). Data processed in table 9.2.

Table 9.3

ZAP-70 level expression with CD19+/CD5+ cells for CLL patients with different disorder course variants after 12 months

ZAP-70 expression	**L** (n – 2)		**LP** (n – 67)		**AP** (n – 28)	
	number	**%**	**number**	**%**	**number**	**%**
ZAP-70–	2	100	19	28	25	89
ZAP-70+	0	0	48	72	3	11

After twelve months in ZAP-70+ subgroup were 51 patients and in ZAP-70- subgroup were 46 patients. From data presented in table, there were11% of patients from group with progressing course common ZAP-70+ expression and 89% from group with progressing course common ZAP-70-. There was no patient in benign group ZAP-70+ marker and in ZAP-70- subgroup was 2 patients that corresponds 100%. In the slowly progressing variant group there were 72% ZAP-70+ expression and 28% of patients were negative (ZAP-70-). Data presented in table 9.3.

9.2.3. CLL Standart Prognosis Factor with Different ZAP-70 Expression

Traditional CLL prognostic parameters were analysing such as gender, lymphocytes absolute number and lymphocyte doubling time, we conclude, that in ZAP-70- subgroup, lymphocyte absolute quantity is $32.6 \pm 0{,}03 \times 10^9$/l, $p < 0.05$; lymphocyte doubling time is > then 12 months, $p < 0.05$. In ZAP-70+ subgroup lymphocyte absolute number is $69.6 \pm 0.32 \times 10^9$/l, $p < 0.05$; lymphocyte doubling period is 9.1 ± 1.1months, $p < 0.05$. In the same time no correlation found between patients gender and subgroups ZAP-70-and ZAP-70+, $p > 0.05$.

9.2.4. Imunephenotypical Marker Expression Rating for CLL Patients with Different ZAP-70 Expression

Markers expression explored superficial in ZAP-70- and ZAP-70+ subgroups. Three imunephenotypical marker clusters were analyzed: differentiating (CD19, CD20), differential diagnostic (CD5, CD23). Differential markers were required for lymphocyte population division to T cells and B cells. Differential diagnostic markers were used to determine diagnosis, because to each disorder corresponds set cell phenotype. B CLL determination markers were CD5, CD23.

Zap-70+ group, all have CD 19, expressed on more than 80% cells, excluding 3 patients. There were only 2 patients in ZAP-70- group, CD19 expressed on <20% cells.

The immunophenotypical analysis for B CLL diagnosis determination by phenotype must contain CD23+ and CD5+ lymphocytes. Data obtained

confirms that both ZAP-70- and ZAP-70+ subgroups for patients number found high of both differentially diagnostically markers percent content. Statistically reliable probability for CD5 marker $p < 0.01$; CD23 $p < 0.05$.

9.2.5. CD25 and CD38 Activation Marker Expression in Different CLL Patients Groups According to ZAP-70 Indicator

There is no important diagnostical role for activation marker in diagnosis determination with flow cytometry, but these markers characterizes lymphocyte activation process. CD38 – multifunctional membrane superficial glycoprotein that expresses with different cells, including T and B lymphocyte determined on their development phase. As a transmembranal receptor that regulates proliferation and T and B lymphocyte differentiation. Among other CD38 ligands effects notes kinesis activation and protein phosphorylation. CD-25 is on activated T and B lymphocytes and macrophages, in case of increased lymphproliferative process activation. That why it is important to analyze link between activation markers and ZAP-70+ and ZAP-70- subgroups for CLL patients.

Results obtained shows that in ZAP-70- group for most of patients are both markers with level not less than 20%. CD38 marker in ZAP-70- group is not higher than 20% is not determined. For CD25 marker is common patient's quantity reduction, increasing the markers level. For ZAP-70+ group is common the biggest patients number in both analyzed marker case, if marker level is 70-90% (respectively CD25 – 23 patients and CD38 – 28 patients). For both markers, if parameters level is higher than 90%, also common same

patients quantity – 5. For both markers are almost same patient's quantity division in subgroups with markers level less than 20% and 20-70%.

9.2.6. Correlation between ZAP-70 and LDH

In table 9.4 presented connections between different ZAP-70 levels and LDH level in different observation periods in studied patients group. Data provided in research, which reflects link between LDH level and different ZAP-70 levels. It is known, that LDH level increase may characterize activation of lymphproliferative process.

Table 9.4

Correlation between ZAP-70 and LDH

ZAP-70, %	LDH, U/l at the beginning	LDH, U/l after 6 mon.	LDH, U/l after 12 mon.
ZAP70– <20	382.10 ± 96.14	380.80 ± 88.70	454.60 ± 199.60
ZAP-70+ >20	520.0 ± 220.20	443.40 ± 94.25	477.10 ± 123.30

Data expressed as average ± standart deviation (SD). p = 0.05, comparing LDH at the beginning and LDH after 6 months.

Data presented in table 6 shows, that ZAP-70 level 0-20% correlations between LDH before treatment beginning, also during therapy were not discovered. For subgroup with level more than 20% (ZAP70+) after 6 treatment months discovered LDH parameters reduction, with reliability p = 0.05.

9.2.7. Correlation between ZAP-70 and β_2 MG

Analyzed connection between different ZAP-70 and β_2 MG levels in three different periods: before treatment, after 6 months and after 12 months of therapy.

In table 9.5 presented connections between different ZAP-70 levels and β_2 MG level in different observation periods in studied patients group. In ZAP-70- subgroup with level 10-20 β_2 MG level before treatment and after 6 months therapy reduced with reliable correlation p = 0.05. However, in ZAP-70+ subgroup with level, which is higher than 20%, comparing β_2 MG level between patients before treatment and after 12 treatment months, was reliable p = 0.0074. Other data presented in table does not correlation between each other.

