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Enhancing detection of central nervous system involvement in multiple myeloma: A novel multidimensional dot-plot based analysis for flow cytometry

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Abstract

Central nervous system (CNS) involvement in multiple myeloma (MM) is a rare but severe complication with a poor prognosis. The identification of malignant plasma cells in cerebrospinal fluid (CSF) is essential for early diagnosis and intervention. However, the sensitivity of traditional diagnostic methods like cytology is low, especially in samples with low-cell counts. This study aimed to develop a multidimensional radar dot-plot analysis using Kaluza software to enhance the sensitivity and specificity of flow cytometry for detecting abnormal plasma cells in CSF. One hundred and twenty-five CSF samples were sent for flow cytometric testing to investigate the central nervous system involvement of MM. Finally, 89 samples from 40 patients were included in our study. Multicolor flow cytometry was performed using an 8-color labeling method, and radar dot-plot analysis was developed using diagnostic bone marrow samples to distinguish normal plasma cells, abnormal plasma cells, and cellular debris. The sensitivity of the novel method was evaluated by diluting myeloma bone marrow cells in pooled CSF samples to simulate low cell counts. Of the 125 CSF specimens, 16 samples from 4 patients showed abnormal plasma cells using both conventional and multidimensional flow cytometry analysis. Discordant results were found in 32 samples (25%), where conventional analysis suggested the presence of abnormal cells, but these were ruled out by multidimensional analysis. Sensitivity testing showed that the multidimensional dot-plot method outperforms conventional two-dimensional dot-plot analysis, as the radar dot plot can be used to identify abnormal cells in samples diluted to 5 WBC/μL, where the cell count of abnormal plasma cells is < 1 cell/μL. Our results showed that the new radar dot-plot analysis can increase the sensitivity and specificity of flow cytometry in MM for the detection of CNS

involvement, even in low-cell-count CSF samples, regardless of whether the sample was obtained in a tube containing special reagent or not (TransFix/EDTA CSF Sample Storage tubes). This approach improves diagnostic accuracy, reduces the number of false positive cases caused by antibodies adhering to cell debris, and provides a reliable tool for assessing neurological complications in MM. Further validation is needed in a larger number of cases and testing of the method on different antibody panels.

KEYWORDS
central nervous system involvement, cerebrospinal fluid, flow cytometry, integrated data visualization, multidimensional dot-plot, multiple myeloma

1 | INTRODUCTION

Flow cytometry plays a crucial role in the diagnosis of hematological malignancies. It allows detailed analysis of individual cell populations in different biological samples, including cerebrospinal fluid (CSF). In multiple myeloma, the identification and quantification of malignant cells in CSF is important for the diagnosis of central nervous system (CNS) involvement, a serious complication of the disease. CNS myeloma has a poor prognosis; the overall survival is 7 months or less (Egan et al., 2020, Jurczyszyn et al., 2016, Rajkumar & Kumar, 2020).

CNS involvement occurs in less than 1% of patients with MM (Marini et al., 2014).

There is no definitive guideline for how to assess central nervous system involvement of MM in most studies using flow cytometry (Egan et al., 2020, Varga et al., 2018), morphology and/or imaging (Chen et al., 2013; Dias et al., 2018; Fassas et al., 2004).

The use of flow cytometric analysis in hematological malignancies can increase the sensitivity and specificity of pathological cell detection in CSF samples (Kraan et al., 2008; Ranta et al., 2014). In the CSF sample, normal plasma cells most often arise in the presence of infection, which can be well distinguished from plasma cells with abnormal phenotypes in myeloma by flow cytometry (Péter, 1967).

The efficiency of the identification of abnormal cell populations can be further enhanced if the flow cytometric assay is based on integrated data visualization in addition to traditional two-dimensional dot plot analysis. The radar dot-plot in the Kaluza software (Beckman Coulter, Brea, CA, USA) allows all markers in a tube to be displayed simultaneously. Previous studies have shown that each population of cells is in a specific location. Thus, it is possible to develop analysis protocols where the position of pathological and normal cell populations is clearly distinguished (Gupta et al., 2021; Jafari et al., 2018; Kárai et al., 2019; Kárai et al., 2021).

