

# Predictive significance of the cut-off value of CD20 expression in patients with B-cell lymphoma

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**Abstract.** The introduction of the anti-CD20 monoclonal antibody, rituximab, into the treatment of patients with B-cell lymphomas has improved the overall response rate, as well as the response duration and the overall survival of these patients. However, only a few studies have addressed the question of whether higher CD20 expression parallels with better treatment outcomes. The aim of this study was to assess the relationship between the level of CD20 expression and overall survival (OS), disease-free survival (DFS) along with the overall response rate (ORR) in B-cell lymphoma patients. The ultimate objective of the study was to determine the cut-off value of CD20 expression together with the predictive significance of better outcome of rituximab treatment. One hundred and fourteen patients with different histological types of B-cell lymphomas treated with rituximab and chemotherapy between 2003 and 2007 were enrolled in the study. All patients had CD20 expression assessed prior to the beginning of treatment. The level of CD20 expression was determined by quantitative flow cytometric measurements, while the OS and DFS were evaluated by means of Kaplan-Meier survival curves. The cut-off value of CD20 expression, which predicts a better response to rituximab in patients with B-cell lymphomas, was determined at 25.000 molecules of equivalent soluble fluorochrome (MESF). Our data show that patients who achieved complete response after rituximab therapy had a significantly higher expression of the CD20 antigen ( $p=0.018$ ) than those whose disease only stabilized after rituximab therapy. No significant difference was observed in the response duration between the patients with CD20 antigen expressed above the cut-off value and those expressing CD20 antigen below the cut-off value [hazard ratio (HR),

0.5667; 95%CI, 0.124 to 3.18,  $p=0.57$ ]. Even though we have proved that patients with a CD20 expression level above the cut-off value treated with rituximab had a significantly longer OS [hazard ratio (HR), 0.4573; 95%CI, 0.1364 to 0.9461,  $p=0.0383$ ] than patients with a CD20 expression level below the cut-off value. Among our study population, 17.5% had a CD20 expression level below the cut-off value. The highest percentage (80%) of the patients with a CD20 expression level below the cut-off value belonged to the group of chronic lymphocytic leukemia (CLL) patients, while the lowest (6.7%) was observed in the follicular lymphoma (FL) patient group. These data indicate that a higher level of CD20 expression correlates with an improved OS in patients treated with rituximab. The cut-off limit of CD20 expression suggested to have the predictive significance of better outcome was in our series set at 25.000 MESF. This cut-off value should be considered when the decision regarding treatment with rituximab is taken. However, these results warrant further studies on larger groups of patients.

## Introduction

B-cell lymphomas are diseases characterized by proliferation of lymphoid tissue and occasionally by the presence of abnormal lymphoid elements in the peripheral blood (<http://medical-dictionary.thefreedictionary.com/lymphoproliferative>). The incidence of these malignancies in Slovenia has been increasing over the past ten years by ~3% per year (1).

Over the last two decades, there has been a significant increase in management options of these patients, consisting of observation in case of indolent lymphomas, various chemotherapies (alkylating agent-based, fludarabine-based therapies, anthracycline-based), hematopoietic stem-cell transplantation and biologic therapies among which also therapy that targets the CD20 antigen, such as rituximab.

Rituximab is a chimeric murine/human monoclonal antibody directed against the surface antigen CD20 expressed on normal and >90% of lymphoma malignant B cells. It induces lysis and apoptosis of CD20-positive B cells and also sensitizes malignant B cells to the cytotoxic effect of chemotherapy (2,3).

The wide application of rituximab in the treatment of patients with B-cell lymphomas has improved the overall response rate (ORR), as well as the response duration and the

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*Key words:* B-cell lymphoma, rituximab, CD20 expression, cut-off value, overall survival, disease-free survival, response quality

Table I. The distribution of patients receiving rituximab according to the histological type of lymphoma.

