Original paper

The role of the CLL-1 protein in disease monitoring in patients diagnosed with acute myeloid leukaemia and myelodysplastic syndrome

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The normal subpopulation of CD34+CD38 – haematopoietic stem cells does not express CLL-1; therefore, the assessment of the expression of this protein can be used for the diagnosis of minimal residual disease. The aim of this study was to evaluate, using multi-colour flow cytometry, the level of CLL-1 protein expression on CD34+CD38- myeloid niche cells in acute myeloid leukaemia (AML) and myelodysplastic syndromepatients at the time of diagnosis and during disease monitoring on the example of 3 practical cases. The following conclusion was drawn: CD34+CD38-CLL-1+ cells in AML patients may serve as a biomarker to predict disease aggressiveness.

Key words: AML, CLL-1, MDS, multi-colour flow cytometry.

Introduction

Minimal residual disease (MRD) is defined as the presence of leukemic cells that remain after treatment without any clinical or haematological signs of disease. Numerous studies report a significant value of MRD in acute myeloid leukaemia (AML), which is used to assess the state of remission, determine the kinetics of response to applied therapies, or to identify potential relapse [1–5]. Currently, research is underway on the use of MRD in the treatment of AML [6]. The incidence of MRD has a strong correlation with recurrence rates and survival [1, 5, 7]. The immunophenotypic method of MRD detection using multiparameter flow cytometry is applicable to over 90% of AML patients [8–13]. The advantage of multi-colour flow cytometry (MFC) compared to molecular techniques is the quick analysis time [11]. A small population of leukemic stem cells (LSCs) may be responsible for AML relapse and chemotherapy resistance. Most current chemotherapy drugs eliminate the rapidly dividing leukaemic blasts that constitute the main mass of the leukaemic cells, leaving intact LSCs in the G0 phase of the cell cycle hidden in the myeloid niche [14–17]. Leukemic stem cells have unlimited self-renewal and they can produce large numbers of blast cells [18]. Identification and monitoring of LSCs in MRD improves the prognostic significance of the impact of MRD using both MFC and polymerase chain reaction (PCR) methods [13, 19]. Current European Leukemia Net (ELN) recommendations are to use MFC as the method of choice in the absence of a standardised molecular target [20–23].

The CLL-1 protein (C-type lectin-like molecule 1) is associated with the cell subpopulation responsible for disease relapse. The malignant nature of the CD34+CD38-CLL-1+ subpopulation was confirmed by studies involving the transplantation of leukaemic CD34+CD38-CLL-1+ cells of human origin into NOD/SCID mice, which resulted

in the development of leukaemia in these animals. The normal subpopulation of CD34+CD38- haematopoietic stem cells (HSCs) does not express CLL-1; therefore, the assessment of the expression of this protein can be used for the diagnosis of MRD. The presence of CLL-1 on AML cells is probably permanent; it is detected before treatment (diagnostic test) and in subsequent MRD tests [19, 24–28].

According to the literature, the identification of MRD-LSC cells in AML patients anticipates overt clinical relapse by up to 3 months, which potentially provides time to prepare for allogeneic transplantation or inclusion of the patient in a clinical trial [29]. The identification of CD34+CD38-CLL-1+ cells also makes sense among myelodysplastic syndrome (MDS) patients, viewing them as a group particularly vulnerable to the development of AML. In the presented work, the MFC method was used to detect LSC with the immunophenotype CD34+CD38-CLL-1+. Identification of LSCs was based on positive expression of the surface marker CLL-1, which is absent in the CD34+CD38- compartment on healthy bone marrow cells. We present the practical use of individual cytometric parameters to assess disease recurrence in 3 clinical cases.

Material and methods

The study was conducted on bone marrow samples of 3 patients treated at the Department of Haematology and Transplantology University Clinical Hospital 1 Pomeranian Medical University (PUM) in Szczecin, who were diagnosed with AML or MDS between March 2018 and March 2020. The diagnosis was established on the basis of morphological, cytochemical, immunophenotypic, cytogenetic, and/or molecular tests, in accordance with the World Health Organisation 2016 classification, from excess samples of material used as part of the diagnostic process at the Department of Pathology SPSK1 PUM in Szczecin in the years 2018–2020. In this project, for detection of LSC with the immunophenotype CD34+CD38-CLL-1+, the MFC method was used. Identification of LSCs was based on positive expression of the surface marker CLL-1, which is absent in the CD34+CD38- compartment on healthy bone marrow cells. In each case, the test material consisted of 1 mL of bone marrow collected into a tube with ethylenediaminetetraacetic acid anticoagulant. Samples for MFC analysis were processed within 24 hours of collection. Cells of the prepared bone marrow were analysed in a BD FACS Canto II flow cytometer equipped with 3 lasers: L1-solid state with a wavelength of 488 nm, L2-HeNe with a wavelength of 633 nm, and L3-solid state with a wavelength of 405 nm. To determine which bone marrow cell populations express the CLL-1 antigen and which bone marrow cells could serve as a negative internal control for the tested CLL-1 antigen, the main cell populations were gated using the SSC/CD45 cytogram and then their mean fluorescence intensity (MFI) value relative to the CLL-1 antigen was calculated, comparing it to the MFI value of the CLL-1 isotype control [30, 31]. The CLL-1 antigen MFI of the study populations was then illustrated compared to the CLL-1 isotype MFI for the same populations. The lymphocyte population and the CD45- cell population was used as an internal control for the CCL-1 antigen [26, 31].

