# The role of the immunophenotype and prognostic markers in progression of chronic lymphocytic leukemia

PhD Thesis

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# 1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries. CLL belongs to the mature B-cell non-Hodgkin's lymphomas according to the WHO classification. CLL is a low-grade indolent lymphoma, which develops in the bone marrow accompanied by leukemic blood picture. In the case of progression lymph nodes and other lymphoid tissues can be affected.

CLL is characterized by **highly variable clinical course**. One-third of CLL patients don't need therapy for years and show long survival. The other patient group is characterized by rapid progression, aggressive clinical course and short survival rates despite the therapies. In 5-30% of the cases CLL transforms into malignant non-Hodgkin's lymphoma, most commonly diffuse large B-cell lymphoma (called Richter syndrome) or prolymphocytic leukemia, rarely Hodgkin's lymphoma. Transformation is associated with more aggressive clinical course and shorter overall survival.

Several **prognostic factors** predict the outcome of the disease. Beside the Rai- and Binet clinical classification many novel prognostic markers were identified in CLL. The ideal prognostic factor predicts the aggressive disease, but also takes part in the pathogenesis and can serve as a potent therapeutic target.

80% of CLL cases carry cytogenetic abnormalities, many of them have also prognostic impact. The deletion of 13q, 11q, 17p, 6q and **trisomy 12** (12tri) are the most common chromosome abnormalities in CLL. Patients with 12tri have an intermediate prognosis. Despite the intermediate risk, 12tri is related with early progression and very aggressive clinical outcome, furthermore show higher incidences of Richter syndrome and secondary tumors.

Analysis of prognostic markers with flow cytometry is quick, reliable and very useful by follow-up studies. In addition, subpopulations can be identified and follow-up by gating. The prognostic marker ZAP-70, CD23, CD38 and CD49d are detected with flow cytometry in CLL.

CLL cells are characterized by high expression of CD23. The **CD23** (FcεRII) is the low affinity receptor for IgE. **Two isoforms** of CD23, namely **CD23a and CD23b**, have been described, which differs only at the N-terminus of the protein, representing the intracytoplasmic domain. The expression of the CD23 isotypes is regulated by the cytokines IL-4 and IFNγ, the NOTCH2 signaling pathway just us the antigen binding of

the B-cell receptor (BCR). CLL is characterized by overexpression and altered regulation of CD23 comparing with normal B-cells. Unlike normal B-cells, CLL cells express both isotype constitutively. CD23a has a role in survival, while CD23b enhances proliferation. The CD23 is a widely used **differential diagnostic marker**, which distinguish the $CD5^+$ CLL and mantle cell lymphoma (MCL). Despite the differential diagnostic role, CLL cells show **variable CD23 expression**. CD23 level has potent **prognostic value**: low CD23 level correlates with shorter overall survival, with bone marrow prolymphocyte infiltration and negatively with absolute lymphocyte count (ALC).

CD49d is the strongest flow cytometry based prognostic factor in CLL. The **CD49d** is the **α4 integrin subunit**, which associated with the CD29 (β1 integrin) composes the VLA-4 adhesion molecule. The CD49d/CD29 complex mediates cell-cell and cell-extracellular matrix interactions. Its ligands are the VCAM-1 adhesion molecule and the fibronectin. The CD49d/CD29 exists in **different conformation states**, which define the ligand binding affinity of the molecule. Resting cells show bent conformation with low affinity for ligands, but various stimuli (chemokines, BCR) can induce the high-affinity form. Ligand-binding of the CD49d/CD29 activates several cytoskeletal components and intracellular signalling pathway related to adhesion, migration, survival and proliferation.

While normal B-cells show high CD49d level, **CLL cells have variable CD49d expression**. CD49d is **an adverse prognostic factor** in CLL. High CD49d level is associated with shorter overall survival, earlier therapy indication, lymphadenopathy, Richter syndrome and advanced clinical state (Rai III, IV). CD49d plays important role in migration and homing of CLL cells. $CD49d^+$ CLL cells are characterized by greater migratory potential, furthermore these cases show more extensive bone marrow infiltration, comparing with $CD49d^-$ CLL cells. The **direct survival and proliferation effect** of CD49d is proved to be **controversial**.

It is well known that the **microenvironment** (along with genetic lesions) plays crucial role in the pathogenesis of CLL. Neoplastic B-cells receive several **anti-apoptotic, proliferation and migration signals from the microenvironment**, which can lead to therapy-resistance or presence of minimal residual disease after therapy. CLL cells (or a subpopulation of the peripheral CLL pool) are in continuous recirculation between the peripheral blood and lymphoid tissues. By progression CLL cells migrate into the tissues,

hence the bone marrow and lymph nodes represent more beneficial niches. Integrins, especially the CD49d/CD29, chemokines and their receptors are key determinants of CLL cell trafficking. Migration toward CXCL12 chemokine gradient is well known in CLL. The receptor of CXCL12 is the **CXCR4** expressed on CLL cells, which induces chemotaxis, actin-polymerisation, moreover survival.

