

The prognostic impact of CD49d protein and mRNA expression in patients with chronic lymphocytic leukaemia

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Abstract

Introduction: This research aimed to study CD49d expression – a potential prognosis marker in chronic lymphocytic leukaemia (CLL) patients – at the protein and mRNA levels. It was analysed in terms of time to first treatment and compared to currently known prognostic markers and novel molecular markers.

Material and methods: Using samples from 199 newly diagnosed CLL patients, we conducted immunophenotypical analyses of CD49d with flow cytometry, and assessed its expression on the mRNA level using quantitative polymerase chain reaction (PCR).

Results: Cytometric analysis showed significantly higher expression of CD49d protein in ZAP-70+ (cut-off of 20%) patients than that in ZAP-70 patients (18.52 vs. 6.57, $p = 0.028$), and a tendency of higher CD49d expression in CD38+ patients than in CD38 patients (20.48 vs. 7.25, $p = 0.072$). CD49d expression significantly correlated with serum β 2-microglobulin ($r = 0.273$, $p = 0.012$) and lactate dehydrogenase activity ($r = 0.159$, $p < 0.01$). Analysed in subgroups divided according to novel mutations, CD49d expression showed a tendency to be higher in MUT *SF3B1* (93.98 vs. 7.86, $p = 0.06$) and MUT *NOTCH1* (18.52 vs. 7.86, $p = 0.140$) groups than in the corresponding UNMUT groups. CD49d expression did not affect the time to first treatment at protein or mRNA level.

Conclusions: High CD49d expression at the protein level accompanies aggressive biological markers in CLL patients, indicating its prognostic potential. It can thus be used to identify patients with poor clinical CLL prognosis.

Key words: chronic lymphocytic leukaemia, *NOTCH1*, CD49d, *SF3B1*.

Introduction

Chronic lymphocytic leukaemia (CLL) is a heterogeneous disease characterised by the clonal expansion of mature, typically CD5-positive B cells that accumulate in the blood, bone marrow, and lymphoid organs.

CLL cells are defined by a distinctive immunophenotype of the surface antigen CD5 together with the B-cell antigens CD19, CD20, and CD23 [1].

The biological heterogeneity of the disease – including chromosomal alterations, gene mutations, the immunoglobulin heavy-chain variable region (*IGHV*) mutation status, as well as immunophenotypic features – determines its variable clinical course, with patient survival ranging from months to decades [1, 2]. The currently most relevant prognostic score is the CLL International Prognostic Index (CLL-IPI), which classifies patients based on clinical stage, age, *IGHV* mutational status, serum β 2-microglobulin, and TP53 dysfunction, summing their hierarchical values based on the hazard ratios for OS [3]. During CLL diagnosis, other prognostic markers are used to predict the outcomes of the patients, the expression of zeta-chain-associated protein kinase 70 (ZAP-70), the expression of CD38, the concentration of lactate dehydrogenase level (LDL), and chromosome aberrations [4]. Hepatitis B surface antigen positivity is another unfavourable prognostic factor that is insufficiently screened in the Polish population of CLL patients [5].

Despite the wide range of markers in CLL, particularly at an early stage, currently available prognostic factors often show low efficiency in predicting the course of the disease. Therefore, more efficient biomarkers are still being sought [6]. To date, there is no comprehensive hierarchical model incorporating all markers.

Recent studies have pointed to the surface adhesion molecule CD49d as one of the strongest of the poor prognostic factors in CLL [7, 8]. CD49d – the α chain of the $\alpha_4\beta_1$ integrin heterodimer, also known as very late antigen 4 (VLA-4), coded by the *ITGA4* gene – is involved in forming supportive microenvironmental interactions of CLL cells and accessory cells such as nurse-like cells, vascular endothelial cells, dendritic cells, and T cells [9, 10]. Functionally, by binding to fibronectin and vascular-cell adhesion molecule-1 (VCAM-1), CD49d mediates cell-cell and cell-matrix interactions. CD49d plays crucial roles in leucocyte trafficking, activation, and survival. It also mediates interactions between leucocytes and stromal cells of marrow or the germinal centre of lymphoid follicles [10–14]. Additionally, CD49d promotes B-cell survival via upregulation of Bcl-2 family members, which may determine progression of CLL [14]. CLL cells have also been found to require CD49d engagement to undergo transendothelial migration and enter the proliferation centres of lymph nodes [15, 16]. Strati et al. [17] showed that CD49d expression was associated with nodal presentation and the subsequent development of lymphadenopathy in CLL patients. Furthermore, the effec-

tiveness of blocking CD49d has been also studied in multiple sclerosis and acute leukaemia with the use of natalizumab - monoclonal antibody against CD49d and ATL1102 - second-generation antisense oligonucleotide to CD49d RNA [18].

In order to be effective, a new negative prognostic biomarker in CLL should also correlate with well-established markers for unfavourable prognosis. According to recent research, this is the case with CD49d, which has shown links with high ZAP70 and CD38 expression, unmutated *IGHV* genes, trisomy 12, high β -2 microglobulin, and LDH [7, 19]. Bulian et al. [8] reported that CD49d showed the predictive value of overall survival and treatment-free survival in CLL patients; they also classified CD49d as the strongest flow cytometry prognostic marker. Another study revealed the association of CD49d with the presence of *NOTCH1* (neurogenic locus notch homologue protein 1) mutations [20]. These studies suggest that CD49d has potential predictive value in CLL, but due to the insufficient number of studies validating features of CD49d and its potential utility in the management of CLL further research is required.