Histological type	Number of patients	% of all patients
DLBCL	42	36.8
FL	30	26.3
CLL	5	4.4
MCL	20	17.5
MZL	2	1.8
Unclassified	15	13.2
Total	114	100.0

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; Unclassified, unclassified B-cell lymphoma.

overall survival (OS) of these patients (2,4). It has also been observed that patients with some histological types of B-cell lymphomas respond better to rituximab than the others. Although there is a substantial difference between these histological types of lymphoma in the CD20 expression level (5-7), the correlation between the level of CD20 expression and survival of these patients still remains unknown and warrants further studies.

Our study therefore assessed the relationship between the level of CD20 expression and response quality, disease-free survival (DFS) along with OS in B-cell lymphoma patients treated with rituximab. Another objective of the study was to determine the cut-off value of CD20 expression with predictive significance of better outcome to rituximab treatment.

## Patients and methods

**Study population.** One hundred and fourteen patients with different histological types of B-cell lymphomas treated with rituximab containing therapy between 2003 and 2007 were included in the study. All of these patients had the level of CD20 expression assessed prior to the introduction of treatment with quantitative flow cytometric measurements. The majority of patients had diffuse large B-cell lymphoma (DLBCL; 42 patients; 36.8%) and follicular lymphoma (FL; 30 patients; 26.3%). The distribution of patients receiving rituximab according to the histological type of lymphoma is given in Table I. Research was approved by the National Ethics Committee.

**Evaluation of outcome.** The treatment response data were retrospectively noted from patient records for 114 B-cell lymphoma patients. The quality of response was assessed by Cheson's criteria (8).

In the next step, the OS data for 114 B-cell lymphoma patients together with the DFS data for 87 B-cell lymphoma patients who achieved complete (CR) or partial response (PR) after treatment were collected.

Table II. The distribution of patients receiving rituximab according to the quality of response to treatment.

Response quality	Number of patients	% of all patients
CR	70	61.4
PR	17	14.9
SD	6	5.3
PD	19	16.7
Unspecified	2	1.8
ORR	87	76.3
Total	114	100.0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, overall response rate.

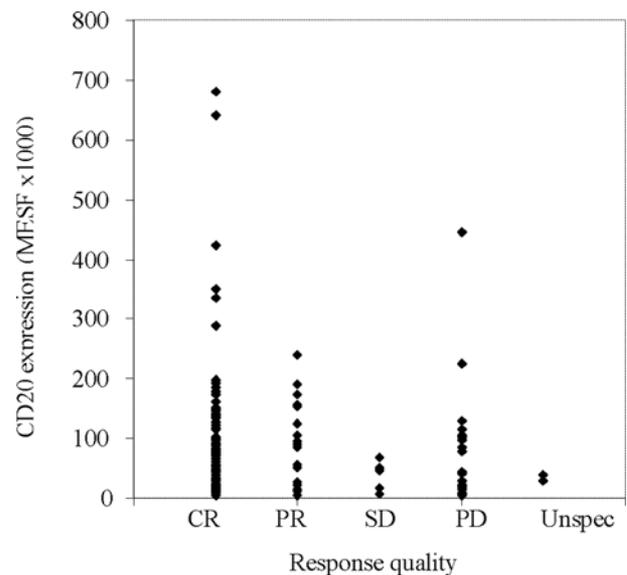


Figure 1. Distribution of the CD20 expression levels according to the response quality. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; Unspec, unspecified response.

The OS was assessed by Kaplan-Meier survival curves from the beginning of the treatment for all 114 patients. The response duration expressed as the DFS was evaluated by Kaplan-Meier survival curves from the end of the treatment for the 87 patients attaining CR or PR after rituximab containing treatment.

