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Measurable residual disease in hematologic malignancies

Edited by
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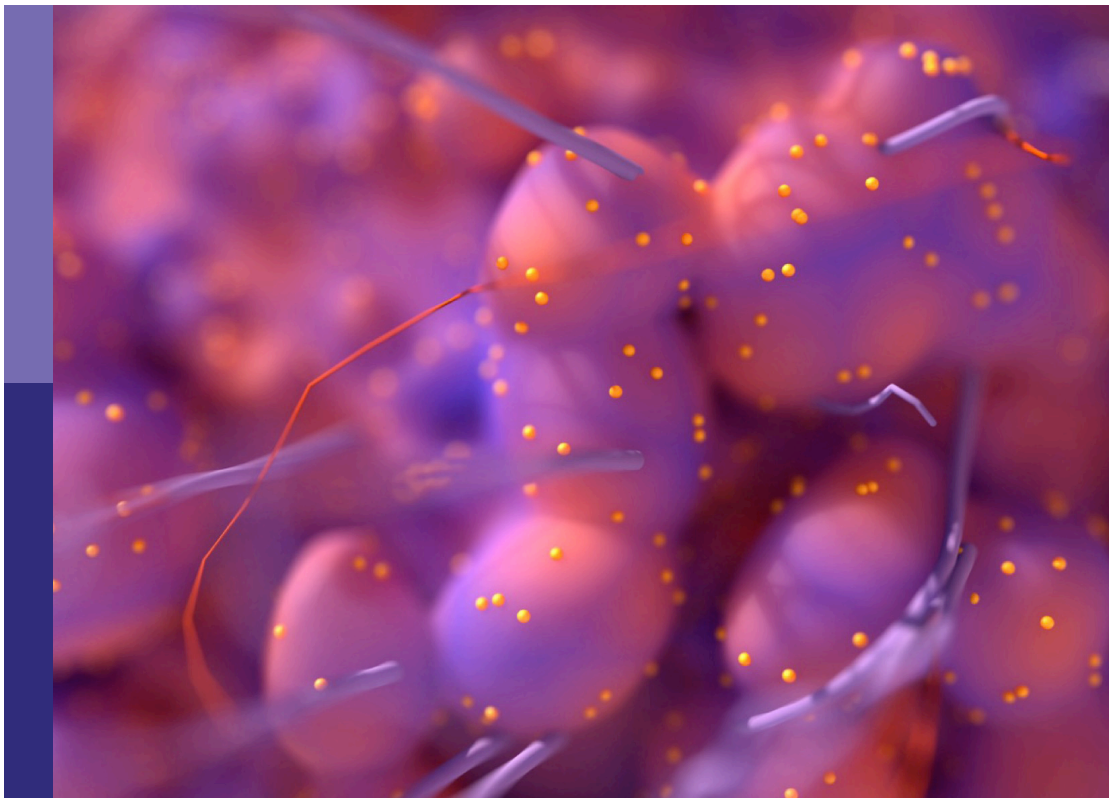

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Measurable residual disease in hematologic malignancies

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Editorial: Measurable residual disease in hematologic malignancies

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KEYWORDS

MRD - measurable residual disease, multiparameter flow cytometry, polymerase chain reaction, surrogate endpoint biomarker, next generation sequencing, next generation flow cytometry (NGF), chimerism after allo-HSCT

Editorial on the Research Topic
Measurable residual disease in hematologic malignancies

Measurable residual disease (MRD) has progressively taken a central role in the field of hematological malignancies, not only as a reliable marker of quality of response to treatment but also as a guide for the decision-making therapeutic choice. Accordingly, MRD is increasingly incorporated in experimental trials and in daily clinical practice, potentially representing a surrogate biomarker to accelerate drug development and approval.

In chronic (Benintende et al.; Robak and Robak) and acute (Tettero et al.) blood malignancies, either in the adult or in the pediatric (Lee et al.) setting, MRD can be considered a reliable prognostic biomarker in that it can provide an estimate of clinical response (Visentin et al.). This information may be particularly relevant in those hematological malignancies that are inherently characterized by a high risk of recurrence, such as acute leukemias (Chiusolo et al., Malagola et al.). In this subset, MRD might be incorporated into clinical trials as a "therapeutic target" to reduce disease burden before curative-intended strategies (including hematopoietic stem cells transplantation) or to indicate treatment de-intensification to spare unnecessary toxicities in patients with no evidence of residual disease (Tecchio et al.). A potential consequence of this assessment is that it may eventually improve the clinical outcome and also have a favorable impact on the financial cost of the overall treatment strategy. Indeed, the probability of hospitalizing patients achieving MRD negativity is usually lower, just as their inpatient stay is shorter.

Besides giving a reliable estimate of the quality of response, MRD monitoring after treatment may also allow to significantly shorten the time to new drug approval if validated as a surrogate endpoint for overall and disease-free survival. Based on this, the U.S Food and Drug Administration (FDA) has recently released a guidance document for the use of MRD in clinical trials testing new drugs for approval. According to this document, the assumption that MRD negativity correlates with a relatively small amount of residual cancer cells, thus representing a "biologically plausible" surrogate for a longer survival,

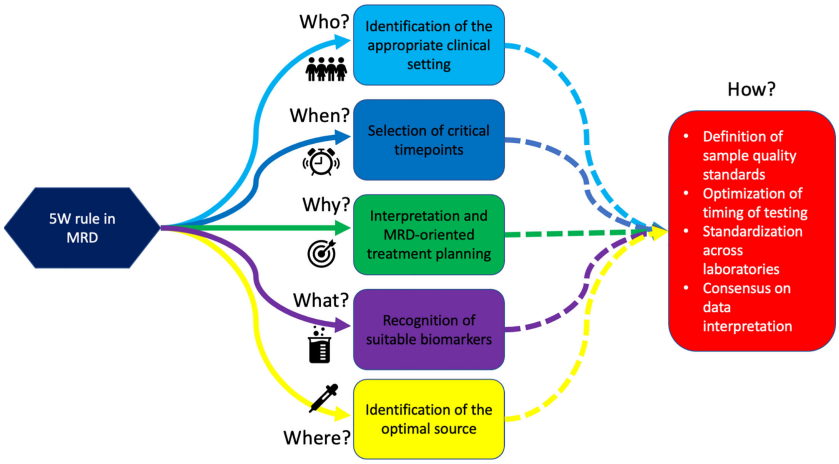

FIGURE 1
The 5W rules in MRD. We are progressively learning *Why* test to MRD, *Who* deserves MRD monitoring, *Which* population should be monitored, *Where* to search for MRD, and *When* to assess it. This will eventually lead to defining "How" we should do it.

should be actively pursued in clinical trials. Therefore, several trials are now incorporating MRD as an endpoint to accelerate new drug testing and approval, particularly in acute and chronic lymphocytic leukemia and multiple myeloma.

From the technical standpoint, the innate heterogeneous nature of hematological malignancies has prompted the improvement of the sensitivity and specificity of the available techniques and also the design of new tools to track cancer populations more efficiently than "standard" MRD might do. This may be particularly relevant for the identification of cancer stem cells, which are thought to be responsible for disease relapse in those cases of apparent MRD negativity, or the refinement of post-transplant chimerism assessment to identify an impending relapse. Similarly, there is accumulating evidence on the role of next-generation sequencing (NGS) and digital polymerase chain reaction (PCR)-based techniques for MRD determination in addition to reverse transcriptase quantitative PCR and multiparametric flow cytometry (Pacelli et al.; Assanto et al.). In which type of disease these novelties will become stand-alone techniques is not yet known.

Irrespective of the clinical subset and of the source to be tested for MRD (peripheral blood rather than bone marrow or other tissues), assessing the quality of the sample is critical to ensure the reliability of the assay. This can be particularly critical when testing the bone marrow, since either the background noise due to normal hematopoiesis or the poor quality of samples (e.g., hemodilution) can significantly reduce the sensitivity and specificity of the tests (Vigliotta et al.). However, since no consensus has been established yet on criteria for samples' quality acceptability, specific guidelines to address this issue are needed in the near future.

In conclusion, thanks to the accumulating evidence from prospective and retrospective MRD-centered trials, the "why" of testing MRD (e.g., the development of MRD-driven strategies) is becoming progressively clear. Nevertheless, until harmonization–standardization efforts are accomplished by the scientific community, "who" (e.g., patients that may benefit from testing), "what" (e.g., which biomarkers are suitable for MRD monitoring), "where" (e.g., which is the optimal source for MRD monitoring), "when" (e.g., which timepoints are crucial for clinical decision-making), and "how" (e.g., which technique is fit for which patient) to assess MRD will remain open questions (Figure 1).

Author contributions

FB and RP wrote the manuscript and drawn the figure, MG and SG critically reviewed the test. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Day +60 WT1 assessment on CD34 selected bone marrow better predicts relapse and mortality after allogeneic stem cell transplantation in acute myeloid leukemia patients

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The aim of this study was to evaluate the role of WT1 expression after allogeneic stem cell transplantation (alloHSCT) in patients with acute myeloid leukemia (AML). We studied WT1 expression in bone marrow cells from 50 patients in complete remission on day +60 after transplant. WT1 was assessed on unfractionated bone marrow mononuclear cells (MNC) and on CD34+ selected cells (CD34+). A ROC curve analysis identified 800 WT1 copies on CD34+ selected cells, as the best cut-off predicting relapse (AUC 0.842, p=0.0006, 85.7% sensitivity and 81.6% specificity) and 100 copies in MNC (AUC 0.819, p=0.007, 83.3% sensitivity and 88.2% specificity). Using the 800 WT1 copy cut off in CD34+ cells, the 2 year cumulative incidence of relapse was 12% vs 38% (p=0.005), and 2 year survival 88% vs 55% (p=0.02). Using the 100 WT1 copy cut off in unfractionated MNC, the 2 year cumulative incidence of relapse 13% vs 44% (p=0.01) and the 2 year survival 88% vs 55% (p=0.08). In a multivariate Cox analysis WT1 expression in CD34 cells proved to highly predictive of relapse (p=0.004); also WT1 expression on unfractionated cells predicted relapse (p=0.03). In conclusion, day-60 WT1 expression after allogeneic HSCT is a significant predictor of relapse, particularly when tested on CD34+ selected bone marrow cells.

KEYWORDS

AML, stem cell transplantation, minimal residual disease, stem cell transplant (SCT), minimal residual disease (MRD), WT1, relapse

Introduction

Despite advances in treatment and supportive care, the prognosis of adult acute myeloid leukaemia (AML) remains poor with about 40% of young patients and less than 20% of elderly patients surviving in the long term (1). Allogeneic hematopoietic stem cells transplantation (alloHSCT) is the best post-remission treatment for prevention of relapse due to the graft versu leukemia effect (GVL), which is effective regardless of cytogenetic subcategory and minimal residual disease (MRD) status (2, 3).

Patients with positive MRD are considered to be at high risk of recurrence and should receive alloHSCT in first complete remission (CR). AlloHSCT is not indicated in patients with a favourable risk profile (2), whereas patients with favourable risk but persistent MRD are eligible for transplantation (4). The results of alloHSCT compared to autoHSCT and chemotherapy have produced conflicting results in intermediate-risk patients, taking into account molecular markers and MRD status as essential parameters (5–7). In fact, one of the main goals of MRD assessment is to identify, as early as possible the subset of patients at risk of relapse, despite being in CR. This means that these patients can be treated with intensified chemotherapy protocols or transplantation. Unfortunately molecular markers and a leukemia aberrant immunophenotype (LAIP), are not always present in AML patients, making it difficult to establish MRD.

The Wilms' tumour gene (WT1) was originally identified as a suppressor gene for paediatric Wilms' kidney cancer. In normal human bone marrow, WT1 is expressed at extremely low levels and is confined to primitive CD34+ cells, but is abnormally expressed in many types of haematological malignancies, making it a molecular marker for leukaemia (8)

The main limitation that prevented the clinical application of this marker for many years was the detection of low transcript levels even in normal haematopoietic cells, suggesting that it could be considered a non-specific marker overexpressed by immature cells. With the introduction of Real Time Quantitative PCR (RQ-PCR) into clinical practice, it became clear that WT1 expression was not only an immaturity marker, but its overexpression was a reliable indicator of the presence of leukemic cells. In particular, WT1 overexpression has been reported in the majority of acute myeloid leukaemia (AML) patients, regardless of the presence of specific fusion transcripts (9).

Several studies have shown that persistence of an abnormal WT1 transcript after chemotherapy, is a strong predictor of subsequent relapse (10). Given the existence of a background WT1 expression in normal bone marrow, qualitative RT-PCR provided conflicting results on the clinical value of this marker (11, 12), whereas RQ-PCR can be used to assess different levels of WT1 transcripts in AML cells, normal hematopoietic cells and normal bone marrow cells regenerating after chemotherapy (13, 14). Therefore, longitudinal RQ-PCR analysis of the amount of WT1 transcript may be clinically relevant for monitoring AML.

In a retrospective study on a cohort of patients submitted to alloHSCT we demonstrated that WT1 expression on bone marrow mononuclear cells (MNCs) is predictive of leukemic relapse, and can be used to initiate immunotherapy with donor lymphocyte infusion using as cut off < 100 WT1 copies normalised to 10^4 Abelson copies (ABL) (14). We found that patients with WT1 copies >100 had a 54% probability of relapse whilst patients with copies <100 had a 16% probability of relapse.

In a more recent study from our group, in addition to confirming the data, we showed that by administering immunotherapy (IT) in two different groups defined by the expression levels of Wt1 copies >180(WT1-180) and Wt1 copies >100(WT1-100) the cumulative incidence of recurrence was 76% in the WT1-180 group compared to 29% in the WT1-100 group, i.e. a significant improvement in MRD positive disease free survival of 23% compared to 74% (15). Therefore, WT1 is a sensitive marker of leukemic relapse, and predictive therapy is feasible by defining an expression level >100 copies as a cut off. Several studies have confirmed that WT1 expression before and/or after allogeneic transplantation predicts leukemia relapse (16–19).

The aim of the present study is to further increase the predictive role of WT1 expression by evaluating selected CD34\+ cells, isolated from bone marrow on day +60 after allo-HSCT.

Methods

Study population

AML patients undergoing alloHSCT at Fondazione Policlinico A. Gemelli IRCCS from June 2018 to July 2020 were prospectively investigated. Healthy bone marrow donors were included as controls. The study was approved by the local Ethic Committee (Prot.4065/21 April 28, 2021).

Patient, donor, and graft data

Patients' variables included demographics, diagnosis and date of diagnosis, date of transplant, disease status (complete remission or not), disease risk index (DRI), European Leukemia Net (ELN) risk, hematopoietic cell transplantation comorbidity index (HCT-CI), date of acute or chronic GVHD (aGVHD and cGVHD), date of relapse, date of death, or last follow-up. Donor variables included HLA match, age, and gender.

Cell samples and quantitative assessment of *WT1* expression

WT1 expression was evaluated on both MNCs and CD34+ cell samples. Mononuclear cells were separated on a Ficoll-

Hypaque (Lymphophlot; Bio-RAD Medical Diagnostics GmBH, Dreireich, Germany) density gradient. Total RNA was extracted using Trizol (Invitrogen, Life Technologies, CA), following the manufacturer's instructions. CD34+ cells were isolated from MNCs by immunomagnetic method (Miltenyi, Biotech, Bergish Gladbach, Germany).

All analysis were performed in triplicate. For quantitative assessment of *WT1* mRNA, a calibration curve with a plasmid containing the *WT1* target sequence was used (ProfileQuant WT1 Kit, European Leukemia Net, Ipsogen, France). The *WT1* ProfileQuant kit includes specific plasmids and primers and probe mixes for *WT1* and Abl. These components have been validated together in the context of a collaborative study led by a group of experts from the European LeukemiaNet consortium (10). RQ-PCR reactions and fluorescence measurements were made on the RotorGene3000 (Corbett Life Science, Sydney, Australia). The *WT1* mRNA levels of expression were normalized with respect to the number of Abl transcripts and expressed as *WT1* copy numbers/10^4 copies of Abl.

For each patient, a bone marrow sample was collected on day +60 after transplantation. WT1 copy number data normalised for 10^4 Abl copies was obtained on selected CD34 + cells in 45 patients and on whole bone marrow mononuclear cells in 40 patients.

In addition, two control groups of healthy bone marrow donors were enrolled and an aliquot of the graft was used for WT1 determination. In one donor group of 42 subjects, WT1 was evaluated on selected CD34+ cells, while in the other group of 18 healthy donors WT1 was determined on whole bone marrow mononuclear cells.

Statistical analysis

The continuous numeric variable WT1 was compared between groups using the Mann-Whitney and Kruskal-Wallis tests. Using the Receiver Operating Characteristics (ROC) curve, the cut-off of the continuous variable WT1 was defined in relation to the relapse outcome, and for this cut-off the percentage of sensitivity and specificity was reported, as well as the area under the curve (AUC) of the ROC and its relative 95% confidence interval. The continuous variable WT1 was then transformed into a categorical variable as a function of the cut-off defined by the ROC curve. Categorial variables were compared by Chi square and Fisher exact test between patients with and without relapse. Univariate and multivariate analysis were performed with the Cox regression model for relapse and survival with the following variables: patients age, donor HLA matching, intensity of the conditioning regimen (myeloablative, reduced intensity), adverse karyotype (yes/no), adverse ELN risk (yes/no), remission status at transplants (yes/no), stem cell source (peripheral blood/(bone marrow), and WT1 expression in CD34+ cells, or WT1 expression in unfractionated BM cells. Cumulative relapse incidence curves were compared by Grays test.

Kaplan Meier curves were drawn for survival and compared with the log-rank test. The statistical analysis was carried out with the NCSS19 software.

Results

In total 50 AML patients and 60 donors were included in the study. Patients and transplant characteristics are shown in Table 1. The median age was 56 years (25–69). The ELN risk groups were as follow: favourable (n=10), intermediate (n=27), adverse (n=13).

Seventeen patients (34%) developed aGvHD after a median of 34 days (range 16-90). Grading was as follows: grade I in 12 patients (70.6%), grade II in 4 patients (23.5%) and grade III in 1 patient (5.9%). Chronic GvHD was diagnosed in 13 (28.3%) of the 46 patients with a follow-up of more than 100 days. Grading was as follows: mild in 9 patients (69.2%) and moderate in 4 patients (30.8%).

Eleven patients (22%) relapsed after a median of 120 days after transplantation (range 73-582), while the others maintained a complete remission at the follow-up time of July 2021. At the same follow-up time, 40 patients (80%) were alive with a median survival of 435 days (range 84-861), while 10 patients (20%) died after a median time of 186 days (range 96-334). The causes of death were as follows: transplant-related mortality in 3 patients (6%) and disease recurrence in 7 patients (14%).

WT1 expression in patients and controls

The expression of WT1 was assessed at day + 60 in 50 AML patients: in 40 patients both CD34+ cells and MNCs were evaluated, while in further 10 patients, WT1 was evaluated only in CD34+ cells (5 patients) or MNCs (5 patients). Moreover, 42 CD34+ cell samples and 18 MNCs samples from healthy bone marrow donors were used as controls.

We first compared WT1 expression in patients and controls. No difference was seen between patients (49.7 copies, 95%C.I 29.6-67.3) and controls (43.2 copies, 95%CI 17.1-59.5) looking at WT1 expression on total bone marrow MNC (p=0.2). On the contrary a statistically significant difference was observed between the median WT1 levels on selected bone marrow CD34+ cells between the two groups: 406.5 copies for patients (95%CI 342.8-634.6) and 252.3 copies in controls (95%CI 188.9-314.2) (p=0.0007).

WT1 expression on total bone marrow MNC was significantly different in patients who remained in remission (37.9 copies - 95%CI 25.5-60.2), as compared to patients who relapsed (135.3 copies 95%CI 21.4-1072.8) and to controls (43.2 copies, 95% CI 17.1-59.5) (p=0.03) (Figure 1A). In CD34 + cells the median WT1 copy number was 389.2 copies for patients

TABLE 1 Patient's characteristics.