This study aims to contribute to this knowledge by studying the expressions of CD49d protein and mRNA in a cohort of newly diagnosed Polish CLL patients. To distinguish between CD49d-positive and -negative patients when testing the prognostic impact of CD49d protein, most previous studies have recommended the cut-off of 30% of positive cells [7, 8, 10, 19]. As benchmarks, we used the most common currently available prognostic factors in CLL: ZAP-70, CD38, *IGHV* gene mutational status, and *IGHV* subsets; and new mutations, such as *NOTCH1*, *SF3B1* (splicing factor 3B subunit 1), and *MYD88* (myeloid differentiation primary response gene 88). We also assessed the prognostic significance of CD49d in terms of the time to first treatment (TTFT).

Material and methods

Participants

The study used 199 newly diagnosed and previously untreated CLL patients. The cohort included 79 women and 120 men, with a median age of 66 (range 38–90) years. The prognostic significance in terms of TTFT was clinically assessed for 90 patients. The time to first treatment was defined as the time interval between the date of diagnosis and the date of first CLL treatment administered according to the International Workshop on Chronic Lymphocytic Leukaemia (iwCLL) treatment indications criteria [1]. The patients' detailed clinical characteristics are displayed in Table I. Ethical approval was granted by local review committees, and written informed consent

was collected from each participant according to the principles of the Declaration of Helsinki.

Peripheral blood mononuclear cell isolation

From the 199 patients, we collected peripheral blood samples. Peripheral blood mononuclear cells were isolated using the intensity gradient centrifuge method, using Ficoll (Biochrom, Germany). Next, the cells were washed with PBS (Biochrom, Germany). The viability of peripheral blood mononuclear cells (PBMCs) was more than 95%. Peripheral blood mononuclear cells were cryopreserved at -80°C until the time of analysis.

mRNA extraction and reverse transcription

Total RNA isolation from PBMCs was performed using QIAamp RNA Kit (Qiagen, Netherlands), according to the manufacturer's protocol. Concentrations and purities were measured using BioSpec-nano (Thermo Scientific, USA). From each sample, 1 μg of total RNA was reverse transcribed to 20 μl of cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Netherlands). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) reactions used 1 μl of cDNA per sample.

Quantitative reverse transcriptase-polymerase chain reaction-

The quantity of the mRNA expression of the transcript of CD49d (*ITGA4*) was assessed in each of the 199 CLL patients using qRT-PCR with the TaqMan Gene Expression Assays methodology (Applied Biosystems, USA). The procedure was performed according to the manufacturer's protocols. The end volume of a particular PCR reaction per well was 20 μl , which included 10 μl of TaqMan Universal PCR Mastermix (Applied Biosystems, USA), 1 μl of Taqman assay for *ITGA4* with the specific primers and probe (ThermoFisher scientific, USA), 8 μl RNase-Free water (Qiagen, Netherlands), and 1 μl of cDNA. Distilled water was used as a negative control.

As a constitutively expressed housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used. Expression of *GAPDH* was assessed using Taqman probes with TaqMan Universal PCR Master Mix (Applied Biosystems, USA) as well as TaqMan *GAPDH* Control Reagents Human Assays (Applied Biosystems, USA). The procedure was performed according to the manufacturer's protocols. The end volume of a particular PCR reaction per well was 25 μl , which included 12.5 μl of TaqMan Universal PCR Mastermix (Applied Biosystems, USA), 0.5 μl of forward primer as well as 0.5 μl of reverse primer for *GAPDH* (Applied Biosystems, USA), 10 μl RNase-Free water (Qiagen, Netherlands), 0.5 μl JOE probe, and 1 μl of cDNA.

Table I. Clinical characteristic of patients

| | CD49d mRNA | CD49d protein |
|-----------------------------|------------|---------------|
| Number of patients | 199 | 106 |
| Sex | | |
| female | 79 | 38 |
| male | 120 | 68 |
| Median age (years) | 66 | 66 |
| Age range (years) | 38–90 | 38–90 |
| CD49d mRNA | | |
| high | 99 | 47 |
| low | 100 | 51 |
| not available | n/a | 8 |
| CD49d protein (cut-off 30%) | | |
| positive ($\geq 30\%$) | 36 | 36 |
| negative ($< 30\%$) | 70 | 70 |
| not available | 93 | n/a |
| ZAP-70 (cut-off 20%) | | |
| positive ($\geq 20\%$) | 58 | 31 |
| negative ($< 20\%$) | 104 | 56 |
| not available | 37 | 19 |
| CD38 (cut-off 30%) | | |
| positive ($\geq 30\%$) | 52 | 27 |
| negative ($< 30\%$) | 116 | 59 |
| not available | 31 | 20 |
| <i>IGHV</i> mutation status | | |
| mutated | 94 | 49 |
| unmutated | 102 | 54 |
| not available | 3 | 3 |

ZAP-70 – zeta-chain-associated protein kinase 70, *IGHV* – immunoglobulin heavy-chain variable region gene, n/a – not applicable.

Thermocycling program was set for 40 cycles of 15 sec at 95°C , 1 min at 60°C , with an initial denaturation step at 95°C for 10 min using the 7300 Real Time PCR System (Applied Biosystems, Life Technologies Corporation, USA).

The particular gene expression levels of *ITGA4* were calculated as an inverse ratio of the difference in cycle threshold (ΔCt), where ΔCt is the Ct value of the transcript of CD49d (*ITGA4*) expression minus the Ct value of *GAPDH*.