Results

Case report 1

A 71-year-old patient diagnosed with AML with cytogenetic abnormalities AML t(8.21) (q21;q22.1), with an unfavourable prognosis (AMLg)

Figures 1–5 show the expression level of the analysed parameters. The X-axis shows the analysed parameter; the Y-axis shows the time in which the bone

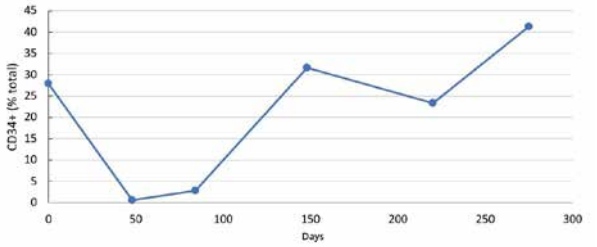

Fig. 1. Percentage of CD34+ blasts (% total) in a patient with acute myeloid leukaemia during subsequent examinations

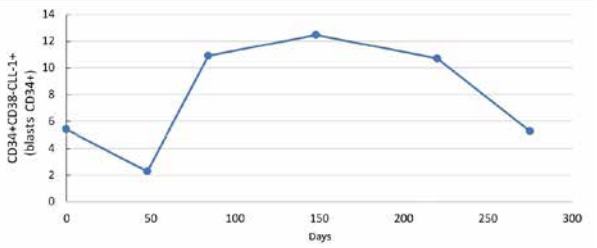

Fig. 2. Expression level of CD34+CD38-CLL-1+ cells (% CD34+ blasts) in a patient with acute myeloid leukaemia during subsequent examinations

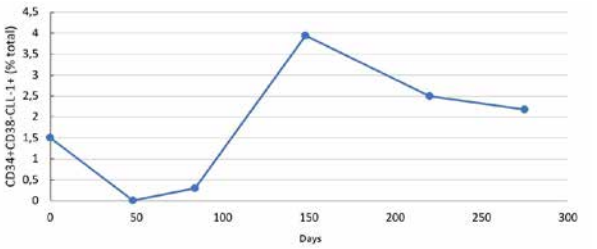

Fig. 3. Expression level of CD34+CD38-CLL-1+ cells (% total) in a patient with acute myeloid leukaemia during subsequent tests

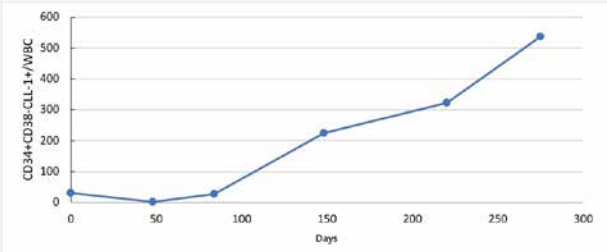

Fig. 4. Expression level of CD34+CD38-CLL-1+/WBC cells in a patient with acute myeloid leukaemia during subsequent tests

WBC – white blood cells

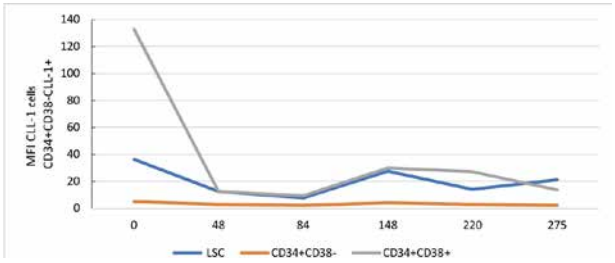

Fig. 5. Mean fluorescence intensity CLL-1 level of CD34+CD38-CLL-1+ cells in a patient with acute myeloid leukaemia during subsequent tests

MFI – mean fluorescence intensity, LSC – leukemic stem cells

marrow examination was performed. The first bone marrow examination in which AMLg was diagnosed was considered point zero, and each subsequent point indicates the number of days until the next examination. A red line has been drawn in Figures 1, 3, 4, cutting off the percentage of blasts below 5%, which means morphological remission. The percentage of CD34+ blasts in relation to the entire bone marrow cell population in the first study indicated the diagnosis of AML (blast percentage above 20%). The second and third tests suggest morphological remission (blast percentage below 5%). The remaining studies showed high CD34+ values (% total), indicating AML recurrence (Fig. 1).

The percentage of CD34+CD38-CLL-1+ cells, relative to blasts expressing CD34+, at the time of AML diagnosis was ~5%. During the second examination (considered a morphological remission), the percentage of analysed cells was twice as low. In the third, fourth, and fifth examinations, the level of CD34+CD38-CLL-1+ cells (% CD34+ blasts) was twice as high as at diagnosis, suggesting AML recurrence (Fig. 2).

All bone marrow examinations of this patient confirmed the presence of CD34+CD38-CLL-1+ cells (% total), the percentage of which was below 5%, indicating their low diagnostic value in the assessment of remission with a blast cutoff threshold of 5% (Fig. 3).