Patients	50
Age, median (range)	56 ys (25-69)
Gender, F/M	24/26
ELN Risk	10 (20%)
Favourable	27 (54%)
Intermediate	13 (26%)
unfavourable	
Molecular Markers	17 (34%)
NPM	14 (28%)
FLT3	1 (2%)
t(8;21)	4 (8%)
Inv(16)	1 (2%)
c-kit	
Time from diagnosis to transplant, median (range)	186 days (50-935)
Disease status at transplant	30 (60%)
1 CR	5 (10%)
2 CR	4 (8%)
PR	11 (22%)
Relapsed/refractory	
Donor match	8 (16%)
Sibling	16 (32%)
Haplo	13 (26%)
MUD	13 (26%)
MMUD	
HCT-CI, median (range)	3 (0-6)
Conditioning regimen	22 (44%)
MA	28 (56%)
RIC	
GvHD prophylaxis	7 (14%)
CSA+MTX+ATG	1 (2%)
CSA+Cy	42 (84%)
CSA+MMF+Cy	
CD34+, median (range)	5.75 x10^6/Kg (0.1-10.8)
Stem cells source	30 (60%)
PB	16 (32%)
BM	4 (8%)
CB	
Donor	24 (48%)
Related	26 (52%)
Unrelated	

ELN = European Leukemia Net risk; HSCT = hematopoietic stem cell transplantation; CR =complete remission; PR =partial remission; MA= myeloablative conditioning; RIC= reduced intensity conditioning; MUD = matched unrelated donor; MMUD= mismatched UD; Haplo= haploidentical donor; sibling= HLA matched sibling; ATG = antithymocyte globulin; PB = peripheral blood; BM= bone marrow; CB= cord blood; aGvHD = acute graft-versus-host disease; CD34+ = selected CD34+ cells on bone marrow samples; MNC= total mononucleated cells in bone marrow.
CSA = cyclosporin; MMF= mycophenolate; CY= cyclophosphamide

who remained in remission (95% CI 246.3-472.2), and 1129.1 copies for patients who relapsed (95% CI 58.8-1918.2) and 252.3 for controls (95% CI 188.9-314.2) (p=0.001) (Figure 1B).

ROC curve for WT1 with relapse outcome

The ROC curve was then used to define a threshold of MRD of WT1 for the relapse outcome. For WT1 level on whole bone marrow MNC the AUC was 0.819 (CI 95% 0.426-0.952). The selected cut-off was 100 copies, with a sensitivity of 83.3% and a specificity of 88.2%. (p=0.007, Figure 2A). For the WT1 level determined on selected bone marrow CD34+ cells, the AUC was 0.842 (95% CI 0.508-0.956). The selected WT1 cut-off was 800 copies, with a sensitivity of 85.7% and a specificity of 81.6% (p=0.0006, Figure 2B). Using the cut-offs identified with the ROC curve, the continuous WT1 levels variable was transformed into a categorical variable.

Univariate analysis

Comparing patients who later relapsed, with patients in continuous remission (Table 2), significant difference were found in the proportion of patients with an adverse Karyotype (p= 0.02), and in the proportion of patients with a high WT1 day +60 expression, both on CD34+ cells as well as on unfractionated BM cells (Table 2). The cumulative incidence of relapse is shown in Figure 3: when using the 800 WT1 copies cut off, on CD34+ cells, the 2 year cumulative incidence of relapse was 12% vs 38% (p=0.005) (Figure 3A); when using the 100 WT1 copies cut off on unfractionated BM cells, the 2 year cumulative incidence of relapse was 13% vs 44% (p=0.01) (Figure 3B).

Cox analysis on relapse

In univariate analysis, significant predictors, were WT1 expression on CD34+ cells and unfractionated cells, as well as an adverse karyotype and adverse ELN risk group. In multivariate analysis WT1 expression was entered either from CD34+ cells or from unfractionated BM cells: both were predictive of relapse (Table 3).

WT1 expression and survival

The two year survival of patients stratified according to WT1 expression on CD34+ cells was 88% vs 59% (p=0.02) (Figure 4A); the survival of patients stratified according to WT1 expression on unfractionated BM cells was 82% vs 55% (p=0.08) (Figure 4B). DFS was also predicted by WT1 expression on CD34+ cells (79% vs 61%, p=0.03, with the 800 copy cut off), and also on unfractionated BM cells (85% vs 56%, p=0.01, with the 100 copy cut off).

In a Cox multivariate analysis on survival, age >60 years was a significant predictor (p=0.03) together with WT1 copy number over 800 for CD34+ cells (RR 18.1, p=0.05) and less so for WT1 copy number over 100 for unfractionated BM cells (RR 4.2, p=0.09). Similarly in a Cox model for disease free survival WT1 expression on CD34+ cells was a better predictor of failure

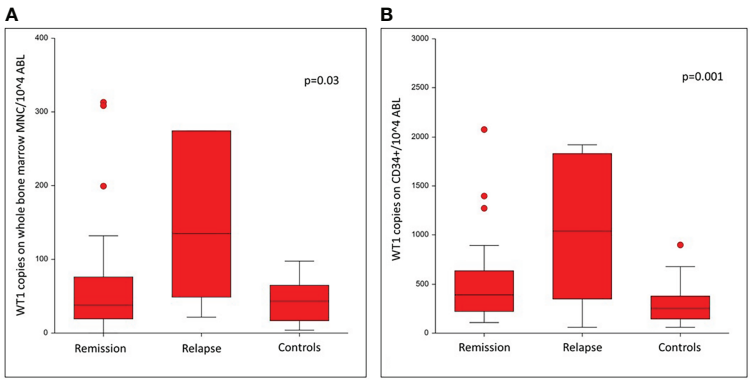

FIGURE 1
Comparison of WT1 levels on day +60 after allogeneic HSCT for patients in remission, patients relapsed and in controls.

(RR 5.8, p=0.01) as compared to WT1 expression on unfractionated BM cells (RR 7.4, p=0.04), together with age >60 years (RR 8.4, p=0.02).

Discussion

The quantification of MRD is considered a powerful, independent predictive factor after HSCT. Monitoring leukemia-specific gene mutation by PCR or LAIP represents the gold standard to stratify patients on the basis of the risk to relapse. Unfortunately, more than 50% of AML cases lack specific genes and 10-30% of them lack LAIP. The National Cancer Institute's second workshop on relapse after HSCT (20) identified several topics for the prevention of leukemia relapse, including "detection and preventive therapy of impending relapse". Three papers addressed the issue of WT1 as a marker of MRD in AML after transplantation (21–23) and were able to identify a predictive association between WT1 levels and relapse.

Rossi et al. found that high WT1 levels at 1 month from the transplant significantly impacted on DFS (.p = 0.010) and had a higher predictive value than WT1 levels on days +90 (21). Israyelyan et al. focused on the period after alloHSCT for predicting relapse onset using WT1 overexpression and looked at WT1 levels on peripheral blood cells and determined a cut-off level that would identify patients at risk of hematological relapse (22). Both cut-off levels of 50 and 20 reproduced high specificity and sensitivity. The WT1/c-ABL transcript ratio of 50 or above demonstrated 100% specificity and 75% sensitivity predicting relapse with an observed average of 29 days, while a lower ratio of 20 or above had lower specificity, but higher sensitivity (84.8% and 87.5%, respectively) and identified more patients who had an hematological relapse, at earlier times, providing an earlier warning with actual average lead time of 49 days. Using the ratio of 20 (HR 58.16, p<0.0001) WT1, together with high risk disease (HR 3.27, p=0.02) and donor age above 34 years (HR 5.12, p=0.01), are listed as predictor variables for relapse occurrence. Among these, multivariate analysis confirmed only WT1 ratio of

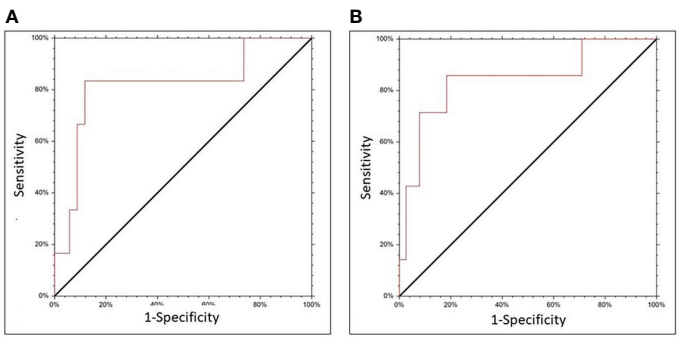

FIGURE 2
ROC curve for WT1 expression on CD34 selected cells **(A)** or unfractionated BM cells **(B)**, and relapse.

TABLE 2 Characteristics of patients who subsequently did or did not relapse.

	RELAPSE Yes	RELAPSE No	P
Recipients age	59 (42-66)	53 (49-57)	0.3
Adverse karyotype	44%	14%	0.02
Adverse ELN	44%	28%	0.2
CR at transplant	55%	73%	0.3
Myeloablative conditioning	84%	81%	0.8
HLA matched donor	11%	35%	0.08
ATG in the conditioning	11%	15%	0.8
Stem cell source PB	81%	74%	0.6
HCT-CI,median (range)	3(2-4)	3 (2-3)	0.4
Acute GvHD II-IV	18%	8%	0.3
WT1 >800 copies *	60%	20%	0.01
WT1 >100 copies **	57%	15%	0.01

HCT-CI =hemopoietic stemc cell transplant- comorbidity index. ATG = anti-thymocyte globulin. * on CD34+ cells; ** on unfractionated BM cells.

20 as associated with decreased time to relapse (22). Yoon et al. (23) examined WT1 transcription levels in bone marrow MNC one month after transplantation in patients with refractory anemia with excess blasts demonstrating that a cut-off level of 154 copies at 1 month was predictive of leukemia relapse. In this study, 47% of patients who exceeded this cut-off level, versus 7% of patients who did not reach 154 copies, relapsed. Multivariate analysis confirmed high WT1 expression (HR 9.94, p=0.002) and poor karyotype before transplant (HR 3.52, p=0.05) as predictive variables for subsequent relapse. A further study showed that low WT1 levels after transplantation were associated with higher and longer-lasting frequencies of WT1-specific cytotoxic T cells (CTLs) in long-term survivors (24). High WT1 levels in autologous peripheral blood apheresis were also shown to predict relapse in AML patients (25).

Pozzi et al. also confirmed that AML patients in CR before transplant and with a median expression of WT1 >100/10^4 ABL after transplant had a higher relapse risk (53% vs 26%) and a lower 5-year survival (36% vs 62%) when compared with patients who had less than this cutoff (14). In multivariate analysis predicting factors for relapse were: disease phase at transplant (RR 2.3, p=0.002), pre-transplant WT1 level (RR 2.2, p=0.01) and post-transplant WT1 level (RR 4.5, p=0.0001) determined on bone marrow samples.

In a more recent study the same group (15) examined the efficacy of IT (consisting of cyclosporine interruption and infusion of donor lymphocytes) triggered at different levels of MRD expression: patients treated at a cut-off level WT1 expression in marrow cells of 100 copies had a significantly lower risk of progressing to hematological relapse than patients treated at a higher cut-off level (180 copies) demonstrating that the greater efficacy of IT in WT1-100 patients is due to the fact that the intervention occurred with a lower disease burden. The greater effect of IT in WT1-100 patients was also demonstrated

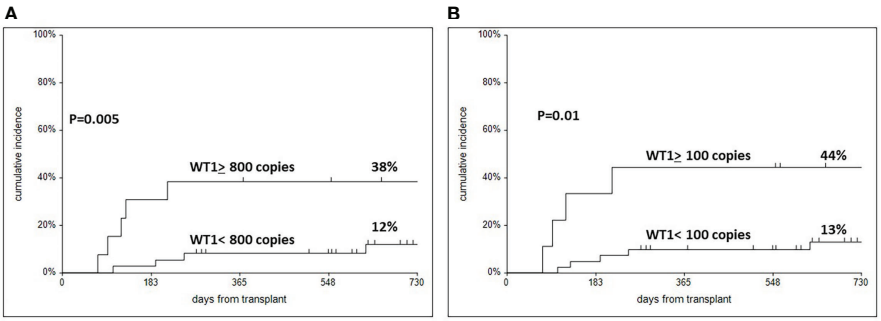

FIGURE 3
Cumulative incidence of relapse in patients according to WT1 expression in CD34 selected cells **(A)** with a cut off of 800, and in unmanupulated mononuclear cells with a cut off of 100 **(B)**.

TABLE 3 Univariate and multivariate Cox analysis on relapse.

	UNIVARIATE			MULTIVARIATE			MULTIVARIATE		
Variable	**RR**	**95%CI**	**P**	**RR**	**95%CI**	**P**	**RR**	**95%CI**	**P**
WT1>800*	5.6	1.5-20	.008	8.5	1.9-37	.004			
WT1 >100**	8.8	1.6-48	.01				6.8	1.1-39	.03
Adverse karyotype	7.2	2.1-23	.001	8.6	0.3-48	.1	6.4	0.1-22	.1
Adverse ELN	3.9	1.2-12	.02	1.3	0.6-29	.8	2.1	.1-18	.6
Age >60 years	1.4	0.3-5.4	.6	–	–	–	–	–	–
CR at transplant	0.4	0.1-1.2	.09	0.8	0.2-3.3	.8	0.3	0.1-8	.7
HLA matched don	2.1	0.6-7	.2	–	–	–	–	–	–
MA regimen	0.5	0.1-2	.5	–	–	–	–	–	–
PB vs BM	1.0	0.2-4	.9	–	–	–	–	–	–

as in Tables 1 and 2

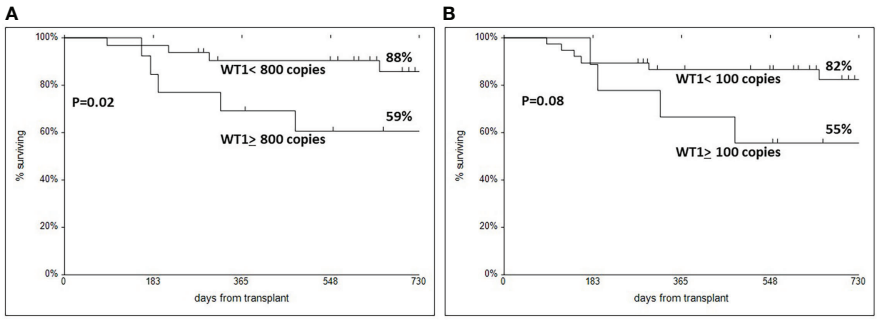

FIGURE 4
Actuarial two year survival in patients with a cut off of WT1 800 copies for CD34 selected BM cells **(A)**, or WT1 100 copies for unfractionated BM cells **(B)**.

by the higher percentage of patients achieving molecular remission: 96% compared to 35% of WT1-180 patients (15).

The goal of our study was to evaluate a greater predictivity of WT1 expression in CD34 + cells as compared to the expression levels on unfractionated MNCs after alloHSCT in AML patients. We evaluated WT1 expression levels in selected bone marrow CD34 + cells of 50 patients at day 60 post HCST.

Using the ROC curve it was possible to define a cut off equal to 800 copies in CD34 + selected from MNC on bone marrow and a cut off equal to 100 copies on unfractionated mononuclear cells from bone marrow, confirming the results of Pozzi et al. (14).

In particular, in a multivariate Cox model, patients with WT1 ≥ 800 copies on selected CD34 + bone marrow cells, had a 8.5-fold higher risk of relapse, as compared to patients with WT1 <800 copies. The predictive value of WT1 expression over 100 copies, on unfractionated bone marrow mononuclear cells, was predictive of relapse (6.8-fold greater risk), but with less statistical power (p=0.03 as compared to p=0.004 for CD34+ cells). WT1 expression on CD34+ cells was also predictive of survival in a multivariate analysis (p=0.05) and disease free survival (p=0.01) together with patients age > 60 years (p=0.03). The predictive role of WT1 expression on unfractionated BM cells was less significant for survival (p=0.09) and disease free survival (p=0.04). So the expression of WT1 on CD34+ cells appeared to provide a higher predictive value in the multivariate Cox model.

In conclusion, the expression of WT1 on CD34 cells selected on day +60 after allogeneic transplantation, is greater as compared to WT1 expression on unfractionated bone marrow MNC, and provides a predictive assay for leukemic recurrence after alloSCT. We would favor CD34 selected cells to assess MRD on day +60 after transplant, and thus predict relapse, in particular in patients not expressing LAIP or molecular markers suitable for MRD monitoring after transplant.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The dataset will be available upon request

to the corresponding author. Requests to access these datasets should be directed to patrizia.chiusolo@unicatt.it.

Ethics statement

The studies involving human participants were reviewed and approved by Comitato Etico-Fondazione Policlinico Universitario Agostino Gemelli IRCCS. The patients/participants provided their written informed consent to participate in this study.

Author contributions

PC and AB designed research. GM, SG, and MR performed research and analyzed data. EM, SG, FS, ML, FF, NP, MB, CG, LT, and SS treated the patients. PC, EM, and AB wrote the paper and all co-authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Clinical implication of minimal residual disease assessment by next-generation sequencing-based immunoglobulin clonality assay in pediatric B-acute lymphoblastic leukemia

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Measuring minimal residual disease (MRD) during treatment is valuable to identify acute lymphoblastic leukemia (ALL) patients who require intensified treatment to avert relapse. We performed the next-generation sequencing (NGS)-based immunoglobulin gene (Ig) clonality assay and evaluated its clinical implication in pediatric B-ALL patients to assess MRD. Fifty-five patients who were diagnosed and treated with *de novo* (n = 44) or relapsed/refractory B-ALL (n = 11) were enrolled. MRD assessment was performed using the LymphoTrack® Dx IGH and IGK assay panels. The percentage of the clonal sequences per total read count was calculated as MRD (% of B cells). The data were normalized as the proportion of total nucleated cells (TNC) by LymphoQuant™ Internal control or the B-cell proportion in each sample estimated by flow cytometry or immunohistochemistry. Clonal Ig rearrangement was identified in all patients. The normalized MRD value was significantly lower than the unnormalized MRD value ($p < 0.001$). When categorizing patients, 27 of 50 patients (54%) achieved normalized MRD <0.01%, while 6 of them did not achieve MRD <0.01% when applying the unnormalized value. The normalized post-induction MRD value of 0.01% proved to be a significant threshold value for both 3-year event-free survival (100% for MRD <0.01% vs. 60.9% ± 10.2% for MRD ≥0.01%, $p = 0.007$) and 3-year overall survival (100% for MRD <0.01% vs. 78.3% ± 8.6% for MRD ≥0.01%, $p = 0.011$). However, unnormalized MRD was not a significant factor for outcome in this cohort. Our study demonstrated that MRD assessment by NGS-based Ig clonality assay could be applied in most pediatric B-ALL patients. Normalized post-induction MRD <0.01% was a significant prognostic indicator.

KEYWORDS

minimal residual disease (MRD), B-acute lymphoblastic leukemia (B-ALL), immunoglobulin clonality, next-generation sequencing (NGS), normalization

Introduction

In pediatric acute lymphoblastic leukemia (ALL), event-free survival (EFS) rate has improved through the accurate identification of prognostic factors, the designation of risk group based on these factors, and treatment of appropriate duration and intensity according to risk group, done within the setting of cooperative clinical trials (1). Measuring minimal residual disease (MRD) during treatment is an additional risk factor to identify patients who require intensified treatment to avert relapse. Recently, it has been shown that the presence and the degree of MRD at specific time points during therapy can be used to guide treatment, demonstrating the clinical significance of detecting MRD (2, 3). Methods of evaluating MRD by reverse transcriptase qPCR (RT-qPCR), quantitative polymerase chain reaction (qPCR), multi-parametric flow cytometry (MFC), and next-generation sequencing (NGS)-based immunoglobulin (Ig) clonality assay have been shown to be promising MRD monitoring tools for B-ALL.

Specifically, NGS-based Ig clonality assay showed excellent analytical performance with high sensitivity and applicability to most B-cell neoplasia (4, 5). The most recent National Comprehensive Cancer Network® recommended that a validated MRD assessment technology should have a sensitivity of at least 10^{-4} (6). In addition to the analytical performance including sensitivity, standardization is the other issue that should be addressed before clinical implication. MRD value could be reported differently according to the method: expression ratio of fusion gene per reference gene for RT-qPCR, patient-specific clonal gene burden calculated by standard curve for qPCR, and % of bone marrow (BM) mononuclear cells or total nucleated cells (TNCs) for MFC. NGS-based Ig clonality assay provides two values: % of B cell, which is calculated by clonal Ig read count per total Ig read count, and % of TNC, which is adjusted according to the proportion of B cells in each sample. However, it remains unclear which value is optimal for risk stratification in each patient and how clinical laboratories should determine the % of TNC when undergoing MRD assessment by NGS-based Ig clonality assay.