**CD20 expression analysis by flow cytometry and quantification of CD20 expression.** For flow cytometric immunophenotyping, samples of lymph node fine needle aspiration biopsies (FNAB), effusions, cerebrospinal fluids, bronchoalveolar lavages (BAL) and samples of peripheral blood were prepared according to the protocol adopted for cytological samples at the Institute of Oncology, Ljubljana, Slovenia (Kloboves Prevodnik V, *et al.*: XXII. Congress of International Society for Analytical Cytology, P122348, 2004). Standard antibody panels usually



	Response				
	CR	PR	SD	PD	Unspec.
Comparable response					
CR		NS	p=0.018 <sup>a</sup>	p=0.063 <sup>c</sup>	NS
PR	NS		p=0.066 <sup>c</sup>	NS	NS
SD	p=0.018 <sup>b</sup>	p=0.066 <sup>c</sup>		NS	NS
PD	p=0.063 <sup>c</sup>	NS	NS		NS
Unspec.	NS	NS	NS	NS	

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; Unspec., unspecified response; NS, no statistical significance between compared response groups. <sup>a</sup>Significantly lower CD20 expression than in compared response group; <sup>b</sup>significantly higher CD20 expression than in compared response group; <sup>c</sup>difference of borderline significance between compared response groups.

applied in the diagnostic of lymphomas were used in the study. For the analysis of CD20 expression, the anti-CD20 antibodies conjugated with allophycocyanin (APC) were utilized (BD Bioscience). All samples were measured with the four-color flow cytometer FACSCalibur (BD Bioscience) and analyzed by means of the CellQuest software (BD Bioscience).

For the quantification of the CD20 expression, the Sphero Rainbow Calibration beads (Spherotech, IL, USA) were employed. These beads have been daily utilized in our laboratory for the monitoring of performance status of the flow cytometer since 2001. The Sphero Rainbow Calibration beads and the samples have always been measured applying equal flow cytometric settings which allows for the use of these beads in the analysis for the quantification of CD20 expression.

The PMT Linearity QC Record software (Spherotech) was utilized to calculate the absolute units of MESF. Firstly, the daily results of the relative channel number of calibration beads were used for the construction of the sphero calibration graph for the APC channel. Secondly, the geometric mean of fluorescence intensity of CD20 was used to calculate the MESF and therefore the quantity of the CD20 expression (9).

**Statistical analysis.** Since the measurement variables did not meet the normality assumption of an ANOVA we used the non-parametric Kruskal-Wallis test while comparing the expression of CD20 antigen in groups of patients with different response to treatment. The Spearman's non-parametric rank statistic as a measure of the strength of the association between two variables was used.

The difference between the survival curves was assessed by log-rank test and the difference between the overall response rates by Chi-square test.

## Results

Our evaluation began with the determination of response quality to rituximab containing treatment in all 114 B-cell lymphoma patients. The distribution of patients receiving rituximab according to the response quality is given in Table II.

The ORR to treatment with rituximab and chemotherapy regardless of the histological type of lymphoma or of the line of treatment was 76.3% (61.4% CR, 14.9% PR).

We continued with the assessment of the relationship between the level of CD20 expression and the response quality. The distribution of the CD20 expression levels according to the response quality are shown in Fig. 1.

Among 114 patients, the group of patients with complete response to rituximab treatment had the highest CD20 expression while the group of patients with an unspecified response to rituximab had the lowest CD20 expression.

According to the confirmed significant difference between the highest and the lowest expression of the CD20 antigen (p=0.051) between various responders, we assessed where among different pairs of response quality groups the difference existed. Significant difference in the CD20 expression between different response quality groups is shown in Table III.

Our data confirm that patients who achieved complete response after rituximab containing therapy had a significantly higher expression of CD20 antigen (p=0.018) than those whose disease was only stabilized. The differences in CD20 expression between complete responders and patients with progressive disease (p=0.063) and between partial responders and patients with stable disease (p=0.066) were borderline.

In advance, we wanted to determine the cut-off value of CD20 expression with predictive significance of better outcome in patients with B-cell lymphomas following rituximab treatment. We applied the OS data to determine the cut-off value. With this intent, we compared different pairs of survival curves obtained through arbitrary set cut-off values in declines of 5000 MESF and assessed where the survival curves significantly diverged. All of 114 patients treated with rituximab and chemotherapy were included in the assessment. The cut-off level of 25.000 MESF was in this manner found to discriminate patients with significantly different overall survival. We also estimated whether the determined cut-off value predicts better response to rituximab treatment and longer DFS.