In the present study, we aimed to develop an analysis system based on radar dot plot to identify abnormal plasma cells. Since CSF samples often have a low cell count, the proportion of cell debris among the collected events is higher. Cell debris can mimic the immunophenotype of abnormal cells by attaching antibodies to cell particles, thus providing false signal. Therefore, in our study, we specifically investigated the location of cell debris and abnormal plasma cells in the radar dot plot.

2 | MATERIALS AND METHODS

2.1 | Study design and flow cytometry

One hundred and twenty-five cerebrospinal fluid (CSF) samples were sent to the Department of Laboratory Medicine from the Clinical Center at the University of Debrecen, between January 2018 and August 2023 for flow cytometry examination. Thirty-six samples had to be excluded from the study because flow cytometry analysis was not performed by a standard panel, but a unique antibody combination was developed based on their diagnostic samples. Thus, 89 samples from 40 patients could be retrospectively reanalyzed (mean age 69 ± 8; 20 male/20 female). The most common reasons for requesting a flow cytometry examination were neurological symptoms: limb weakness/walking instability (9 cases), headache and nausea/vomiting (7 cases), visual impairments (5 cases), speaking problem (3 cases). In some patients, imaging findings indicated the need for flow cytometry (7/40 patients). None of the patients were diagnosed with plasma cell leukemia. None of the patients had received anti-CD38 therapy prior to flow cytometry analysis of the CSF sample. Samples from the University were received in CSF sampling tubes without special transport media (Vacuette Z No Additivie Tube, Greiner Bio-One GmbH, Austria; SarStedt Monovette, SarStedt AG, Germany) and processing started within 1 h (12/89 samples). The remaining samples were from other cities (77/89 samples), so to protect the integrity of the white blood cells, samples were collected in TransFix/EDTA CSF Sample Storage Tubes (Cytomark, Caltag Medsystems, Buckingham, UK) and sent to our laboratory. In these cases, the average time between lumbar puncture and cytometric CSF analysis was 24 h (range from a few hours to 5 days). The median amount of CSF sample was 3 mL (1–4.7 mL). White blood cell count (WBC) was determined on Sysmex XN2000 (Sysmex, Kobe, Japan) hematology analyzers in body fluid mode. If the white blood cell count was < 10 cells/μl, cell counting was performed manually in a Fuchs Rosenthal chamber. The red blood cell (RBC) count was also determined manually in all cases. In all cases, the complete sample was processed as follows: The whole CSF sample was centrifuged at 1200 RPM for 5 min. The supernatant was removed, and the precipitate was washed with 1 mL of bovine serum albumin phosphate buffered saline (BSA-PBS) for the intracytoplasmic tube and PBS for the surface tube. The sample was then centrifuged

TABLE 1 Antibody combinations used in flow cytometric examination for the diagnosis of multiple myeloma.

	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7	PB	PO
Tube 1	CD38	CD56	CD27	CD19	CD117	CD81	CD138	CD45
Tube 2	cyκ	cyλ	CD28	CD19	CD38	CD20	CD138	CD45

Note: Tube 1 is used to label surface antigens, tube 2 contains the intracellular antigens.
Abbreviations: APC, allophycocyanin; APC-H7, conjugation allophycocyanin-alexa fluor H7; FITC, fluorescein isothiocyanate; PB, pacific blue; PC7, phycoerythrin cyanin 7; PE, phycoerythrin; PerCP-Cy5.5, peridinin chlorophyll protein 5.5; PO, pacific orange.

again. The supernatant was removed, and the precipitate was resuspended in 0.2–0.3 mL PBS.

After that, CSF samples were examined routinely by 8 color labeling method in two tubes, one for the labeling of surface antigens (Tube 1), and one for the intracellular antigens (Tube 2). The antibodies we examined are shown in Table 1. CD38, CD27, CD117, CD81, κ/λ, CD28, CD38, and CD20 were purchased from Beckton Dickinson (San Jose, CA, USA); CD19 and CD138 were purchased from Beckmann Coulter (Marseille, France); CD56 was purchased from Dako (Santa Clara, CA, USA); CD45 was purchased from Exbio (Praha, Czeh Republic).