Immunophenotypic analyses of CD49d, ZAP-70, and CD38-

Flow cytometry was used to characterise the surface expression of Cd49d on CLL cells using combination of fluorescent-labelled monoclonal antibodies: anti-CD19 FITC (BD Biosciences, USA),

anti-CD5 PerCP5 (BD Biosciences, USA), and anti-CD49d APC (BD Biosciences, USA). A fluorescence minus one (FMO) control was used for PE. Samples were measured using an FACSClyric (BD Biosciences, USA) flow cytometer. Data were analysed using the BD FACSuite software (BD Biosciences, USA). A minimum of 100,000 cells were collected and analysed. The representative flow cytometry analysis is presented in Figure 1.

Evaluation of CD38 and ZAP-70 expression in CD19+/CD5+ leukemic cells in all tested samples was performed according to the previously described procedure [21]. Based on a CD38 cut-off value of 30%, CLL patients were categorised into CD38-positive ($\geq 30\%$) and CD38-negative subgroups ($< 30\%$). For ZAP-70 the cut-off value was defined as 20%, and patients were categorised into ZAP-70-positive ($\geq 20\%$) and ZAP-70-negative ($< 20\%$) subgroups. The cut-off values used in the present report were selected in accordance with previously published studies [21].

NOTCH1, MYD88, SF3B1, and IGHV mutations

NOTCH1 c.7544_7545delCT in PEST domain (exon 34) and *MYD88* L265P mutations were investigated by amplification refractory mutation system PCR. *SF3B1* mutations (K700, E622/R625, and H662/K666) were confirmed by Sanger sequencing method. Detection of *SF3B1*, *NOTCH1*, *MYD88* L265P, and *IGHV* mutation status were assessed as previously described in detail [22]. A cut-off of 98% germline homology was used to assess *IGHV* mutation status. The sequences with a germline homology of 98% or higher were considered unmutated, and those with a homology less than 98% were considered mutated.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (La Jolla, CA, United States). Because the data did not follow the normal distribution, we used nonparametric methods. Dif-

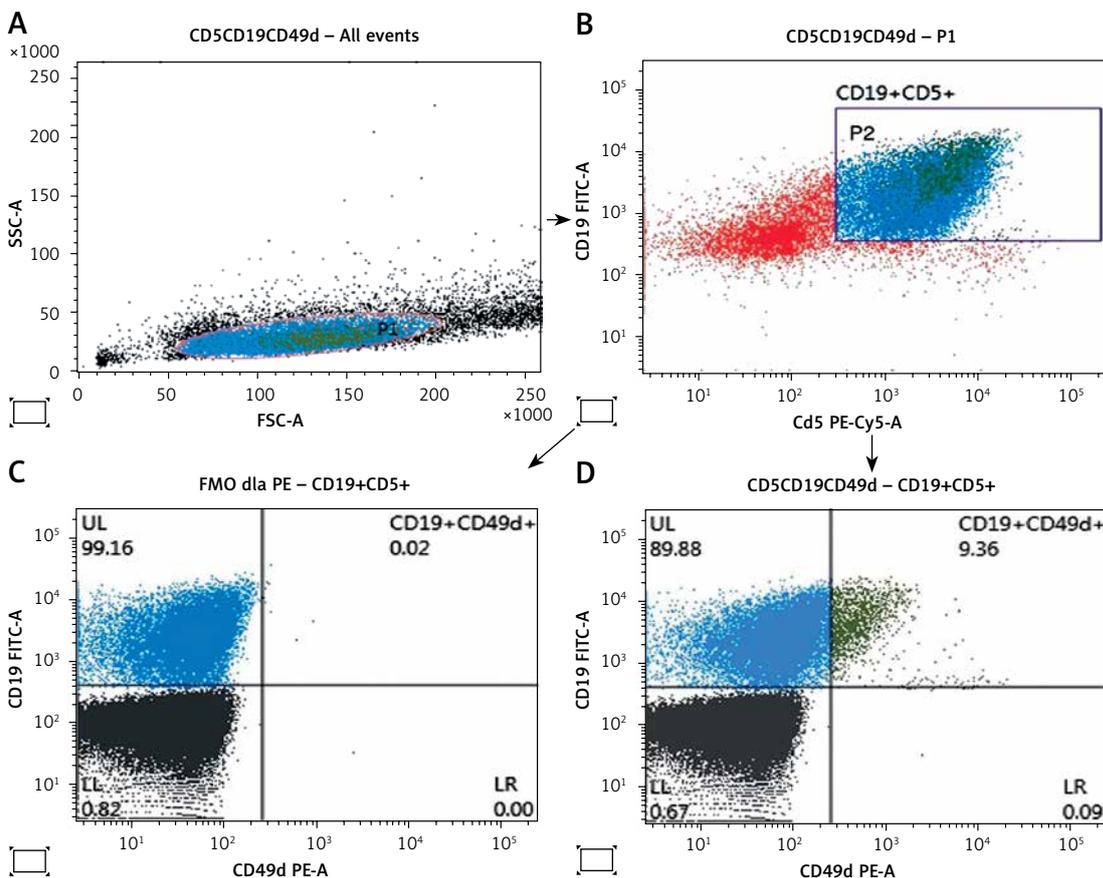


Figure 1. Cytometric analysis of CD49d expression on CD19+CD5+ cells. CD49d expression was normalised against fluorescence minus one (FMO) control. The dot plots (A, B, C, D) represent an example of the analysis of CD49d expression on chronic lymphocytic leukaemia (CLL) cells. **A** – The lymphocyte population was gated (region P1) based on side vs. forward scatter (FSC vs. SSC) distribution. **B** – From the P1 region CLL cells were gated (region P2) as double positive cells for CD19 and CD5. CLL were gated (region P2) as double positive cells for CD19 and CD5. **C** – Compared to FMO control, the CD49d expression on CLL cells was evaluated. **D** – The final dot plot was acquired using combined gating of events using P1 and P2 regions; thereby, all CD19+CD49d+ cells were CD5+. The final dot plot was acquired using combined gating of events using P1 and P2 regions; thereby, all CD19+CD49d+ cells were CD5+ number in the upper right quadrant in the dot plot represents the percentage of CD19+CD49d+ (9.36%)