Regarding the cut-off value of 25.000 MESF, we determined that only 55% of patients with a CD20 expression level below the cut-off value responded to rituximab therapy

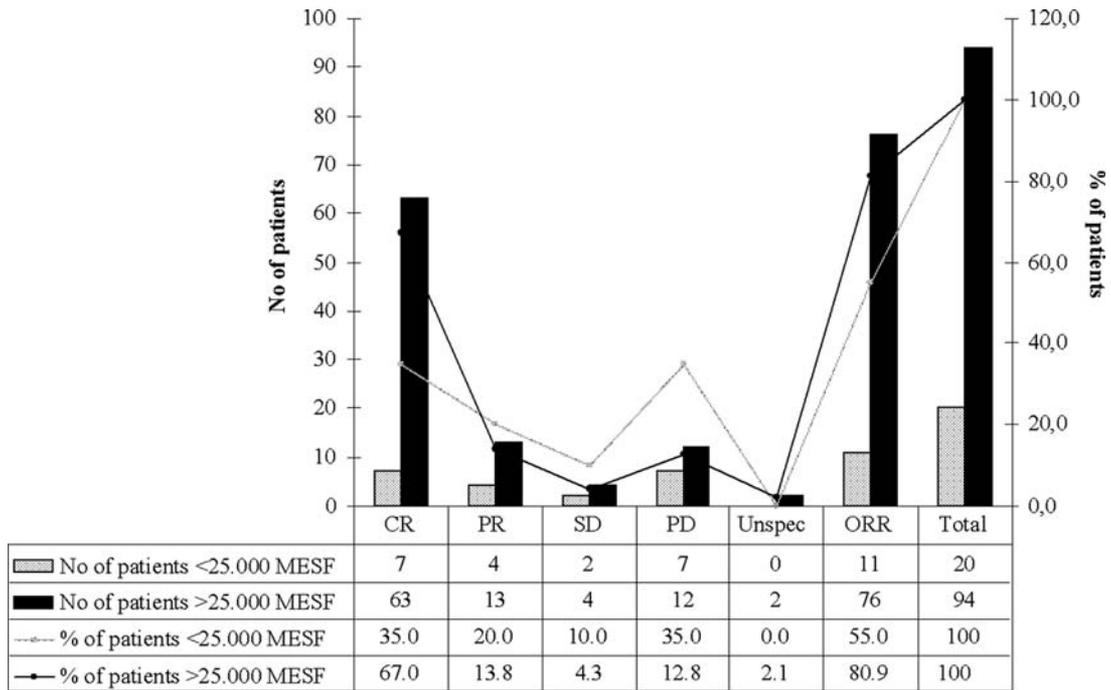


Figure 2. Distribution of patients receiving rituximab according to the response quality and cut-off value. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; Unspec, unspecified response; ORR, overall response rate.

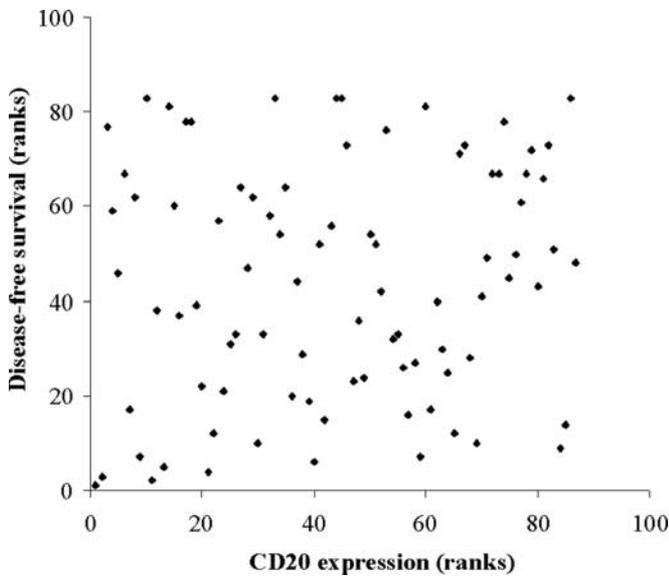


Figure 3. Correlation between the CD20 expression and disease-free survival.

in comparison with 80% of the patients with a CD20 expression level above the cut-off value as can be seen from Fig. 2, which shows the distribution of patients receiving rituximab according to the response quality and the cut-off value. The difference in the overall response rate between the two groups was statistically significant ( $p < 0.001$ ).