Table 9.5

Correlation between ZAP-70 and β_2 MG

ZAP-70, %	β_2 MG at the beginning	β_2 MG after 6 month	β_2 MG after 12 month
ZAP-70– <20	2.83 ± 3.18	2.32 ± 0.63	2.65 ± 0.88
ZAP-70+ >20	3.09 ± 2.84	2.51 ± 0.76*	2.40 ± 0.80

Data expressed as average ± standard deviation (SD). p = 0.05,

9.2.8. Correlation between ZAP-70 and CD38

However, in this chapter showed connection between ZAP-70 level and different CD38 quantity in different observation periods. In ZAP-70– subgroup with level 0–20% observed CD38 level increase tendency – between initial CD38 level and level after 6 months correlation was p = 0.01. However, to compare CD38 level before treatment start and after 12 months, correlation

is p = 0.006. In ZAP-70+ subgroup with level >20% obtained CD38 level reduction after 6 observation months, but correlation not observed. Comparing CD38 level before treatment and after 12 months, correlation was only p = 0.0001. Data presented in table 9.6.

Table 9.6

Correlation between ZAP-70 and CD38

ZAP-70, %	CD-38 at the beginning	CD-38 after 6 month	CD38 after 12 month
ZAP-70– <20	9.86 ± 13.87	10.87 ± 9.41	15.15 ± 14.40
ZAP-70+ >20	13.83 ± 3.18	12.32 ± 0.63	22.65 ± 0.88

Data expressed as average ± standard deviation (SD).

9.2.9. Correlation between ZAP-70 and Disease Stage

In table 9.7 presented ZAP-70 level data on different control periods in all Rai classification stages. Correlation between ZAP-70 level and disorder stage has not been found.

Table 9.7

Correlation between ZAP-70 and disease Rai stage

Stage	ZAP-70 at the beginning	ZAP-70 after 6 month	ZAP-70 after 12 month
0	4.30 ± 3.46	3.98 ± 2.02	4.30 ± 3.43
I	12.37 ± 12.14	11.79 ± 13.15	10.33 ± 10.36
II	18.98 ± 18.74	19.11 ± 17.18	20.81 ± 19.82
III	17.06 ± 18.36	18.27 ± 17.57	20.31 ± 20.98
IV	28.38 ± 29.55	27.30 ± 27.62	19.67 ± 21.12

Data expressed as average ± standard deviation (SD).

9.2.10. Correlation between ZAP and TK

In table 9.8 reflected data of TK levels and different obtain periods in different ZAP-70 level case. In ZAP-70– level from 0 till 20% case, there is TK level reduce tendency in all control periods, p = 0.02, comparing TK at the beginning and TK after 6 months therapy. p = 0.01, comparing TK at the beginning and TK after 12 months therapy. However, if ZAP-70+ level is higher than 20%, there is no TK level reduce after 6 months tendency obtained, but, comparing TK levels after 6 months and after 12 months.

Table 9.8

Correlation between ZAP-70 and TK

ZAP-70, %	TK at the beginning	TK after 6 month.	TK after 12 month.
ZAP-70– <20	2461 ± 4064	1126 ± 1602	803 ± 1130
ZAP-70+ >20	4098 ± 4676	2746 ± 3270	3883 ± 5146

Data expressed as average ± standard deviation (SD).

Data obtained shows, that before treatment TK level in all ZAP-70 subgroups was high. Superlative analyzed marker parameter observed in ZAP-70+ subgroup with level which is higher than 20%. Control after 6 months shows that in all ZAP-70 subgroup levels TK significantly decreased. Control after 12 months shows, that TK level decrease remains only in subgroup with ZAP-70- (0-20%). In ZAP-70+ subgroup TK level comparing with control before treatment, decreased and increased, comparing with TK level after 6 months.

10. PROGNOSTIC MARKER DYNAMICS DURING THERAPY

10.1. Therapy Variants

All CLL patients, who took part in research, after therapy were divided into two subgroups. Patients group, which have not get any therapy – without therapy (w.th.) and patients group, which got chemotherapy course variants, that includes fludarabine (F, FC), patients group, which got polychemotherapy without fludarabine (COP, CHOP). Huge patients group have been treated with *Chlorambucil* (leukeran) (L). Chemotherapy variant data presented in table 10.1.

Table 10.1

Therapy variants depending on treatment period

Therapy period	**Without therapy Abs. number**	**Leukeran containing therapy Abs. number**	**Fludarabine containing therapy Abs. number**	**Fludarabine-free therapy Abs. number**	**Total patient number Abs. number**
0 month (Beginning of therapy)	16	52	35	17	120
After 6 month	17	50	35	7	109
After 12 month	18	55	16	8	97

Analyzing patient groups, according to treatment periods and basing on previously treatment variants, after first-time visit to doctor and CLL diagnosis approval, patients were divided into subgroups, according to Rai classification, also prescribed therapy. Selected treatment variants in dependence on subgroup presented in table 10.2. Therapy prescribed by

treating physician hematologist. The fact, that patient is included in this research, didn't influence therapy choice.

Table 10.2

Chosen treatment options variant depending on subgroup

Stage subgroup	Without therapy	Leukeran containing therapy	Fludarabine containing therapy	Fludarabine – free therapy	Total patient number
0–I	13	25	7	0	45
II	3	17	15	4	40
III–IV	0	8	13	13	35
Total	16	52	35	17	120

In table 10.3 presented data about treatment variants in subgroups after 6 months.

Table 10.3

Treatment options in subgroups after 6 month

Stage subgroups	Without therapy	Leukeran containing therapy	Fludarabine containing therapy	Fludarabine-free therapy	Total patient number
0–I	13	21	7	0	41
II	3	24	10	1	38
III–IV	1	5	18	6	30
Total	17	50	35	7	109

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After 12 month there was a group of 97 patients. Therapeutical treatment got 79 patients, 17 patients were only observed. Data about therapy otions in subgroups presented in table 10.4.

