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REVIEW

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Flow cytometric evaluation of measurable residual disease in chronic lymphocytic leukemia: Where do we stand?

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Abstract

Measurable residual disease (MRD) has emerged as a relevant parameter of response to therapy in chronic lymphocytic leukemia (CLL). Although several methods have been developed, flow cytometry has emerged as the most useful and standardized approach to measure and quantify MRD. The improved sensitivity of MRD measurements has been paralleled by the development of more effective therapeutic strategies for CLL, increasing the applicability of MRD detection in this setting. Chemotherapy and chemoimmunotherapy have firstly demonstrated their ability to obtain a deep MRD. Combined targeted therapies are also demonstrating a high molecular response rate and prospective trials are exploring the role of MRD to guide the duration of treatment in this setting. In this review we briefly summarize what we have learned about MRD with emphasis on its flow cytometric detection.

KEYWORDS

chronic lymphocytic leukemia, flow cytometry, measurable residual disease

Luca Laurenti and Antonella Aiello authors contributed equally to the work.

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1 | INTRODUCTION

The International Workshop on Chronic Lymphocytic Leukemia as well as the European Society of Medical Oncology guidelines consider the evaluation of measurable residual disease (MRD) not mandatory outside clinical trials in assessing the response depth to therapy in CLL patients.[1,2] Despite this, great attention has been paid on MRD evaluation due to the demonstration of the relevant prognostic impact of undetectable (u)-MRD in immuno-chemotherapy and venetoclax plus CD20-antibody combinations (Table 1).[1–23] Furthermore, the detection of u-MRD status before discontinuation therapy is crucial when targeted agents, such as B cell receptor (BCR) inhibitors, are used.[24–26] Actually, the advent of BCR inhibitors initially reduced the relevance of MRD measurements, because these drugs as single agents aim more at the control rather than at the eradication of the disease itself. However, combination therapies of targeted agents have brought the relevance of MRD back to the forefront.[27] Moreover, MRD-guided therapeutic protocols have been designed that allow suspension of therapy when u-MRD is achieved rather than its continuation until the loss of response or drug intolerance. Finally, in patients undergoing allogeneic stem cell transplantation, the detection of MRD may justify the reduction of immunosuppressive therapies, the administration of donor lymphocyte infusion and antileukemic maintenance treatment.2.

As examples of the relevance gained by MRD detection in driving therapy in CLL, we report two studies whose results have been recently published.[22,23] Firstly, in the CAPTIVATE multicenter phase 2 study investigating combined ibrutinib plus venetoclax in first-line treatement of CLL/Small Lymphocytic Lymphoma, patients were randomized according to MRD status to receive placebo or ibrutinib in the confirmed uMRD cohort or receive ibrutinib or ibrutinib plus venetoclax in those not achieving u-MRD.[22] Huber et al reported data on CLL2-GIVe trial testing ibrutinib, venetoclax and obinutuzumab triple combination to treat previously untreated CLL patients with del17(p) and/or TP53 mutation.[23] The treatment protocol consisted of an induction phase with obinutuzumab, ibrutinib and venetoclax, a consolidation phase with ibrutinib and venetoclax and a maintenance phase with ibrutinib monotherapy according to response and MRD level.

Progression free-survival and overall survival are two clinical parameters too long to be assessed in patients with CLL due to the low grade nature of the disease and this would lead to unjustified delays in the approval of new drugs. Measurable residual disease detection has been demonstrated to be a useful surrogate for response to therapy since the preliminary reports that chemotherapy (fludarabine and cyclophosphamide) combined with the anti-CD20 monoclonal antibody rituximab (FCR) is able to achieve undetectable MRD in CLL.[4,14,16,28] These data, taken together, led the European Medicines Agency in 2016 to authorize the use of u-MRD as an intermediate point in randomized clinical trials for the approval of new drugs.[29]

At present MRD detection should not yet guide clinical decisions in the daily practice. However, clinicians treating CLL patients should be prepared to go into this direction creating a proper context of expertise to be ready to use MRD when it will become formally proven as a "must" for clinician decisions.