The majority of patients with a CD20 expression level above the cut-off value (67%) classified as complete responders after rituximab therapy. In comparison, only 35% of the patients with a CD20 expression level below the cut-off value achieved complete response after therapy with rituximab. On

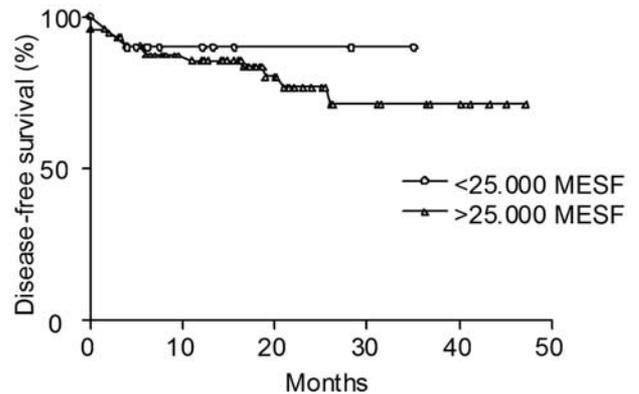


Figure 4. Disease-free survival of 87 patients after treatment with rituximab and chemotherapy according to the CD20 expression.

the contrary, more than twice as many patients with a CD20 expression level below the cut-off value (35%) experienced progressive disease when compared to patients with a CD20 expression level above the cut-off value (12.8%).

While evaluating the DFS, just 87 patients (76.3%) achieving CR and PR to treatment with rituximab and chemotherapy were included in the assessment. No significant correlation between the level of CD20 expression and the response duration was perceived (Fig. 3).

The survival curves were drawn according to the previously set cut-off value of CD20 expression and are given in Fig. 4. Even though the curves diverged we proved no significant difference in the response duration between the patients with CD20 antigen expressed above the cut-off value and those expressing CD20 antigen below the cut-off value [hazard

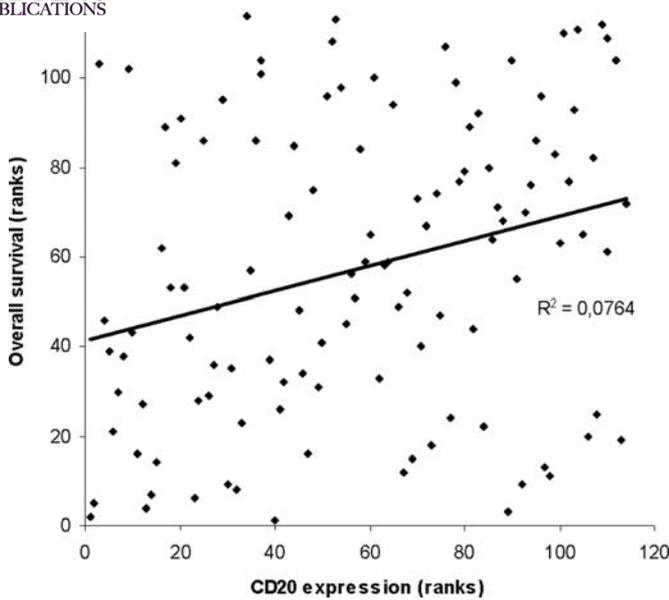


Figure 5. Correlation between the CD20 expression and the overall survival.

Table IV. The distribution of patients with different histological types of B-cell lymphomas according to the cut-off value of CD20 expression.