ferences between the groups were verified using the Mann-Whitney and Kruskal-Wallis tests, while correlations between the variables were assessed using the Spearman rank correlation coefficient. The Kaplan-Meier method was used to analyse the TTFT of CLL patients. Univariate and multivariate analyses were performed using the Cox regression model. All the analyses assumed a significance level of 0.05.

Results

The prognostic value of CD49d expression in chronic lymphocytic leukaemia

To determine the prognostic value of CD49d in CLL, we divided the patients into groups according to the presence of certain prognostic factors. CD49d expression tended to be higher in patients characterised by unmutated (UM) *IGHV* gene status than in those with mutated (MUT) *IGHV* (14.33 vs. 5.09, $p = 0.151$) (Figure 4 A). ZAP-70+ (cut-off of 20%) patients had higher Cd49d expression than did ZAP-70-patients (Figure 2 A); likewise, CD38+

(cut-off of 30%) patients tended to have higher expression than did CD38-patients (Figure 2 B). CD49d expression weakly correlated with serum level of β 2-microglobulin ($r = 0.273$, $p = 0.012$) (Figure 3 A) and activity of lactate dehydrogenase (LDH) ($r = 0.159$, $p < 0.01$) (Figure 3 B).

We ascertained whether CD49d expression depended on new molecular markers in CLL: *SF3B1*, *NOTCH1*, and *MYD88*. The analysis revealed a tendency for CD49d to be more highly expressed in MUT *SF3B1* cases than in UNMUT *SF3B1* (93.98 vs. 7.86, $p = 0.06$) (Figure 4 B). We observed a tendency in MUT *NOTCH1* versus UNMUT *NOTCH1* cases (18.52 vs. 7.86, $p = 0.140$) (Figure 4 C). CD49d expression, however, did not depend on the mutational status of *MYD88* (4.65 vs. 11.03, $p = 0.227$) (Figure 4 D). Likewise, CD49d had similar expression in patients for whom the stereotyped B-cell receptor (BCR) sequences provided adverse prognosis (subsets #1, #2, and #8) and those with favourable prognosis (the remaining subsets) (6.42 vs. 8.32, $p = 0.484$).

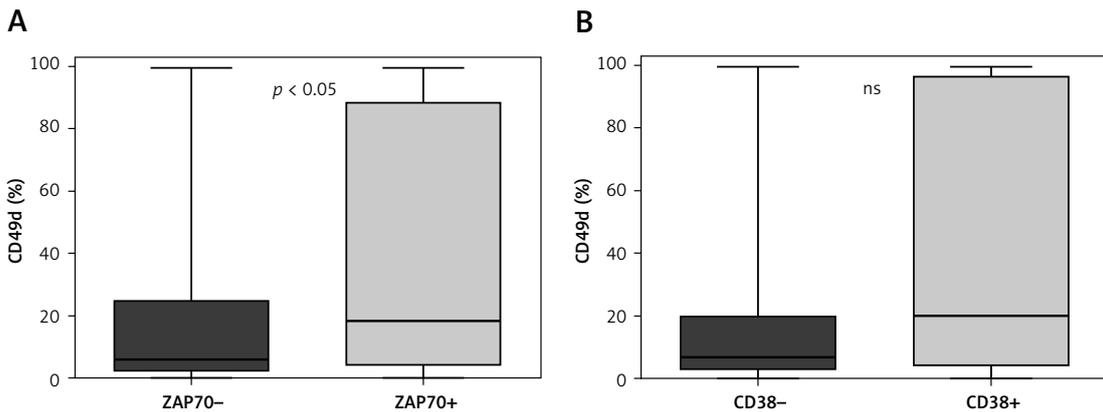


Figure 2. Expression of CD49d protein in patients depending on ZAP-70 (A) and CD38 (B) expression. **A** – CD49d protein expression was higher in ZAP-70+ patients than in ZAP-70 patients (18.52 vs. 6.57, $p = 0.028$). **B** – CD49d protein expression tends to be higher in CD38+ patients than in CD38 patients (20.48 vs. 7.25, $p = 0.072$)