Table 10.4

Treatment options in subgroups after 12 month

Stage subgroup	Without therapy	Leukeran containing therapy	Fludarabine containing therapy	Fludarabine-free therapy	Total patient number
0–I	12	21	2	1	36
II	6	20	5	1	32
III–IV	0	14	9	6	29
Total	18	55	16	8	97

10.2. Leukocytes Number Changes during the Therapy

Most notably 12 months containing therapy reduced leukocyte number. 6 or 12 months therapy statistically reliably do not effect leukocyte number in blood for chronic lymphoid leucosis patients. For patients after 6 and 12 months fludarabine containing therapy, we observed statistically reliable leukocyte number reduction, comparing with pre-therapy data – $77.18 \times 10^9/l \pm 28.91 \times 10^9/l$ against $5.3 \times 10^9/l \pm 1.40 \times 10^9/l$ and $77.18 \times 10^9/l \pm 28 \times 10^9/l$ against $5.35 \times 10^9/l \pm 1.43 \times 10^9/l$, ($p = 0.035$ and $p = 0.04$).

Furthermore, for patients after 6 months fludarabine-free therapy, we observed leukocyte number reduction tendency, comparing with pre-therapy data – $86.87 \times 10^9/l \pm 35.96 \times 10^9/l$ against $12.1 \times 10^9/l \pm 4.60 \times 10^9/l$, ($p = 0.1$). But after 12 months fludarabine-free therapy, we observed statistically reliable leukocyte number reduction, comparing with pre-therapy data – $86.87 \times 10^9/l \pm 35.96 \times 10^9/l$ against $8.6 \times 10^9/l \pm 4.40 \times 10^9/l$, ($p = 0.04$). Leukocyte number in subgroups without therapy remains without special changes.

10.3. Lymphocytes Number Changes during the Therapy

Results obtained during research shows, that without therapy in subgroup seen small tendency for lymphocyte number to increase without statistical reliability. Results obtained shows, that 12 months leukeran therapy increases lymphocyte number, but 6 months therapy with big statistical reliability reduces lymphocyte number, comparing with pretherapy data (11.60 x 10^9/l ± 3.56 x 10^9/l against 21.40 x 10^9/l ± 2.78 x 10^9/l, p =0.03). 12 months fludarabine containing therapy better reduces lymphocyte number then 6 months therapy, comparing with pre-therapy data (9.40 x 10^9/l ± 1.71 x 10^9/l against 28.0 x10^9/l ± 5.63 x 10^9/l, p =0.01). Similar also fludarabine-free 12 months therapy, but not 6 months therapy, reduces lymphocyte number, comparing with pre-therapy data (3.10 x 10^9/l ± 0.88 x10^9/l against 44.32 x 10^9/l. ± 0.96 x 10^9/l, p = 0.048).

10.4. β_2 mikroglobuline Level Changes during the Therapy

In research we obtained, that in subgroup without therapy β_2 MG level does not increase. But to compare 6 and 12 months leukeran therapy, we obtain, that 12 months therapy increases β_2 MG level, comparing with 6 months therapy (4.28 mg/ml ± 0.7 mg/ml against 2,86 mg/ml ± 0.24 mg/ml, p = 0.045). During research we obtained, that 12 months fludarabine containing therapy reduces β_2 MG level, comparing with pre-therapy data (2.12 mg/ml ± 0.47 mg/ml against 4.80 mg/ml ± 0.53 mg/ml, p = 0.02). Also 6 months fludarabine-free therapy increases β_2 MG level, comparing with pre-therapy data (2.23 mg/ml ± 0.17 mg/ml against 3.55 mg/ml ± 0.59 mg/ml, p = 0.04).

10.5. LDH Level Changes during the Therapy

Results obtained during research shows, that as 6, as 12 months leukeran therapy has tendency to increase LDH level. Fludarabine containing 12 months therapy reduces LDH level, comparing with pre-therapy data (354.0 U/l ± 13.77 U/l against 438.0 U/l ± 18.76 U/l, $p = 0.01$). But after 12 months polychemotherapy without fludarabine containing medicine, LDH level increased, comparing with pre-therapy data (449.0 U/l ± 31.00 U/l against 361.70 U/l ± 10.87 U/l, $p = 0.04$).

10.6. ZAP-70 Level Changes during the Therapy

Results obtained during research a show, that for chronic lymphoid leukemia patients without therapy, also with leukeran, fludarabine containing therapy and fludarabine-free therapy after 6 or 12 months ZAP-70 concentration does not change statistically significantly, comparing with patients at the beginning of observation period.

10.7. CD38 Level Changes during Therapy

Results obtained during research shows, that for patients with chronic lymphoid leukemia, fludarabine containing 12 months therapy reduces CD38 level, comparing with pre-therapy data (16.56% ± 5.47% against 34.5% ± 6.98%, $p = 0.049$). Leukeran and fludarabine-free 12 months therapy has tendency to increase CD38 level. But CD38 level without therapy not change.

10.8. TK Level Changes during the Therapy

Results received during research show that for chronic lymphoid leukemia patients without therapy, 6 and 12 month therapy has liability to reduce TK level. Leukeran therapy, 6 and 12 month therapy has liability to reduce TK level without reliability (3016 ± 7609 to 2001 ± 3997, p = 0.0567 and 3016 ± 7609 to 2124 ±4206, p = 0.0768).

Results received during research show that for chronic lymphoid leukemia patients fludarabine 6 month containing, but with higher reliability 12 month therapy, reduces TK concentration, comparing with pre-therapy data (843.3 ± 249.7 to 4861.0 ± 1815.0, p = 0.049 un 597.0 ± 267.4 to 4861.0 ± 1815.0, p = 0.045).