Histological type	No. of patients below cut-off value/ Total no. of patients	% of patients below cut-off value
DLBCL	6/42	14.3
FL	2/30	6.7
CLL	4/5	80.0
MCL	3/20	15.0
MZL	0/2	0.0
Unclassified	5/15	33.3
Total	20/114	17.5

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; Unclassified, unclassified B-cell lymphoma.

ratio (HR), 0.5667; 95%CI, 0.124 to 3.18,  $p=0.57$ ]. The median DFS has not been reached in any of the patient groups.

Contrary to results of the DFS, we acquired evidence of a significant ( $p=0.003$ ) correlation between the level of CD20 expression and the OS, which is shown in Fig. 5. While evaluating the OS, all 114 B-cell lymphoma patients were included in the assessment.

Fig. 6 gives the overall survival curves after treatment with rituximab and chemotherapy according to the newly set cut-off value of CD20 expression. The B-cell lymphoma patients whose expression of CD20 antigen was above the cut-off value had a significantly longer OS than those expressing CD20 antigen below the cut-off value [hazard ratio (HR),

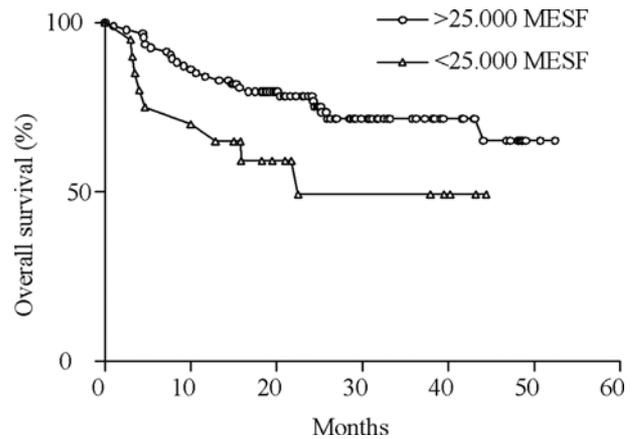


Figure 6. Overall survival after treatment with rituximab and chemotherapy according to the cut-off value of CD20 expression.

0.4573; 95%CI, 0.1364 to 0.9461,  $p=0.0383$ ]. The median OS in patients with the CD20 antigen expressed below the cut-off value was 22.5 months, while the median OS in patients with the CD20 antigen expressed above the cut-off value has not been reached yet at a median duration of observation of 53 months.

Among our study population, 17.5% of patients had a level of CD20 expression below the cut-off value. The highest percentage (80%) of the patients with a CD20 expression level below the cut-off value was noted in the group of patients with chronic lymphoid leukemia (CLL) while the lowest (6.7%) was observed in the group of patients with FL (Table IV).

## Discussion

Results of our study regarding response quality and CD20 expression relationship provide strong evidence for their correlation as complete responders to rituximab therapy had a significantly higher expression of CD20 antigen ( $p=0.018$ ) than those whose disease was only stabilized after rituximab therapy. Due to the lack of comparable studies, it is very difficult to make a comparison. To date the prognostic and predictive significance of CD20 expression in lymphoma patients remains controversial and a matter of ongoing debate. Some clinical studies found no significant association between the CD20 expression and the outcome (10,11), whereas the others reported a significantly better clinical outcome in patients whose tumors were CD20 positive (12-15). For this purpose, we assessed the relationship between the level of CD20 expression and OS, DFS along with response quality in B-cell lymphoma patients. To accomplish this, we determined the cut-off value of CD20 expression with predictive significance of better outcome to rituximab treatment. In this manner, the cut-off level of 25,000 MESF was found to discriminate patients with significantly different overall survival. The patients whose expression of CD20 antigen was above the cut-off value had a significantly ( $p=0.0383$ ) longer OS than those expressing CD20 antigen below the cut-off value. This is in agreement with the conclusions of the study by Johnson *et al* (14) that reduced CD20 expression in

primary DLBCL is associated with an inferior survival. In this study, the association between CD20 expression and clinical outcome was determined in 272 patients with DLBCL. The patients (16%) who were found to have a reduced CD20 expression also had a markedly inferior survival when treated with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) or rituximab-CHOP. Interestingly, the poor prognostic effect of reduced CD20 expression was also seen in the CHOP-treated patients, suggesting that CD20 expression correlates with the cellular biology of the malignant lymphocytes and that the CD20 antigen is important beyond merely serving as the rituximab target (14). Similar to the percentage of patients with reduced CD20 expression reported in the study of Johnson *et al* (14), 17.5% of patients among our study population had the level of CD20 expression below the cut-off value. On basis of our results, we expect that approximately one-fifth of the B-cell lymphoma patients, mostly patients with CLL and the least number of patients with FL will not respond to rituximab treatment at all or will not respond optimally.