1.1 | Recommended nomenclature for reporting Measurable residual disease

Measurable residual disease in CLL is defined as the number of clonal cells detectable in peripheral blood (PB) or bone marrow (BM) after treatment. In particular, MRD negativity (MRD-neg) is used when <1 CLL cell in 10.000 lymphocytes ($<10^{-4}$) is found (1). Of interest, the CLL8 clinical trial by the German CLL Study Group identified 3 levels of MRD with a different prognostic relevance in *naive* patients treated with FCR: low: $<10^{-4}$, intermediate: 10^{-4}-10^{-2}; and high: $>10^{-2}$.4 Very recently, an expert panel proposed recommandations on the terminology to be used (Table 2).[30,31] Briefly, to standardize the way of describing MRD, a logarithmic level has been suggested to be used. 'Measurable' instead 'minimal' in defining residual disease, because the latter is subjective while 'measurable' is unambiguous when the limit of detection is reported. Moreover, the final report must include information on the percentage of disease involvement, the detection limit of the sample, the method used [flow cytometry, Allele Specific Oligonucleotide Polimerase Chain Reaction (ASO-PCR), Next Generation Sequencing (NGS)], and the tissue tested (PB or BM). 'U-MRD' is also to be preferred to 'MRD-negative' or 'MRD-' to underline the inability in detecting measurable disease at a specific reporting threshold, meaning that the disease may be detectable below the level of sensitivity adopted. The experts recommend to use only validated assays, such as European Research Initiative on CLL (ERIC)-standardized approach and EuroMRD-compliant Real-Time Qualitative Polimerase Chain Reaction (RQ-PCR), depending on the rationale for MRD measurement. Measurable residual disease should be tested in both PB and BM in clinical trials. Testing BM is not required if MRD is detectable in PB. On the contrary, BM evaluation has to be made to confirm u-MRD in PB. Response to therapy should be assessed at least 2 months after completing the last treatment in fixed-duration therapies. In continuous treatment, however, the assessment should be made at the best clinical response achieved.[32]

2 | METHODS FOR MEASURABLE RESIDUAL DISEASE DETECTION

Multicolor flow cytometry is the most common method used because it is relatively quick and has wide availability (Table 3). Several antigenic panels have been proposed, based on the typical immunophenotypic profile of CLL (Figure 1 and Table 4). ERIC proposed the first consensus method based on a standardized 4-color flow cytometry (4 tubes) with a sensitivity below 10^{-4} (Table 4).[33] Of relevance, high concordance (95%) with ASO-PCR was also found. An ERIC and European Society for Clinical Cell Analysis Harmonization Project was then proposed in order to improve the sensitivity of flow

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TABLE 1 MRD and outcome in patients with chronic lymphocytic leukemia (CLL)