11. RESEARCH FINDINGS AND DISCUSSION

Several researchers state that chronic lymphoid leukemia is heterogeneous after clinical manifestations and patients' life expectancy, ranging from several months till years. In 2008 F. Caligaris-Cappio in his work described contemporary views not only on CLL pathogenesis but also on different disorder course variants. M. Horwitz conducted CLL family case research and proved that in every next generation disorder develops heavily and affects younger patients. Consequently, disorders heterogeneity depends on many factors. With the change of therapy, which allows to achieve full remission, changes patients monitoring tactics, that is why on earlier disorder stages it is very important to determine which CLL form has a patient. Every individual case allows choosing right monitoring tactics, carrying out individual treatment and control. Both prognostic factors (disorder stage

determination, lymphocytes doubling time, β2 MG, LDG and others) and new prognostic markers – CD38, ZAP-70, and TK help to find a solution to a problem.

Our research has been based on RAKUS, in Chemotherapy and hematology clinics from 2007 till 2009 year. During the research 120 CLL patients, 60 patients (50%) men and 60 (50%) women, were observed. Average men's age is 68.51 ± 8.72, women's – 65.42 ± 1.56. Analyzed patients' group was the same age and formed the same subgroups of men and women. However, all the scholars state that more often men get ill then women. For example, E. Montserrat in 1988 conducted the research where there were 261 patients in a group, 74% of them were men. All the patients in the research were divided according to Rai classification into stages: in 0 stage were 7 patients; I stage – 38 patients; II stage – 40 patients; III stage – 19 patients and in IV stage – 16 patients. There are no described disorder stages in publications for first-time CLL patients, but described patients division after disorder progressing risk Principe. Due to prognosis predicting, it is much better to divide CLL into progressing variants. According to publications data, taking into account progressing risk, as described in 2006. T.Seiler work, patients were divided into 3 groups: early (Rai 0), middle (Rai I, II), progressive (Rai III, IV).

First patients group included 7 people (6%) with benign disorder course. Second patients group included 78 people (65%), disorder had slowly progressing character. Third group included 35 patients (29%), disorder had fast progressing character.

All patients made blood test. In work performed statistical analysis and discovered link between blood test parameters and disorder stage. The dependency is important and that have been described by many authors, because disorder stage allows to choose further patients observation tactics. J.Hus with co-authors provided analysis for 156 CLL patients and proved

laboratory parameter connection with disorder stage temps. Results obtained during our research shows that statistically reliable link exists and there is a positive correlation between leukocytes level increase and stage ($r = +0.33$; $p = 0.0002$), discovered negative correlation between hemoglobin level reduction and disorder stage ($r = –0.36$; $p = 0.0001$), also negative correlation and reliable link between erythrocyte level and disorder stage ($r = –0.38$; $p = 0.0001$), between thrombocyte level and disorder stage there is negative correlation ($r = –0.42$; $p = 0.0001$), between lymphocyte level and disorder stage there is positive correlation and reliable link ($r = +0.32$; $p = 0.0003$). Statistically reliable data obtained between blood parameter indicators and disorder stage consistent with the W. G. Wierda et al. published data. In period from 1981 until 2004 analyzed more than 1500 patients. Group of researches published results that show leukocyte raise, increasing the stage and erythrocyte and thrombocyte level reduction, increasing the stage.

Similarly, in research carried out traceable statistically reliable link between tumor mass size and blood parameters. Traceable statistical reliability and positive correlation between leukocytes level increase and spleen size ($r = +0.19$; $p = 0.049$), between erythrocyte quantity reduce and spleen size determined negative correlation and reliable link ($r = –0.25$, $p = 0.005$), between hemoglobin level reduce and spleen size is negative correlation ($r = –0.30$, $p = 0.0009$), negative correlation and reliable link is also between thrombocytes level reduce and spleen size ($r = –0.32$, $p = 0.0004$), between lymphocyte quantity increase and spleen size is positive correlation and reliable link ($r = +0.20$, $p = 0.03$). Tumor mass dependence from disorder stage, that influence on disorder course and CLL progress speed are described in C. Rozman research in 1995. Data received in our research coincides with data published in C.Rozman article where analyzed patients treatment conception. Tumor mass size is important CLL progress evidence.

Many researches indicate the importance of prognostic marker, such as β_2 MG and LDH. T. Seiler et al. in his work describes this parameter level dependence and disorder stage, but there have been no data found on these parameters connection with tumor mass amount. We conducted a statistical analysis. There have been discovered a reliable link and positive correlation between β_2 MG quantity and disorder stage ($r = +0.23$; $p = 0.009$), between β_2 MG quantity a maximal lymph node diameter is a reliable link and positive correlation ($r = +0.39$; $p = 0.0035$) and between β2 MG quantity and spleen size is reliable link and positive correlation ($r = +0.17$; $p = 0.05$).

Similar tendency has been used for analyzing LDH. We established reliable, positive correlation and significant connection between LDH level and disorder stage ($r = +0.25$; $p = 0.006$), statistically reliable, positive correlation and significant link between LDH level and spleen size ($r = +0.22$; $p = 0.014$) and correlation tendency and a reliable link between lymph node diameter and LDH ($r = +0.27$; $p = 0.056$).

According to A. Krober (2006) and M. Crespo (2003) mutative status or that surrogate marker ZAP-70 is very important since that allows to distinguish patients subgroups with different prognosis, especially within the early stage. ZAP-70 task is to activate thyrozine kinase and phosphorilate intracellular CD3 complex protein. T lymphocytes activation happens with T cell receptor connection with antigen containing cell intermediation (macrophages, dendrite cells and B lymphocytes). This combination phosphorilates transmembrane protein and activating T lymphocyte. It turns out that one of the most important gens, that distinguish chronic B cell lymphoid leukemia variants with different mutation status, is the gene which coordinates proteinkinase ZAP-70, that sends signals which come from T cell receptor. This gene expresses in that chronic B cell lymphoid leucosis' variant, which has no mutation in variable domen and not expressing in variant with mutations.