In this study, we performed the NGS-based Ig clonality assay and evaluated its clinical implication in pediatric B-ALL patients to assess MRD. We further clarified the method of normalization to calculate the clonal burden of % of B cells into % of TNC and elucidated the significance of both MRD values when applied to clinical decision-making.

Materials and methods

Patients and therapy

This study was approved by the institutional review board of Seoul St. Mary's Hospital, which is affiliated with The Catholic University of Korea (IRB No: KC17TESI0187). Study participants were patients diagnosed with *de novo* or relapsed/refractory ALL at our institution from June 2016 to December 2018. Overall, 55 patients were enrolled: *de novo* ALL (n = 44), BM relapse (n = 10), and refractory (n = 1) (Table 1). One patient was considered refractory due to lack of response after 2 courses of remission induction chemotherapy. Diagnosis of ALL was based on BM pathology, immunophenotyping, cytogenetics, and molecular genetics, as shown in the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues (7). Recurrent genetic abnormalities were diagnosed according to previously reported methods (8). For the 44 *de novo* ALL patients, initial risk group classification was done according to our institutional regimen (9), and patients were classified as follows: low risk (n = 9, 20%), standard risk (n = 11, 25%), high risk (n = 11, 25%), and very high risk (n = 13, 30%). For the 10 relapsed patients, the median time from diagnosis to relapse was 40.5 months (range: 12.1–68.9 months).

Patient treatment and time point of MRD monitoring

The 44 *de novo* ALL patients were classified and treated according to an institutional protocol, the details of which have been previously reported (9). Forty-two patients achieved complete remission (CR) after remission induction chemotherapy, while two patients achieved delayed CR after additional chemotherapy. All except one patient were treated with chemotherapy only, while the remaining patient received allogeneic hematopoietic stem cell transplantation (HSCT) in the first CR due to molecular relapse prior to the delayed intensification phase of chemotherapy.

For the 11 relapsed/refractory patients, the reinduction regimens were as follows: vincristine, steroid, asparaginase, and anthracycline (daunorubicin or idarubicin) (four drug regimens, n = 6); four drug regimens with imatinib (n = 1); vincristine, steroid, and imatinib (n = 1); fludarabine, cytarabine, and idarubicin (n = 2);

TABLE 1 Patient characteristics.

	n = 55 (%)
Median age at diagnosis (range)	7.2 years (2.3–17.0)
Median initial WBC count (range)	19.70 × 10^9/L (1.29–207.34)
Disease status	
De novo	44 (80)
Relapsed	10 (18)
Refractory	1 (2)
Genetics	
High hyperdiploidy	10 (18)
ETV6-RUNX1	8 (15)
E2A-PBX1	4 (7)
BCR-ABL1	2 (4)
Normal	18 (33)
Others	13 (24)

WBC, white blood cell count.

clofarabine, cyclophosphamide, and etoposide (n = 1). Ten patients achieved CR with reinduction chemotherapy, and these patients proceeded to allogeneic HSCT.

Samples were retrospectively retrieved for MRD assessment at the time of diagnosis, after induction [4 weeks, time point 1 (TP1)], consolidation (14 weeks, TP2), and 24–25 weeks (TP3). Patients who relapsed during follow-up were evaluated for Ig rearrangement again. MRD assessment was done in 50, 40, and 22 patients at TP1, TP2, and TP3, respectively, depending on the availability of samples for MRD analysis (Supplementary Table 1).

MRD monitoring using NGS

Genomic DNA (gDNA) was isolated from BM aspirates using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Samples were quantified using Qubit dsDNA BR assay (Thermo Fisher Scientific, Waltham, MA, USA). The LymphoTrack® IGH FR1/2/3 and LymphoTrack® IGK assay panels (InVivoScribe Technologies, San Diego, CA, USA) were used for the analysis of initial samples to determine clonal rearrangements and MRD samples to detect previously characterized clonotypic rearrangements. For MRD testing, low-positive controls were also included in every run.

All experiments were performed according to the manufacturer's guidelines, which were previously reported (10). Briefly, amplification by PCR was performed using 240 ng of gDNA per sample, and master mixes contain primers designed with barcoded sequence adaptors. Next, we purified the amplicons using an Agencourt® AMPure XP system (Beckman Coulter, Brea, CA, USA) and quantified the amplicons with a Qubit® dsDNA HS Assay Kits (Thermo Fisher Scientific), High Sensitivity D1000 Reagents, and High Sensitivity D1000 ScreenTape (Agilent Technologies, Santa Clara, CA). The libraries were sequenced on a MiSeqDx instrument (Illumina, San Diego, CA, USA) using the MiSeq Reagent Kit version 2 (500 cycles), aiming at 500,000 reads per sample. We prepared one replicated libraries from the gDNA sample to analyze MRD; each library had 240 ng of gDNA input. Percentage confidence for which the searched sequence was not detected was 97.17% at 10^{-4}.

The FASTQ files were analyzed using the LymphoTrack MRD software v2.0.2 (InVivoScribe Technologies) for clonality assessment and sequence tracking. Clonal rearrangement was determined according to the manufacturer's guidelines. When the total number of reads for each sample was ≥20,000 and the top merged sequence had ≥2.5% of the total reads or when the total number of reads for each sample was ≥10,000 but <20,000 and the top merged sequence had ≥5% of the total reads, these results were interpreted as clonal. For MRD assessment, the clone of exact sequence matches and similar sequences (up to two mismatched nucleotides) were sought after chemotherapy according to the manufacturer's guideline. If any sequences exact or similar to the initial clone were found, the amount of residual Ig clone was described as the proportion per total Ig read counts (% of B cell). All clonal rearrangements found at diagnosis in each patient were evaluated in subsequent MRD samples.

We tried to estimate the MRD clone in each sample by normalization using the following methods. LymphoQuant™ Internal control was added to each PCR reaction at 100 cell equivalency when testing these follow-up samples to allow the estimation of cell equivalents within each sample. The proportion of the MRD clone in each sample was calculated as % of TNC using the formulas provided by the manufacturer. Alternatively, we estimated the CD19-positive B-cell proportion in each sample using flow cytometry [FACSCanto II Flow Cytometer and FACSDiva software (Becton Dickinson, San Jose, CA, USA)] or immunohistochemical stain (IHC, mouse monoclonal anti-human CD19 antibody; NovoCastra, Newcastle upon Tyne, UK). The interchangeability among the methods for normalization has been evaluated in advance. The CD19-positive B-cell proportion analyzed by flow cytometry and IHC showed good correlation (R^2 = 0.9518, p < 0.001) and could be used interchangeably (Supplementary Figure 1A). In addition, we compared the MRD results that were normalized by LymphoQuant™ Internal control with those normalized by flow cytometry or IHC and found that they showed good correlation (R^2 = 0.8558, p < 0.001) (Supplementary Figure 1B).

The percentage of TNC was calculated using the following formula: (% of B-cell) × (CD19-positive B-cell proportion in sample)/100. For convenience, we annotated % of B cell and % of TNC as unnormalized and normalized MRD, respectively.

Statistics and outcome measures

Event-free survival (EFS) was defined as time from diagnosis of ALL to last follow-up in CR, or first event. Relapse, early death,

primary refractory disease, death in CR, and secondary malignancy were considered events. Patients with primary refractory disease or those who died during remission induction chemotherapy were considered to have events at time zero. For the relapsed patients, EFS was defined as time from relapse to last follow-up in CR, or subsequent event. Overall survival (OS) was defined as time from diagnosis (or relapse for the 10 relapsed patients) to last follow-up, or death from any cause. Comparison of EFS in the *de novo* cohort was done for the following variables: age (<10 years old vs. ≥10 years old), initial white blood cell (WBC) count (<50 × 10^9/L vs. ≥50 × 10^9/L), prephase steroid response during remission induction chemotherapy, presence of good prognosis genetic abnormalities (high hyperdiploidy or *ETV6-RUNX1*), and MRD at TP1 (<0.01% vs. ≥0.01%). Probabilities of EFS and OS were calculated using the Kaplan–Meier method, and comparison of survival curves according to risk factors was done with the log-rank test. Comparison of end of induction MRD value (negative vs. positive with a threshold of 0.01% normalized value) according to patient disease status (*de novo vs.* relapsed/refractory) was done with chi-square test. Patient follow-up was done up till 30 June 2021. Comparison between normalized and unnormalized MRD was performed by Wilcoxon signed-rank test, and their correlation was done by Spearman's rho correlation. p-value <0.05 was considered significant.

Results

Clonal Ig rearrangement was identified in all patients. *IGH* FR1 was useful in most patients (n = 49), and *IGH* FR2 and *IGK* were useful in three patients each. Twenty-four patients had one Ig clone and 20 patients had two. The other 11 patients showed more than three Ig clones. *IGH* V3-J4 rearrangement was most common followed by V3-J6 and V3-J5 (Supplementary Figure 2). The mean proportion of Ig clone at diagnosis was 54.153% ± 22.859%. During MRD assessments, we derived two MRD values: unnormalized (% of B cell) and normalized MRD (% of TNC). These two MRD values showed good correlation with a correlation coefficient of 0.968 (p < 0.001). The average and standard deviation (SD) of unnormalized MRD was 10.397% ± 23.253%, 1.311% ± 3.196%, and 1.535% ± 3.557% at TP1, TP2, and TP3, respectively. The normalized MRD value was significantly lower than unnormalized MRD (p < 0.001). The average and SD of normalized MRD was 2.649% ± 10.545% at TP1, 0.059% ± 0.173% at TP2, and 0.058% ± 0.189% at TP3.

Then, we categorized patients according to the MRD value 0.01%, 0.1%, and 1%. We observed that there was a difference between before and after normalization. At TP1, 27 of 50 patients (54%) achieved normalized MRD <0.01%, while 21 (42%) showed unnormalized MRD <0.01%. At TP2, 34 of 40 patients (85%) showed normalized MRD <0.01% while 29 (72.5%) showed unnormalized MRD <0.01%. At TP3, 16 (73%) and 15 (68%) of 22 patients showed normalized and unnormalized MRD <0.01, respectively. Overall, 12 patients were recategorized from MRD ≥0.01% to MRD <0.01% after normalization. Considering that therapy adjustment decisions may be made based on MRD <0.01% threshold at TP1, the results of six patients indicated the need for more intensified treatment due to MRD ≥0.01% prior to normalization (Supplementary Figure 3).

Events in the *de novo* cohort of patients included eight patients who relapsed at a median of 22.4 months from diagnosis (range: 17.1–47.6 months). Two patients died of relapsed/refractory disease. The estimated 3-year EFS and OS of the *de novo* cohort was 88.6% ± 4.8% (36/44) and 95.3% ± 3.2% (42/44), respectively. All 11 patients followed from the point of relapsed/refractory ALL achieved subsequent CR. However, 6 of the 11 patients experienced further events: subsequent relapse (n = 5) and secondary malignancy (n = 1). Overall, four patients died: three from relapsed/refractory disease and one from acute respiratory distress syndrome in CR. The 3-year EFS and OS of the relapsed/refractory cohort were 45.5% ± 15.0% (5/11) and 63.6% ± 14.5% (7/11), respectively. Utilizing a normalized MRD threshold of 0.01%, 26 of 40 *de novo* ALL patients (65%) with evaluable data were TP1 MRD negative, while only 1 of 10 relapsed/refractory patients (10%) were TP1 MRD negative (Table 2, p = 0.003 when comparing the two patient groups).

In combining the *de novo* and relapsed/refractory ALL cohorts, normalized TP1 MRD value of 0.01% proved to be a significant threshold value for both 3-year EFS (100% for MRD <0.01% vs. 60.9% ± 10.2% for MRD ≥0.01%, p = 0.007) and 3-year OS (100% for MRD <0.01% vs. 78.3 ± 8.6% for MRD ≥0.01%, p = 0.011). However, unnormalized TP1 MRD was not a significant factor for EFS in this cohort (3-year EFS 100% for MRD <0.01% vs. 69.0 ± 8.6% for MRD ≥0.01%, p = 0.125) (Figures 1A–D). When limiting the analysis to the *de novo* ALL cohort, the initial WBC count proved to be a significant factor for EFS: 3-year EFS of 96.7% ± 3.3% (initial WBC count <50 × 10^9/L) vs. 71.4% ± 12.1% (initial WBC count ≥50 × 10^9/L), p = 0.027. Patients with a normalized TP1 MRD <0.01% had superior outcome compared with those with MRD ≥0.01%, although without statistical significance (3-year EFS 100% vs. 78.6% ± 11.0%, p = 0.229).

Discussion

The prognostic significance of MRD, measured from the BM at specific time points after therapy, is well-established. Basically, cellular MRD counts have general prognostic value at the cutoff level of 0.01% MRD cells (10^{-4}), indicating 1 in 10,000 cells in a specimen. Because MRD values are reported in various ways according to the assessment technology, standardization is essential to establish the strategy for monitoring patients. In terms of NGS-based Ig clonality assay, data normalization and the quality control (QC) of robust amplification, library preparation, and sequencing are technically important. Several

TABLE 2 Correlation between patient disease status and end of induction minimal residual disease value using a threshold value of 0.01.

		End of induction normalized MRD value		Total
		<0.01%	≥0.01%	
Disease status	*De novo*	26	14	40
	Relapsed/refractory	1	9	10
	Total	27	23	50

MRD, minimal residual disease.

procedures were established to address the normalization issue such as a central in-tube QC spiked to each tube as library control and calibrator and central polytartget QC (11, 12). The LymphoQuant™ internal controls are used for in-tube QC and are probed to be optimal for normalization, showing better correlation with the MFC results (Supplementary Figure 4).

In this study, we could identify clonal Ig rearrangement in all pediatric B-ALL patients by an NGS-based Ig clonality assay. A total of 89% of cases were successfully characterized using FR1 primer sets, similar to the results of previous studies in B-cell neoplasia (4, 5). The frequency of common V-J rearrangements was also in line with our previous study (10). Unnormalized and normalized MRD values showed good correlation, which was predicted because both values were calculated based on clonal Ig read count. The values, however, differed with regard to prognostic relevance. The most significant factor resulting in difference was the proportion of B cells in each sample. At diagnosis, most cells were B cells with clonal Ig rearrangement. After treatment, normal hematopoietic components of erythroid and granulocytic lineages were reconstructed, leading to a relatively lower B-cell proportion. Accordingly, total Ig read count was low in those samples even though the amount of input DNA was sufficient, resulting in a relatively high unnormalized MRD value. For example, we found that six TP1 MRD-negative patients were categorized as having persistent MRD before normalization. More importantly, unnormalized TP1 MRD

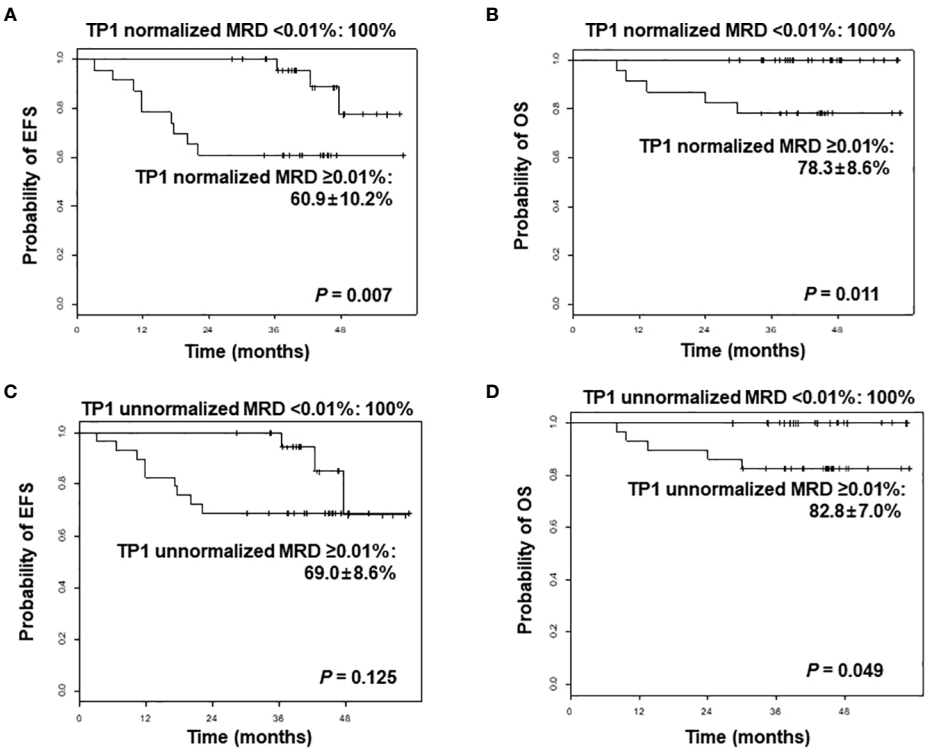

FIGURE 1
Comparison of event-free survival (EFS) and overall survival (OS) of overall patients according to normalized minimal residual disease (MRD) values **(A, B)**, and unnormalized MRD values **(C, D)** after induction, analyzed by next-generation sequencing-based immunoglobulin clonality assay. TP1, time point 1.

did not predict patient outcome whereas the normalized TP1 MRD was a significant prognostic factor for both EFS and OS. Therefore, normalization is a pivotal process for MRD assessment to remain an efficient prognostic indicator and a factor in therapy modification in ALL, as well as to prevent chemotherapy intensification of limited value (13). Post-therapy MRD should be able to indicate prognosis in *de novo* ALL patients. A clear limitation of our results was that for the 44 patients with *de novo* ALL, patients with normalized TP1 MRD <0.01% had higher EFS than those with MRD ≥0.01%, but without statistical significance. Initial WBC count was the only significant factor for EFS, with the threshold WBC count set at 50×10^9/L as defined in the National Cancer Institute/Rome criteria for high-risk ALL (14). At present, we are implementing NGS-based MRD measurement in all of our ALL patients, and a subsequent, larger-scale study may clarify the role of MRD at TP1 using this modality in determining patient outcome.

The important prognostic role of end of induction MRD detected in the BM has been established through both flow cytometry and PCR detection of Ig and T-cell receptor gene rearrangements (15, 16). NGS-based Ig clonality assay is likely more sensitive than previous methods of MRD detection, and may also be able to predict patients with worse outcome. One recent study comparing NGS-based MRD assessment and flow cytometry with a threshold of 0.01% found that NGS identified 38.7% more patients as MRD positive (17). Importantly, these patients had significantly lower EFS than those who were MRD negative according to NGS, indicating overall that NGS had a lower false-negative rate than flow cytometry. Further studies are necessary to determine the prognostic role of MRD assessment using an NGS-based Ig assay, as well as the optimum threshold for risk group classification.

Consequently, our study demonstrated that MRD assessment by NGS-based Ig clonality assay could be applied in most pediatric B-ALL patients. TP1 MRD with a threshold of 0.01% could be a valid prognostic factor. Importantly, normalization of MRD measurements as % of TNC using LymphoQuant internal control or the B-cell proportion in the sample allowed for NGS-based MRD to become a significant prognostic indicator.

Data availability statement

The original contributions presented in the study are included in the Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

This study was reviewed and approved by Institutional review board of Seoul St. Mary's Hospital(IRB No: KC17TESI0187). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

YK, N-GC, and MK were responsible for the study concept and project administration. JWL, AA, JML, JWY, SK, BC, and N-GC acquired resources. JWL, AA, and MK analyzed and interpreted data. JWL, YK, N-GC, and MK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.957743/full#supplementary-material

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The *ALLgorithMM*: How to define the hemodilution of bone marrow samples in lymphoproliferative diseases

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Introduction: Minimal residual disease (MRD) is commonly assessed in bone marrow (BM) aspirate. However, sample quality can impair the MRD measurement, leading to underestimated residual cells and to false negative results. To define a reliable and reproducible method for the assessment of BM hemodilution, several flow cytometry (FC) strategies for hemodilution evaluation have been compared.

Methods: For each BM sample, cells populations with a well-known distribution in BM and peripheral blood - e.g., mast cells (MC), immature (IG) and mature granulocytes (N) – have been studied by FC and quantified alongside the BM differential count.