References	Line of therapy	Therapy	MRD	Sample tested	Outcome
Egle et al.[6]	I	FR + lenalidomide	FC (MRD3)	PB	PFS: 76.1 versus 46.4 months (p 0.007)
Abrisqueta et al.[7]	I	R-FCM	FC (MRD4)	PB and BM	PFS (at 4 years) PB: 89.5% versus 27% ($p < 0.01$) BM: 86% versus 60% (p 0.027)
Appleby et al.[8]	I	FCR	FC (MRD4)	BM	TTF: 85.3 versus 59.2 months (p 0.0008)
Fischer et al.[9]	I	BR	FC (MRD4)	PB	EFS: 32.4 months versus 11.8 months versus NR ($p < 0.01$)* OS: 23.2 months versus NR versus NR*
Frankfurt et al.[10]	I	AlemR	FC (MRD4)	BM	PFS: 41.3 versus 16.9 months (p 0.026)
Short et al.[11]	I	FCR3	FC (MRD2)	PB	TTP: No significant differences
Strati et al.[12]	I	FCR	FC (MRD4)	BM	PFS: HR 0.1; p 0.04 OS: HR 0.7; p 0.05
Thompson et al.[13]	I	FCR	PCR (MRD4)	PB/BM	PFS: 13.7 versus 4 years
Bottcher et al.[4]	I	FCR versus CF	FC (MRD4)	PB	PFS: HR 2.49; $p < 0.0001$; OS: HR 1.36; p 0.36
Goede et al.[14]	I	GClb arm	ASO-PCR	PB/BM	PFS: Not reached versus 19.4 months
Greil et al.[15]	I and II	CIT ± R (mantenance)	FC (MRD4)	PB	PFS: HR 0.4 (PB); $p < 0.0001$; HR 0.26 (BM); $p < 0.0001$
Kovacs et al.[16]	I	FCR versus CF FCR versus BR	FC (MRD4)	PB/BM	PFS: 60.7 versus 54.2 versus 35.4 versus 20.7 months per U-MRD CR, U-MRD PR, MRD + CR e MRD + PR
Santacruz et al.[17]	I	Any	FC (MRD4)	PB/BM	TFS: 76 versus 16 months; $p < 0.001$ OS: 108 versus 78 months; p 0.014
Jones et al.[18]	R/R	Venetoclax	FC (MRD4)	PB	PFS: HR 0.23; p 0.021
Moreton et al.[3]	R/R	Alemtuzumab	FC (MRD4)	BM	TFS: Not reached versus 20 months for U-MRD CR and MRD + CR; $p < 0.0001$ OS: Not reached versus 60 months for U-MRD CR and MRD + CR; p 0.0007
Fraser et al.[19]	R/R	Ibr + BR versus PBO + BR	FC (MRD4)	PB/BM	PFS (36 mesi): Ibr + BR: 88.6% versus 60.1% PBO + BR: 54.5% versus 11.2%
Kater et al.[5]	R/R	VenR versus BR	FC (MRD4) ASO-PCR	PB	PFS: VenR: HR 0.48 (U-MRD vs. b); HR 0.15 (U-MRD vs. a); BR: HR 0,44 (U-MRD vs. b), HR 0.08 (U-MRD vs. a)
Stilgenbauer et al.[20]	Any	Venetoclax	FC (MRD4)	PB	PFS (18 months): 78% versus 51%
Kwok et al.[21]	Retrospective	Any	FC (MRD4)	PB	PFS: 7.6 versus 3.3 versus 2.0 years for U-MRD, b, a OS: 10.6vs 5.3 versus 3.6 for U-MRD, b, a
Wierda WG et al.[22]	Untreated	Ibr + Venetoclax	FC (MRD4)	PB/BM	DFS 95% (12-month)
Huber H et al.[23]	Untreated	Obinutuzumab, Ibrutinib, Venetoclax	FC MRD4	PB/BM	CR 58.5%; both PFS and OS 95.1% (at 24 months)

Note: Modified from Wierda et al.[30]

Abbreviations: AlemR: alemtuzumab, rituximab; BM: bone marrow; BR: bendamustine, rituximab; EFS, event-free survival; FC: flow cytometry; FCR: fludarabine, cyclophosphamide, rituximab; FR: fludarabine, rituximab; GClb: CIT + -R: obinotuzumab, chlorambucil; chemoimmunotherapy + rituximab; Ibr: ibrutinib; OS: overall survival; PB: peripheral blood; PFS: progression-free survival; R/R: relapsed/refractory; R-FCM: rituximab, fludarabine, cyclophosphamide, mitoxantrone; TTF: time to first treatment; TTP: time to progression; VenR: venetoclax, rituximab.

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TABLE 2 Recommended nomenclature for reporting Measurable residual disease (MRD) in chronic lymphocytic leukemia (CLL)

Recommended	Rationale
Measurable residual disease (MRD)	More objective than 'residual'
Undetectable-MRD (U-MRD)	More accurate than MDR-negative or MRD-
MRD4, MRD5, etc.	<0.01%/10^{-4}; <0.001%/<10^{-5}, etc
Detectable (d) **	Residual disease below the stated threshold but measurable above the next MRD threshold (i.e., MRD4d: <0.01%/10^{-4} but ≥0.001%/10^{-5})
Undetectable (u)**	Residual disease is not detectable, but the assay/sample si no suitable for detection of disease at the next threshold (i.e., MRD4u: <0.01%, assay limit of detection does not reach 0.001%/10^{-5})
Assay method (e.g., flow cytometry) and analysis tecnique (e.g., ERIC protocol)	Results may differ by assay method and analysis
Tissue assayed (PB, BM) must be reported	MRD may differ in different tissues from the same patient
MRD rate as % U-MRD in intention-to-treat population in clinical trials must be reported	To avoid confusion with the rate in MRD-tested population (e.g., MRD4 rate = number of patients with <0.01% MRD as % of the intention-to-treat population)

Note: *modified from Wierda WG et al.[30], **within an MRD category.