A. A. Wiestner et a1. (2003) in a group of 107 chronic lymphoid leucosis' patients, proved that ZAP-70 is a gene, which is the best to divide chronic lymphoid leukemia subtypes after mutative status: for patients without VH genes mutations, ZAP-70 gene expresses 5,54 times stronger than for patients with VH gene mutations. In this research for 93% of patients ZAP-70 expression correctly predicted mutative status. 120 first-time patients group was analyzed before treatment and in dynamics.

In 2006 A. C. Bakke described ZAP-70 determination method and noted that this parameter is stable. There have not been found statistically reliable difference in ZAP-70 parameters after 6 and 12 months therapy. First group included 87 patients, stages were determined according to Rai classification – 56 male patients and 31 female patients on different disorder stages. ZAP-70 level control was performed before treatment and after treatment course.

In our work, we have observed the tendency of positive correlation between stage and ZAP-70 ($r = +0.18$, $p = 0.05$). However, there are no data in publications on ZAP-70 level dependency from disorder course, but described ZAP-70- and ZAP-70+ subgroup dependency from other factors. In 2008 this subgroup analysis was made by M. Ertault-Daneshpouy et al. In this work are described two subgroups of patients in A stage after Binet classification, patients' gender was taken into account, age and CD38 level. ZAP-70- included 40 patients, ZAP-70+ - 54 patients. Previously mentioned scholars' works was not found the connection between patients' gender in different ZAP-70 subgroups.

In our research patients' group was divided into subgroups, one subgroup was of 61 ZAP-70+ patients, another subgroup was of 59 ZAP-70- patients. 4 patients or 11% of patients from the group with progressing course had ZAP-70+ expression. Patient number in benign group after ZAP-70+ marker was 5 patients or 72% of total patients' number selection. 52 patients or

67% of slowly progressing variant group characterized by ZAP-70+ expression and in ZAP-70– subgroup are 26 patients or 33%. Analyzing CLL traditional prognostic parameters, such as gender, lymphocytes absolute quantity and lymphocyte doubling time, we have concluded, that in ZAP-70– subgroup lymphocyte absolute quantity is $32.6 \pm 0.03 \times 10^9$/l, $p < 0.05$; lymphocyte doubling time is > for 12 months, $p < 0.05$. In ZAP-70+ subgroup lymphocytes absolute quantity is $69.6 \pm 0.32 \times 10^9$/l, $p < 0.05$; lymphocytes doubling period is 9.1 ± 1.1 months, $p < 0.05$. At the same time there was no correlation discovered between patients' gender and subgroups ZAP-70– and ZAP-70+, $p > 0.05$.

It is known that for CLL determination with the help of immunophenotypical analysis, phenotype must contain CD23+ and CD5+ lymphocytes. Data obtained, demonstrated that in both ZAP-70- and ZAP-70+ subgroups for patients basic number have been found high percentage content of differential diagnostic markers. Statistically significant probability of CD5 marker $p < 0.01$; CD23 $p < 0.05$. Data obtained coincides with analyzed results in M. Ertault-Daneshpouy et al. 2008 research. The author points out that these markers determination may be used as CLL diagnostics routine method.

Results obtained during the research shows that for patients with chronic lymphoid leukemia without therapy, also with leukeran, fludarabine containing therapy and fludarabine-free therapy after 6 or 12 months, ZAP-70 concentration statistically has no significant change, comparing with patient beginning of the observation period. Considering stability of the indicator, that may be used for CLL variant determination at the beginning of disorder. Several authors note that ZAP-70 level during disorder course period does not change. Among those authors is *F. Van Bockstael* who in 2009 with co-authors carried out a prognostic marker research, which is used for CLL patients on earlier stage. Our research has reached the same conclusions. There is author's opinion, for example M. I. Del Principe, that ZAP-70 level reduces treating

with corticosteroids. On a way to this conclusion, researchers group made observations for 3 years period. In our research corticosteroids were used in subgroups with fludarabine containing therapy and fludarabine-free therapy, but ZAP-70 level remained unchanged. Observing stability of this indicator that might be used for CLL diagnostics together with other parameters.

One more of prognostic parameter is CD38. After C. Chang (2003) information, CD38 has quality under certain conditions, stimulate or prevent apoptosis in human lymphocytes. From other ligand CD38 effects, notes kinase activation and protein phosphorylation. In this work CD38 level dynamical changes for CLL patients are analyzed.

In our work all patients were divided into ZAP-70- and ZAP-70+ subgroups. In these groups CD38 level analysis was made. In ZAP-70– subgroup for all patients CD38 level is lower for about 20%, p=0.05, at the same time in ZAP-70+ subgroup the greater patients number with CD38 level 70-90% (28 patients). Comparing CD38 level dynamics in subgroup with ZAP-70- level till 20%, observed CD38 expressed incensement (comparing CD38 at the beginning and CD38 after 12 months therapy 9.86 ± 13.87 and 15.15 ± 14.40, $p = 0.006$; 10.87 ± 9.41 and 15.15 ± 14.40, $p = 0.01$, comparing CD38 after 6 and 12 months therapy; 13.83 ± 3.18 and 22.65 ± 0.88, $p = 0.0001$, comparing CD38 and CD38 after 12 months therapy) comparing with ZAP-70+ subgroup.

M. I. Del Principe in 2006 carried out these parameter connection analysis and came to a conclusion: if in ZAP-70+ group is high CD38, for these patients prognosis is worse, and vice versa, if in ZAP-70- subgroup is low CD38 level, for these patients prognosis is good. In 2002 T. J. Hamblin discovered a strong correlation between these 2 parameters and suggested to use it as standard in CLL diagnostics. In our research analyzed connection between CD38 level and disorder stage.

There was not found statistically significant correlation between CD38 and disorder stage during research, which coincides with M. Montillo et al. (2005) opinion, even though author analyzes treatment results, noting dependency between CD38 level and disorder stage, in his work. At the same time, there are is no data in scientific publications about dependency between CD38 level, spleen size and lymph nodes size. In this work have been researched CD38 level connection with the above parameters, but statistically significant correlation between CD38, spleen size and lymph node diameter was not found.