Results: The frequencies of cells' populations were correlated to the IG/N ratio, highlighting a mild correlation with MCs and erythroblasts (R=0.25 and R=0.38 respectively, with p-value=0.0006 and 0.0000052), whereas no significant correlation was found with B or T-cells. The mild correlation between IG/N, erythroblasts and MCs supported the combined use of these parameters to evaluate BM hemodilution, hence the optimization of the *ALLgorithMM*. Once validated, the *ALLgorithMM* was employed to evaluate the dilution status of BM samples in the context of MRD assessment. Overall, we found that 32% of FC and 52% of Next Generation Sequencing (NGS) analyses were MRD negative in samples resulted hemodiluted (HD) or at least mildly hemodiluted (mHD).

Conclusions: The high frequency of MRD-negative results in both HD and mHD samples implies the presence of possible false negative MRD

measurements, impairing the correct assessment of patients' response to therapy and highlighs the importance to evaluate BM hemodilution.

KEYWORDS

minimal residual disease, multiple myeloma, acute lymphoblastic leukemia, hemodilution, hemodilution/methods, flow cytometry, measurable (minimal) residual disease

Introduction

The study of minimal residual disease (MRD) provides critical information for the management of hematological patients affected by lymphoproliferative diseases, representing the best biomarker to monitor treatments' efficacy and to define the eradication of residual tumor cells. Moreover, MRD is increasingly taking hold for the choice of therapy, whether for the modulation of therapeutic intensity, for the indication of stem cell transplantation or eventually for treatment discontinuation (1–7). International guidelines are recommending the use of bone marrow (BM) aspirate, with specific sensitivity thresholds, as gold standard for MRD measurements in most hematological malignancies, in particular Multiple Myeloma (MM) (1, 2, 8–10) and Acute Lymphoblastic Leukemia (ALL) (4, 11–13).

However, one of the main pitfalls of the MRD quantification is represented by the quality of the sample itself, as the dilution of the tumor cells with peripheral blood (PB) (defined as hemodilution) that occurs within BM sampling can cause an underestimation of residual disease cells, and lead to biased MRD evaluation. Considering the growing role of MRD evaluation both for prognosis and for treatment tailoring (14, 15), it seems more and more necessary to investigate whether the cases of negativity would not actually be linked to bad sampling of the BM specimen, particularly in those settings involving T cell redirecting therapies (e.g., engineered chimeric antigen receptor (CAR) T-cells).

To date, no consensus has been established yet, both on criteria for samples' quality acceptability and on the set up of standardized protocols for hemodilution evaluation. In fact, over time, several parameters have been investigated to define hemodilution in different diseases by distinct groups, such as the Holdrinet index (16), the frequencies of plasma cells and CD34+ cells (17), the ratio between immature and mature granulocytes (18), mast cells and hematogones presence (19) among others.

Thus, the aim of the current study was to define a robust and reproducible method for the assessment of BM hemodilution, applicable to lymphoproliferative diseases, in order to ensure the best quality of MRD measurements and interpretation.

Materials and methods

Patient cohort

All patients included in the study provided written informed consents for biological studies and have been treated following local recommendations for clinical trials or routine clinical practice at the Seràgnoli Institute, IRCCS Azienda Ospedaliero-Universitaria of Bologna, Italy. An initial cohort of 78 Multiple Myeloma (MM) and 18 Acute Lymphoblastic Leukemia (ALL) patients (104 and 34 samples, respectively) was used to set-up the experimental plan to evaluate the cellular immunophenotype by flow cytometry (FC) of the most representative BM cell types and to create the matrix to compare diverse hemodilution strategies, previously described by other groups (16–20). ALL patients are represented by Philadelphia (Ph)-negative B-ALL or T-ALL patients, for whom minimal residual disease (MRD) is measured molecularly by Next Generation Sequencing (NGS). NGS was employed for MRD monitoring in MM patients, as well. All patients and cohort characteristics are shown in Table 1. Briefly, patients had a median age of 46 (range 20-77) and 61 (range 39-76) years old for ALL and MM, respectively; as for the gender, 65% (39/60) of ALL patients and 56% (74/133) of MM were male. ALL patients were mostly treated with chemotherapy by pediatric-like regimens and/or by reduced regimens in elderly patients (10/25; 40%); 8/25 patients (32%) were relapsed/refractory or MRD-positive under Inotuzumab ozogamicin and/or Blinatumobab at the time of BM sampling, whereas in 6/25 patients (24%) MRD was evaluated after BM transplant. One patient was not under treatment, when BM was collected. Most MM patients (82/91; 90%) were transplant-eligible: in most cases (84%) MRD samples were collected during maintenance, whereas in the remaining (16%) post-induction. The majority (54%) of MM patients were under Immunomodulatory drugs (IMiDs) regimens at the time of BM sampling, whereas others were under treatment with anti-CD38 (15%) or Proteasome Inhibitors (PI; 23%) in the context of outpatient regimen and/or within clinical trials.

In the validation phase of the study, CAR-T-treated MM patients (9/100; 9%) (i.e., anti-B-cell maturation antigen (BCMA) CAR-T) have been added to the cohort. In these cases, since CAR-T cells therapy, preceded by lymphodepletion chemotherapy,

TABLE 1 Patients' overview. Three main sub-groups of patients have been included in the study: Acute Lymphoblastic Leukemia (ALL) patients, Multiple Myeloma (MM) patients and MM patients treated with chimeric antigen receptor (CAR)- T cells.

	Patients	Samples	Age (range)	Gender
ALL	25	69	46 (20-77)	F[2] 35%-M 65%
MM	91	150	61 (39-76)	F 44%-M 56%
CAR-T[1]	9	14	54 (38-64)	F 36%-M 64%
Tot	125	233	54 (20-77)	F 38%-M 62%

[1]CAR-T is referred only to MM patients who were sampled after at least 56 days after anti-BCMA CAR-T infusion (median: 171 days; range: 56-365 days); [2]F, female and M, male.

could cause a modulation of BM cell populations (19), we decided not to perform hemodilution evaluations on BM samples taken from patients who had undergone the CAR-T infusion within 56 days (median: 171 days; range: 56-365 days).

For each patient, at least 4 ml of BM aspirates were collected, deriving from a single-site iliac crest sampling and samples were preserved in EDTA tubes for subsequent analyses (e.g. MRD evaluation).

Flow cytometry strategy

Cell distribution within BM aspirates, in terms of quantification and characterization, was assessed FC and all analyses were performed within 24 hours from BM sampling. Cellular immunophenotype was analyzed *via* FACSCanto™ II (BD Biosciences, San Jose, CA, USA), using a combination of antibodies provided by BD Biosciences: CD45-V500, CD56-APC, CD16-V450, CD10-PECy7 and APC-H7, CD19-PerCP-Cy5.5, CD81-FITC, CD38-PECy7, CD138-PE, CD71-FITC, CD117-APC and adding CD117-BrilliantViolet 421 (BioLegend, San Diego, CA, USA). Briefly, 5 µl of each antibody was mixed to 100 µl of fresh BM sample and incubated for 15 minutes. The sample was then lysed and washed before acquisition. A median of 75 000 events were acquired and no less than 5 events were used to define a cell population. We decided to exclude CD34+ cells from our subset populations, because of the disease's context (lymphoproliferative diseases) and the fact that CD34+ cells amounts are also related to patient gender and age (17), as well as to circadian cycles (21–23). Alongside FC analyses of cell populations, a BM differential blood count *via* both cytological analysis (data not shown) and Sysmex XN-1000™ Hematology Analyzer (Sysmex America Inc., IL, USA) (shown in Table 2) was performed.

The antibody combinations employed to define each cell type is described in Table 3, and the result of gating strategy is shown in Figure 1.

IG/N ratio definition

The immature granulocytes (IG)/mature (neutrophils, N) ratio is a mathematical relationship between IG and N aimed at simplifying the hemodilution evaluation of BM samples. Considering that Sorigue et al. and Julie Pont herself had already studied both the correlation between Pont's IG/N and the formula defined by Holdrinet et al. (HI) (18, 20), defining an R of 0.8 (p-value <0.001) in healthy individuals, we decided to use IG/N ratio as reference for our considerations. We excluded the possibility to employ the HI as a reference, even though it is considered as the gold standard to assess hemodilution by most authors, since it requires both a parallel PB sampling and a parallel characterization and count of leukocytes and erythrocytes. Conversely, the IG/N ratio is straightforward and, particularly in the lymphoproliferative disease setting, is not biased by the pathology considered and/or by MRD-positive results. Thus, according to Pont et al. a cut-off IG/N ratio of 1.2 was employed to distinguish hemodiluted (<1.2) from non-hemodiluted samples (≥1.2).

Minimal residual disease assessment

For all patients MRD measurement was performed by NGS to investigate the IgH/TCR rearrangement(s). Analyses have been conducted *via* LymphoTrack® Dx IgH (FR1/FR2/FR3)/IgK/TCR assays on MiSeq™ System (Illumina Inc, San Diego, CA, USA) on DNA extracted from BM samples. MRD measurements have been quantified at a sensitivity of at least 10^{-5}, using a LymphoQuant B-cell Internal Control. Data analysis was performed by the LymphoTrack® MRD Software

TABLE 2 BM cell populations evaluation. Assessment of the presence of bone marrow cell populations by an automated analyzer.

Type of Cells	Median (range)	Median Percentage (range)
WBCs[1]	9940 (80-68420)	na*
Neutrophils	na*	59.1 (23.6-89.6)
Monocytes	na*	26.09 (0-79)
Lymphocytes	na*	9.2 (1.8-37.6)
NRBCs[2]	na*	13.1 (0-44.1)
IG[3]	na*	15.85 (0-44.1)

[1]White Blood Cells (WBCs) expressed in cell/µl; [2]Nucleated Red Blood Cells (NRBCs); [3]Immature granulocytes (IG); *Not applicable (na)/not evaluated.

TABLE 3 Flow cytometry definition of BM cell populations. Distribution of the main BM cell populations, according to their immunophenotype.

Type of Cells	Immunophenotype[1]	Median (range)
Immature granulocytes	SSC^{++}/FSC^{++}; $CD45^{dym/+}/CD16^{low}/CD10^{neg}$	22.85% (0.4-57.2)
Mature granulocytes	SSC^{++}/FSC^{++}; $CD45^{dym}/CD16^{++}/CD10^{++}$	12.6% (0.2-57.8)
IG/N ratio	na[2]	1.26 (0.02-5.2)
Mast cells	SSC^{++}/FSC^{++}; $CD117^{hi}/CD45^{dym}$	0.006% (0-0.11)
Plasma cells	SSC^{low}/FSC^{+}; $CD138^{hi}$	0.2% (0-3)
Hematogones	SSC^{low}; $CD81^{hi}/CD10^{+}/CD45^{dym}$	0.9% (0-9)
B lymphocytes	SSC^{low}; CD45hi/$CD19^{+}/CD56^{neg}$	1.6% (0-10)
NK-like T cells/NK	SSC^{low}; CD45hi/$CD19^{neg}/CD56^{hi}$	0.4% (0.3-16)
Erythroblasts	SSC^{low}/FSC^{low}; $CD45^{neg}/CD71^{+}$	5.3% (0.4-12.4)

[1]According to the EuroFlow consortium; [2]Not applicable (na).

(*In vivo*scribe Inc, San Diego, CA, USA) (24). Undetectable MRD was assessed with at least 90% of confidence.

In ALL patients, MRD was performed also by FC, in parallel with NGS, employing FACSCanto™ II (BD Biosciences, San Jose, CA, USA). Quality control of the instrument was daily performed using FACSDiva™ CST IVD beads (BD Biosciences, San Jose, CA, USA). The panel used to measure MRD comprised CD45, CD19, CD20, CD10, CD58, CD123, CD34, CD22 for lineage B, and CD3, CD5, CD7, CD2, CD4, CD8, CD1a and TCRγ for the T-lymphoid compartment. The FC overall sensitivity was at 10^{-4}, and MRD negativity was set under the 0.01%, according to standardized guidelines (25–27).

Statistical and bioinformatic analyses

All bioinformatics and biostatistics analyses were conducted using personalized scripts and R packages. Pearson and Kruskal-Wallis tests were employed to evaluate correlations between cell populations included in the study, as well as parameters related to the patient's cohort, such as age, gender and therapy regimens at the time of the BM aspirate sampling. Medians were used to define cut-offs for flow cytometry analysis of cellular sub-sets. The confidence interval considered was 95%. All results obtained shown a p-value of at least 0.001.

Results

This was a multi-step study, initially focused on the comprehensive comparison of various FC-based approaches to assess BM hemodilution (according to the comparative analyses of different cell populations previously employed by several authors to this purpose), and then aimed at the development of a novel, original algorithm (named *ALLgorithMM*) for the hemodilution assessment in patients' BM samples dedicated to MRD evaluations.

Definition of cell types within BM aspirates

For each BM sample, cell populations with a well-known distribution in both PB and BM (i.e., plasma cells, mast cells, lymphocytes, erythroblasts and granulocytes) were characterized and quantified by FC, as described in Materials and Methods section. As shown in Table 3 (for gating strategy refer to Figure 1), relevant sub-sets of marrow cells were defined by a specific immunophenotype and quantified as absolute percentage, according to the major EuroFlow consortium operating procedures (28). For immature granulocytes all the maturation stages are intended, including promyelocytes, myelocytes and metamyelocytes.

As explained in Table 2 (see Materials and Methods), BM aspirates have been also analyzed *via* an automated counter to obtain the differential blood count together with the percentage of each cell population.

Correlation between major populations present in BM samples

Several approaches to define hemodilution have been reported in the literature over time, mostly based on flow cytometric analysis and particularly in the context of acute leukemias (either ALL or Acute Myeloid Leukemia) (16–19, 29). These studies were taken as a starting point to define the most suitable approach to be applied to lymphoproliferative disease during post-treatment follow-up. Using the bioinformatic and statistical strategy described in section Materials and Methods (paragraph 2.5), all populations present in BM aspirates – previously employed for hemodilution assessment – were correlated, considering as reference the immature (IG)/mature granulocytes (neutrophils, N) ratio defined by Pont in 2018 (18), to highlight which one best contributes to the definition of BM hemodilution.

In the initial cohort of patients, IG/N ratio was <0.5 in 33/138 (24%) cases, between 0.5 and 1.2 in 36/138 (26%) samples

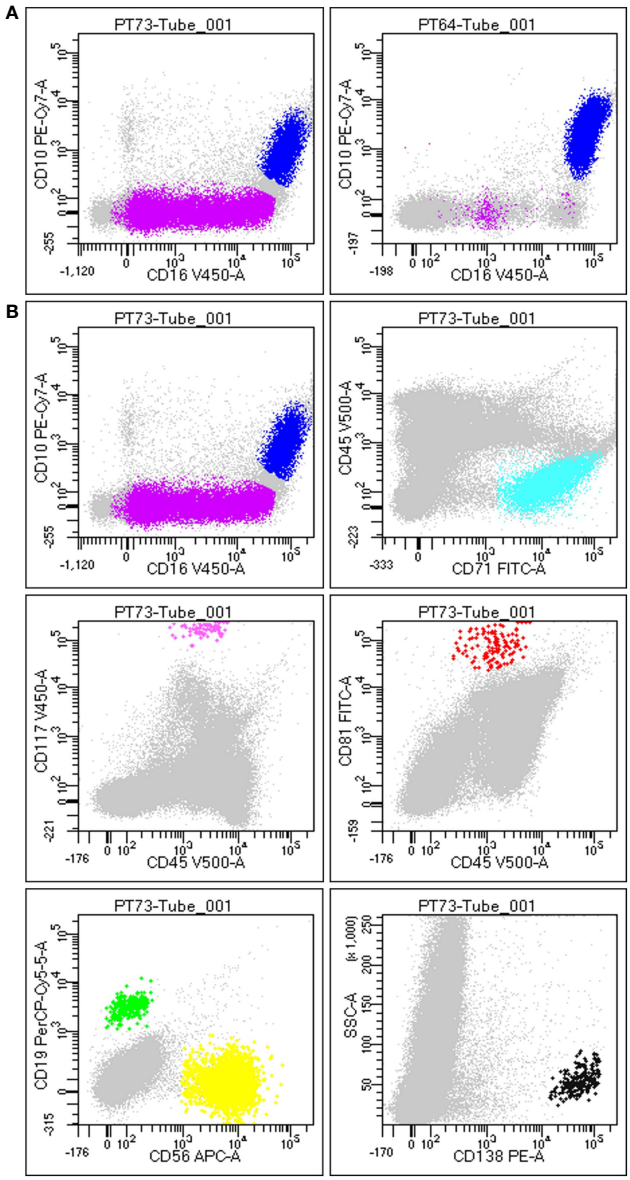

FIGURE 1
Gating strategy to define BM population. **(A)** Difference between a non-hemodiluted sample (left) and a hemodiluted sample (right), as shown by the presence of both immature (purple) and mature granulocytes (blue): the maturation line of granulocytes (including promyelocytes, myelocytes and metamyelocytes) is highlithed in purple and is highly present in nonHD samples (on the left), wherease it is almost absent in HD samples (on the right). **(B)** Each plot is the result of the different gating strategies used to define different populations, according to FSC/SSC and the specific markers. In details, plot 1 displays immature (SSC++/FSC++; CD45dim/+/CD16low/CD10neg) and mature granulocytes (SSC++/FSC++; CD45dim/+/CD10+/CD16+), plot 2 shows erythroblasts (SSClow/FSClow; CD45neg/CD71+), plot 3 displays mast cells (SSC++/FSC++; CD45dim/CD117hi), plot 4 exhibits hematogones (SSClow; CD45dim/CD10+/CD81hi), plot 5 shows B-cells (SSClow; CD45hi/CD19+/CD56neg) and T-cells (NK-like T-cells/NK; SSClow; CD45hi/CD19neg/CD56hi), and plot 6 displays plasma cells (SSClow/FSC+; CD138hi).

and ≥1.2 in the remaining 69/138 (50%) cases, without distinction among the different diseases.

The amount of different cell populations (as described above and listed in Table 3), such as B-lymphocytes precursors and erythroblasts, was correlated to the IG/N ratio. Results are shown in Figure 2, highlighting a mild but highly significant correlation with mast cells and erythroblasts (R=0.25 and R=0.38, p-value = 0.0006 and 0.00000526, respectively), regardless of the hematological disease studied; on the contrary, no correlations were found with B- nor with T-cells. Hematogones showed a lower correlation (R=0.24), as compared to the other cells' population. However, due to the pathological

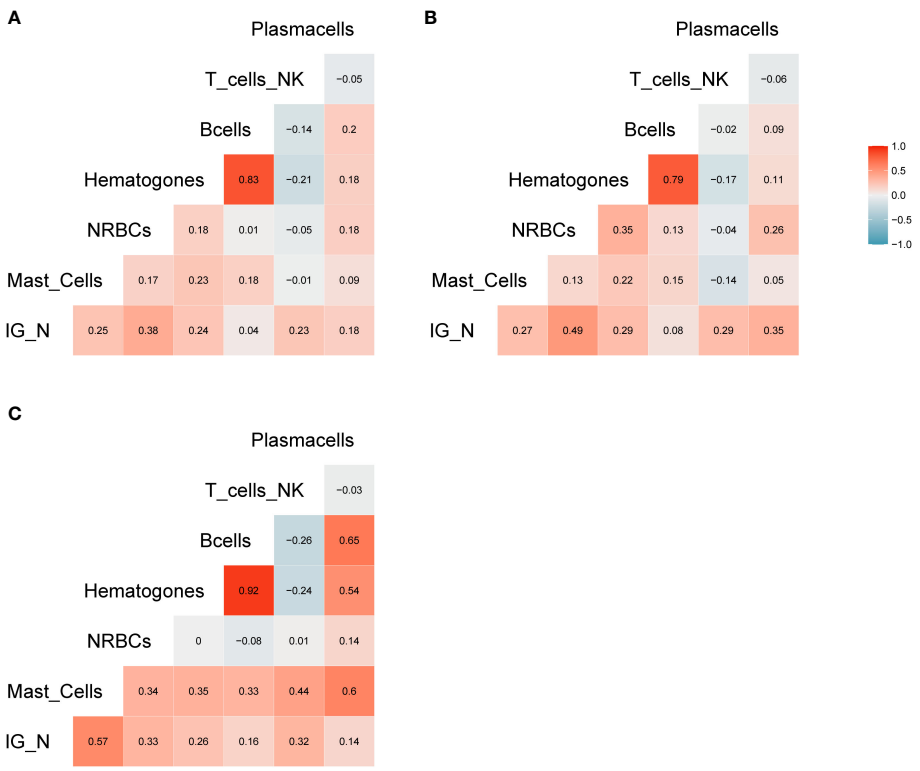

FIGURE 2
Correlation analysis. **(A)** Correlation matrix related to all samples included in the study; the relative matrices for MM **(B)** and ALL patients **(C)**. Positive correlations are displayed in red, while negative correlations are displayed in blue. Color intensity is defined by the correlation coefficient. Overall, the Pearson's rho (*R*) for IG/N vs. mast cells and erythroblasts was ~0.3, with p<0.0006.

context (mainly involving the lymphoid lineage) and to the immunomodulatory effects of therapies employed, we decided not to take hematogones into consideration for further development of the study.