The measurements were performed on a FACS Canto II flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) at a low collection rate. In all cases, the whole sample was measured. A mean of 2875 (621–300,000 events) was collected in the first tube and 4508 (1150–300,000 events) in the second tube. Multidimensional analysis was performed using Kaluza Software (Beckman Coulter, version 1.2).

2.2 | The development of multidimensional dot-plot analysis

Diagnostic bone marrow samples from patients with MM were used to develop a multidimensional dot-plot based analysis. First, two-dimensional dot plots were applied to select singlets (FSC-A/FSC-H). Plasma cells are defined as CD38+/CD138+ cells, then CD19 expression was used to separate normal (CD19+) and abnormal plasma cells (CD19-). Forward, side-scattered light and CD56, CD117 were used to clearly identify the selected cells. In the tube with intracellular labeling, light chain expression helped to identify abnormal cells. The cell populations were then visualized on a radar dot-plot of Kaluza software. Since it is possible to change the number and position of the parameters of interest, we looked for the setting where abnormal and normal plasma cells, and cellular debris were best separated. For the final analysis system, six parameters were considered for both the first and second tubes, in addition to the intensity of light scattered forward and sideways. For ease of analysis, we set the axes so that the abnormal cells in Tube 1 were on the left and in Tube 2 at the bottom. Cell debris was placed on the right side in Tube 1 and on the top in Tube 2 (Figure 1). Since CD56, CD117 expression may differ in myeloma cells, our analysis protocol was evaluated on several bone marrow samples where abnormal cells expressed these markers differently. Considering that the positions of CD56-/CD117- myeloma cells and cell debris on the radar dot-plot overlapped, a second radar dot-plot was also constructed on Tube 1. On this one, the myeloma cells are positioned on top, while the cell debris is on the bottom (Figure 2).

Using the developed radar dot-plot, the CSF samples were retrospectively reanalyzed. For patients where diagnostic bone marrow samples were available ($n = 23$ patients), we compared the positions of abnormal cells in the radar dot-plot for bone marrow and CSF samples.

2.3 | Investigation of the sensitivity of multidimensional dot-plot based analysis

To evaluate the sensitivity of the multidimensional dot-plot-based assay, we used a bone marrow sample from a patient with MM who had a remaining sample after routine diagnostic tests. The percentage of myeloma cells was 17.2% in the BM of this patient. Mononuclear cells were separated from the sample using Ficoll- Histopaque (Sigma Aldrich, Darmstadt, Germany). The cell count was then adjusted to 10×10^9/L with phosphate-buffered saline (PBS) working solution. The remaining CSF samples from three patients after routine diagnostic tests were pooled. The pooled sample was colorless, clear, with a white blood cell count of 1 cell/μL. The pooled CSF sample was then used to dilute the mononuclear cell count to 100, 50, 10, 5, 1 cell/μL. These samples were labeled using the 8-color labeling technique also used in routine diagnostics. Measurements were performed on FACS Canto flow cytometer (Beckton Dickinson Biosciences, San Jose, CA, USA). Samples were analyzed using conventional two-dimensional dot-plot and the new multidimensional dot-plot based protocol (Supplementary Figure 1).

Normal distribution was tested using the Shapiro–Wilk test. P values < 0.05 were considered as associated with statistical significance. Statistical calculations and descriptive statistics were carried out using GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA).

2.4 | Impact of preanalytical factor on multidimensional dot-plot analysis

Most of the samples were received in TransFix/EDTA CSF Sample Storage tubes (77/89 samples), and therefore we investigated whether the fixative in the tube affected the position of the abnormal cells on radar dot plots. Mononuclear cells were separated from a