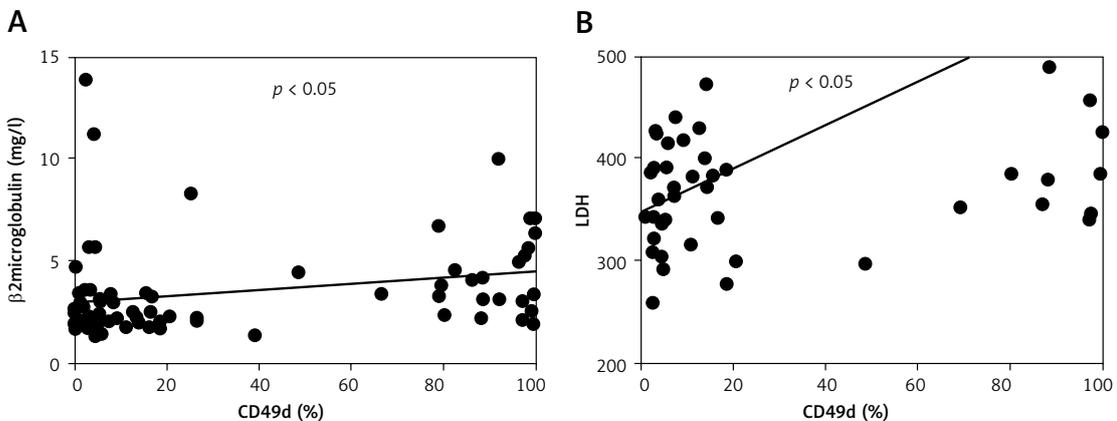


Figure 3. Correlation between CD49d protein expression and the serum level of β 2-microglobulin (A) and activity of lactate dehydrogenase (LDH) (B). **A** – Correlation between CD49d protein expression and the serum level of β 2-microglobulin was low ($r = 0.273$, $p = 0.012$). **B** – Correlation between CD49d protein expression and the activity of LDH was also low ($r = 0.159$, $p < 0.01$)

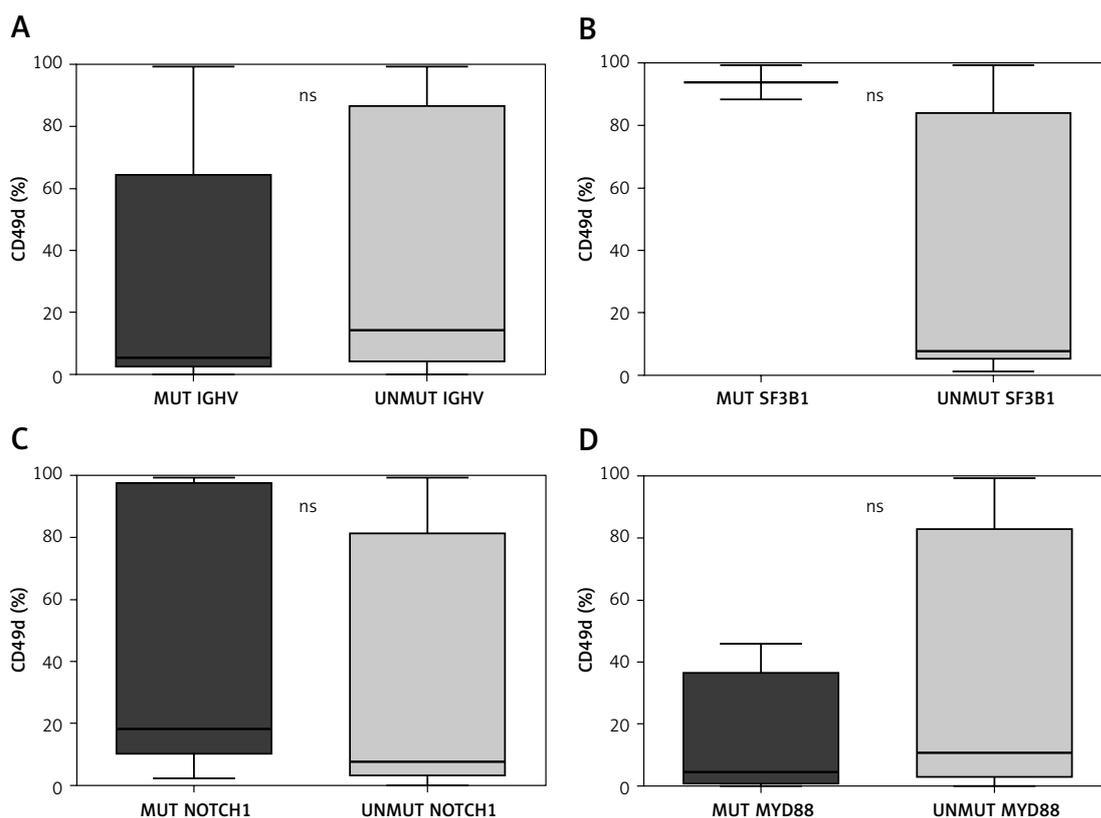


Figure 4. CD49d protein expression depending on mutational status of *IGHV* (A), *SF3B1* (B), *NOTCH1* (C), and *MYD88* (D). A – Expression of CD49d protein tends to be higher in patients with the UNMUT *IGHV* gene status than in those with the MUT *IGHV* gene status (14.33 vs. 5.09, $p = 0.151$). B – Expression of CD49d protein tends to be higher in the MUT *SF3B1* patients than in the UNMUT *SF3B1* ones (93.98 vs. 7.86, $p = 0.06$). C – Expression of CD49d protein tends to be higher in the MUT *NOTCH1* patients than in the UNMUT *NOTCH1* patients (18.52 vs. 7.86, $p = 0.140$). D – Expression of CD49d protein was similar in MUT *MYD88* and UNMUT *MYD88* patients (4.65 vs. 11.03, $p = 0.227$)

The influence of the cut-off on the impact of Cd49d expression on the clinical outcome in chronic lymphocytic leukaemia

As the survival curves show (Figure 5 A), the expressions of ZAP-70 and CD38 proved to be efficient prognostic values in CLL ($p = 0.001$). We divided the patients into 4 groups according to positive and negative expressions of both markers, for which we compared TTFT predictions. We found worse prognosis for patients with ZAP-70+ and CD38+. The recommended cut-off level of CD49d expression is 30%. Because the preliminary analysis of our data suggested other cut-off levels of CD49d expression, we used 3 of them: 30%, 20%, and the median (9.9%). Time to first treatment did not significantly differ in patients with CD49d+ expression and those with CD49d-expression, irrespective of the cut-off point used: 30% ($p = 0.501$) (Fig. 4B), 20% ($p = 0.256$) (Figure 5 C), and the median of 9.9% ($p = 0.821$) (Figure 5 D).