The number of CD34+CD38-CLL-1+ cells in 1 μl of bone marrow, expressed as the CD34+CD38-CLL-1+/white blood cells (WBC) parameter, was ~ 30 cells/μl in the first study. In the second study (considered morphological remission), it dropped to the level of 1 cell/μl. The third test (also considered morphological remission) showed another increase in the number of analysed cells to a level close to the diagnosis of ~28 cells/μl, which suggested AML recurrence. Each subsequent study showed high values of the analysed parameter, and thus no remission (Fig. 4).

The CLL-1 MFI values of CD34+CD38-CLL-1+ cells were at a similar intensity level to CD34+CD38+ cells, suggesting the stability of the CLL-1 antigen at every stage of the study (Fig. 5).

The patient with acute myeloid leukaemia with an unfavourable prognosis achieved morphological remission after chemotherapy with the percentage of CD34+ blasts in relation to the entire bone marrow cell population (% total) below the threshold of 5% in the second and third examination. In each subsequent bone marrow examination, the percentage of CD34+ cells (% total) exceeded the threshold of 5%, which indicated the recurrence of the disease. The percentage of CD34+CD38- myeloid niche cells (% total) was parallel to the CD34+ population (% total) in all available bone marrow studies. Particular attention was paid to the second and third bone marrow examinations, which revealed morphological remission. At the time of the third examination, molecular MRD was also determined at the level of 0.01%, which confirmed complete remission, although the disease recurred after a month. In the third control study, the percentage of myeloid niche cells with the CD34+CD38- immunophenotype, in relation to the percentage of blasts expressing CD34+ (% CD34+ blasts), increased fourfold compared to the second study and reached a level similar to the diagnosis of AMLg, which means that the level of myeloid niche cells increased significantly. Similarly, the percentage of LSCs defined as CD34+CD38-CLL-1+ (% CD34+ blasts) in the third study increased noticeably compared to the percentage of these cells at diagnosis. This allows us to observe an increase in the percentage of CD34+CD38-CLL-1+ cancer cells in the third test, which was considered a complete remission in the molecular test. LSC cells constitute a negligible population in MRD studies, and the interpretation of the CD34+CD38-CLL-1+ population in relation to the entire population of bone marrow cells (% total), taking into account the 5% threshold (morphological remission), is difficult. In the third bone marrow examination, the percentage of CD34+CD38-CLL-1+ cells (% total) did not exceed the threshold of morphological remission; therefore, it did not clearly indicate recurrence. Only the level of LSC expressed as the number of CD34+CD38-CLL-1+ cells in 1 μl of bone marrow with the adopted cut-off threshold of 0.03% showed that in the third bone marrow collection (considered as remission) the number of LSC cells reached the level before diagnosis. This means that the number of malignant cells increased until