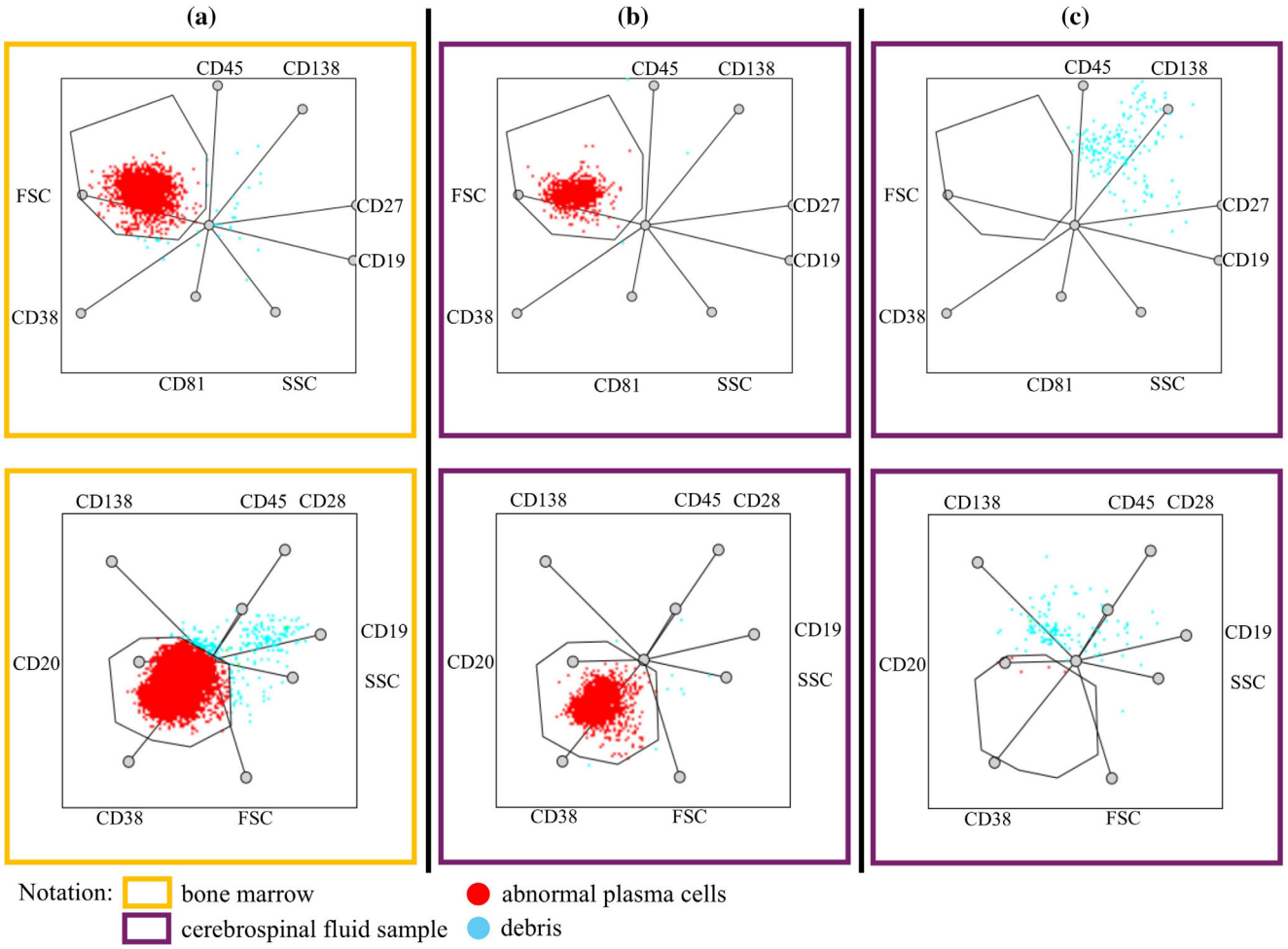

FIGURE 1 Multidimensional dot plot for surface and intracellular tubes to distinguish pathological plasma cells from debris. The multidimensional analysis pattern of the bone marrow sample is shown in a yellow frame; the CSF sample is shown in a purple frame. Red color indicates abnormal plasma cells, and blue color represents debris. The B column shows the result of a CSF sample where pathological plasma cells could be identified. In the sample shown in the C column, no abnormal plasma cells were detected. [Color figure can be viewed at wileyonlinelibrary.com]

residual bone marrow sample, where 9% had detectable myeloma immunophenotype cells. Separation was performed in a similar method to the sensitivity testing. These cells were mixed with the remnant of a normal CSF sample. The remnant of the normal CSF sample after performing the diagnostic tests was clear, colorless, with a WBC count of 4 cells/μL. The sample, now containing myeloma cells, was divided into two parts, and half was placed in a TransFix/EDTA CSF Sample Storage tube. Cell counts were measured on a Sysmex XN2000 hematology analyzer in body fluid mode. Both samples were then labeled with the standard MM panel. For both samples, 100,000 events were collected on a FACS Canto II flow cytometer.