All variables were assessed in a univariate Cox regression model, and those which were statistically significant were considered for inclusion in multivariate analysis (Table II). There was no sta-

tistically significant difference in CD49d protein and mRNA expression in univariate analyses, respectively ($p = 0.51$, hazard ratio HR = 1.26, 95% CI: 0.6–2.5) and ($p = 0.83$, HR = 1.16, 95% CI: 0.3–4.8).

The prognostic value of transcript of CD49d (ITGA4) expression on mRNA levels in chronic lymphocytic leukaemia and its impact on clinical outcome in chronic lymphocytic leukaemia

To characterise the prognostic value of *ITGA4* in CLL, we studied whether *ITGA4* expression affected the clinical outcome, defined as TTFT. Time to first treatment did not depend on CD49d expression at the mRNA level ($p = 0.763$) (Figure 6). *ITGA4* expression also did not show significant prognostic value in the groups of patients divided according to CD38 and ZAP-70 expression factors (Figure 7 A, B).

Discussion

Recent advances in CLL research – such as the discovery of its genomic landscape and the

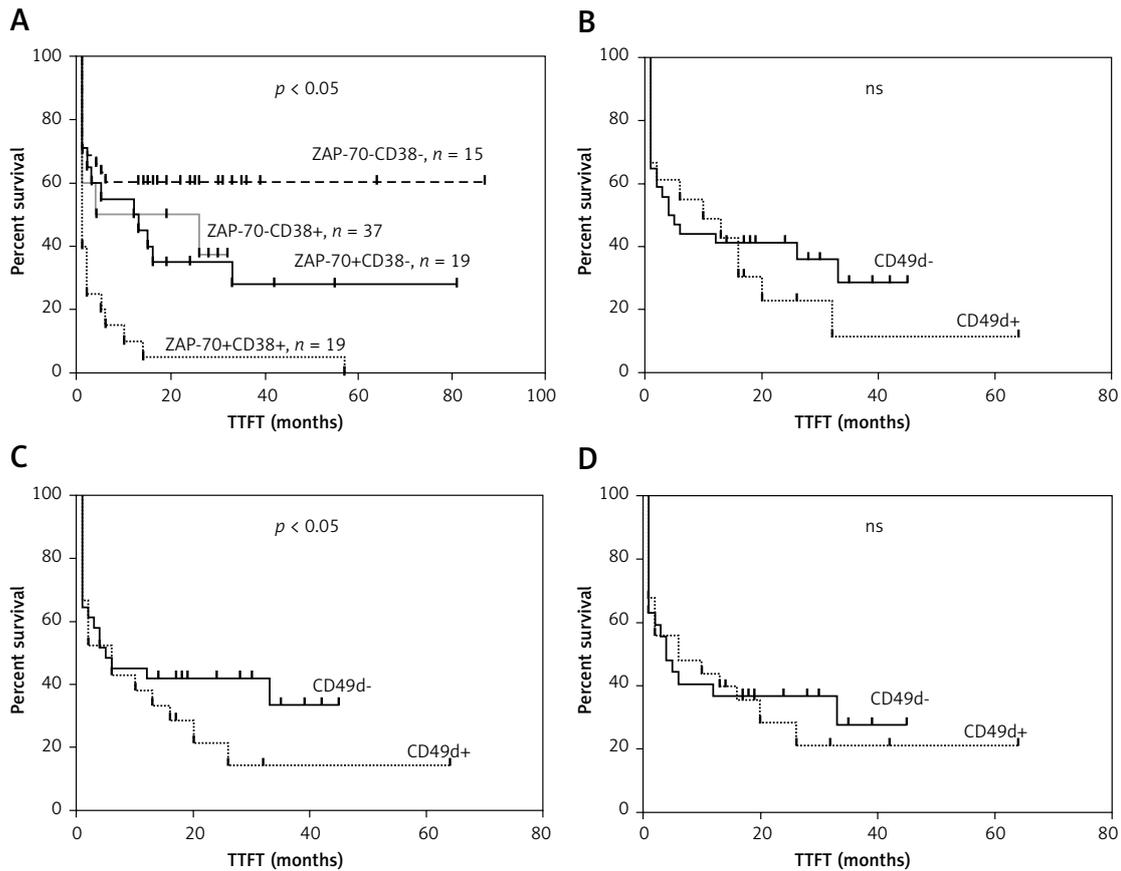


Figure 5. Prognostic values in terms of time to first treatment (TTFT) of ZAP-70 and CD38 expression (A), CD49d protein expression (cut-off 30%) (B), cut-off of 20% (C), and the median cut-off of 9.9% (D). A – There were statistically significant differences in terms of TTFT in patients divided into 4 groups according to positive and negative expressions of ZAP-70 and CD38 ($p = 0.001$). B – There were no statistically significant differences in terms of TTFT in patients with the positive and negative CD49d protein expression (cut-off 30%) ($p = 0.505$). C – There were no statistically significant differences in terms of TTFT in patients with positive and negative CD49d protein expression (cut-off 20%) ($p = 0.26$). D – There were no statistically significant differences in terms of TTFT in patients with positive and negative CD49d protein expression (median cut-off of 9.9%) ($p = 0.821$)