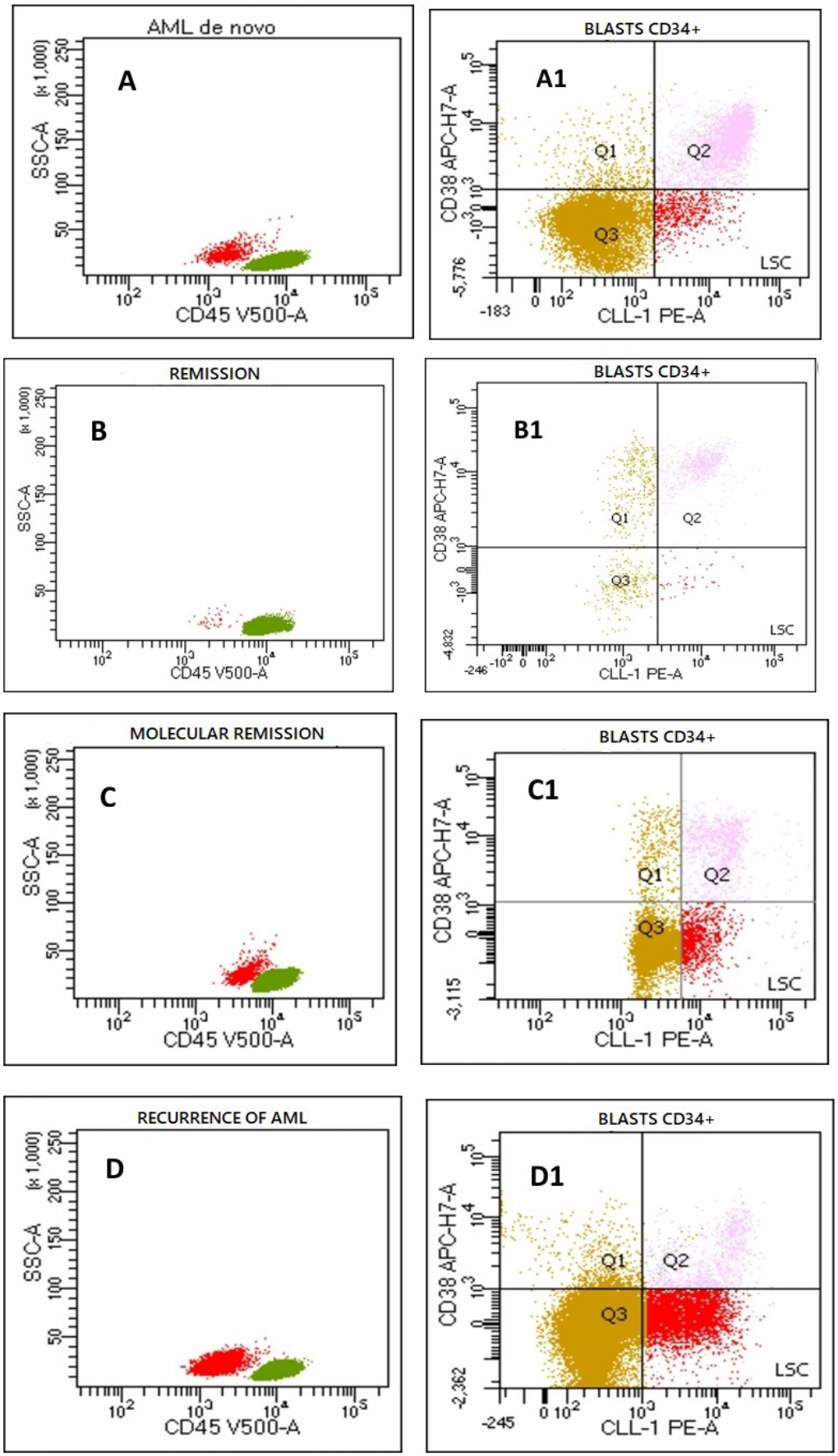

Fig. 6. Expression pattern of CD34+ blasts at different stages of acute myeloid leukaemia (AML) disease of a relapsed patient. The lower right quadrant (red) consists of CD34+CD38-CLL-1+ cells. A, A1) Diagnosis of AML. B, B1) Morphological remission. C, C1) Molecular remission. D, D1) Recurrence of AML. CD34+CD38-CLL-1+ cells are visible at all stages of the disease

AML – acute myeloid leukaemia

AML was diagnosed, which indicates a poor prognosis of the disease despite the lack of morphological symptoms. In subsequent studies (studies 4, 5, 6) the malignant cells could not be eliminated – LSCs were still present. The mean fluorescence intensity of the CLL-1 molecule of LSCs in all bone marrow studies was similar to the MFI of the CLL-1 molecule of CD34+CD38+ progenitor cells and remained relatively stable, suggesting the stability of the CLL-1 antigen at all stages of disease [31–33].

The cytometric expression pattern of CD34+CD38-CLL-1+ cells at various stages of the disease of the described AMLg patient, who experienced remission of the disease and then relapsed of AMLg, is shown in Figure 6.

Case report 2

A 63-year-old patient diagnosed with myeloproliferative diseases (MPN) developed AML after a period of 2 years.

Figure 7 shows the results obtained in the study. The X-axis shows the analysed parameter, and the Y-axis the year in which the bone marrow examination was performed. In graphs relating to total leukocytes, values below the red line indicate morphological remission. The percentage of CD34+ blasts and the percentage of CD34+CD38- myeloid niche cells relative to all bone marrow cells, and CD34+CD38- myeloid niche cells relative to CD34+-expressing cells, increased markedly between the diagnosis of MPN and AML. Similarly, there was a significant increase in the percentage of malignant CD34+CD38-CLL-1+ cells, relative to the following: total bone marrow cells, CD34+ expressing cells, and the number of LSCs in 1 μl of bone marrow, at the time of AML.

The cytometric expression pattern of CD34 +CD38-CLL-1+ cells at different stages of the disease of a patient with MPN who developed AML is shown in Figure 8.