3 | RESULTS

The 125 CSF samples had a median white blood cell count of 1 cell/μL (< 1–23.4 cell/μL) and a median red blood cell count of 2 RBC/μL (< 1–1602 RBC/μL).

The RBC count was greater than 10 RBC/μL in 21 cases. Of these, nine samples were negative and nine samples were positive by both two-dimensional and multidimensional analysis. In three cases, the two-dimensional analysis was positive while the multidimensional analysis was negative.

In 41 samples from 32 patients, no abnormal plasma cells could be detected with either the conventional or the novel analysis protocol (the median WBC count: 1 cell/μL (< 1–21 cell/μL); the median RBC: 2 RBC/μL (< 1–654 RBC/μL)). In 16 samples (4 patients), abnormal cells could be detected with both analysis protocols. In these cases, the median WBC count was 5 cells/μL (1–1076 cells/μL), and the median RBC count was 19 RBC/μL (< 1–13,867 RBC/μL). Discordant results were obtained for a total of 32 samples from 16 patients. In these cases, conventional analysis based on two-dimensional dot plots raised the possibility of the presence of abnormal cells, while multidimensional dot plots ruled this out. The median WBC count was the lowest in the discordant cases, 1 cell/μL (WBC: < 1–7 cells/μL). In this group, the median RBC was 1 RBC/μL (< 1–19 RBC/μL) (Supplementary Table 1).