Table II. Univariate and multivariate Cox regression analysis model

| Variable | Univariate analysis | | | Multivariate analysis | | |
|----------------------------|---------------------|----------|----------|-----------------------|----------|---------|
| | Hazard ratio | CI (95%) | p-value | Hazard ratio | CI (95%) | p-value |
| Gender | 0.79 | 0.5–1.3 | 0.36 | | | |
| Age | 1.00 | 1.0–1.0 | 0.77 | | | |
| SF3B1 gene | 0.40 | 0.1–1.8 | 0.22 | | | |
| CD49d status | 1.26 | 0.6–2.5 | 0.51 | | | |
| CD49d mRNA | 1.16 | 0.3–4.8 | 0.83 | | | |
| LDH | 1.00 | 1.0–1.0 | 0.06 | | | |
| β2-microglobulin | 1.25 | 1.1–1.4 | 0.0001 | 1.11 | 0.8–1.5 | 0.53 |
| ZAP-70 | 2.35 | 1.4–4.1 | 0.002 | 2.12 | 0.9–5.2 | 0.10 |
| CD38 status | 2.12 | 1.2–3.6 | 0.005 | 0.73 | 0.3–1.9 | 0.52 |
| IGHV status | 4.37 | 2.3–8.3 | 0.000006 | 2.13 | 0.7–6.5 | 0.19 |
| NOTCH1 gene | 1.79 | 1.0–3.2 | 0.05 | 3.44 | 1.3–9.1 | 0.01 |
| Disease stage at diagnosis | 4.46 | 2.0–10.2 | 0.0004 | 2.93 | 0.8–10.5 | 0.10 |

CI – confidence interval, SF3B1 – splicing factor 3B subunit 1, LDH – lactate dehydrogenase, ZAP-70 – zeta-chain-associated protein kinase 70, IGHV – immunoglobulin heavy-chain variable region gene, NOTCH1 – neurogenic locus notch homologue protein 1. The cut-off levels used in the analysis were: SF3B1 gene: mutated vs. non mutated; CD49d status: 30% positive cells; ZAP-70: 20% positive cells, CD38 status: 30% positive cells; IGHV status: mutated vs. non mutated, NOTCH1 gene: mutated vs. non mutated; disease stage at diagnosis: RAI scale 0–2 vs. 3–4.

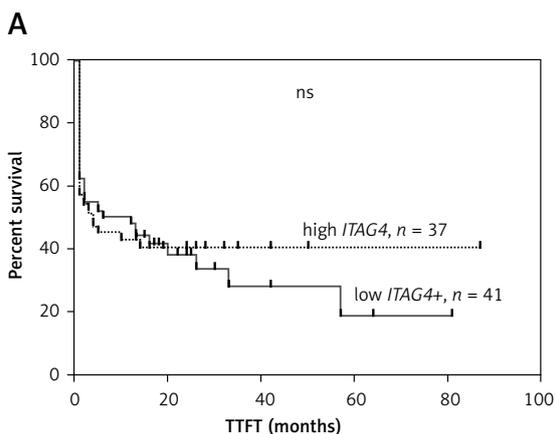


Figure 6. Time to first treatment (TTFT) in patients with low and high *ITGA4* expression. There were no statistically significant differences in terms of TTFT in patients with the positive and negative CD49d expression at mRNA level ($p = 0.763$)

development of prognostic tests – have changed the way CLL is diagnosed, managed, and treated. The International Workshop on CLL published in 2018 updated recommendations on the diagnosis and management of CLL. Among the changes was an update of the list of potential biomolecular markers with a new potential immunophenotypic marker with clinical relevance: CD49d [3].

In order to learn whether CD49d should be used not only as a potential prognostic marker but also as an effective one, we analysed in detail CD49d expression at both protein and mRNA levels in the context of currently established prognostic factors and TTFT in a representative cohort of CLL patients. Although its prognostic value has already been confirmed at the protein level, this knowledge has yet not been transferred to routine practice [7–9, 12].

According to 2 landmark papers presented in 1999, one of the most powerful prognostic markers discovered to date is *IGHV* mutational status [23, 24]. Chronic lymphocytic leukaemia patients

with the MUT *IGHV* gene usually have a low-risk disease and good prognosis, while those with the UNMUT *IGHV* genes (defined as 98% or more sequence homology to the nearest germline gene) usually have progressive disease and respond poorly to therapy, both shortening their survival [7, 23]. Our study has shown higher CD49d expression in patients with unmutated *IGHV* status, a result consistent with previous studies that revealed a correlation between CD49d expression and *IGHV* status [7, 13, 19]. We have also confirmed that CD49d expression correlates with other aggressive biological markers, such as high β 2-microglobulin and LDH [19]. CD49d has been proven to correlate with the expressions of ZAP-70 and CD38, both related with bad prognosis – yet another result that is consistent with previous researches [7, 8, 19, 25].

Next-generation sequencing has identified new genomic abnormalities in CLL patients, such as *NOTCH1* and *SF3B1* mutations. Both have negative prognostic significance, but the *SF3B1* mutation has already been accepted as an independent prognostic factor [22]. Affecting up to 15% CLL patients and significantly overrepresented in the trisomy 12 cytogenetic, *NOTCH1* mutations are known to show poor prognosis [26]. Moreover, Benedetti et al. showed that CD49d expression correlates with *NOTCH1* mutation, indicating that CD49d expression can be up-regulated by the activation of the *NOTCH1* pathway through the involvement of NF- κ B [20]. In our study, CD49d expression tended to be higher in patients with *NOTCH1* mutations and those with *SF3B1* mutations. Our data partly corroborate the results of Baumann et al. [7], who showed a significant correlation between CD49d and both mutations, while we have observed a tendency. In our study, we did not show an association between CD49d expression and *MYD88* mutations, a result contributing to various data indicating that this ab-