Analyzing treatment results and changes of CD38 level, following data observed: six months chemotherapy increases CD38 number for chronic lymphoid leucosis' patients, comparing with pre-therapy data – 16.01% ± 1.474% against 13.66% ± 1.526 % ($p = 0.01$). But 12 months therapy with greater statistical reliability increases CD38% quantity (21.99% ± 2.008% against 13.66% ± 1.526%, $p = 0.0001$) and comparing with pre-therapy data and with 6 months therapy results 21.99% ± 2.008% against 16.01% ± 1.474%, ($p=0.03$). In P. Chevallier et al. (2002) and U. Thrumberg (2001) opinion, CD38 level increase associates with disorder progress. Our data coincided with the author's data from the above.

L. Z. Rassenti in 2008 with co-authors, analyzing first-time patients CD38 level changes in different Binet stages, discovered aggressive subgroups with high level of CD38.

Looking through the separate types of made therapy, following results obtained: for patients with chronic lymphoid leukemia fludarabine containing 12 months therapy reduces the CD38 level, comparing with pre-therapy – 16.56% ± 5.47% against 34.5% ± 6.98%, ($p = 0.049$). Leukeran and fludarabine free 12 months therapy tends to increase CD38 level. Meanwhile, without therapy CD38 level does not change in subgroup. I. Del Giudice et al. in 2005 determined ZAP-70 and CD38 level in 201 non-treated patients group. Re-

analysis was conducted after 26 months. Author notes that in case of high CD38 level, especially in earlier CLL stages, the therapy must be started immediately.

In our research TK analyze made for 120 primary CLL patients, whose average age is 66.87 ± 1.007. In group were 60 women and 60 men. There is no consensus about demographic link with CLL course severity, and prognostic factor expression for women and men. Meanwhile, research shows data: differences between patients groups in 0 stage (2718 ± 4008 ng/l), in I, II stage (3712 ± 6798 ng/l) and in III, IV stage (4259 ± 5230) are reliable ($p = 0.05$). If there were not found statistically important differences in TK content, considering patients gender (men 2025 ± 3714 ng/l; women 2574 ± 3598 ng/l), then in I, II stage (men 4567 ± 6471ng/l, women 2805 ± 4276 ng/l) and in III, IV stage (men 9559 ± 1323 ng/l, women 3930 ± 4369 ng/l) observed this marker double increase for men, comparing to women. There are no scientific works where TK level might be analyzed depending on patients gender. E. Montserrat (1988) notes that CLL most commonly found for men. It is characterized by more severe course and existence of unfavorable prognostic factors.

There are researches, showing that TK level reflects total tumor mass. T.Seiler with co-authors (2006) in 3 year period explored process activities for CLL patients in different disorder stages according to Rai classification. Group of authors came to a conclusion, that tumor mass growth with disorder progressing, researchers associate with dividing cells in greater quantities, respectively, TK level increase in blood serum. Consequently, TK output growth provides a significant proliferative advantage in tumor cells.

In our work, patients were analyzed in accordance with Rai classification before starting treatment. Discovered reliable statistical connection between TK and Rai stage. In our research, in period of patients examination, made TK level analysis for 120 patients, connection and positive

correlation between TK level and Rai stage found (r = + 0.36; p = 0.0001). In our research discovered the connection between TK level and tumor mass size. During patient examination period there was discovered the connection between TK level and spleen size (r = + 0.23; p = 0.015) and between TK level and lymph node diameter (r = +0.30; p = 0.03).

The role of TK in leucosis process programming has been mentioned in many works. After M.Hallek (1999) opinion, TK level determination can be used as independent prognostic factor soaring and quickly progressing CLL variant determination. C. Magnac (2000) analyzed two prognostic factors and came to a conclusion that TK level can provide information about mutative status. In practice, mutative status determination is long and expensive process, but nowdays suggested ZAP-70 determination method is easier. Both parameters in dynamics were analyzed during the research. Data shows, that in ZAP-70- subgroup is statistical reliability (p = 0.01), comparing TK at the beginning and TK after 12 months, in ZAP-70+ subgroup connection between TK level in dynamics is not seen. There is no data about analysis between TK level in ZAP-70+ and ZAP-70- subgroups from other scientific publications.

According to M.Hallek (1999) chemotherapy slows tumor clone cell active division process and reduces TK level. Similar results have been obtained in our research. TK level analysis in dynamics showed studied marker reduction. Results received during research shows that 6 months chemotherapy reduces TK level for chronic lymphoid leukemia patients, comparing with pre-therapy data – 1557.0 ± 213.70 ng/l against 2821.0 ± 374.70 ng/l (p = 0.0001). 12 months therapy significantly reduces TK level – 1775.0 ± 312.40 ng/l against 2821.0 ± 374.70 ng/l (p = 0.02).

Received results shows that TK level after 12 months increases, comparing with 6 months control. Using a variety of treatment variants and assessing TK level changes, the received results show that patients with chronic lymphoid leukemia without therapy, with leukeran therapy, fludarabine

containing 6 and 12 months therapy tend to reduce the concentration of TK, comparing with pre-therapy data.

However, 6 month fludarabine containing therapy and 12 months therapy, significantly reduces the concentration of TK , comparing with pre-therapy data, respectively, 843.3 ± 249.7 ng/l against 4861.0 ± 1815.0 ng/l ($p = 0.049$) and 597.0 ± 267.4 ng/l against 4861.0 ± 1815.0 ng/l ($p = 0.045$).

Fludarabine- free 6 and 12 months therapy tends to increase TK concentration, comparing with pre-therapy data. *B. Simonsson* (1985) was one of the first who described TK level changes, andaccording to *M. Hallek* (1996) TK level changes may contain important information in evaluating the results of treatment. The author drew to this conclusion after analyzing 113 patients with different disorder stages.