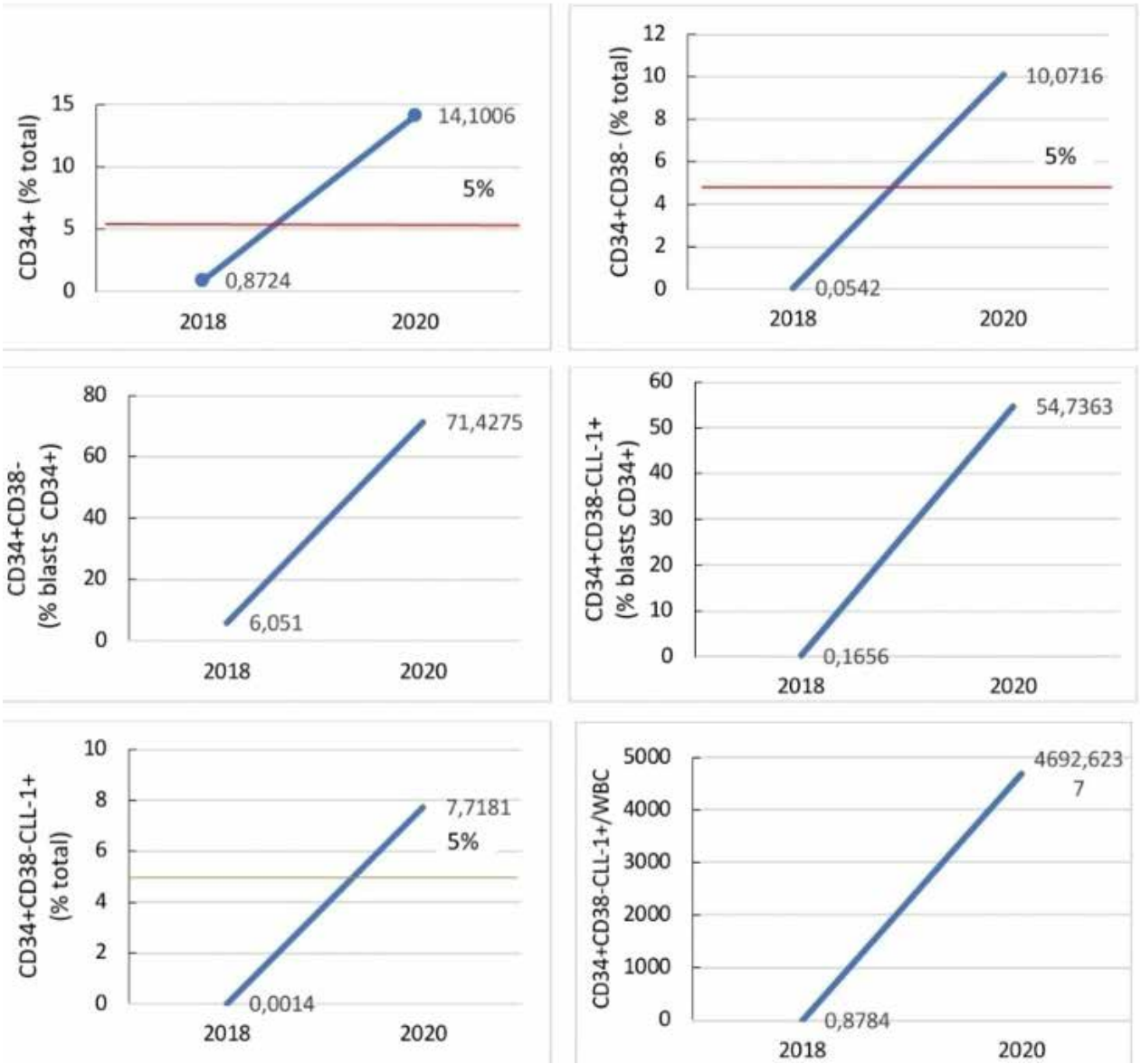

Fig. 7. Values: CD34+ (% total), CD34+CD38- (% total), CD34+CD38- (% CD34+ blasts), CD34+CD38-CLL-1+ (% total), CD34+CD38-CLL-1+ (% CD34+ blasts), CD34+CD38-CLL-1+/WBC in a patient with myeloproliferative diseases who developed acute myeloid leukaemia