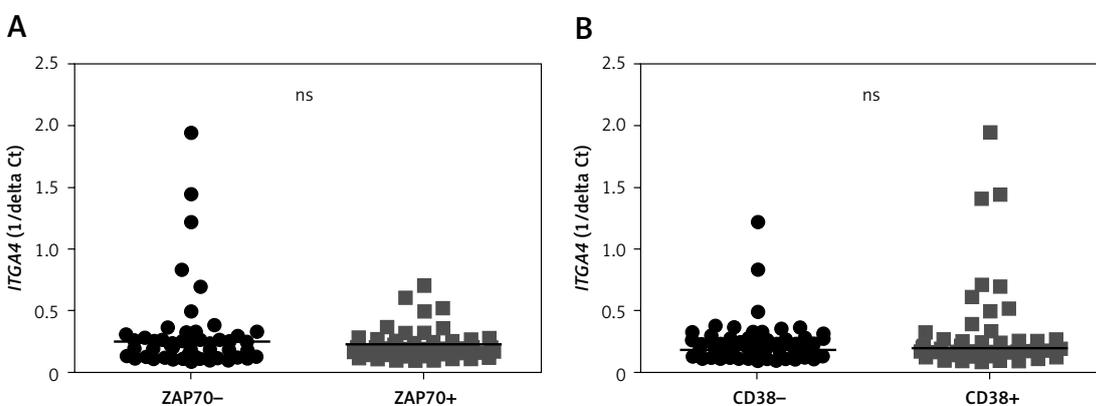


Figure 7. *ITGA4* expression in patients with negative and positive expression of ZAP-70 (A) and CD38 (B). A – Expression of CD49d at mRNA level was similar in ZAP-70-positive and ZAP-70-negative patients (0.1859 vs. 0.1894, $p = 0.988$). B – Expression of CD49d at mRNA level was similar in CD38-positive and CD38-negative patients (0.1976 vs. 0.1869, $p = 0.295$)

erration has no clinical implication in CLL patients [27–29]. Some studies have reported longer TTFT and overall survival in CLL patients with *MYD88* mutations [30]; however, the relevance of *MYD88* mutation remains unclear and requires further investigation.

We used TTFT to analyse the prognostic value in the context of the patients' clinical outcomes. TTFT not show significant differences for any of the assessed cut-off levels: 30%, 20%, and the median (9.9%). On the other hand, analysing TTFT against the background of currently accepted immunophenotypic factors revealed significant differences in the CD38 and ZAP-70 subgroups, with worse prognosis in positive patients in both subgroups (ZAP-70+ and CD38+). The univariate and multivariate Cox regression model also did not prove the negative prognostic value of CD49d, despite results of analyses of other prognostic factors which confirmed that our cohort included a representative group of CLL patients. These data disagree with previous reports that pointed out negative clinical implications (TTFT and OS) of high CD49d expression in CLL patients [7, 8, 19]. Moreover, in the study by Bulian et al. CD49d emerged as the strongest flow cytometry-based prognostic marker, even better than CD38 and ZAP-70 [8].

CD49d may also be expressed in a bimodal pattern in CLL patients with the copresence of negative and positive groups of cells in the same sample. Recent multicentre research showed that CLL patients with CD49d expression exceeding the 30% cut-off as well as with bimodal expression of CD49d had negative clinical implications. Both subpopulations, regardless of the 30% cut-off in a bimodal pattern of expression, experienced similar clinical behaviour in the context of a chemoimmunotherapy and ibrutinib setting [31].

Targeting CD49d could represent an interesting approach in the management of CLL. The monoclonal humanised IgG4 anti-CD49d antibody – natalizumab – demonstrated the potential to overcome stromal cell-induced resistance of B cell lymphoma cells against rituximab and cytotoxic drugs in vitro [9, 32]. CD49d playing a key role in adhesion and migration of cells and is related with BCR signalling by inside-out activation [10]. To date, the BCR signalling pathway has emerged as one of the most important targets for novel therapies in CLL using BCR kinase inhibitors, such as ibrutinib [33]. The therapeutic effects of BCR inhibitors in CLL may contribute to similar clinical activity of integrin inhibitors. It is also hypothesised that these agents could synergise with chemotherapeutic agents and monoclonal antibodies [9, 33].

Flow cytometry is the common laboratory technique; however, it is associated with disadvantages such as inconsistent performance depending on the monoclonal antibodies and

fluorochromes used and different cut-off levels reported. The expression of CD49d could also be assessed by qRT-PCR, which is a widely used, sensitive, and accurate method for monitoring gene expression [34]. Another prognostic marker in CLL patients – the expression of ZAP-70 – was confirmed to have useful prognostic value both in protein and mRNA levels [35]. Therefore, we performed an analysis of CD49d expression using both methods. In our study, we results of expression of CD49d analysed by Quantitative reverse transcriptase PCR (CD49d mRNA) was compared to other prognostic factors and have showed no statistical difference.

On the other hand analysis of CD49d expression by cytometry method showed statistical difference comparing to other prognostic factors in CLL. That's why cytometry method should be used to assess prognostics of CLL results may contribute to numerous post-transcriptional and translational regulation mechanisms in protein expression, such as phosphorylation, ubiquitination, methylation, acetylation, or GlcNAcylation [36].

In conclusion, this study has shown that high CD49d expression is associated with other aggressive biological markers in CLL patients, confirming what previous studies have suggested, i.e. that CD49d is a promising novel prognostic marker.

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Conflict of interest

The authors declare no conflict of interest.

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