## 2. Aims

1. Analysing the role of CD23 expression in prognosis of CLL:
    - Investigating the CD23 isotypes (CD23a and CD23b) expression at mRNA level in CLL cells and in other $CD23^+$ lymphoma cells
    - Analysing the correlation of CD23 expression with prognostic and immunophenotypic markers
    - Comparing the clinical parameters of cases with different CD23 expression furthermore studying whether patient subgroups can be determined based on the CD23 expression

2. Investigating the role of high CD49d expression:
    - Analysing the correlation of CD49d expression with prognostic markers and molecules mediating microenvironmental interactions
    - Investigating the direct effect of the CD49d-VCAM-1 interaction in survival, proliferation, actin-polymerisation and immunophenotype-change
    - Studying the active conformation of the CD49d/CD29 complex

## 3. Methods

**Patients and samples:** lymphoma cells were isolated from peripheral blood samples of CLL and other $CD23^+$ lymphoma (MCL-mantle cell lymphoma, MZL-marginal zone lymphoma) patients, by some investigations bone marrow aspirates were used.

**Molecular analysis:** to investigate the mRNA expression of CD23 isotypes conventional and quantitative real-time PCR analysis were performed after RNA isolation and reverse transcription. To quantify the CD23 mRNA levels TaqMan® probes were used. The relative expression of the CD23 isotypes was analysed by $\Delta\Delta C_T$ method.

**Immunophenotype analysis by flow cytometry:** samples were stained with fluorochrome-conjugated antibodies. Stained cells were measured on FACSCalibur flow cytometer and analysed using CellQuest Pro software.

**FISH:** in CLL cases del11q23, del17p13, del13q, 12tri, in MCL samples t(11;14) aberration were verified by FISH analysis.

**Cell culture:** CLL cells were cultured alone, on VCAM-1 covered surface or in BMSC (bone marrow stromal cell) co-culture for 7 days.

**Apoptosis measurement:** after culturing CLL cells were stained with annexinV/propidium-iodide (PI). Samples were measured by flow cytometry.

**Cell cycle analysis and expression of proliferation markers:** the rate of proliferating cells was determined based on the PI fluorescence following fixation and alkalic extraction. In addition, anti-Ki67 and anti-cyclinD2 stainings were performed detected by flow cytometry.

**Real-time investigation of the conformation-change of CD29 by flow cytometry:** the conformation of the CD49d/CD29 complex was investigated using the high affinity conformation-specific anti-CD29 (clone HUTS-21) antibody. The activation process was analysed by real-time binding of HUTS-21 antibody assessed by flow cytometry after adding VCAM-1, CXCL12 or TS2/16 monoclonal antibody, which directly activates CD29. $MnCl_2$ served as technical control, which induce the maximum amount of the active conformation.

**Investigation of actin polymerisation:** phalloidin-staining was performed. Samples were visualized by confocal microscopy then analysed using ImageJ 1.48k program.

**Statistical analysis:** Wilcoxon-, Mann-Whitney-test or Spearman's correlation coefficient, by categorical variables (Rai, Binet stage) $X^2$-test was used for statistical evaluation using the SPSS software. Statistical significance was set at $p<0.05$.

# 4. Results

## The role of different CD23 expression in CLL prognosis

### Analysing the expression of CD23 isotypes

First, using conventional PCR two groups of CLL patients were determined based on the CD23 isotype expression. CLL1 cases expressed only the CD23a isotype, while in most of the cases (called CLL2 group) both CD23a and CD23b isotype was detected. Interestingly, analysing by quantitative real time PCR, CLL1 cases expressed also CD23a and CD23b. We found that relative expression of CD23a was higher than CD23b in all CLL cases. Comparing the CLL1 and CLL2 groups, significantly lower CD23a and CD23b level was detected by the CLL1 cases. However, CLL1 samples showed higher expression of CD23 isotypes, than MCL, MZL cells and normal B-cells.

### Immunophenotype of CLL1 and CLL2 cases

CLL1 cases showed lower CD23, higher CD38, CD20 and CD22 expression, comparing with CLL2 samples. Considering the overlap between the phenotype of CLL1 cases with the CD23 positive MCL cases, we examined the t(11;14) translocation in CLL1 cases by FISH to exclude the possibility that these cases represent MCL. All CLL1 cases were negative for t(11;14) translocation.

### Comparing the clinical data of CLL1 and CLL2 cases

CLL1 cases showed more aggressive disease course: they had more advanced Rai stage at diagnosis, higher ratio for patient treatment necessity and lower ALC, comparing with CLL2 group. However, the differences did not reach statistical significance.