Contrary to the results of the OS, we acquired no evidence of significant difference in the response duration between the patients with CD20 antigen expressed above the cut-off value and those expressing CD20 antigen below the cut-off even though the curves diverged. Yet, we observed a highly significant ( $p < 0.001$ ) difference in the ORR between the two groups. According to this, we conclude that the higher level of CD20 expression determines the better ORR but has only minor impact on the duration of response. Those patients with the expression of CD20 below the cut-off limit who despite the low expression of target antigen achieved partial or complete response to rituximab treatment are likely to have a comparable duration of response as the patients with the expression of CD20 above the cut-off limit.

Our findings are consistent with the initial consideration that the degree of CD20 expression on malignant B cells would influence the magnitude of response to rituximab treatment. However, some other studies have provided dissimilar results demonstrating that the intensity of CD20 expression does not predict response to treatment (16,17). The clinical experience described in one of the studies gave evidence that mantle cell and diffuse large B-cell lymphomas have similar or even higher CD20 expression than follicular lymphoma but lower response rates to rituximab, which means that staining intensity alone does not predict the response. Furthermore, if CD20 expression alone determined rituximab responsiveness, then one would expect development of CD20-negative relapses after rituximab treatment, and this circumstance has rarely been reported (18). Thus, binding of rituximab to CD20 might not be sufficient to kill many lymphoma cells indicating that there could be other mechanisms influencing the degree of response.

The reasons for controversial results may be due to many events that occur prior to rituximab binding to tumor cell surface CD20. These events determine whether rituximab reaches a sufficient percentage of lymphoma cells in sufficient quantities to lead to cytotoxicity. The results of these studies reported that serum rituximab levels correlate with treatment response with the levels being higher in good responders (19). Still, whether the higher levels lead to a better response

rate or whether responders have fewer CD20 molecules due to lower tumor burden and therefore higher circulating antibody levels has not been unequivocally determined. Another factor that might affect response to rituximab is also its unequal distribution in various compartments within the body as well as within a malignant lymph node or extranodal tumor mass. Some studies indicate that rituximab may be more successful in removing cells from blood and marrow than from the rest of the body (20).

An alternative explanation for resistance to rituximab is that perhaps malignant B cells undergo mutations that render them less susceptible to rituximab. It has been observed that cross-linking of rituximab molecules bound to the surface of rituximab resistant cells does not lead to their apoptosis (21). Furthermore, rituximab resistant cell lines have been shown to display changes in the intracellular domain of the CD20 antigen resulting in a decrease in intracellular  $Ca^{++}$  mobilization and inhibition of apoptosis (22). The events that occur prior and during rituximab binding to CD20 antigen may be multifactorial and can cause occurrence of mechanisms of non-responsiveness. Thus, the identification and understanding of these mechanisms should lead to improved treatment.

According to the beneficial influence on the long-term prognosis, rituximab has become the standard of treatment of patients with B-cell lymphomas. However, the success of rituximab treatment in line with our results depends upon the level of CD20 expression both in terms of response quality and in terms of overall survival. The impact of the level of CD20 expression on the duration of the response seems to be less pronounced in patients who despite the low expression of target antigen achieve a partial or complete response. The cut-off limit of CD20 expression suggested to have the predictive significance of better outcome was set at 25.000 MESF. This cut-off value should be evaluated individually and considered when the decision regarding treatment with rituximab is taken. However, these results warrant further studies on a larger group of patients.

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