TABLE 3 Tecniques used for Measurable residual disease (MRD) detection and their main features

Method	Features	Sensitivity	Advantages	Disadvantages
Flow cytometry	Detection of surface markers by monoclonal antibody panels	4-color: 10^{-4} 6-color: 10^{-5} 8-color: 10^{-6}	Wide availability; relatively quick; no need for individual sequencing for primer creation; ERIC consensus guidelines; high concordance (95%)with ASO-PCR (at 10^{-4} detection level)	Sensitivity lower than PCR or NGS; fresh (<48 h) samples and sufficient number of cells required
ASO PCR	Quantification based on clone-specific complementary determining region 3 (CDR3) og IgH hypervariable region.	10^{-5}	No need of fresh material: high sensitivity	Patient-specific primers needed; time and labor intensive due to the need of patient-specific primer
NGS	Measurement of CLL-specific IgH sequences based on consensus primers.	10^{-5}	High sensitivity	More expensive than FC and ASO-PCR; not widely used

Note: NGS is rarely applied due to the methodological limits (i.e., sequencing depth, starting material, spike-ins, calibrators, etc.). The potential to reach 10^{-6} would be done with precise optimization of this methodology.[32]

cytometry with a two-tubes 6-color panel.[34] With this approach, a sensitivity ranging from 10^{-4} to 10^{-5} was reached. Finally, ERIC proposed a single-tube 6-color assay with the most specific monoclonal antibodies used in the previous 6-color method.[35] With this approach sensitivity raised to 10^{-5}.

The last approach proposed by ERIC is very flexible. For that reason, several other groups, based on the International Standardized Approach (ISA) methodology and introducing new tumor-specific markers for CLL, have developed multi-color analyses with the use of one or at most two antibody combinations that allow to include all markers in a single tube, thus reducing the number of total lymphocytes required for the analysis (Table 4). As a consequence, this approach can be used also in cytopenic samples commonly seen after therapy. Furthermore, using CD160, that is not really a CLL-specific marker, although seems to be less intensely expressed in other malignancies, Farren and collaborators developed a 6-color test with a high concordance with the standardized 4-color ERIC approach.[36] Clinically, the same authors demonstrated the prognostic relevance of MRD negativity in CLL patients treated with chemo- or chemo-immunotherapy.[37] Patients who achieved MRD negativity after therapy had greater disease-free survival and longer time to subsequent treatment than those with MRD positivity. Using a 0.01% positivity cut-off, Stehlikova and collaborators demonstrated 100% agreement between the results obtained with the traditional 4-color ISA approach and a new 8-fluorescence test.[38] The former antibody combination is used to detect MRD while the latter to validate the result obtained. The second antibody combination also has a strong correlation with the results obtained with the RQ-PCR (r = 0.94).

Within an international project of the ERIC, Dowling and co-workers optimized and validated an 8 fluorescence combination