### Prognostic rating of CLL1 and CLL2 cases

Analysing the presence of cytogenetic prognostic markers most of the CLL1 cases carried 12tri, while none of the CLL2 samples had 12 tri aberration.

## Investigating the role of high CD49d expression

### Correlation of CD49d expression with prognostic and microenvironmental markers

CD49d expression showed positive correlation with CD38, CD29 and CD19 levels. Regarding the expression of CD23 and CXCR4 significant negative relation was found with the level of CD49d.

### Analysing the direct survival role of CD49d

Comparing cases with different CD49d expression VCAM-1 stimulation did not inhibit spontaneous apoptosis either in $CD49d^+$ or in $CD49d^-$ samples. Mimicking the bone marrow microenvironment BMSC co-culture was used. BMSCs increased the viability of CLL cells. The protective effect of BMSCs was independent of the CD49d expression of CLL cells but showed correlation with CXCR4 level: CLL cells with $CD49d^+$ and low CXCR4 phenotype displayed increased sensitivity to apoptosis and to the protective effect of BMSCs.

### Investigating the proliferation inducing effect of CD49d-VCAM-1 interaction

According our data, either VCAM-1 or BMSCs did not induce proliferation of $CD49d^+$ or $CD49d^-$ CLL cells.

### Study of the active conformation of the CD49d/CD29 complex

According our results using high-affinity conformation specific antibody the CD49d/CD29 is in inactive, low-affinity state on the surface of CLL cells. But after stimulation by various agents (VCAM-1, CXCL12) the complex underwent activation process and high-affinity conformation was induced.

### Actin-polymerisation of $CD49d^+$ and $CD49d^-$ CLL cells upon VCAM-1 engagement

In $CD49d^+$ CLL cells greater F-actin formation was detected as adhered to VCAM-1-coated slides comparing with control (BSA) slides. CLL cells with $CD49d^-$ phenotype did not show significant alteration in actin reorganization after VCAM-1 contact.

**Immunophenotype change of CD49d$^+$ and CD49d$^-$ CLL cells after VCAM-1 treatment or in BMSC co-culture**

VCAM-1 did not induce phenotypical change in groups with different CD49d expression. BMSCs induced higher CD5, CD49d, CD44, CD19, CD126 level and decreased the expression of CXCR4. We did not find significant changes in the expression of CD38, CD80, CD86 and ROR1. Compared the two groups with different CD49d levels, greater increase in CD5 expression was observed after BMSC contact by CD49d$^+$ CLL cells.

**Analysing the clinical data of patients with different CD49d and CXCR4 expression**

The poor prognostic effect of CD49d was confirmed by our clinical data: CD49d$^+$ cases had more advance Rai and Binet stage at diagnosis, showed higher LDH level, had shorter time-to-treatment and showed greater ratio of treatment necessity and having cytogenetic aberrations comparing with CD49d$^-$ patients. Lower ALC was detected in CD49d$^+$ cases, but it did not reach statistical significance. Regarding the CXCR4 expression of CLL patients no significant difference was detected in any clinical parameters between the CXCR4$^{low}$ and CXCR4$^{high}$ cohorts.

## 5. Discussion

CLL is an indolent, non-Hodgkin's lymphoma, but a patient group is characterized by rapid progression and transformation into aggressive lymphoma. In our work we investigated the role of the expression of CD23 and CD49d prognostic markers in the progression of CLL cells.

We studied the level of CD23 isotypes in CLL cases with different CD23 expression. The level of CD23a was higher than CD23b in CLL, in other $CD23^+$ lymphomas (MCL, MZL) and in normal B-cells. According previous results, CD23a mediates survival, while CD23b enhances proliferation. The high CD23a and lower CD23b levels are in agreement with the well-known characteristics of CLL cells, which showed apoptosis-inhibition and limited proliferation in the peripheral blood. In a subset of CLL cases (CLL1) the CD23 isotypes can only be detected at a lower level and showed different immunophenotype as well: lower CD23, higher CD20, CD22 and CD38 expression, moreover most of the cases carried 12tri. It is well studied in CLL, that patients with 12tri have atypical phenotype, morphology and biological characteristics, hence this group may need different therapy. We suppose that the lower CD23 expression in combination with high CD20 and CD38 expressions, detected by flow cytometry may be a useful tool in predicting trisomy 12. Despite 12tri indicate intermediate prognosis, we found more aggressive disease course by the CLL1 cases. Previous reports showed that 12tri is associated with early progression and very aggressive course, moreover CD23 is negatively correlate with overall survival and bone marrow prolymphocyte ratio. During Richter transformation the phenotype of CLL cells can change: loss of CD5 and CD23 was reported. The decreased CD23 isotype expression together with 12tri detected in CLL1 cases represent the first step in loss of surface CD23 during transformation.