12. CONCLUSIONS

1. To determine malignant tumor spread, ZAP-70 level may be used at the chronic lymphoid leukemia diagnosis moment, because ZAP-70 correlates with stage ($p = 0\ 05$) and for ZAP-70– positive patients with greater differentiation marker CD19 and CD20 ($p = 0.05$), CD5 ($p = 0.01$), CD23 ($p = 0.05$), CD25 ($p = 0.05$) and CD38 ($p = 0.05$) expression degree and ZAP-70 – negative patients with smaller number absolute lymphocyte number ($p = 0.05$).

2. For ZAP-70– negative patients, ZAP-70 may be used as prognostic marker to begin therapy, because in this group lymphocyte doubling period is longer ($p = 0.05$), but cannot be used to control the dynamics during the therapy.

3. Thymidine kinase may be used at the chronic lymphoid leucosis diagnosis moment to determine malignant tumor spread, where the higher TK

level, the greater disorder stage ($p = 0.0001$), the larger the spleen USI size ($p = 0.015$), the bigger the maximal lymph node diameter ($p = 0.02$).

4. Thymidine kinase control in dynamics may be used for chronic lymphoid leukemia therapy monitoring for ZAP-70– negative patients after 6 months ($p < 0.02$) and 12 months ($p = 0.01$) therapy, and patients who get chemotherapy with fludarabine according to the following schemes – both in a 6-month-period ($p = 0.049$) and in a 12-month-period ($p = 0.045$) therapy.

5. The percentage of CD38 at the chronic lymphoid leukemia diagnosis moment can be used for malignant tumor spread determination, because CD38% level correlates with a disorder stage, it increases CD38% composition.

6. The percentage of CD38 can be used for therapy control, because it increases both in a 6-month-period ($p = 0.03$) in a 12-month-period ($p = 0.0001$) therapy, particularly, it is expressed in case of therapy with fludarabine containing schemes.

7. At the moment of chronic lymphoid leukemia determination, LDH differentiation can be used for malignant tumor spread determination, because LDH level is higher on later stages of disorder ($p = 0.006$), increased spleen USI size ($p=0.014$), and in increased maximal lymph node diameter case ($p = 0.056$).

8. LDH dynamic cannot be used as a control indicator during chronic lymphoid leukemia therapy.

9. β_2 mikroglobuline level at chronic lymphoid leukemia diagnostic moment, can be used for malignant tumor spread determination, because β_2 MG is higher in case of later stages of disorder ($p = 0.009$), increased spleen USI size ($p = 0.05$) and increased maximal lymph node diameter case ($p = 0.0035$).

10. Chronic leukemia patients who were treated with fludarabine containing schemes β2 MG may be used for efficiency control, because this

parameter strongly decreases in a 12-month treatment (p= 0.02) – both in ZAP-70-positive and in ZAP-70-negative subgroups.

13. PRACTICAL RECOMMENDATIONS

1. At chronic lymphoid leukemia diagnostic moment should be determined ZAP-70, TK, CD 38, LDH and β_2 MG, because these indicators are correlated with tumor spread and stage.

2. For chronic lymphoid leukemia therapy control in dynamics may be used TK and β_2 MG reduction, CD38% increase, also for ZAP-70 negative patients, fludarabine containing therapy control dynamics.

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15. PUBLICATIONS RELATED ON TOPIC

Publications:

1. **Rivkina A.**, Vitols G., Murovska M., Lejniece S. Identifying the stage of new CLL patients using TK, ZAP-70, CD38 levels. Exp Oncol. 2011 Jun; 33 (2): 99-103. PMID 21716207.

2. Vidmane-Ozola I., Boka V., Tsunskis E., Leynietse S., **Rivkina A**., Kalnihnsh I. Experience of laparascopy application in the treatment of the patients, suffering hematological diseases in Latvia. Клінічна хірургія. 2012. № 3 (29): УДК 616. 15: 616—072.1—089.819.

3. **Rivkina A.**, Lejniece S., Murovska M., Udre I. ZAP-70, CD38 and Beta-2-microglobulin in CLL patients, first diagnosed in 2007. Riga Stradins University, 2008 publications of medical research papers, Riga, Latvia, 2008; 83-87.

4. **Rivkina A.**, Lejniece S., Murovska M., Vitols G., Udre I. Levels of Thymidin Kinase in blood of primery patients with Chronic Lymphocytic Leukaemia diagnosed in 2007. Riga Stradins University, 2008 publications of medical research papers, Riga, Latvia, 2008; 80-82.

5. **Rivkina A.**, Murovska M., Vitols G., Lejniece S. The Thymidine Kinase's Level Correlation with Different Clinical Parametrs of Chronic Lymphocytic Leukemia Patients.Riga Stradins University, 2010 publications of medical research papers, Riga, Latvia, 2010; 29-34.

6. Holodnuka I., Kozireva S., **Rivkina A.**, Lejniece S., Murovska M., Imreh S. Transcription of B-cell Surfece Chemokine Receptors CCR1 and CCR2 in EBV-negative Malignant and EBV-positive Non-malignant B-cell Lines.Riga Stradins University, 2010 publications of medical research papers, Riga, Latvia, 2010; 122-127.

7. Spaks A., Birkenfelde R., Spaka I., **Rivkina A.**, Upmane M., Sasoveca I., Lejniece S., Kalnina V., Holodnuka I. Presence of HHV-6A, HHV-6B and EBV DNA in pereferal blood of primary Chronic lymphocytic leukemia patients and its influence on B-cell subpopulation profile. Riga Stradins University, August Kirchenstein institute of microbiology and virology, workshop. Immunomodulating human herpesviruses and their role in human patologies, 2011 publications of medical research papers, Riga, Latvia, October 13-14, 2011; 30-35.

Patent:

„Hroniskas limfoleikozes variantu prognozēšanas paņēmiens" **Alla Rivkina** (LV), Sandra Lejniece (LV), Aivars Lejnieks (LV). Patenta Nr. 14164, spēkā no 15.02.2010.