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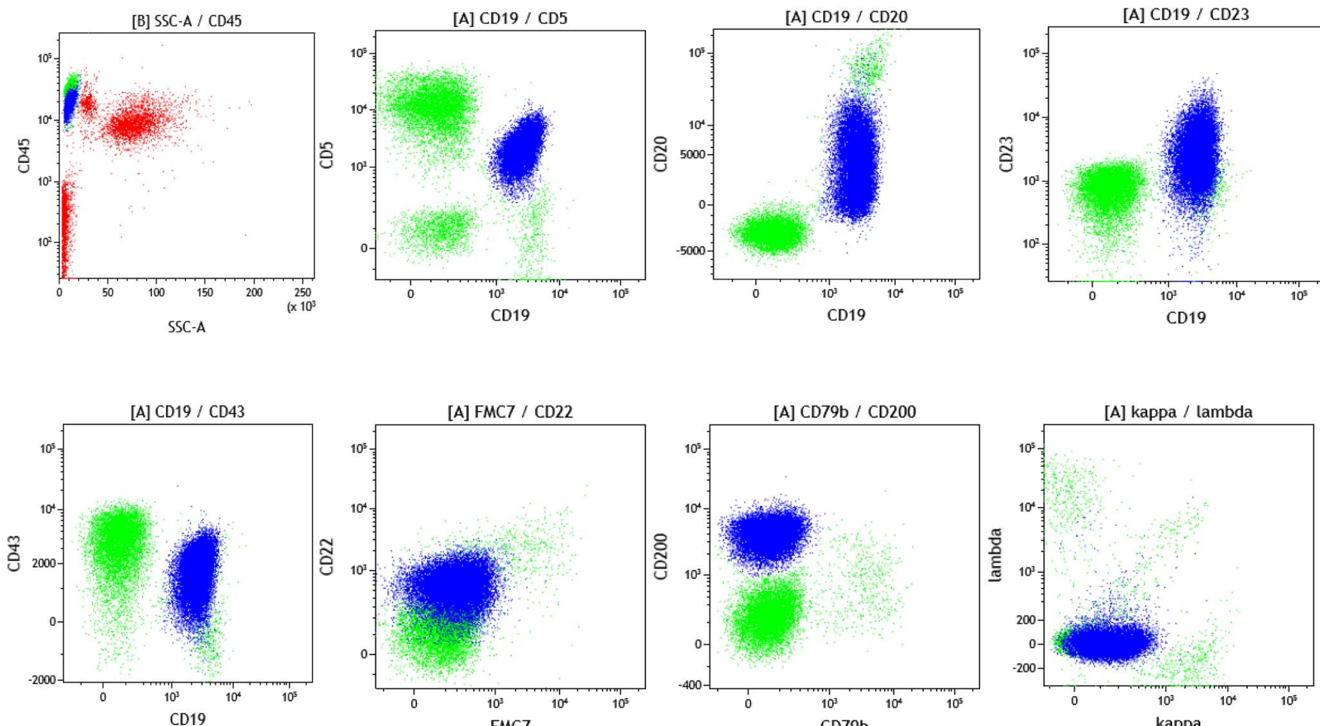

FIGURE 1 Typical immunophenotypic profile of chronic lymphocytic leukemia (CLL) according to the panel used at our Centers. Flow cytometric evaluation of a bone marrow (BM) sample from a CLL patient: clonal B-cells co-express CD19, CD5, CD23, CD200, CD43. Kappa light chain, CD20, and CD22 are expressed at low density, while FMC7 and CD79b are negative

TABLE 4 Multicolor flow cytometric protocols proposed for Measurable residual disease (MRD) detection in CLL

Reference	No. colors	Tubes (no.)	Monoclonal antibody panels used	Analyzed events/tube	Sensitivity
Rawstron, 2007[33]	4	4	Tube 1: CD5/CD19/kappa/lambda	500.000	10^{-4}
			Tube 2: CD5/CD19/CD20/CD38		
			Tube 3: CD5/CD19/CD43/CD79b		
			Tube 4: CD5/CD19/CD22/CD81		
Rawstron, 2013[34]	6	2	Tube 1: CD3/CD5/CD19/CD20/CD38/CD79b	≥500.000	10^{-4}
			Tube 2: CD5/CD19/CD29/CD22/CD43/CD81		
Rawstron, 2016[35]	6	1	CD5/CD19/CD20/CD43/CD79b/CD81	$\geq 2 \times 10^6$	10^{-5}
Farren, 2015[36]	6	1	CD2/CD5/CD19/CD23/CD45/CD160	≤500.000	10^{-4}-10^{-5}
Stehlikovà, 2014[38]	8	2	Tube 1: CD3/CD5/CD19/CD22/CD43/CD45/CD79b/CD81	≥700.000	10^{-4}
			Tube 2: CD5/CD14/CD19/CD20/CD38/CD43/CD45/CD79b		
Dowling, 2016[39]	8	1	CD3/CD5/CD19/CD20/CD22/CD43/CD79b/CD81	10^6	7×10^{-5}
Patz, 2016[40]	8	1	CD5/CD19/CD20/CD43/CD45/CD79b/CD81/ROR-1	$\leq 2 \times 10^6$	7×10^{-5}
Sartor, 2013[41]	10	1	CD3/CD5/CD19/CD20/CD22/CD38/CD43/CD45/CD79b/CD81	$\geq 1{,}8 \times 10^6$	10^{-5}
Bento, 2020[42]	8	1	CD5/CD19/CD81/CD79b/ROR-1/CD43/CD20/CD45	>700.000	10^{-4}
Aiello (ongoing)	8	1	CD5/CD19/CD20/CD43/CD45/CD79b/CD81/CD200	3×10^6	8×10^{-6}