WBC – white blood cells

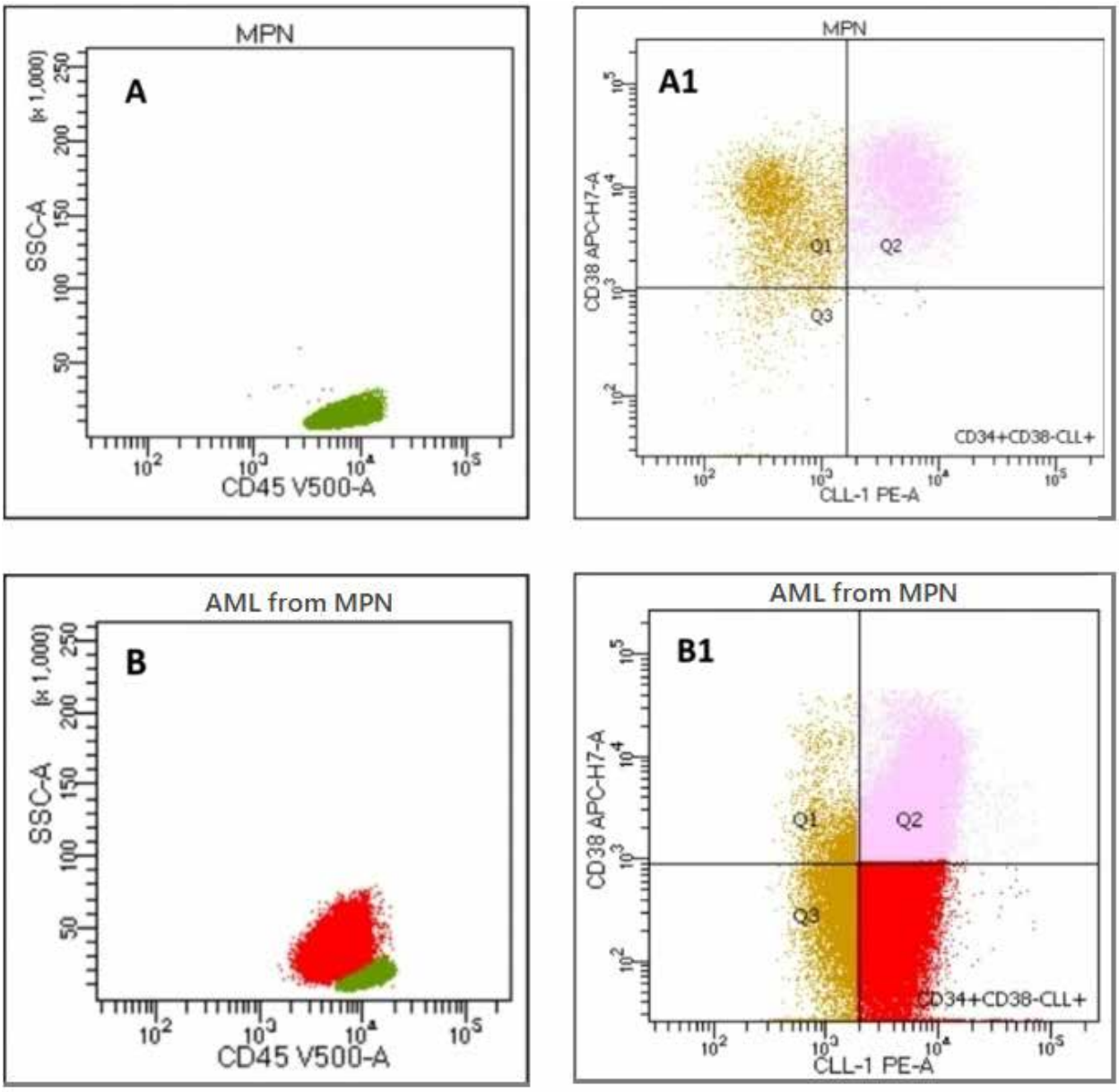

Fig. 8. Expression pattern of CD34+ blasts at different stages of the disease in a patient with myeloproliferative diseases (MPN) who developed acute myeloid leukaemia (AML). The lower right quadrant (red) consists of CD34+CD38-CLL-1+ cells. A, A1) Diagnosis of MPN. B, B1) Diagnosis of AML (after 2 years)

AML – acute myeloid leukaemia, MPN – myeloproliferative diseases

Case report 3

A 71-year-old patient diagnosed with myelodysplastic syndrome with ring sideroblasts (MDS-RS) with progression to AML.

Figure 9 shows the results obtained in the study. The X-axis shows the analysed parameter, and the Y-axis is the year in which the bone marrow examination was performed. In the graphs referring to total leukocytes (% total), values below the red line indicate morphological remission. The percentage of CD34+ blasts and the percentage of CD34+CD38- myeloid niche cells increased markedly between the diagnosis of MDS and AML, relative to all bone marrow cells. The percentage of CD34+CD38- myeloid niche cells also increased relative to cells expressing CD34+. There was an increase in the percentage of malignant CD34+CD38-CLL-1+ cells in relation to the following: total bone marrow cells and the actual number of LSC cells in 1 μl of bone marrow in MDS before the development of AML. Only the percentage of CD34+CD38-CLL-1+, relative to cells expressing CD34+, decreased slightly in AML compared to MDS.

The cytometric expression pattern of CD34+CD38-CLL-1+ cells at various stages of the disease of the described patient with MDS-RS with progression to AML is shown in Figure 10.