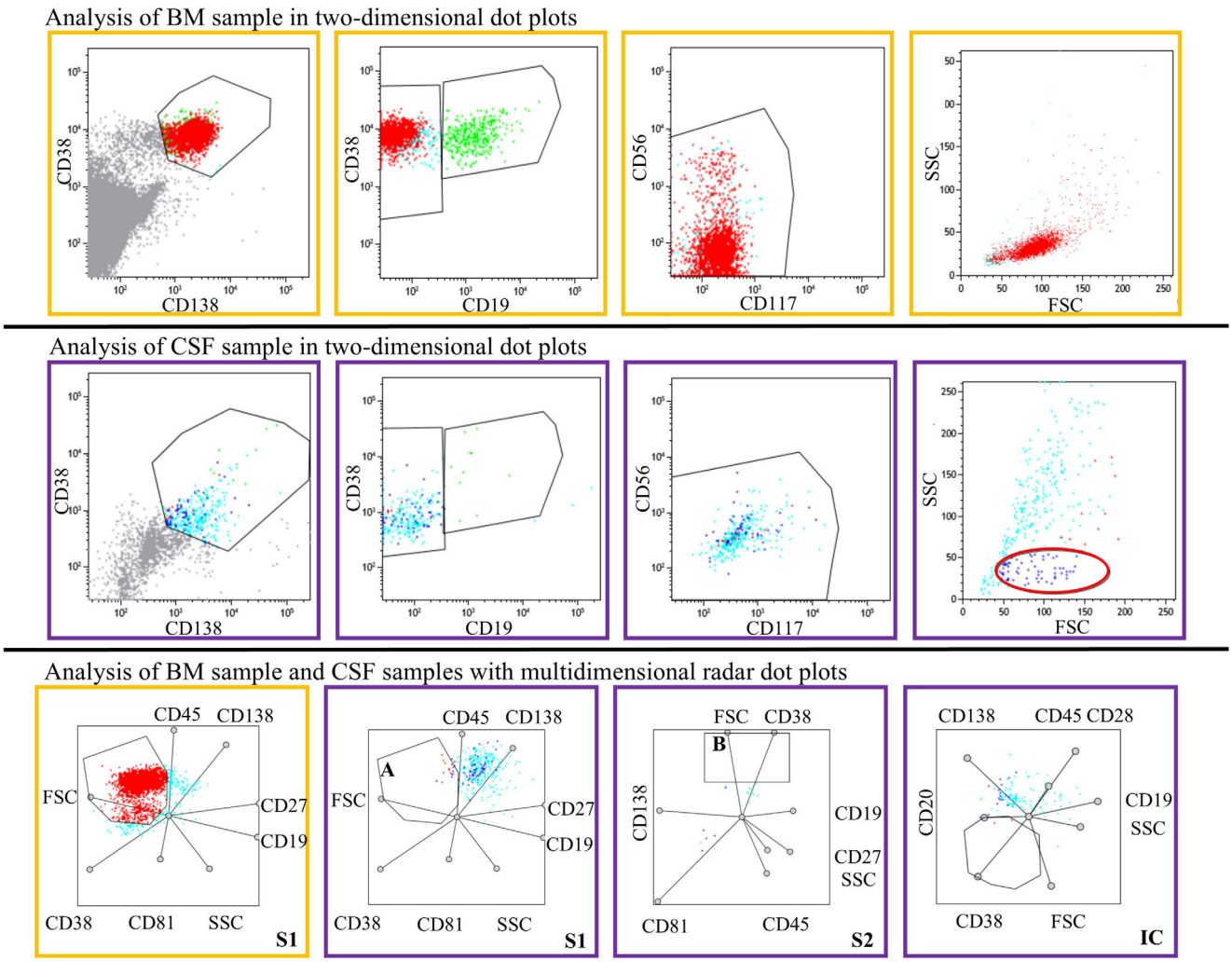

FIGURE 2 Representative dot-plot of a discordant case. In this case, plasma cells were unusually CD56 and CD117 negative in the bone marrow sample (first row, yellow frame). Red color indicates true abnormal plasma cells; apoptotic cells (CD138 dim negative events) are excluded based on the light scattering character, normal plasma cells are green, and light blue represents debris. Dot plots of two-dimensional analysis of the CSF sample are shown in the second row (purple frame). The last dot plot in the second row shows the intensity of light scattered forward and sideways by the gated cells. For the cells in the red circle (dark blue), the two-dimensional dot plots do not allow a clear statement of whether they are abnormal or normal cells. The third row shows the result of the multidimensional dot plot analysis. Since some events in the CSF sample fell in the position of abnormal plasma cells in the bone marrow (gate A), these events were examined separately with a second multidimensional dot plot (S2), where abnormal plasma cells are positioned above the dot plot (gate B). It can be seen that the cells in gate A are not positioned in gate B, so these events are not abnormal plasma cells. The events in the red circle marked in dark blue are not located in the gate of abnormal plasma cells in a multidimensional dot plot. S1: Surface tube 1, S2: Surface tube 2, IC: Intracellular tube. [Color figure can be viewed at wileyonlinelibrary.com]

Since the WBC count in a sample of normal, healthy people was < 5 cells/μL, we examined separately cases with WBC counts below this number (Figure 3).

In 50% of cases with abnormal cells by both analysis protocols ($n = 8$), the WBC count was < 5 cells/μL. In 93% of negative cases ($n = 34$), the WBC count was < 5 cells/μL. WBC count below 5 cells/μL occurred in 94% of discordant cases ($n = 30$).

Dilution of pooled CSF samples with myeloma positive bone marrow samples to evaluate the specificity and sensitivity of the multidimensional dot-plot based assay showed that abnormal plasma cells can be identified safely using the two-dimensional dot plots from samples with 100 and 50 cells/μL. With this assay, the abnormal cells and cell debris cannot be reliably distinguished in a sample with a cell count of 10 cells/μL or less. Using integrated data visualization, the identification of abnormal plasma cells is unambiguous at cell counts set at 10 and 5 cells/μL. At 1 cell/μL, no abnormal plasma cells could be detected by either method (Supplementary Figure 1).