Note: The harmonized protocols validated by ERIC34-36 are recognized by EMA to authorize the use of u-MRD as an intermediate point in randomized clinical trials for the approval of new drugs.

which, with the acquisition of 10^6 events, can allow to reach an analytical sensitivity of 0.007%.[39] Again, using an ISA panel-based 8-color tube produced in dried form, ROR-1 was introduced as a highly discriminating marker for the detection of CLL residual cells.[40] The authors demonstrated the specificity of the test and a sensitivity that reaches 0.007%, which is very close to that of

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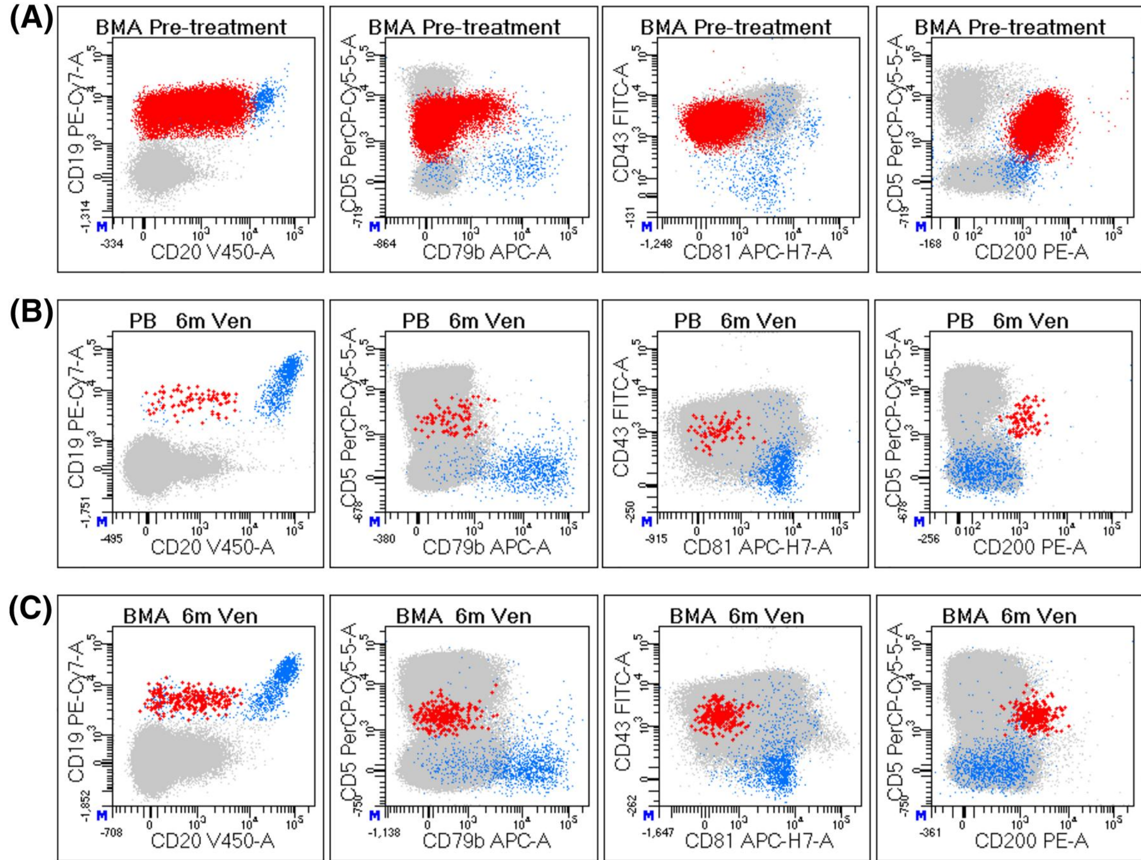