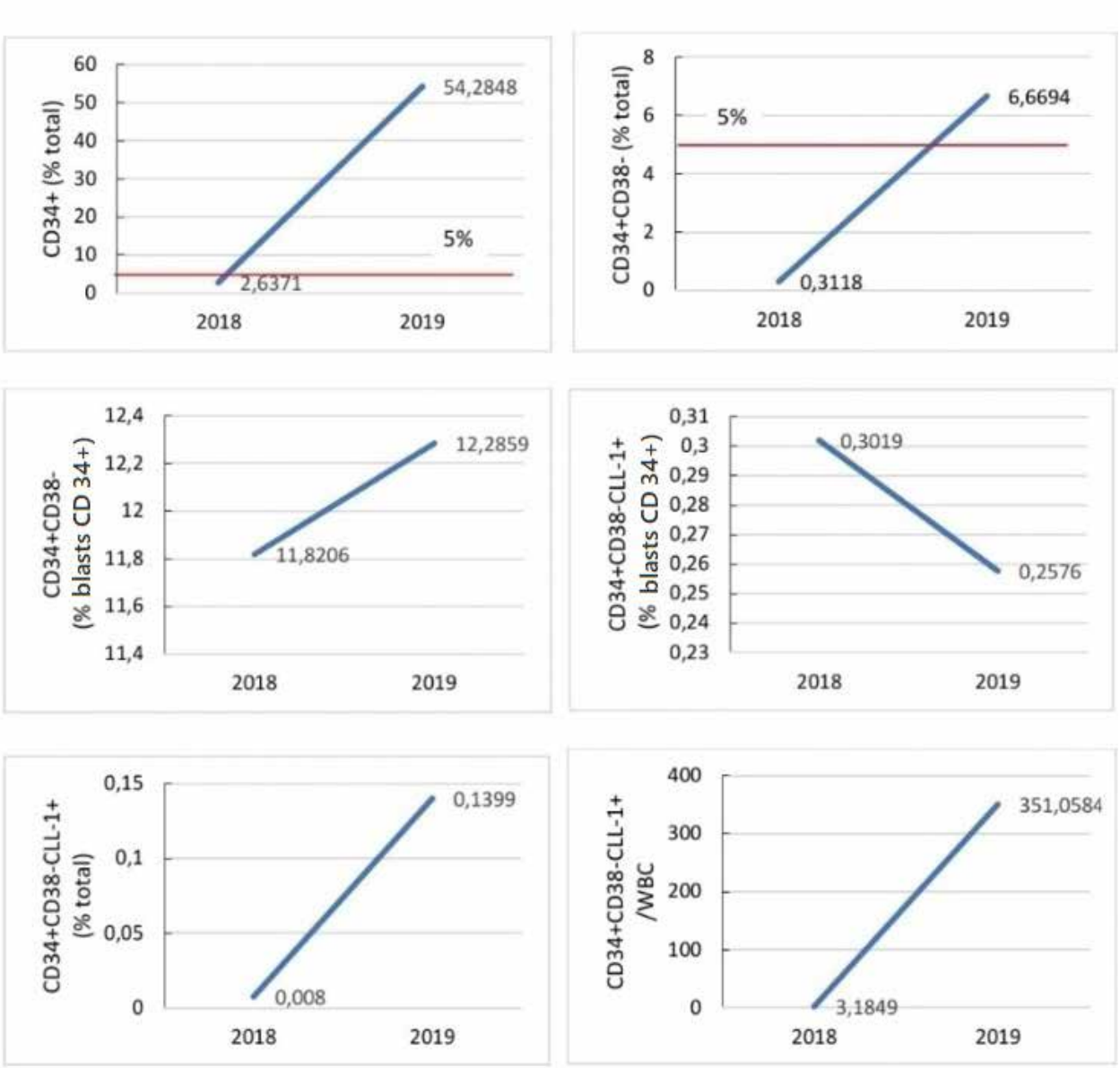

Fig. 9. Values: CD34+ (% total), CD34+CD38- (% total), CD34+CD38- (% CD34+ blasts), CD34+CD38-CLL-1+ (% total), CD34+CD38-CLL-1+ (% CD34+ blasts), CD34+CD38-CLL-1+/WBC in a patient with myelodysplastic syndrome with ring sideroblasts with progression to acute myeloid leukaemia

MDS – myelodysplastic syndrome, WBC – white blood cell

Discussion

To correctly identify LSCs, determine their prognostic significance, and precisely determine the percentage of these cells at diagnosis and during treatment monitoring, it is extremely important to precisely identify the features that distinguish LSCs from HSCs. To detect LSC cells, an antibody against CLL-1 protein was used in the presented work.

CLL-1 protein is absent on HSCs and in the regenerating bone marrow after chemotherapy. This property is very useful in identifying MRD cells using this single marker. Many researchers have confirmed the usefulness of the CLL-1 antibody for detecting LSCs in AML both at diagnosis and during treatment [11, 19, 31, 34]. The role of the CLL-1 protein in MDS is not fully understood. In one study on the identification of CLL-1 cells in the CD34+CD38- compartment in MDS patients, their presence was found in 71% of all MDS subtypes and in all IPSS-R risk groups [35]. The results of long-term colony-initiating cell assays in this study showed that although CD34+CD38-CLL-1+ cells were self-renewing and atypical, analysis of CLL-1 expression did not help distinguish HSCs from malignant stem cells because the clonal abnormalities associated with MDS was also detected in the CD34+CD38-CLL-1- subpopulation. Differences between MDS and AML regarding