The results of measuring the effect of the preanalytical factor (the type of sampling tube) showed that although the intensity of CD138 expression decreased on plasma cells, this did not affect their position on the radar dot plots. Thus, the analysis protocol can also

all cases (n=89)		multidimensional analysis	
		positive cases	negative cases
2D analysis	possible positive	32	16
	negative cases	0	41

<5 WBC/µL (n=72)		multidimensional analysis	
		positive cases	negative cases
2D analysis	possible positive	30	8
	negative cases	0	34

>5 WBC/µL (n=17)		multidimensional analysis	
		positive cases	negative cases
2D analysis	possible positive	2	8
	negative cases	0	7

FIGURE 3 Evolution of white blood cell count in CSF samples for bivariate and multivariate dot-plot based analysis. Dark blue color indicates the number of cases where no abnormal plasma cells could be detected by any of the analytical protocols. Red indicates the number of cases where abnormal plasma cells could be identified by both methods. Light red indicates the number of cases where the bivariate dot-plot based analysis raised the possibility of the presence of abnormal plasma cells, but this was not supported by the multidimensional dot-plot based analysis. Light blue indicates the number of discordant cases where abnormal plasma cells could only be detected by multidimensional dot-plot analysis. (Abbreviation: WBC: White blood cell). [Color figure can be viewed at wileyonlinelibrary.com]

be used for samples collected in TransFix/EDTA CSF sample storage tubes (Supplementary Figures 2A, 2B).

4 | DISCUSSION

The main message of our study is that analysis by integrated data visualization further increases the specificity and sensitivity of flow cytometry in diagnosing neurological involvement in patients with multiple myeloma.

CNS involvement in hematological malignancies is associated with poor prognosis; therefore, the identification of pathological cells in CSF samples is crucial. Several studies have demonstrated the efficacy of flow cytometry compared to cytopathology and imaging (Nückel et al., 2006; Sammartano et al., 2022; Zeiser et al., 2004). Imaging (MRI) can identify CNS involvement of leukemia/lymphoma in 40%–60% of cases (Chamberlain et al., 2009). Although the specificity of morphological examination is high (>95%), the sensitivity is below 50% (Del Principe et al., 2021).

However, flow cytometry also has its drawbacks as sample size is limited and cell counts are often low. The results of our study highlighted that especially in the case of low cell counts, the interpretation of flow cytometric test results becomes uncertain. In CSF samples, cells are vulnerable, cell debris often gives a false signal and can mimic the immunophenotype of abnormal cells. Therefore, we focused on the difference between cellular debris and abnormal plasma cells, since with the increase in the number of markers, the ability to distinguish between abnormal and normal plasma cells is clear even with conventional analysis.

The evaluation of CSF samples is further complicated by the fact that sampling is not always adequate, and the sample may be contaminated with peripheral blood. In our study, 21 patients had an RBC count above 10 RBC/microliter, but none of the patients included in the study were diagnosed with plasma cell leukemia. However, if the RBC count is elevated and abnormal plasma cells can be detected, we recommend that the hematologist should request a peripheral blood sample in parallel with the CSF sample to exclude a peripheral blood origin.

Another important finding of this study is that, although the CSF sample is considered normal if the cell count is < 5 cells/µL, it is still recommended to perform flow cytometry. In eight cases, myeloma immunophenotypic cells were found in samples with cell counts below < 5 cells/µL, which was confirmed by multidimensional dot-plot based analysis.

Despite the low cell count, we recommend the examination of both the surface and the intracellular tube. In some cases ($n = 8$), the cell debris in the surface tube showed not only false signal for some markers, but for several markers, so that even in the integrated data display the cell debris was located at the position of the abnormal plasma cells. In these cases, examination of the intracellular tube made it clear that the result was merely artificial.

The main limitation of our study is that the developed multidimensional dot-plot based assay system is only applicable to cases where the same antibody panel is used as in our study. Thus, if a

different antibody combination is used for labeling, we propose to develop our own analysis system using diagnostic bone marrow samples. Another limitation of our study is the small sample size, so validation of our results is needed for larger sample sizes, especially for low cell count cases and MM cases with rare immunophenotypes (e.g., CD19 positive MM).

In conclusion, flow cytometry is an objective, rapid, reliable method to assess CNS involvement in multiple myeloma. Analysis based on integrated data visualization further enhances the sensitivity and specificity of the method and can provide reliable results even at low cell counts.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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