FIGURE 2 CLL measurable residual disease (MRD) detection by 8-color flow cytometry. Example of chronic lymphocytic leukemia (CLL) MRD detection with the 8-color panel described in Table 4 (Aiello, unpublished), containing CD43 fluorescein isothyocianate, CD200 phycoerythrin, CD5 PerCP-Cy5.5, CD19 PE-Cy7, CD79b allophycocyanin, CD81 APC-H7, CD20 V450, CD45 V500 antibodies (BD Biosciences, Italy). CLL cells (highlighted red dots) were identified within total B cells (blue dots) following the ISAC/ERIC gating strategy.[33-35] Accordingly, after doublets and debris exclusion, B cells were selected among mononuclear leukocytes by CD19 positivity and CD20 heterogeneous expression. CLL cells were then identified in sequential dot plots according to their CD5, CD43, CD200 positivity and CD20, CD79b, CD81 low expression compared to normal mature B cells and were enumerated by combining the appropriate gates with Boolean logic. MRD was then calculated dividing the CLL cells by the number of total CD45-positive leukocytes. Two different levels of MRD were found in peripheral blood (PB) and bone marrow (BM) aspiration (BMA) of a patient after 6 months (6m) of Venetoclax treatment. A, BMA pre-treatment status (CLL = 19.8%). B, PB 6m Venetoclax: MRD = 0.0054%, with a test sensitivity (LOD) of 0.0013%. The residual disease is undetectable according to the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) threshold of 0.01%, while is classified d-MRD4 according to the new nomenclature proposed by Wierda et al.31 **C)** BMA 6m Venetoclax: MRD = 0.0131%, with a test LOD of 0.0010%. The disease is detectable according to the iwCLL threshold of 0.01%, and classified d-MRD3 according to Wierda et al.[30]

molecular techniques. Sartor and collaborators have proposed a single 10-color tube that includes all ISA markers and which involves the acquisition of 1.5–1.8 $\times$ 10^6 cells to identify one pathological cell out of 100,000 (10^{-5}).[41] Furthermore, Bento et al showed a good performance of dry reagents in detecting MRD with 10^{-4} sensitivity.[42] In an ongoing observational study on patients with relapsed/refractory (R/R) disease treated with Venetoclax alone or in combination with Rituximab, an 8 fluorescence tube has been set up containing CD200 and CD45 in addition to the markers recommended by ERIC in their single-tube 6-color assay.[35] The cytometric test has proved to be specific and, for acquisitions of 3 $\times$ 10^6 cells, can reach a sensitivity of 8 $\times$ 10^6 (Aiello A, unpublished data). This study aims at comparing the MRD results obtained with flow cytometry (FC) and molecular techniques in order to evaluate the association between MRD status and achievement of complete or partial remission. An additional purpose is to compare the MRD status obtained in clinical pratice with data reported in clinical trials. Figure 2 shows an example of the FC evaluation of MRD in the peripheral and medullary blood of a patient included in this study at a specific time-point.

3 | CONCLUSIONS

Chronic lymphocytic leukemia therapy has increasingly evolved in recent years. The new drugs have resulted in achievement of u-MRD in a substantial proportion of patients and, as a consequence, have raised the relevance of MRD detection in the clinic. Evolution in technology, methodologies, and nomenclature have been made. FC is the most widespread technique for MRD evaluation. Very relevant in

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this context is the gating strategy procedures used and the antibody panel including the standardization/harmonization of the method in relationship to their impact on feasibility, accuracy and sensitivity. ERIC, the worldwide leader of these studies, has proposed protocols that have now become a standard. Current efforts are aiming at increasing the sensitivity of the methods used by reducing the inter and intra-laboratory variability. To this purpose, international External Quality Assessment programs have been concurrently developed to monitor and ensure the quality of clinical tests in this field.

AUTHOR CONTRIBUTION

Giovanni D'Arena, Dimitar Efremov, Luca Laurenti and Antonella Aiello wrote the manuscript. Fiorella D'Auria, Teodora Statuto Luciana Valvano, Giuseppe Pietrantuono, and Antonella Aiello provided the flow cytometric images. Giovanni D'Arena, Alessandro Sgambato, Silvestro Volpe Giuseppe Coppola, Rachele Amodeo, Virginia Tirino, Silvia Deaglio, Dimitar Efremov Luca Laurenti, and Antonella Aiello revised and discussed critically the published literature. All Authors revised and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable – no new data generated.

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