Reports on the Results:

1. **Rivkina A.** Diferenciācijas klasteru izmaiņas hroniskas limfoleikozes ārstēšanas gaitā. Latvijas Hematologu asociācijas sēde, Rīga, Latvija, 2008. gada 31. oktobris.

2. **Rivkina A.** ZAP-70 un CD38 – prognostiskie rādītāji hroniskas limfoleikozes gadījumā. Latvijas Hematologu asociācijas sēde, Rīga, Latvija, 2010. gada 26. februāris.

3. **Rivkina A.** Diagnostika ar plūsmas citometrijas palīdzību B šūnu limfoproliferācijas gadījumā. Latvijas Hematologu asociācijas sēde, Rīga, Latvija, 2011. gada 28. oktobris.

Presentations at International Scientific Conferences in an Oral Presentation:

Rivkina A. Prognostic factors for CLL. 6th Baltic States Haematologist's Congres, Vilnius, Lithuania, May 8-10, 2008.

Speaking Latvian-scale Conferences with an Oral Presentation:

1. **Rivkina A.**, Lejniece S., Ūdre I., Vītols G., Murovska M. Timidīnkināzes koncentrācijas izmaiņas hroniskas limfoleikozes ārstēšanas

gaitā. Rīgas Stradiņa universitāte, 2011. gada zinātniskā konference, Rīga, Latvija, 2011. gada 14.–15. aprīlī; 278.

2. **Rivkina A.**, Murovska M., Ūdre I., Lejniece S. ZAP-70 kā nemainīgs prognostiskais rādītājs hroniskas limfoleikozes gadījumā. Rīgas Stradiņa universitāte, 2011. gada zinātniskā konference, Rīga, Latvija, 2011. gada 14.–15. aprīlī; 279.

Presentations at Scientific Conferences with Posters:

1. Kholodnyuk I., Kalnina V., Kozereva S., Piskura I., **Rivkina A.**, Lejniece S., Murovska M., Imrech S., Kashuba E. Expression of chemokine receptors CCR1 and CCR2 in B-cell lymphoma cell lines and on CD10-positive B-cells in peripheral blood of patients with B-cell lymphoproliferative disorders. 16th Congress of the European hematology association London, United Kindom, June 9-12. 2011; 0360.

2. **Rivkina A.**, Murovska M., Vitols G., Lejniece S. Dynamic change of serum Thymidin kinase levels in patients with CLL. 15th Congress of the European hematology association Spain, Barselona, June 10-13. 2010; 1275.

3. **Rivkina A.**, Vītols G., Murovska M., Lejniece S. Determination levels of TK, ZAP 70, CD38 in new cases of CLL. Type I hematology tutorial diagnostic work-up Focus on Acute Malignancies. Cascais, Portugal, November 5–7, 2010; 34.

4. **Rivkina A.**, Murovska M., Vitols G., Lejniece S. Level of timidinkinase in blood of primary patients with chronic lymphocytic leukemia.6th Baltic States Haematologist's Congres, Vilnius, Lithuania, May 8-10, 2008; 26.

5. **Rivkina A.**, Murovska M., Vitols G., Lejniece S. Levels of thymidin kinase in blood of primary patients with chronic lymphocytic leukaemia diagnosed in 2007. International conference Chronic lymphocytic leukaemia, Barcelona, Spain, 7-9 November, 2008; poster 11.

6. **Rivkina A.**, Lejniece S., Murovska M., Vītols G., Ūdre I. Timidīnkināzes daudzums asinīs pirmreizējiem hroniskas limfoleikozes slimniekiem. Rīgas Stradiņa universitāte, 2008. gada zinātniskā konference, Rīga, Latvija, 2008. gada, 13.–14. martā; 180.

7. **Rivkina A.**, Murovska M., Ūdre I., Lejniece S. ZAP-70, CD38, B-2 mikroglobulīns kā prognostiskie marķieri pirmreizējiem hroniskas limfoleikozes slimniekiem. Rīgas Stradiņa universitāte, 2008. gada Zinātniskā konference, Rīga, Latvija, 2008. gada, 13.–14. martā; 173.

8. Kholodnyuk I., Kozireva S., **Rivkina A.**, Lejniece S., Murovska M., Imreh S. Transcription of C-CLL, surface chemokine receptors CCR1 and CCR2 in EBV-positive non-malignant B-CLL lines. Riga Stradins University, 2010 publications of medical research papers, Riga, Latvia, 2010; 122-127.

9. **Rivkina A.**, Murovska M., Ūdre I., Vītols G., Lejniece S. Timidīnkināzes daudzuma izmaiņas dinamikā hroniskas limfoleikozes slimniekiem. Rīgas Stradiņa universitāte 9. zinātniskā konference, Rīga, Latvija, 2010. gada 18.–19. martā; 285.

10. Holodnuka I., Kozireva S., **Rivkina A.**, Lejniece S., Murovska M., Imreh S. Transcription of C-CLL, surface chemokine receptors CCR1 and CCR2 in EBV-negative B-CLL lines. Rīgas Stradiņa universitāte 9. zinātniskā konference, Riga, Latvija, 2010.gada 18.–19. martā; 288.

11. Piskura I., **Rivkina A.**, Spaks A., Birkenfelde R., Lejniece S., Murovska M., Saulite V., Holodnuka I. Expression of cell surface chemokine receptors CCR1 and CCR2 in B-cell sub-populations of chronic lymphocytic leukemia patients. Riga Stradins University, 2011 publications of medical research papers, Riga, Latvia, 2011; 317.

12. **Rivkina A.**, Lejniece S., Murovska M., Vītols G., Ūdre I. Timidīnkināzes daudzums asinīs pirmreizējiem hroniskas limfoleikozes slimniekiem. Rīgas Stradiņa universitāte, 2008. gada zinātniskā konference, Rīga, Latvija, 2008. gada, 13.– 14. martā; 180.