We found correlation between high CD49d expression and low CD23 level. Furthermore, CD49d showed positive correlation with the prognostic marker CD38 and CD29, which exists in the VLA-4 complex with CD49d. Positive correlation was detected with the B-cell co-receptor CD19, which can regulate the signalling of the CD49d/CD29 complex. We investigated the direct role of CD49d in preventing apoptosis, which is a main characteristic of CLL cells. We found that the CD49d does not mediate survival signals by VCAM-1 stimulation. Furthermore, we detected that the anti-apoptotic effect of BMSCs on CLL cells was independent of the CD49d expression. Along the missing

anti-apoptotic effect, the CD49d did not induce proliferation or immunophenotype change, but it is not a result of impaired VLA-4 activation. According our data, the CD49d/CD29 complex is expressed in inactive, low-affinity conformation on the surface of CLL cells but undergoes the normal activation process after ligand binding and other stimulation. In our experiments, VCAM-1 induced robust actin-remodelling in $CD49d^+$ CLL cells. CD49d is a key mediator of processes associated with cytoskeleton remodelling, like adhesion, migration and homing. Previous results reported greater migration and bone marrow homing capacity by the $CD49d^+$ CLL cells, comparing with $CD49d^-$ CLL cells. We found lower ALC at diagnosis by $CD49d^+$ CLL patients, but it did not reach statistical significance. The higher ALC can be explained by the greater migration and retention in the lymphoid tissues. We detected inverse correlation between CD49d and CXCR4 level. CLL cells with $CD49d^+$ and low CXCR4 expression showed higher spontaneous apoptosis rate. CLL cells are continuous recirculation between the peripheral blood and the tissue microenvironment, a more beneficial niche for malignant B-cells. CLL cells, which received anti-apoptotic and proliferation signals from the microenvironment may be more susceptible to the lack of the favouring stimuli. We suppose this "death by neglect" effect can be resulted in the observed high apoptosis rate of $CD49d^+$, low CXCR4 CLL cells, which might be a recently emigrated population from the supportive microenvironment. The protective effect of the stroma was independent from CD49d expression but showed correlation with the low CXCR4 level. According our data, the low CXCR4 expression may indicate the microenvironmental dependency, hence CXCR4 detection may be useful before microenvironmental-targeted therapy. The low CXCR4 expression does not have prognostic impact at diagnosis.

As summary, the CD49d prognostic marker may contribute to the aggressive disease of $CD49d^+$ cases by mediating migration and adhesion to the supportive microenvironment, and not by direct inhibition of apoptosis or induction of the proliferation of CLL cells.

**Statements about the CD23 molecule**:

1. CLL cells express CD23a and CD23b constitutively, the relative expression of CD23a is higher than the expression of CD23b.
2. Based on the lower CD23a and CD23b expression a subgroup of patients can be distinguished, which is characterized by lower CD23, higher CD38, CD20, CD22 expression, more aggressive disease course and frequent presence of 12tri.
3. The low CD23, high CD38, CD20 expression, detected by flow cytometry may be a useful tool in predicting 12tri.

**Statements about the CD49d molecule:**

4. VCAM-1, the ligand of CD49d, does not induce survival effect, proliferation or immunophenotype change, but resulted in actin-remodelling in $CD49d^+$ CLL cells. We suppose that the poor prognosis of $CD49d^+$ cases is caused by greater homing and adhesion to the microenvironment and not by preventing apoptosis.
5. The CD49d/CD29 is expressed in inactive conformation, but ligand binding and various stimuli (chemokines) induce the high-affinity state, the complex undergoes normal activation process.
6. CD49d shows inverse correlation with the CXCR4 chemokine receptor, which expression is associated with microenvironmental dependency, but does not have independent prognostic impact.

## 6. Publications

**Publications in context of the thesis:**

1. **Kriston C**, Bodor C, Szenthe K, Banati F, Bankuti B, Csernus B, Reiniger L, Csomor J, Matolcsy A, Barna G. Low CD23 expression correlates with high CD38 expression and the presence of trisomy 12 in CLL. Hematol Oncol. 2017;35(1):58-63. **IF: 3.193**

2. Mark A, Varga G, Timar B, **Kriston C**, Szabo O, Deak L, Matolcsy A, Barna G. The effect of microenvironmental factors on the development of myeloma cells. Hematol Oncol. 2017;35(4):741-5. **IF: 3.193**

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**Other publications:**

4. Jeney A, Hujber Z, Szoboszlai N, Fullar A, Olah J, Pap E, Mark A, **Kriston C**, Kralovanszky J, Kovalszky I, Vekey K, Sebestyen A. Characterisation of bioenergetic pathways and related regulators by multiple assays in human tumour cells. Cancer Cell Int. 2016;16:4. **IF: 2.74**

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