The construction of the *ALLgorithMM*

The aforementioned correlations highlighted an average but highly significant correlation between IG/N ratio, erythroblasts and mast cells; the absolute percentages of cell populations can be variable in BM regardless of hemodilution, and must be taken into consideration. Thus, we integrated these three parameters to develop a simple (one tube-based), reliable and reproducible algorithm able to define the degree of BM hemodilution, named ALLgorithMM. This integration was also rupported by the categorization of the reference variables, that highlighted a stronger correlation (R>0.44; $p<9.14e10^{-8}$), and confirmed we were able to correctly define BM samples hemodilution (Figure 3).

Starting from the IG/N ratio and factoring for the percentages of erythroblasts and mast cells, ALLgorithMM allows to define three types of BM samples: hemodiluted (HD), mildly hemodiluted (mHD) and non-hemodiluted (nonHD), as highlighted in red, orange and green, respectively, in Figure 4. The cut-offs used in the ALLgorithMM derived from the previously described Pont IG/N ratio, also confirmed in our dataset, whereas the cut-offs for mast cells and erythroblasts where obtained from the median of our collected data. Notably, a biomarker-based cut-off was chosen, instead of and outcome-based one, since no association with outcome could be defined due to the short median patients' follow-up and the lack of survival events.

As resumed in Figure 4, this algorithm sequentially evaluates the IG/N ratio, the mast cells and the erythroblasts: the decisional ALLgorithMM proceeds by stratifying the attained results according to pre-defined cut-offs (i.e., 1.2 for IG/N ratio, 0.006% for mast cells and 5% for erythroblasts/NRBCs), providing the final definition of samples quality. To efficiently analyse the selected parameters, we developed a 1-tube 5-colors FC panel, easily managable and simple to analyse, including CD10-APCH7, CD16-V450, CD45-V500, CD117-APC and CD71-FITC (antibodies provided by BD Biosciences, San Jose, CA, USA). Notably, the use of only 5 markers leaves room to analyse additional, pathology-related markers, according to the need to evaluate the presence of

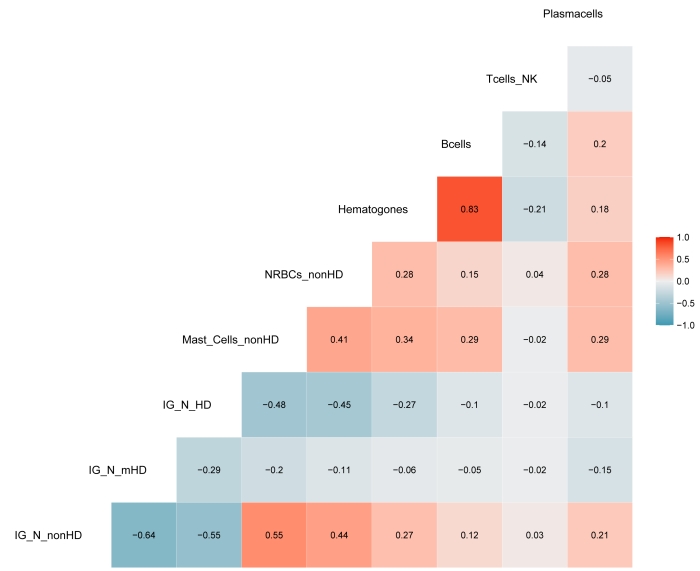

FIGURE 3
Correlation analysis with categorized parameters. Positive and negative correlations are presented in red and blue, respectively. Correlation coefficients are related to the color intensity. Once defined the cut-offs for the three parameters (IG/N, mast cells and erythroblasts (nucleated red blood cells, NRBCs), a higher correlation was observed between these categorized variables as compared to the same continuous variables shown in Figure 2.

residual cells (e.g., in MM, CD38 and CD138 could be added and used to define and to verify the presence of plasma cells).

Finally, we observed that in most cases (220/233, 94.4%), the combined employment of IG/N, mast cells and erythroblasts allowed to confirm the data derived just from the IG/N ratio evaluation. In the remaining cases (13/233, 5.6%), IG/N ratio did not adequately describe BM hemodilution; in fact, in these samples, the presence of just metamyelocytes, in absence of the other elements of granulocyte maturation line, misled the "IG" count, thus resulting in a wrongly defined not-hemodiluted result. Therefore, the combination of different parameters contributed to an improved definition of BM hemodilution, which might be critical, particularly in mildly hemodiluted samples.

IG/N, mast cells and erythroblasts define 3 well-distributed groups

Once defined, the *ALLgorithMM* was prospectively validated on samples consecutively collected from 42 MM and 27 ALL patients (57 and 38 samples, respectively), whose MRD was measured either by NGS or by FC, in the context of daily clinical practice. Overall, 12/57 MM and 10/38 ALL samples resulted HD, and 18/57 MM and 5/38 ALL were instead mHD.

A Kruskal-Wallis test was used to confirm the ability of the *ALLgorithMM* to define significantly homogeneous clusters of samples, defined as HD, as nonHD or mHD. The results shown in Figure 5 enlighten how the three parameters included in the *ALLgorithMM* (a - IG/N; b – erythroblasts; c- mast cells, as in the figure) have been able to significantly stratify patients in distinct groups (i.e., HD, mHD and nonHD) (p-value<2.4e10^{-9}). The other investigated cell populations did not show this behavior (data not shown) and, therefore they were not taken into consideration for the *ALLgorithMM*.

We finally investigated whether clinical variables (such as the therapy provided at the time of BM sampling, patients' age or gender) might influence the hemodilution evaluation by *ALLgorithMM*. As shown in Figure 6, there was no significant correlation between hemodilution definition and the variables aforementioned, supporting the robustness of the validated *ALLgorithMM* approach.

The impact of hemodilution on MRD measurement

To validate the *ALLgorithMM*, we performed *in vitro* serial dilutions of BM in PB derived from two MM patients, aiming also at measuring MRD according to the diverse hemodilution levels. Starting from the non-hemodiluted BM sample, we simulated 4 different scenarios: a) sample as-it-is, after BM collection (nonHD), b) sample diluted with PB, to obtain an MHD sample, c) sample diluted with PB to attain a fully hemodiluted sample (HD), d) sample diluted with PB, to obtain a nearly HD-MHD sample (MHDlow). The 4 samples *per* patient were then analyzed by using the *ALLgorithMM*, to

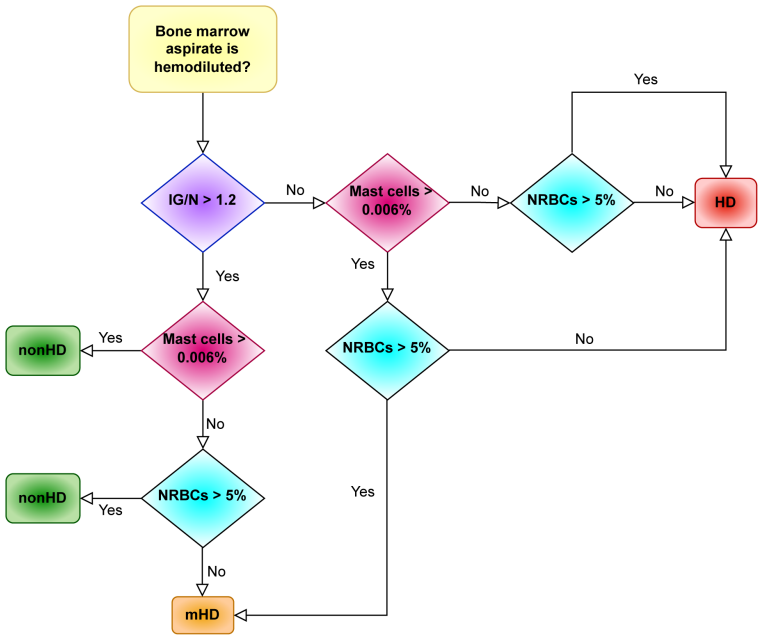

FIGURE 4
The *ALLgorithMM* sequence. For each BM aspirate, the definition of either hemodiluted or non-hemodiluted sample is the result of at least 2 out of 3 parameters, as analyzed by flow cytometry in the BM sample. Immature granulocytes (IG); mature granulocytes/neutrophils (N); erythroblasts (nucleated red blood cells, NRBCs); hemodiluted (HD); mildly hemodiluted (mHD); non-hemodiluted (nonHD).

confirm the hemodilution status, as shown in Table 4, including the relative *in silico*-derived measures. Briefly, the initial BM sample was diluted with patient-derived PB to get the above-mentioned four scenarios, still maintaining the original BM WBC count for each sample. *In silico* and *in vitro* data were then tested to assess the results' linearity, obtaining a Pearson correlation coefficient of 1 with $p=4.2e^{-09}$ for IG/N ratio, a $R=0.99$ with $p=1.4e^{-06}$ for mast cells and $R=0.99$ with $p=3.7e^{-07}$ for erythroblasts, as shown in Figure 7.

MRD was then analyzed by NGS in the same samples and results are shown in Table 5: as expected, the artificial impairment of BM aspirate quality caused an overall underestimation of residual disease measurements. In fact, in patient 1 (pt1), whose residual disease was measured in the order of 10^{-5} cells in nonHD sample, MRD measurement was not reliable in both MHD cases, and also underestimated in the HD sample. On the contrary, in patient 2 (pt2) MRD was undetectable in nonHD sample, with a confidence of 97% at 10^{-5}: this result was progressively less confident in hemodiluted samples, suggesting that low quality BM aspirates might impair also the reliability of MRD-negative results.

According to the *ALLgorithMM*, a range of hemodilution grades could be highlighted, with high percentage of HD and/or mHD samples (22% and 25%, respectively) among all evaluated patients, as shown in Table 6, section a. These BM samples were also addressed to MRD measurement. MRD was assessed by FC (for ALL patients only) and by NGS for MM and ALL Ph-negative patients, as described in the Materials and Methods section.

In general, FC MRD measure resulted undetectable in 25/48 (52%) cases and positive in 23/48 (48%) ALL samples. Molecular MRD measurements were negative in 26/46 (57%), positive in 13/46 (28%) cases and positive non-quantifiable (PNQ) in 7/46 (15%) ALL BM aspirate samples. In MM patients, molecular MRD was undetectable in 38/86 (44%), PNQ in 32/86 (37%), and positive in 16/86 (19%) cases.

Of the 26 ALL samples tested negative for molecular MRD, 6 (23%) were hemodiluted and/or mildly hemodiluted. Of the ALL samples tested by FC, 8/25 (32%) negative and 9/23 (39%) positive cases were either mildly or highly hemodiluted. In MM, undetectable MRD results were reported in 27/38 hemodiluted cases (71%).

All MRD results are summarized in Table 6, section b. For CAR-T MM patients, MRD was not provided for this study.

Overall, 52% of MRD-negative cases evaluated with NGS were HD or mHD (17/64, 27% and 16/64, 25%, respectively), whereas 48% were nonHD. For MM, only 29% (11/38) of BM used to measure MRD passed the hemodilution quality control assessment, whereas 34% (13/38) of cases were highly

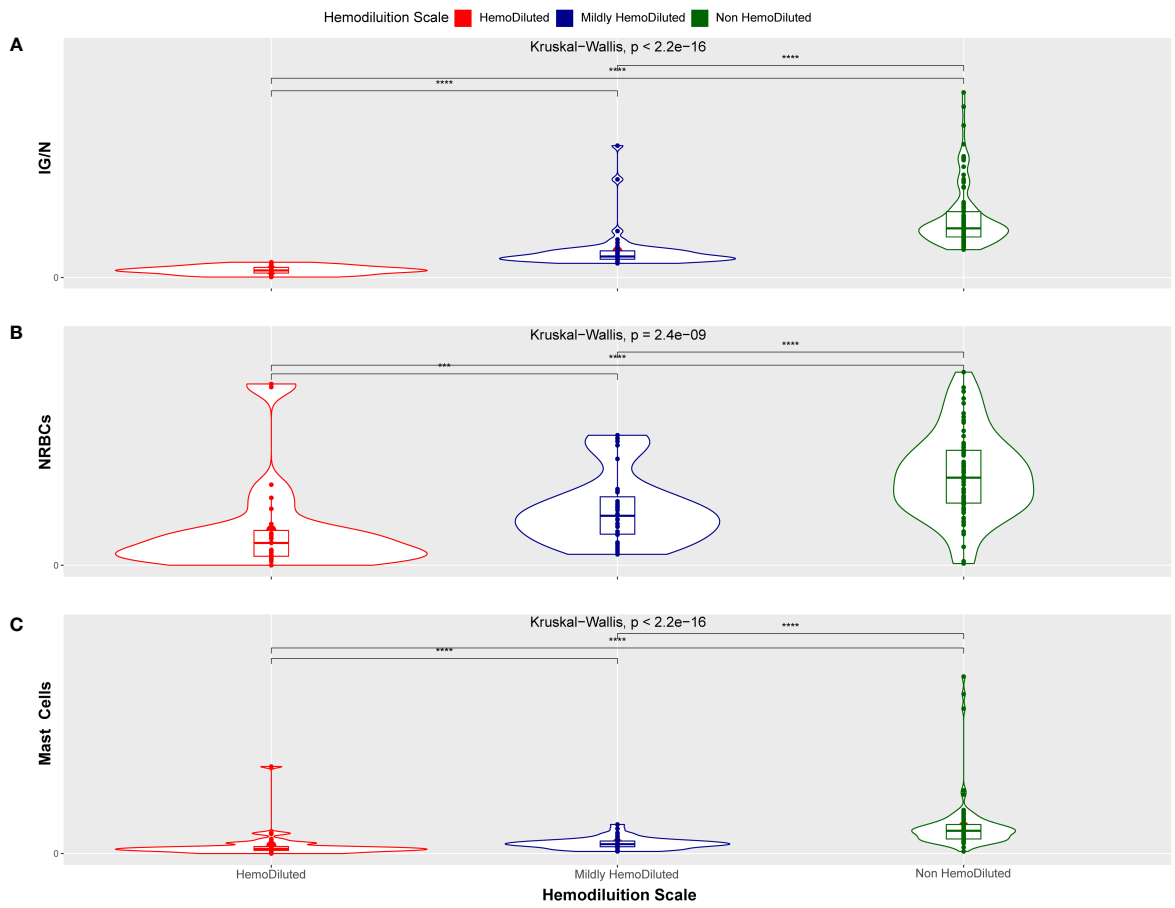

FIGURE 5
Kruskal-Wallis distribution. Hemodiluted (HD, red), mildly hemodiluted (mHD, blue) and non-hemodiluted (non-HD, green) sample distribution related to the IG/N ratio **(A)**, to the erythroblasts (nucleated red blood cells, NRBCs) **(B)** and to the mast cells presence **(C)**. The distribution between groups has a p-value inferior to 2.4e10-9, indicating a statistically highly significant tendency. (***p < 0.0005 and ****p < 0.00005).

hemodiluted and 37% (14/38) were mildly HD. ALL samples were instead HD in the 11% of cases (3/26), mHD in 3 cases over 26 (11%) of evaluation and the majority (77%, 20/26) were non-hemodiluted.

Discussion

Over the last years, the advancement of molecular and flow cytometry methods to assess MRD in lymphoproliferative disorders has led to a game-changer scenario, where it has become fundamental to obtain well-prepared samples to provide a comprehensive and representative snapshot of the BM tumor landscape and distribution. Nevertheless, several non-standardized protocols, mainly based on flow cytometry, have been investigated to evaluate the quality of bone marrow sampling, without anyhow leading to a well-defined and reliable method, easily applicable in lymphoproliferative disease regardless of therapy, age and/or gender of the patient.

Here, we set-up and validated a novel approach to assess BM hemodilution in lymphoproliferative disorders, named *ALLgorithMM*, based on the measurement of three parameters (immature (IG)/mature granulocytes (neutrophils, N) ratio, mast cells and erythroblasts amount), which can easily and objectively stratify BM samples according to the extent of hemodilution.

The *ALLgorithMM* has been validated on BM samples of patients after therapy, excluding BM with active disease (defined as > 5% blast cells and >5% of plasma cells for ALL and MM samples, respectively).

The combination of the three selected parameters derived from the observed mild, but highly significant correlation between IG/N ratio, nucleated red blood cells and mastocytes, which prompted the combined employment of these parameters to

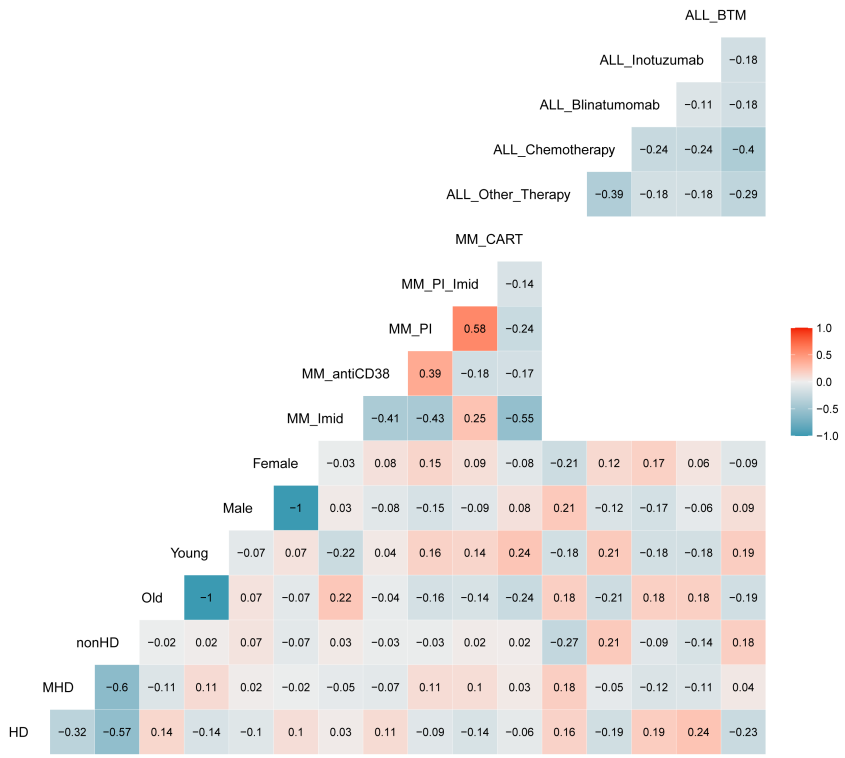

FIGURE 6
Correlation matrix between *ALLgorithMM*-defined BM hemodiluted (HD), mildlyHD (mHD) and non-hemodiluted (nonHD) vs. age (old or young), gender (female or male) and therapy used at the time of the sampling. Blue and red stand for negative or positive correlations, respectively. Color intensity is proportional to the correlation coefficients. The figure shows an absence of correlation between the 3 HD groups and others variables, confirming the robustness of the *ALLgorithMM*.

evaluate BM hemodilution; on the contrary, neither lymphocytes nor hematogones (both commonly employed to evaluate hemodilution) seemed informative enough to this purpose. The peculiar choice of these parameters to assess hemodilution was supported by several observations. First of all, the lymphoproliferative hematological diseases are characterized by an alteration in the number of cells of the lymphoid lineage. Secondly, in this clinical context, the immunomodulatory/lymphodepletive role of treatments might cause alterations and/or changes in the composition of the BM niche as well as of the microenvironment. Moreover, either a decrease of B-cell precursors or the expansion of B and T cells can occur after stem cell transplant and/or CAR-T infusion (19, 30). Thirdly, the typical absence of mast cells and of erythroblasts in PB supports their role as references in this approach.

All the above-mentioned reasons strongly supported the choice to focus on the integration of information deriving from mast cells, erythroblasts and IG/N ratio, confirming the originality and the reliability of *ALLgorithMM*, particularly in the context of lymphoproliferative diseases.