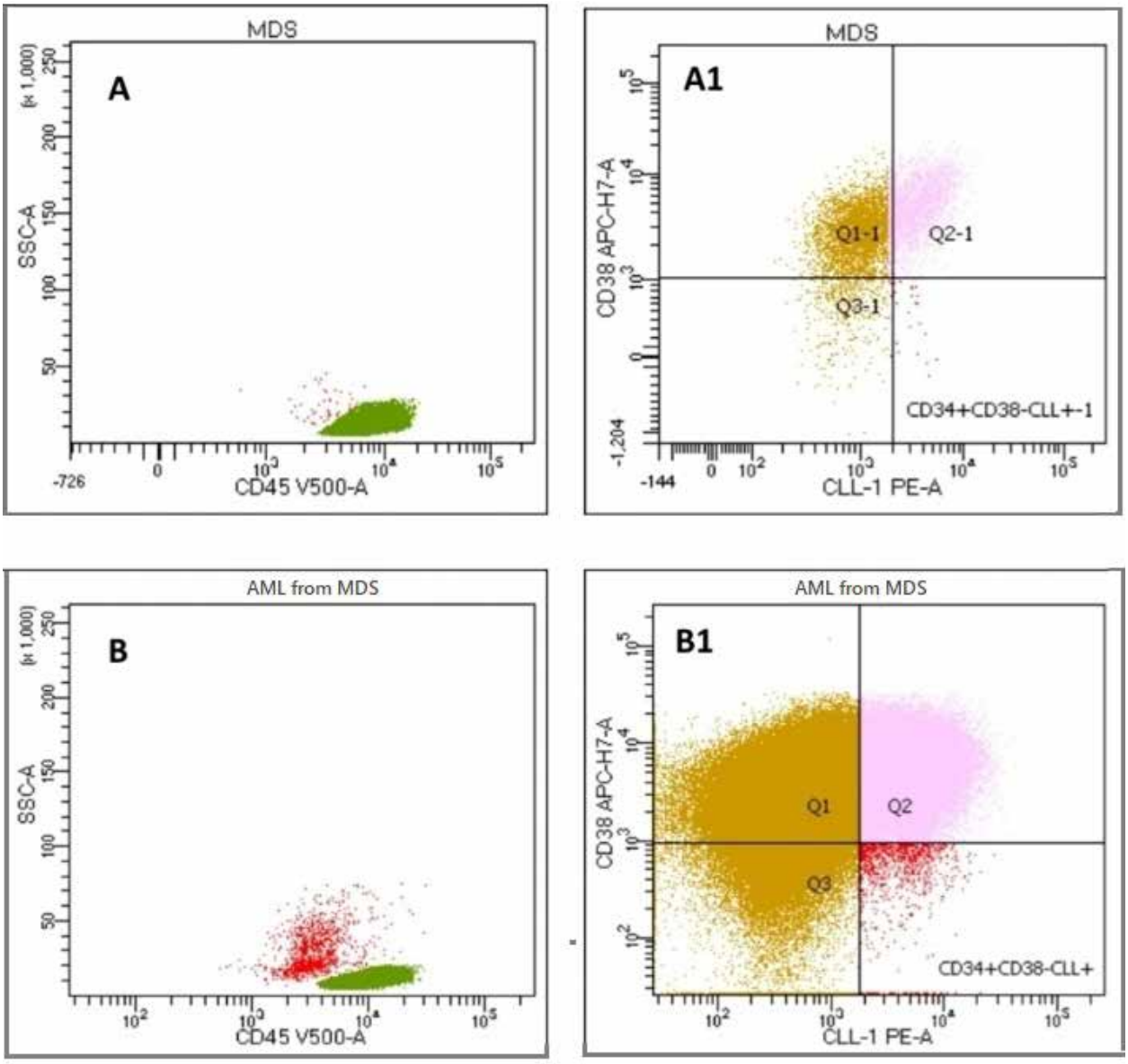

Fig. 10. Expression pattern of CD34+ blasts in a patient with myelodysplastic syndrome with ring sideroblasts (MDS-RS) with progression to acute myeloid leukaemia (AML). The lower right quadrant (red) consists of CD34+CD38-CLL-1+ cells. A, A1) Diagnosis of MDS-RS. B, B1) Diagnosis of AML

AML – acute myeloid leukaemia, MDS – myelodysplastic syndrome

the identification of LSC cells using the CLL-1 protein in the group of CD34+ CD38– cells may result from differences in the biology of these 2 disease entities. Woll *et al*. showed that MDS comes from the earliest stem cell (Lin-CD34+CD38-CD90+), which was also described by other researchers [36–38]. Meanwhile, recent evidence suggests that most AML cases originate from more mature progenitor cells that have acquired the ability to self-renew [39, 40]. In another study conducted by Morsink *et al*., the overall expression of CLL-1 on CD34+CD38- cells among MDS EB1/EB2 patients did not differ from the control group ($p = 0.50$) [41]. Identification of CD34+CD38-CLL-1+ cells using the MFC method in our own studies showed high sensitivity and accuracy comparable to the PCR technique. Analysis of an AML case including diagnosis, remission and relapse using the MFC method revealed the presence of LSC cells in the stage of complete remission, confirmed by PCR tests. While molecular studies did not confirm the presence of LSCs, the MFC method proved to be sufficient. It has been shown that CD34+CD38-CLL-1+ cells are indeed aggressive and predict disease recurrence in the remission phase. The presence of the unique CLL-1 marker in the LSC population in the group of CD34+CD38- cells in the absence of its presence on HSCs has generated great enthusiasm in the field of haematology and oncology. The efforts of many researchers have been di-

rected towards creating a drug that targets CLL-1-positive cells. Currently, the most advanced work on bispecific MCLA-117 antibodies and T-CAR cells is at the stage of early clinical trials [41–43]. Novel therapies are an opportunity for patients with a very poor prognosis who probably do not tolerate high-dose treatment (conditioning). CLL-1 CART cells could potentially be used as a post-chemotherapy consolidation regimen to eliminate MRD prior to all-HSCT or as an adjunct to chemotherapy [44, 45]. It is extremely important to select patients who may benefit from this type of treatment. Many authors report that in the case of CLL-1-directed therapy, it may be necessary to select CLL-1-positive patients [8, 26, 32, 46-48].

Conclusions

The aim of this study was to evaluate, under strictly diagnostic conditions, the level of CLL-1 protein expression on CD34+CD38- myeloid niche cells in AML and MDS at the time of diagnosis and during disease monitoring. Our own research, similarly to other reports, shows that CD34+CD38-CLL-1+ cells in AML patients may serve as a biomarker to predict disease aggressiveness [18]. CLL-1 expression present on AML cells without significant differences in antigen density qualifies CLL-1 as a stable marker for disease monitoring [26, 49]. According to the ELN AML MRD 2018 recommendations, CLL-1 is an appropriate marker for monitoring and predicting the risk of AML recurrence [23, 34].

Disclosures

1. The study received approval from the Research Ethics Committee of the Pomeranian Medical University in Szczecin (no. KB-0012/158/17).
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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