In addition, by using three different parameters, *ALLgorithMM* might be potentially applied also to other

TABLE 4 Changes in the composition of BM cell populations in serial hemodilution experiments. Distribution of parameters used in the *ALLgorithMM* to define hemodilution of BM samples, within 4 different scenarios simulated for each patient: nonHD (sample as it is), MHD (sample diluted with PB to obtain an MHD sample), MHDlow (sample diluted with PB to obtain an MHD sample nearly HD), and HD (full hemodiluted sample). In parentheses are represented *in silico*-derived values, as expected by applying serial dilutions of samples to simulate the 4 different cases.

Type of Cells	[1]pt1 nonHD	pt1 MHD	pt1 MHDlow	pt1 HD	pt2 nonHD	pt2 MHD	pt2 MHDlow	pt2 HD
IG/N ratio	2.79	0.89 (0.8)	0.66 (0.6)	0.43 (0.4)	1.61	0.76 (0.8)	0.62 (0.6)	0.41 (0.4)
Mast cells	0.003%	0.001% (0.0008%)	0.001% (0.0006%)	0.001% (0.0004%)	0.039%	0.023% (0.02%)	0.02% (0.015%)	0.012% (0.01%)
Erythroblasts	9.5%	6.2% (5.72%)	4.1% (4.04%)	3.2% (3.36%)	9.2%	4.1% (4.5%)	3.8% (3.42%)	2.2% (2.28%)

[1]pt: patient.

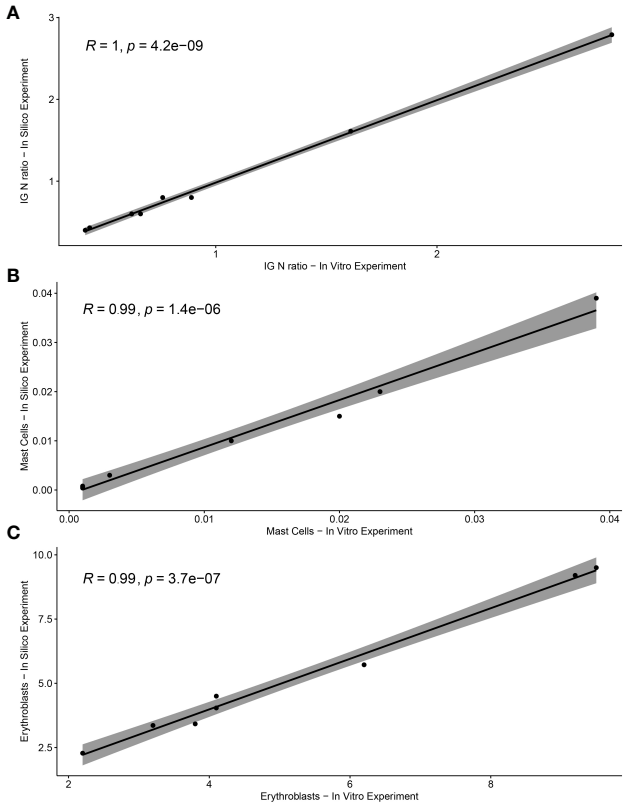

FIGURE 7
Correlation between *in vitro* and *in silico*-calculated data of *ALLgorithMM* parameters. The Pearson's rho (R) accompanied by the relative p-value for **(A)** IG/N ratio, **(B)** mast cells, and **(C)** erythroblasts.

hematological disorders, without incurring in biased data, possibly caused by the over-representation of cells' population directly involved in the disease. Finally, the *ALLgorithMM* prevents all issues related to the intra- and inter-patient intrinsic differences in the BM distribution of cells populations thanks to the combined employment of three parameters, i.e., the IG/N ratio and two cell populations (mastocytes and erythroblasts) percentages.

As a major added value, *ALLgorithMM* refines the results obtained just by the IG/N ratio, particularly when the immature granulocytes configuration is hard to be defined, for instance when the granulocyte maturation and/or composition might be compromised or when the granulopoiesis is just represented by the presence of metamyelocytes. In the present study, the hemodilution assessment has been re-adjusted with respect to the evaluation performed by the IG/N ratio in 5.6% of cases (13/233), thanks to the inclusion of both mastocytes and erythroblasts evaluations. Of these, 5/13 (38%) were defined hemodiluted thanks to mast cells and erythroblasts frequencies clearly under the cut-offs defined in the *ALLgorithMM* (0.006% and 5%, respectively). Similarly, the other 8/13 samples (62%) defined non-hemodiluted just according to IG/N ratio, were re-defined mildly hemodiluted thanks to the use of mast cells and erythroblasts; in fact, in these samples, the presence of metamyelocytes as the only granulocytic cell lineage-representing cells, caused and incorrect assessment of the immature granulocytes count, thus suggesting the importance of additional parameters to correctly define such borderline situations.

Furthermore, the *in vitro* simulation of BM hemodilution scenarios, described in paragraph 3.5, allowed both to validate the *ALLgorithMM* and to highlight the impact of hemodilution on MRD assessment. In fact, the BM serial dilution with PB, by simulating either mildly or fully hemodiluted situations, caused an overall underestimation of residual disease measurements, with a progressively decreased MRD values and/or NGS results' confidence in low quality BM samples.

The need to perform good-quality BM sample aspirates, highly representative of the tumor cells distribution, both not diluted and not contaminated by PB cells, is increasingly higher in hematologic diseases, mainly due to the growing role gained by MRD measurements after therapy for prognostication and for treatment tailoring. The high frequency of MRD-negative measurements observed in this study in both mildly and fully hemodiluted samples indicates a possible recurrent

TABLE 5 MRD evaluation in hemodiluted BM samples. NGS-based MRD measurements for two MM patients (pt) whose BM samples were progressively diluted with PB, to obtain 4 different cases: nonHD, MHD, MHDlow, and HD. MRD measures are quantified by the LymphoTrack® MRD Software (*In vivo*scribe Inc, San Diego, CA, USA). In bold: Results are an estimate of the clonal frequency of the prevalent rearrangement(s) detected at diagnosis. For each sensitivity level indicated, the statistical confidence of the result is reported.

	[1]pt1 nonHD	pt1 MHD	pt1 MHDlow	pt1 HD	pt2non HD	pt2 MHD	pt2 MHDlow	pt2 HD
MRD measure	$1.74x10^{-5}$	$2.44x10^{-5}$	$1.46x10^{-5}$	$9.11x10^{-6}$	0	0	0	0
Confidence 10^{-3}	100%	100%	100%	100%	99.99%	99.99%	99.99%	99.99%
Confidence 10^{-4}	99.74%	99.99%	99.99%	99.99%	99.99%	99.99%	99.99%	84.71%
Confidence 10^{-5}	80.37%	10.75%	15.12%	25.86%	96.68%	77.01%	80.51%	14.51%
Confidence 10^{-6}	0.18%	1.52%	0.06%	0.11%	12.45%	7.57%	3.71%	1.52%
Result	**[2]POS (80% at 10^{-5})**	**[3]NEG (99% at 10^{-4})**	**NEG (99% at 10^{-4})**	**NEG (99% at 10^{-4})**	**NEG (97% at 10^{-5})**	**NEG (99% at 10^{-4})**	**NEG (99% at 10^{-4})**	**NEG (99% at 10^{-3})**

[1]pt: patient; [2]POS (positive); [3]NEG (negative).

TABLE 6 a) Hemodilution assessment through the *ALLgorithMM*. Samples are stratified into three main groups, defined as hemodiluted (HD), mildly hemodiluted (mHD) and non-hemodiluted (nonHD) by the result of the *ALLgorithMM* application. b) The impact of hemodilution on MRD evaluation. For each category, the number and the relative percentage are referred to hemodiluted or mildly hemodiluted samples, enlightening the potential impact of hemodilution on the minimal residual disease (MRD) measurement results, as assessed by flow cytometry (FC) or by molecular (mol, *via* NGS) approaches.

a)		**HD**	**mHD**	**nonHD**	
ALL		17/69 (25%)	8/69 (12%)	44/69 (63%)	
MM		33/150 (22%)	46/150 (31%)	71/150 (47%)	
CAR-T*		2/14 (14%)	4/14 (29%)	8/14 (57%)	
Tot		52/233 (22%)	58/233 (25%)	123/233 (53%)	
b)	**FC MRD neg**	**FC MRD pos**	**mol MRD neg**	**mol MRD pos**	**mol MRD pnq**
ALL	25 (8; 32%)	23 (9; 39%)	26 (6; 23%)	13 (7; 54%)	7 (2; 29%)
MM	na**	na	38 (27; 71%)	16 (4; 25%)	32 (18; 56%)
Tot	25 (8; 32%)	23 (9; 39%)	64 (33; 52%)	29 (11; 38%)	39 (20; 51%)

*CAR-T is referred only to MM patients underwent to CAR-T therapy in general (i.e., anti-BCMA CAR-T) after at least 56 days from the infusion; **Not applicable (na), as our center does not perform MRD evaluations by flow cytometry for MM patients.

underestimation of MRD measurements, which overall might impair the correct assessment of the depth of response to therapy and therefore possibly cause wrongly supported prognostications and/or clinical decisions. This highlights the importance to include a reliable evaluation of BM hemodilution in the daily practice as an important quality control step, allowing to point out low quality BM samples and to report them to clinicians, to possibly plan a new BM evaluation, if needed.

In this context, the *ALLgorithMM* proved to be reproducible and easily applicable to evaluate hemodilution in BM samples of patients affected by lymphoproliferative diseases, such as ALL and MM. The routine application of this method can support a correct assessment of MRD, reducing the possibility of false negative results, providing essential samples quality information. Moreover, the *ALLgorithMM* can be employed also for samples collected both at diagnosis and at relapse (i.e., in highly infiltrated samples), to correctly assess the disease burden at that specific time-point, since it might be underestimated, due to bad-quality sampling. This becomes especially relevantwhen the effective amount of tumor burden is diriment for patients' inclusion in protocols and/or in clinical trials, or is crucial for clonotype assessment by NGS and in the identification of the disease grade.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conception: IV. Experimental analyses: IV, SA, MB, BT, and IP. Manuscript preparation: IV, EB, and CT. Statistical analysis VS and IV. MM, AP, GM, AC, IR, LP, PT, CS, CP, KM, SR, EZ, MA, and MC provided patients and tissue materials. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Concordance in measurable residual disease result after first and second induction cycle in acute myeloid leukemia: An outcome- and cost-analysis

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Measurable residual disease (MRD) measured using multiparameter flow-cytometry (MFC) has proven to be an important prognostic biomarker in acute myeloid leukemia (AML). In addition, MRD is increasingly used to guide consolidation treatment towards a non-allogenic stem cell transplantation treatment for MRD-negative patients in the ELN-2017 intermediate risk group. Currently, measurement of MFC-MRD in bone marrow is used for clinical decision making after 2 cycles of induction chemotherapy. However, measurement after 1 cycle has also been shown to have prognostic value, so

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the optimal time point remains a question of debate. We assessed the independent prognostic value of MRD results at either time point and concordance between these for 273 AML patients treated within and according to the HOVON-SAKK 92, 102, 103 and 132 trials. Cumulative incidence of relapse, event free survival and overall survival were significantly better for MRD-negative (<0.1%) patients compared to MRD-positive patients after cycle 1 and cycle 2 ($p \leq 0.002$, for all comparisons). A total of 196 patients (71.8%) were MRD-negative after cycle 1, of which the vast majority remained negative after cycle 2 (180 patients; 91.8%). In contrast, of the 77 MRD-positive patients after cycle 1, only 41 patients (53.2%) remained positive. A cost reduction of −€571,751 per 100 patients could be achieved by initiating the donor search based on the MRD-result after cycle 1. This equals to a 50.7% cost reduction compared to the current care strategy in which the donor search is initiated for all patients. These results show that MRD after cycle 1 has prognostic value and is highly concordant with MRD status after cycle 2. When MRD-MFC is used to guide consolidation treatment (allo vs non-allo) in intermediate risk patients, allogeneic donor search may be postponed or omitted after cycle 1. Since the majority of MRD-negative patients remain negative after cycle 2, this could safely reduce the number of allogeneic donor searches and reduce costs.

KEYWORDS

acute myeloid leukemia, measurable residual disease (MRD), multiparameter flow cytometry (MFC), prognostic value, earlier detection, guided therapy

Introduction

Acute myeloid leukemia (AML) is characterized by an abnormal proliferation of myeloid progenitor cells. AML is usually treated by two cycles of intensive induction chemotherapy ("3+7"), followed by post-remission consolidation therapy after achieving complete remission (CR) (1, 2). This may either be an allogeneic stem cell transplantation (allo-SCT), one or more cycles of conventional chemotherapy, or an autologous stem cell transplantation (auto-SCT). Choosing the appropriate consolidation treatment is based on estimations of risks of treatment related mortality versus mortality due to relapse of the disease. Commonly, a genetics-based risk classification (mainly the ELN-2017) is used to facilitate this assessment at the time of diagnosis (3, 4). For ELN intermediate risk patients, measurable residual disease (MRD) during therapy is increasingly used as an additional marker to further stratify consolidation choices (5–7). MRD measured *via* multiparameter flow cytometry (MFC), or molecularly, by either quantitative PCR based techniques or next generation sequencing is used to determine leukemic burden after initial treatment (8, 9). MFC-MRD is most frequently used as it is applicable for almost all AML patients (>90%). In HOVON-SAKK trials, a positive MRD result after induction chemotherapy is defined as ≥0.1% of CD45-expressing cells with a leukemia associated immunophenotype (LAIP) for MFC-MRD or, for AML with mutated NPM1, $>10^{-4}$ NPM1 copies using reverse transcriptase polymerase chain reaction. MRD positivity is associated with a significantly increased risk of relapse, shorter event-free survival (EFS) and inferior overall survival (OS) (10–16). The ELN MRD working party recommends MFC-MRD assessment after induction, which is often after two cycles of chemotherapy, and is closest to the consolidation time point, but there is still debate about the optimal time point (8, 17, 18). Several publications have shown that MRD also has prognostic value after one cycle of chemotherapy (19–23). Having a prognostic marker determined earlier during therapy can be helpful for earlier consolidation therapy decisions and clarity towards the patient. This applies in particular to patients of the intermediate risk category, as in this category consolidation therapy is increasingly being guided by MRD results. The earlier clarity *via* a MRD result can be used to be more restrictive in performing allogeneic donor searches and change the current practice to only initiating a search for MRD-positive patients, which can subsequently lead to a cost reduction. Here, we evaluate the concordance of MRD status measured by MFC in AML patients where MRD was assessed at both time point after first and second cycle of induction chemotherapy. In addition,

we calculated potential cost reductions by depending the initiation of HLA-typing and donor search on the MRD result after cycle 1 and comparing it to the current practice of as early as possible after diagnosis.

Materials and methods

Patients and treatment

Patients included for analysis were treated according to the HOVON-SAKK AML92, AML102, AML103 and AML132 trials (6, 24–26), who achieved CR after cycle 1 and had a valid MRD result after 1st and 2nd chemotherapy cycle. These trials consist of newly diagnosed AML (APL excluded) patients between the age of 18 and 65, except for the AML103 study which consisted of patients older than 65 who were fit enough for high dose chemotherapy. All patients younger than 65 years were given two cycles of standard intensive "3 + 7" regimens as initial induction therapy consisting of idarubicin for 3 days and cytarabine for 7 days (overview per study can be found in Supplementary Table S1). Consolidation therapy was based on the risk classification applicable at the time. Only for ELN-2017 intermediate risk patients in the AML132 trial, this choice was guided by the MRD result after cycle 2 (6). All studies were reviewed and approved by the ethics committees of the participating institutions and were conducted in accordance with the Declaration of Helsinki. All patients provided their written informed consent to participate in the study.

Multiparameter flow cytometry MRD assessment

Immunophenotyping was performed in the same way across all studies as previously described (27). Flow cytometry was performed on a FACS CANTO (BD Biosciences, San Jose, CA, USA) for all studies with either 6- or 8-color antibody panels, consisting of four or five different tubes (for details see Supplementary Table S2) (28). These panels have CD45, CD34, CD117, CD13 and HLA-DR as backbone markers. Leukemic population comprises of CD45 expressing cells (WBC) in combination with a primitive marker (CD34, CD117) and myeloid markers (CD13, CD33, or HLA-DR). Additional markers are used to define the leukemia associated phenotype (LAIP, e.g. CD2, CD7, CD36, CD22, CD19, CD15, CD11b, CD14, CD56). MRD was assessed after cycle 1 and cycle 2 in patients in morphologic CR/CRi. MRD percentage was defined as the percentage of LAIP-positive cells of the total WBC (CD45-expressing) population. Both MRD assessment and gating strategy were comparable for all included studies and following a strict protocol as previously published (29, 30).

Cost-effectiveness analysis

We used decision trees to evaluate the impact of initiating the donor search based on the MRD result after cycle 1 on costs. We defined the following strategies: 1) the current care strategy with initiation of donor search for all patients at time of diagnosis; and 2) the MRD-based strategy with initiation of donor search based on MRD result after cycle 1 and no allo-SCT for MRD-negative patients. The decision trees are depicted in Figure 1. The probabilities of having a MRD-negative result after cycle 1 and cycle 2, and the availability of finding an HLA-matched donor or matched unrelated donor (MUD) were based on results from the included patients in this pooled set of patients and current practice (31, 32). Of the AML intermediate risk patients, we assumed to find a HLA-sibling match for approximately 30% of patients, MUD match for 60% of patients and no search for 10% because they are already deemed not fit for allo transplant. The HLA-sibling search was performed for more patients without a match, but these were not included in the cost analysis to keep it feasible. Furthermore, if a patient had a MRD-positive result after cycle 1, an search is initiated with the same ratio as the current strategy (60% MUD, 30% HLA-sib and 10% not eligible for transplant) and regardless of the status at a later time point.

We considered all costs related to the diagnostic process to find the right consolidation treatment, namely costs of the bone marrow (BM) aspiration and MRD measurement, HLA-typing and search for a suitable allo-SCT donor. An overview of the prices used can be found in Supplementary Table S3. Costs were based on the fixed tariffs negotiated between health insurers and hospitals from the Dutch Health Insurance Council and are from 2022 in euros (33, 34).

Statistical analyses

Chi-square or Fisher exact test was used to assess differences at baseline for categorical variables, and the Mann-Whitney U test was used to analyze continuous variables. For cumulative incidence of relapse (CIR) a competitive risk framework was used with correction for competing risk (non-relapse mortality), where patients alive in continuing CR were censored at the date of last contact. EFS was defined as the time between MRD measurement after cycle 1 and the date of hematologic relapse or death. Overall survival was defined from the time of MRD measurement 1 until death from any cause or last follow-up. Survival differences were analyzed using the log-rank test and visualized with Kaplan-Meier curves for EFS and OS. Cox regression analysis was used to determine if MRD was independently associated with EFS and OS, both univariate- and multivariate. The proportional hazard assumption was tested on the basis of Schoenfeld residuals (35). Since the data

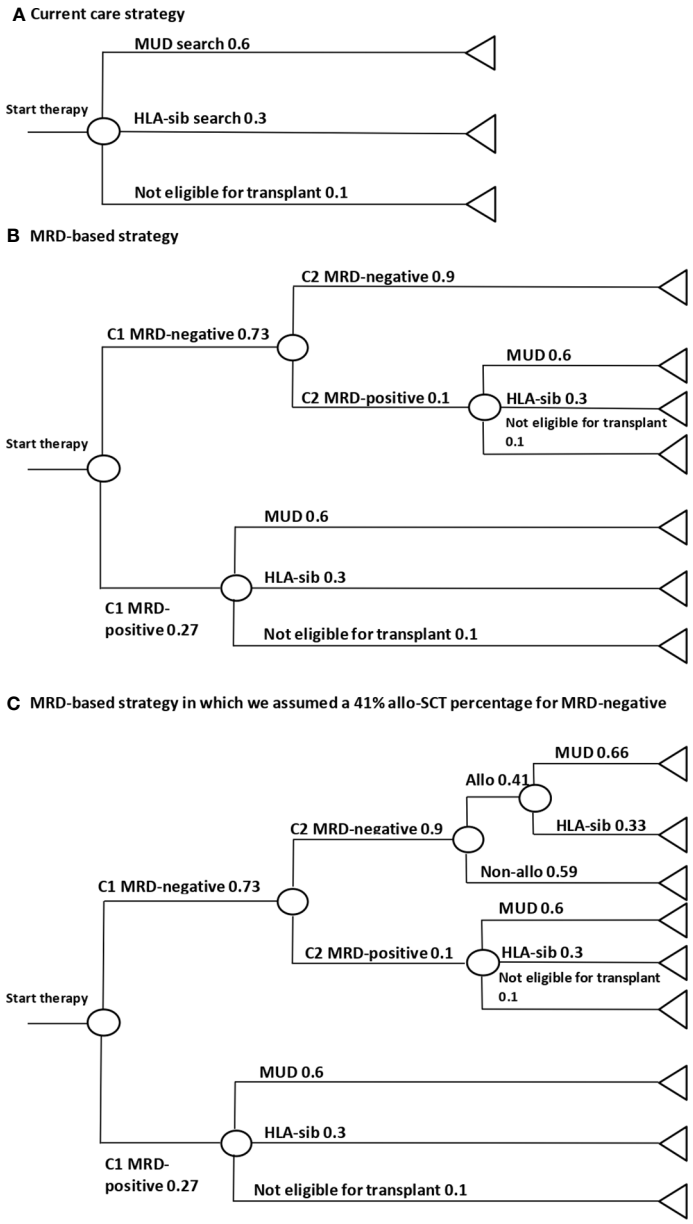

FIGURE 1
Decision trees of the three different strategies. **(A)** Current care strategy for intermediate risk patients were a donor is search is initiated for about 90% of patients of which 60% are match unrelated donors (MUD) and 30% siblings with HLA-match (HLA-sib). **(B)** MRD-based strategy were MRD-negative patients do not receive an allogeneic stem cell transplantation (allo-SCT). **(C)** Combination of MRD-based strategy with addition of treating physicians discretion, in which 41% of the MRD-negative patients after cycle 2 still receive an allo-SCT. This is the same allo-SCT percentage for MRD-negative patients as in this cohort.

consists of multiple clinical studies, we evaluated the heterogeneity between studies using the I^2 statistic (36). All tests were two-tailed at a significance level of 0.05, unadjusted for multiplicity. Statistical analyses were performed using SPSS software (version 28; IBM Corporation, Armonk, NY) and the R software environment for statistical computing and graphics (version 4.0.3, Vienna, Austria) (37).

The expected costs of the two strategies were assessed using the decision trees of Figure 1. First, we calculated the average costs accumulated by a patient following a specific branch of the decision tree. Then, for each branch the unit costs were multiplied with the probability of a patient following a specific branch. Total cost per strategy were calculated by summing up the total expected costs of each branch and subsequently compared. To

evaluate the impact of parameter uncertainty on the total expected costs, we conducted a probabilistic sensitivity analysis (PSA). A beta distribution was fitted to the MRD outcome parameter. For all other parameters, we assumed a 10% relative variance. Next, using Monte Carlo simulations, 1,000 draws were taken from these distributions. Uncertainty surrounding the expected costs was estimated using 95% credibility intervals (CrI) by estimating the 2.5% and 97.5% percentiles.

In addition, we conducted a threshold analysis to determine the maximum cost of the MRD measurement at which the total costs of the MRD-based strategy were equal to the current care strategy. Furthermore, we conducted a sensitivity analysis to assess if the MRD-based strategy would still be cost-efficient if physicians would deviate from the proposed non-allo consolidation treatment for MRD-negative patients. In this analysis, we assumed that the initiation of donor search was based on both MRD result after cycle 1 and treating physicians discretion. Based on the results of our cohort, we assumed that 41% of MRD-negative patients still received an allo-SCT despite ELN-2017 recommendation.

Results

A total of 273 patients from the AML92 (34; 12.5%), AML102 (175; 64.1%), AML103 (12; 4.4%) and AML132 (52; 19%) trials met all inclusion criteria. The precise number of patients enrolled in different trials and reasons why patients were excluded in the present analysis can be found in Supplementary Figure S1. The analysis of heterogeneity for 5-year mortality demonstrated that trials are homogeneous (Supplementary Figure S2) with a percentage of heterogeneity on total variability (I^2) of 0% (p=0.80). The baseline characteristics of the MRD-negative and MRD-positive patients after first and second induction cycle are shown in Table 1.

MRD after cycle I

Of the 273 patients who were in CR(i) and had a valid MRD result at both time points, 196 (72%) were MRD-negative after 1 cycle of chemotherapy and 77 (28%) patients were MRD-positive. A total of 38/77 (49.4%) of the MRD-positive patients relapsed at a median time of 8 months (range 2-38), compared to 62/196 (31.6%) of the MRD-negative patients with a median time of 13 months (range 2-82) (Figure 2A; Hazard Ratio (HR), 2.11; 95% CI, 1.41-3.16; P<0.001). At 5 years, MRD-positive patients both had a significantly worse EFS (Figure 2C; HR, 2.10; 95% CI, 1.46-3.02; P<0.001) and 5-year OS (45% for MRD-positive and 69% for MRD-negative patients (Figure 2E; HR, 2.12; 95% CI, 1.43-3.15; P<0.001)). MRD status after cycle 1 was

TABLE 1 Characteristics of patients by MRD-status after cycle 1 and cycle 2.

		MRD status after cycle 1			MRD status after cycle 2		
Characteristics		**MRD-, N=196**	**MRD+, N=77**	***p-value***	**MRD-, N=216**	**MRD+, N=57**	***p-value***
Age in 3 categories	<=45	47 (24%)	25 (32.5%)	0.108	54 (25%)	18 (31.6%)	0.558
	46-60	96 (49%)	27 (35.1%)		98 (45.4%)	25 (43.9%)	
	>60	53 (27%)	25 (32.5%)		64 (29.6%)	14 (24.6%)	
Sex	M	97 (49.5%)	38 (49.4%)	0.983	110 (50.9%)	25 (43.9%)	0.343
	F	99 (50.5%)	39 (50.6%)		106 (49.1%)	32 (56.1%)	
WHO performance status	WHO 0	101 (51.5%)	35 (45.4%)	0.066	107 (49.5%)	29 (50.9%)	0.044
	WHO 1	65 (33.2%)	24 (31.2%)		71 (32.9%)	18 (31.6%)	
	WHO 2	3 (1.5%)	6 (7.8%)		4 (1.9%)	5 (8.8%)	
WBC count at diagnosis	<20	104 (66.7%)	46 (70.8%)	0.566	116 (67.8%)	34 (68%)	0.993
	20-100	41 (26.3%)	13 (20%)		42 (24.6%)	12 (24%)	
	>100	11 (7.1%)	6 (9.2%)		13 (7.6%)	4 (8%)	
ELN-2017 risk	Favorable	87 (44.4%)	25 (32.5%)	0.092	92 (42.6%)	20 (35.1%)	0.165
	Intermediate	60 (30.6%)	22 (28.6%)		68 (31.5%)	14 (24.6%)	
	Adverse	48 (24.5%)	30 (39%)		55 (25.5%)	23 (40.4%)	
FLT3ITD x NPM1	Pos x pos	33 (16.8%)	7 (9.1%)	0.040	32 (14.8%)	8 (14%)	0.252
	Pos x Neg	17 (8.7%)	8 (10.4%)		20 (9.3%)	5 (8.8%)	
	Neg x pos	50 (25.5%)	10 (13%)		53 (24.5%)	7 (12.3%)	
	Neg x neg	81 (41.3%)	45 (58.4%)		96 (44.4%)	30 (52.6%)	
Consolidation treatment	None	20 (10.2%)	6 (7.8%)	0.172	23 (10.6%)	3 (5.3%)	0.070
	Cycle 3	63 (32.1%)	17 (22.1%)		65 (30.1%)	15 (26.3%)	
	Auto-HSCT	39 (19.9%)	14 (18.2%)		46 (21.3%)	7 (12.3%)	
	Allo-HSCT	74 (37.8%)	40 (51.9%)		82 (38%)	32 (56.1%)	

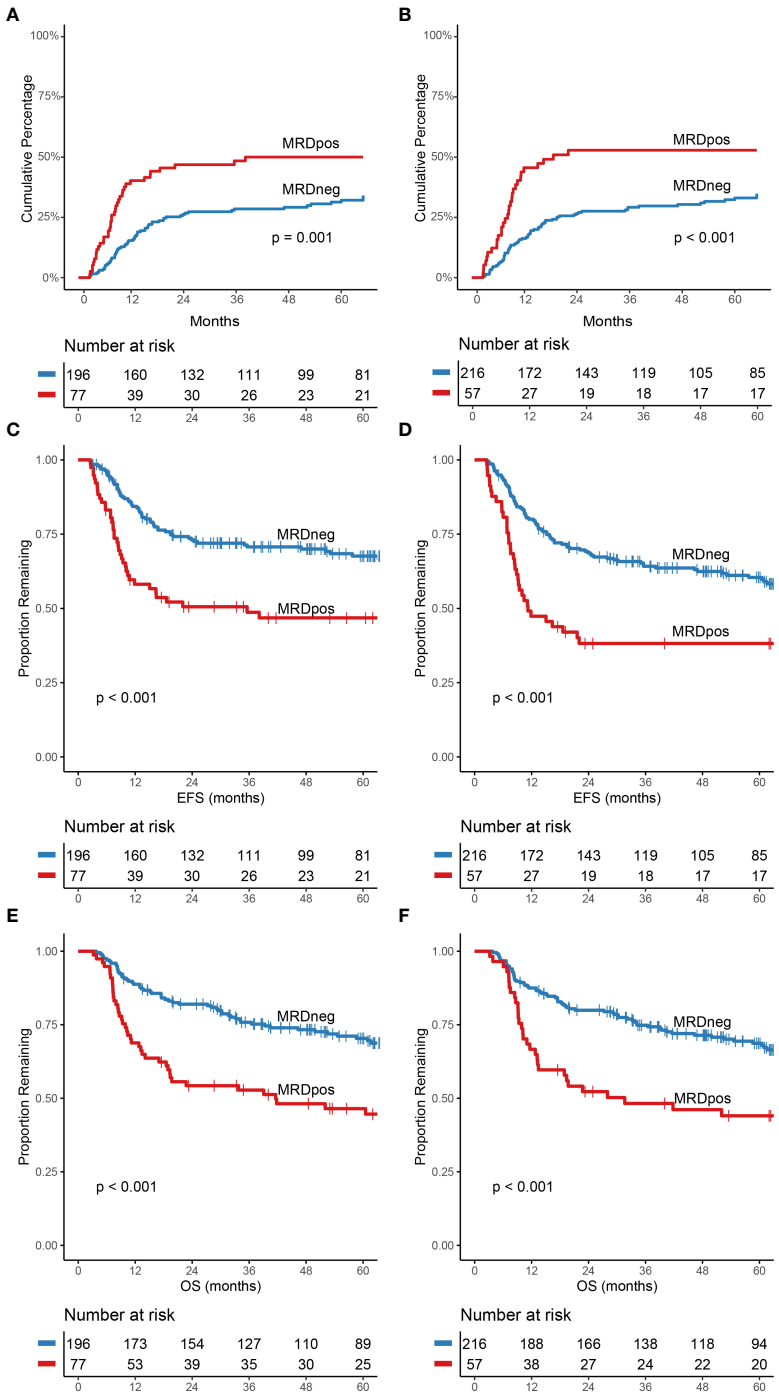

FIGURE 2
Cumulative incidence of relapse, event-free survival and overall survival stratified for MRD-status after cycle 1 and cycle 2. **(A)** CIR after cycle 1 and **(B)** CIR after cycle 2. **(C)** difference in EFS after cycle 1 and **(D)** after cycle 2. **(E)** OS difference for MRD status after cycle 1 and **(F)** after cycle 2. All curves were significantly different based on MRD-status (p<0.002).

significantly associated with FLT3-ITD/NPM1 status at diagnosis (Table 1). In univariate Cox regression analyses, age above 60 years at diagnose and ELN-2017 adverse risk was also significantly associated with worse EFS and OS (Supplementary Table S4). MRD-status after cycle 1 remained a significant prognostic factor in the multivariate model (p<0.001) along with age above 60 years at diagnosis and ELN-2017 adverse risk (Supplementary Table S5).

MRD after cycle II

MRD-positive status after cycle 2 was significantly associated with the WHO performance status at diagnosis (Table 1). More patients were MRD-negative (216/273; 79.1%) compared to the time point after 1 cycle of chemotherapy. MRD-negative patients after cycle 2 had a significantly lower chance of relapsing in the first five years after therapy (Figure 2B; p<0.001) compared to MRD-positive patients. EFS (Figure 2D; HR, 2.03; 95% CI, 1.37-3.01; P=0.001) and OS (Figure 2F; HR, 2.02; 95% CI, 1.33-3.09; P=0.001) were also significantly better for patients who were MRD-negative after cycle 2. In multivariate Cox regression analyses, MRD-status remained a prognostic factor (p<0.001) for EFS and OS together with age above 60 years at diagnose and ELN-2017 adverse risk (Supplementary Table S6).

Combining MRD after cycle 1 and cycle 2

By combining the results of MRD after cycle 1 and cycle 2, we categorized the patients in four groups (Figure 3). 180 patients were MRD-negative at both time points (group I; MRD1-MRD2-), 36 patients were MRD-positive after cycle 1 and converted to MRD-negative (group II; MRD1+MRD2-), 16 patients were MRD-negative after cycle 1 and converted to MRD-positive after cycle 2 (group III; MRD1-MRD2+) and 41 patients were MRD-positive at both time points (group IV; MRD1+MRD2+). No distinct differences in baseline characteristics were found between the four groups (Supplementary Table S7). See Figure 2 for an overview of the fluctuations of MRD status after combining the MRD results after cycle 1 and cycle 2. Of the 196 patients who were already MRD-negative after cycle 1, most remained negative after cycle 2 (180; 91.8%). This concordance was not found for MRD-positive patients, were 41 of the 77 MRD + patients after cycle 1 (53.2%) remained positive, whereas 36 patients converted to MRD-negativity. A higher MRD value after cycle 1 was associated with a higher chance of remaining MRD-positive at cycle 2, although no value could be found above which everyone remained MRD-positive. Of the 16 patients with an MRD value of 1.5% or higher after cycle 1, 11 (68.8%) remained positive after cycle 2 and this was 8/10 (80%) of the patients with an MRD level of 2.5% and higher.

The cumulative incidence of relapse (CIR) was significantly different between MRD-negative patients at both time points (group I; MRD1-MRD2-) and patients who were MRD-positive at both time points (MRD1+MRD2+; p<0.001, Figure 4A). There was no significant difference between group I and patients who were positive at one of the two time points (MRD1+MRD2- and MRD1-MRD2+). For EFS, there was a difference between MRD1-MRD2- patients and MRD1+MRD2+ patients (p<0.001), but also between MRD1-MRD2- patients and MRD1+MRD2- (p=0.044, Figure 4B). These differences were also seen for OS with 73.9% of MRD1-MRD2- patients surviving five years after start of treatment compared to 52.8% of MRD1+MRD2- patients (p=0.014), 50% of MRD1-MRD2+ patients (not significant; p=0.100) and 43.9% of MRD1+MRD2+ patients (p=0.001, Figure 4C).

Decision tree analysis

Of the 273 patients included, 82 were classified as ELN-2017 intermediate risk, of which 60 patients (73%) were MRD-negative after cycle 1 and 54 (54/60; 90%) of these remained negative after cycle 2. The decision trees of the two strategies and the sensitivity analysis are depicted in Figure 1. Using decision tree analyses, we calculated an expected total cost of €1,127,342 per 100 patients for the current care strategy, in which for 90% of

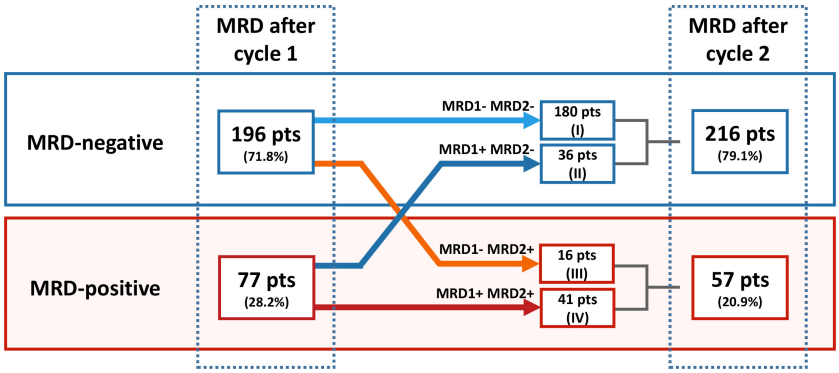

FIGURE 3
Fluctuations of MRD status between measurement after induction chemotherapy cycle I and the time point after chemotherapy cycle II. After one cycle of chemotherapy, 196 patients (71.8%) became MRD-negative and 77 patients were MRD-positive. After two cycles of chemotherapy, 216 patients (79.1%) were MRD-negative of which 180 were already MRD-negative after cycle 1 and 36 converted from MRD-positive to MRD-negative.

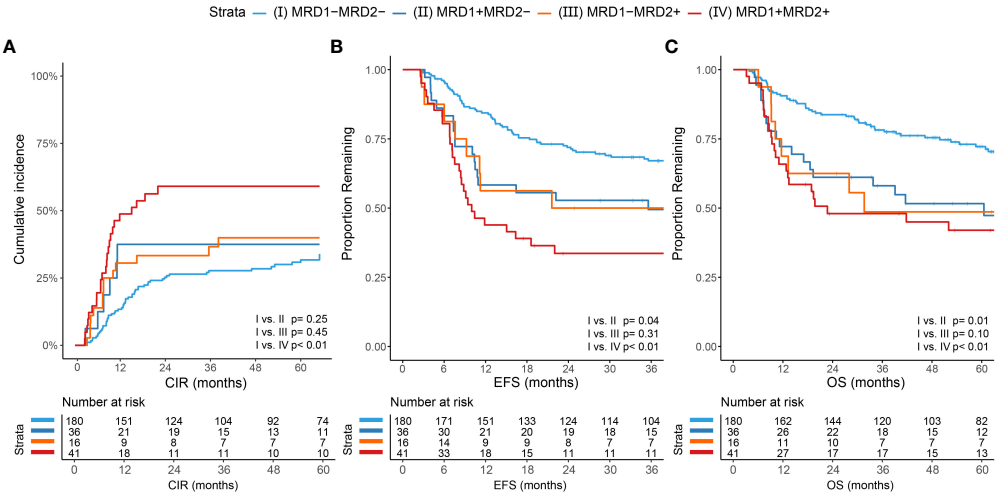

FIGURE 4
Cumulative incidence of relapse, event-free survival and overall survival compared for four groups based on combined MRD status of after cycle 1 and cycle 2. Four different groups were made based on the MRD status at time point after cycle 1 and cycle 2: (I) MRD negative at both time points (MRD1-MRD2-); (II) MRD-positive after cycle 1 and MRD-negative after cycle 2 (MRD1+MRD2-); (III) MRD-negative after cycle 1 and MRD-positive after cycle 2 (MRD1-MRD2+); and (IV) MRD-positive at both time points (MRD1+MRD2+). **(A)** Cumulative incidence of relapse (CIR) between the four groups with only a significant difference between MRD1-MRD2-(I) and MRD1+MRD2+(IV) (p<0.001). **(B)** Event-free survival (EFS) difference of the four groups with a significant difference between group (I) and group (IV) (p<0.001), but also between group (I) and group (II) (p=0.044). **(C)** Overall survival (OS) was also significantly different both between group (I) and group (IV) (p=0.001), and between group (I) and group (II) (p=0.014).

patients an allogeneic donor search is initiated (Figure 1A). In the MRD-based strategy in which MRD-negative intermediate risk patients do not receive an allo-SCT, the search is not initiated for 65.7% of total intermediate risk patients with an expected cost of €555,591 per 100 patients (Figure 1B). This strategy results in a cost reduction of –€571,751 (95% CrI: –€705,309 to –€464,698) per 100 patients, which equals to a 50.7% reduction compared to the current care strategy. The PSA showed that the proposed MRD strategy was consistently cheaper compared to the current care strategy. The threshold analysis showed that the combined cost of the BM aspiration and MRD-measurement could increase to €7,406 (+438%), in order for the MRD-based strategy to be equally expensive as the current care strategy. The sensitivity analysis in which the choice to start a donor search is based on the MRD-result after cycle 1 and treating physicians discretion (third decision tree), resulted in 34.2% less initiation of donor searches with in an expected cost of €789,406 per 100 patients. This means a cost reduction of –€337,936 (95% CrI: –€470,207 to –€222,322) compared to the current care strategy (Figure 1C).

Discussion

MRD-negative status after both one- and two cycles of chemotherapy was significantly associated with less chance of relapse, better EFS and OS (Figure 2; $p \leq 0.002$, for all comparisons). Curves on both time points had similar fits, which suggests similar prognostic value. Comparable results were found after grouping the patients based on the MRD results at both time points, where patients negative at both time points had a significantly better outcome (CIR, EFS and OS) compared to patients positive at both time points (Figure 4). Also evident was the difference in EFS (p=0.044) and OS (p=0.014) between patients who achieved MRD-negativity only after cycle 2 (MRD1+MRD2-) compared to patients who were MRD-negative after both cycles (MRD1-MRD2-). MRD-negative after cycle 1 and positive after cycle 2 (MRD1-MRD2+) was the least observed, with only 5.9% of patients. Likely due to the small sample size, this group was not significantly different from MRD1-MRD2- despite showing similar curves when compared to the MRD1+MRD2- subgroup. These results underline that MRD status after 1 cycle of chemotherapy has strong prognostic implication with failure to achieve MRD-negativity after 1 cycle being associated with a clearly worse outcome.

In addition, because a MRD-negative result after cycle 1 is highly concordant with a negative MRD result after cycle 2 of chemotherapy, it can be used to postpone the initiation of a transplant donor search for intermediate risk patients. This alternative strategy will result in a decrease in donor searches of between 34.2%-65.7% for intermediate risk patients and

average cost savings of €571,751 per 100 patients. Therefore, the proposed alternative strategy can be considered as a valuable alternative approach, especially for countries with more limited budgets. However, a downside to a later search initiation is the potential delay of an allo-SCT in the 10% of MRD-negative patients after cycle 1 who do convert to MRD-positive after cycle 2. The sensitivity analysis showed that even with 41% of MRD-negative patients still receiving an initial allo-SCT, our proposed strategy would be more cost efficient. This analysis however, does not take into account the possible allo-SCT as second consolidation therapy needed after relapse. The decision tree strategy considers all other vital variables in our situation, but caution is warranted when results are being extrapolated to other countries as they could face different conditions.

Up to now, although the prognostic value of MRD after one cycle of chemotherapy has been demonstrated before, information about MRD concordance between the two time points has been sparse (19, 20, 23). One notable exception is the UK-NCRI AML17 study, which showed corresponding results in MRD concordance despite having slightly different inclusion criteria (NPM1+ patients were excluded) (20). The AML17 trial also showed a high degree of concordance between MRD-negative results at the two time points, with 90% of the patients achieving MRD-negativity after cycle 1 remaining MRD-negative after cycle 2. Furthermore, this study also showed the lack of concordance between MRD-positive results at the two time points, with almost 50% conversion from MRD-positive after cycle 1 to MRD-negative after cycle 2, which even more suggests that the second cycle of chemotherapy is an important part of the treatment sequence in these patients.

In general, MRD is not routinely measured after one cycle of chemotherapy since centers have less experience with this time point and it is not generally recommended by the ELN MRD working party (17). Our study only included patients who had a valid MRD measurement after 1 and 2 cycles of chemotherapy, which means that all patients had to be in CR after cycle 1. As a result, conclusions from this study cannot be translated to all AML patients but only to patients already in CR after cycle 1. Moreover, since MRD was not systematically collected after 1 cycle of chemotherapy, relatively many patients were not eligible for inclusion in our study and this could potentially form a selection bias.

Measuring MRD after one cycle of induction chemotherapy has the benefit of giving prognostic value at an early stage of therapy and due to the high concordance with the measurement after cycle 2, a high degree of clarity for the recommended consolidation therapy in the case of an intermediate risk patient. Therefore, we would recommend to incorporate this time point into upcoming studies. However, given the limited experience with measuring MRD after cycle 1, we do not value this point as a replacement for the current "gold standard" after two cycles of chemotherapy. The high degree of concordance between MRD-negativity between the two time points signifies the question if adverse risk patients who reach MRD-negativity after cycle 1, do still benefit from the second induction course or whether they should immediately proceed to transplantation if a donor is available (38). Future (randomized) studies to address this hypothesis are warranted. In addition, when opting for allo-SCT, the risk for nonrelapse mortality is an important factor that needs to be considered next to the ELN risk classification and MRD status (39).

In conclusion, our findings highlight two facets of measuring MFC-MRD after one cycle of chemotherapy. First, achieving MRD-negative CR after one cycle of chemotherapy gives a prognostic advantage in terms of EFS and OS compared to patients who are in CR but are MRD-positive or who are persistent MRD-positive at both time points. Secondly, there is a high concordance between MRD-negative result after cycle 1 and cycle 2 which can be used to pre-sort intermediate risk patient sooner to a recommended consolidation therapy. The early time point of response data can be used to postpone or omit the search for an allogeneic donor, which will result in a cost-reduction and provide patients with more certainty about the course of their further treatment.

Data availability statement

The raw data supporting the conclusions of this article contain too much identifiable data that it will not be made available by the authors. Requests to access the datasets should be directed to j.cloos@amsterdamumc.nl.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethical Committee Erasmus MC. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JT, WA-B, CE, GG, JJ, and JC contributed to conception and design of the study. DB, TF, BG, LG, GJ, JM, MM, TP, JP, KP, BL, GO, and JJ collected the data. JT and WA-B organized the database. JT and MG performed the statistical analysis. PG provided statistical consultation. JT wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.999822/full#supplementary-material

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Measurable residual disease in hairy cell leukemia: Technical considerations and clinical significance

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Hairy cell leukemia (HCL) is a rare type of chronic lymphoid leukemia originating from a mature B lymphocyte. A diagnosis of HCL is based on cytology, confirmed by multiparametric flow cytometry (MFC) studies using anti-B-cell monoclonal antibodies, together with a panel of antibodies more specific to HCL, such as CD11c, CD25, CD103 and CD123. Recently, the *BRAF* V600E mutation has been described as a disease-defining genetic event. Measurable residual disease (MRD) is defined as the lowest level of HCL cells that can be detected accurately and reproducibly using validated methods; as MRD negativity is associated with high rates of durable complete response, by clearing MRD, the long-term outcome may be improved in patients with advanced HCL. MRD is typically detected using bone marrow, and in some cases, peripheral blood; however, in HCL, discrepancies frequently exist between MRD results obtained from blood, bone marrow aspirate and core biopsy. Among the methods used for MRD detection, MFC appears to be a more sensitive technique than immunohistochemistry. Molecular tests are also used, such as real-time quantitative PCR for unique immunoglobulin heavy chain (IgH) gene rearrangements and PCR techniques with clone specificity for *BRAF* V600E. Clone-specific PCR (spPCR) is able to detect one HCL cell in 10^6 normal cells, and is particularly suitable for patients found to be negative for MRD by MFC. Recently, the Hairy Cell Leukemia Consortium created a platform to work on a definition for MRD, and establish the optimal time point, tissue type and method for measuring MRD. This

KEYWORDS

***BRAF*, cladribine, hairy cell leukemia, flow cytometry, immunohistochemistry, minimal residual disease, moxetumomab pasudotox, PCR**

1 Introduction

Hairy cell leukemia (HCL) is a rare type of chronic lymphoid leukemia originating from a mature B lymphocyte (1, 2). Its incidence is 0.3 cases per 100,000 individuals, and median age at diagnosis is 58 years. Approximately 1000 new cases of HCL are diagnosed each year in the United States (3). HCL is four times more common in men than women (4).

A diagnosis of classical HCL is based on morphological, characteristics of hairy cells and immunologic phenotype in multiparametric flow cytometry (MFC) and immunohistochemistry (IHC) in the trephine biopsy and the presence of $BRAF^{V600E}$ somatic mutation (5). Anti-B-cell monoclonal antibodies (MoAb) such as CD19, CD20 or CD22, are used together with antibodies more specific to HCL including CD11c, CD25, CD103 and CD123. More recently, CD200 and LAIR1 were introduced as important markers of HCL (6). Classic HCL is characterized by mutation of the BRAF serine/threonine protein kinase (V600E) with an incidence of nearly 100% of HCL cases at diagnosis (7, 8).

Purine nucleoside analogues (PNA), pentostatin (deoxycoformycin, DCF) and cladribine (2-Chlorodeoxyadenosine, 2-CdA), are recommended for first-line treatment in classic HCL (9, 10). These agents induce durable and unmaintained complete response (CR) in more than 70% of patients, and the relapse rates are about 30% to 40% after 5 to 10 years of follow-up, with overall survival (OS) frequently longer than 20 years (11–13). Patients may expect a normal lifespan when treated with PNA, irrespective of their pretreatment history (13). While 2-CdA and DCF demonstrate similar efficacy and safety (14), 2-CdA is a more common choice than DCF due to its shorter treatment duration (15).

Although median time to relapse following 2-CdA treatment is 16 years, disease-free survival (DFS) and relapse-free survival (RFS) curves have not yet reached a plateau, suggesting that most patients who live long enough will eventually relapse. A recent multicenter analysis in Europe confirmed that 2-CdA used as frontline treatment in HCL patients permits disease control in a significant proportion of cases, given that more than 50% of treated patients require no further therapy. Good quality responses may be maintained for more than 20 years in up to 35% of patients (12).

Rituximab is an effective drug in HCL, especially when used in combination with other agents (16, 17). When rituximab was combined with 2-CdA in early relapsed HCL, CR was achieved in 89-100% of patients, with a 5-year progression-free survival (PFS) of 100% and a 3-year risk of relapse only 7% (16). Recently, several new drugs have been introduced for the treatment of patients with HCL (18). Among these, clinical trials have confirmed the anti-CD22 immunotoxin moxetumomab pasudotox (Moxe), BRAF kinase inhibitors (vemurafenib and dabrafenib), MEK inhibitors (trametinib and cobimetinib) and the Bruton's kinase inhibitor ibrutinib as useful agents in the treatment of patients refractory to PNAs (19–24).

Measurable residual disease (MRD) is defined as the lowest level of neoplastic cells that can be identified using validated methods i.e. their detection below the level of conventional cytomorphology using more sensitive methods, including IHC, MFC, cytogenetics and molecular techniques (25–30). Several studies have indicated that the detection of MRD after therapy for HCL has prognostic value. In particular, clearing MRD may improve long-term outcome in patients with advanced disease (27). It has been shown that in patients treated with 2-CdA, the appearance of positive MRD in bone marrow (BM) may predict disease recurrence in most patients (25, 26). Clinical trials exploring the potential value of MRD evaluation in HCL patients treated with novel drugs, including monoclonal antibodies (MaAbs), immunotoxins and BRAF inhibitors, alone or in combination with other agents, are ongoing. This review presents the current state of knowledge on MRD in HCL, including methodology, clinical results and future directions.

2 Methods of MRD detection in HCL

Measurable residual disease (MRD) is becoming an important investigative tool in the clinical management of several hematologic malignancies, including forms of acute leukemia, chronic myeloid leukemia, chronic lymphocytic leukemia (CLL) and multiple myeloma. In many hematologic neoplasms, especially CLL, MRD has been indicated as a biomarker in clinical trials (31). In HCL, MRD is defined as the lowest level of leukemic cells that can be identified using validated methods (28). Currently, MRD detection in hematological malignancies is based on sensitive methods, such as identifying tumor-associated immunophenotypic characteristics by MFC, or evaluating specific genetic markers by PCR-based methods and next-generation sequencing. In HCL, MRD is evaluated in peripheral blood (PB) and BM aspirate or core biopsy. The presence of MRD should be determined in the context of the sensitivity of the used techniques and the ability of participating laboratories to accurately and reproducibly detect it. The sensitivity of each method is carefully specified (32). In particular, the lowest level of detectability (LLOD) and lowest level of quantitation (LLOQ) should be taken into account. For each illustrated analytical approach, the ranges of attainable LLOD and LLOQ should be carefully specified, since they vary remarkably both with technology and with time. It is widely believed that using $0.01\%/10^{-4}$ as a threshold is less relevant in most hematologic neoplasms, and that future MRD analyses should use a lower LOD (preferable $<0.001\%/10^{-5}$) (32, 33). A consensus report on the potential application of MRD assessment in front-line and relapse settings and recommendations on the future role of MRD assessment in HCL has been recently developed by the

International Group of Experts on Measurable Residual Disease in Hairy Cell Leukemia and should be published soon.

2.1 Immunohistochemistry

Several studies have performed immunohistochemical staining of BM core biopsy. Bengio et al. compared IHC with MFC with for MRD detection in HCL patients after therapy with 2-CdA (29). The procedure used the CD20 monoclonal antibodies L26 and DBA.44 to detect MRD by IHC, and CD20, CD22, CD25, Sig, CD11c and CD103 Mo Abs by MFC. The definition for positive MRD was 1–10% CD20/DBA44 scattered or clustered cells with tricoleukocyte morphology for IHC, and any expression of CD11c/CD25/CD103 in the BM or PB for FC. The MRD positivity rate for IHC was 46% compared with 64% for MFC, suggesting that MFC is a more sensitive technique than IHC.

A recent study by Gupta et al. evaluated the potential of two IHC staining assays to detect HCL involvement in core biopsies (30). Bone marrow IHC was performed using PAX5/CD103 and PAX5/tartrate-resistant alkaline phosphatase (TRAP) dual IHC stains. The sensitivity of the dual IHC stains was found to be 81.4%, positive predictive value was 100% and negative predictive value 81.7%. Simultaneously-performed MFC found the dual IHC allowed the detection of HCL cells even when the disease burden was as low as 0.02% of all identified lymphoid cells. In this study, some of the patients found to be positive with dual IHC staining were also negative for morphologic evidence of disease based on CD20 and H&E stains, suggesting that MRD detection by dual IHC stains is more sensitive than single IHC stains. However, one of the cases with an extremely low disease burden was found to be negative by dual IHC staining, and positive by MFC.

2.2 Flow cytometry

In several hematologic malignancies, the most commonly-used procedure for detecting MRD is flow MFC. In recent years, significant progress has been made in the methodology and interpretation of MFC results. Flow cytometry evolved from a basic (4-color) method to the modern MFC multidimensional cell analysis with ≥6–8 colors (31). In MRD detection, MFC allows the simultaneous recognition of several phenotypic markers (usually 6–8 antigens), and the capacity to analyze large numbers of cells in only a few minutes. The method now offers similar sensitivity to the most sensitive molecular techniques. In HCL, MRD detection by MFC is usually performed by immunophenotyping based on antibodies reacting with antigens characteristic for HCL: CD19, CD20, CD22, CD25, CD79a, CD11c, CD103 and surface immunoglobulin (32). The use of MFC with these markers has sensitivity, typically in the range of one HCL cell per 100 000 cells ($1x10^{-5}$) (32). MFC is the most commonly-used method, as it is the most practical and informative. In 2012, the EuroFlow consortium, presented novel consensus protocols, for standardization of MFC in the diagnosis of hematologic diseases (34). Based on these guidelines, recently specific procedural recommendations for sample collection. An adequate BM or PB sample (1-2 ml) and an extensive antibody panel with backbone and lineage markers is needed is needed for MFC analysis of MRD. In addition, millions of clean CD45+ cell events should be acquired to ensure adequate LLOD and LLOQ levels (35). More recently, the International Group of Experts on Measurable Residual Disease in Hairy Cell Leukemia developed specific guidelines for evaluation of MRD in HCL (manuscript submitted). These specify that high-quality first-pull BM aspirate samples are required for MFC, as hemodilution can prevent the correct quantification of MRD in the BM, and to a greater degree than in other leukemias, mainly due to the limited PB involvement by HCL cells.

2.3 Molecular methods

Originally, molecular methods based on qualitative PCR and real-time quantitative PCR (RQ-PCR) were used for MRD detection (28, 36–38), while some studies have used RQ-PCR for unique immunoglobulin heavy chain (IgH) gene rearrangements. Recently, more advanced PCR techniques have been introduced, including droplet digital PCR and whole-genome sequencing methods known as next generation sequencing (NGS). Amplification using consensus V primers (most commonly for the framework 1-3 regions of IgH, [cpPCR]) has a sensitivity ranging from $1x10^{-4}$ to $1x\ 10^{-5}$ (32); however, PCR methods with clone specificity offer greater sensitivity ($1 x\ 10^{-6}$), and some centers use PCR for *BRAF* V600E mutation (29).

In a study of previously-treated HCL patients, Sausville et al. found PB MFC (CD19, CD22, CD103, FMC7, CD23, CD19, CD20, CD11c, CD25, CD45, CD4, CD8, CD3, CD5, CD7, CD2) to be more sensitive than clonal analysis using consensus primer PCR (cpPCR) for the heavy chain gene (37). The results indicate that 31% of the MFC-positive cases were found to be negative by cpPCR, and only 1% of the cpPCR-positive cases were negative by FC. To improve the sensitivity of detection of MRD, consensus primers assay with CD11c sorting can be used (28). This method, called real-time quantitative PCR, was able to detect one HCL cell in 10^6 normal cells.

Arons et al. compared the sensitivity of MFC and RQ-PCR assay based on patient-specific primers and probes for the IgH gene rearrangement, for detecting MRD (38). In this study MRD assessed by MFC was compared with consensus primer PCR (cpPCR) and splinkerette PCR (spPCR) after therapy with the recombinant immunotoxin BL22 (38). The MRD positivity rates were found to be 74% by FC, 55% by cpPCR, and 98% by spPCR. Moreover, quantitative levels of spPCR correlated with disease

status. These findings suggest that spPCR may be most useful once negativity for MRD has been established by MFC. RQ-PCR was more sensitive than MFC and the quantified relative level of MRD correlated with disease status. This study suggests that patient-specific RQ-PCR is a very sensitive test for MRD in HCL patients and could be used to monitor maximal response in patients treated with antileukemic drugs.

Digital droplet PCR (ddPCR) has recently been applied for the detection of $BRAF^{V600E}$ mutation in HCL (39). This is a molecular method that allows quantification of DNA mutations and the detection of the *B-RAF* V600E mutation and MRD status. Guerrini et al. used the ddPCR in retrospective study of 47 HCL patients including 27 with classic HCL, two with HCLv and 18 with splenic marginal zone lymphoma (SMZL) (12, 39). The study found the sensitivity of dd-PCR to be about half a logarithm superior to QT-PCR (5×10^{-5} vs. 2.5×10^{-4}). Moreover, the specificity of the dd-PCR was similar to QT-PCR in classic HCL. The authors suggest that dd-PCR can be a useful method in the detection and monitoring of MRD in HCL patients. At the end of the treatment, 33% of patients in CR were found to be still MRD-positive after 12 months by dd-PCR, and 28% by QT-PCR. These findings suggest that dd-PCR may be more sensitive than quantitative PCR and can be useful for detecting MRD in HCL. In a similar study, Broccoli et al. measured *BRAF* V600E burden by ddPCR in PB and/or BM in 35 HCL patients at diagnosis, relapse, and CR (12). In PB, the mean fractional abundance values were 12.26% at diagnosis, 16.52% at relapse and 0.02% at CR, with the corresponding values in BM being 23.51%, 13.96%, and 0.26%. In addition, four of six patients evaluated at response were molecularly negative for $BRAF^{V600E}$ in PB. The mean fractional abundance in PB evaluated in 14 patients with long lasting CR was 0.05%, and 10 were *BRAF* V600E negative, indicating that some patients in CR demonstrate a molecular CR. These results indicate that ddPCR for $BRAF^{V600E}$ is a useful method for monitoring MRD in classic HCL.

3 Minimal residual disease in clinical trials

Clinical trials exploring the potential for purine analogs, MoAbs, immunotoxins and BRAF and MEK inhibitors to eradicate MRD have been performed in HCL patients. The results have been published over recent decades (Tables 1, 2).

3.1 Purine nucleozide analogs

Clinical studies have evaluated MRD in patients with HCL treated with cladribine and pentostatin, used alone or in combination with rituximab, and the results are available.

3.1.1 Cladribine

An early study performed by Konwalinka et al. evaluated MRD in 11 HCL patients in CR after treatment with 2-CdA (41). MRD was detected by IHC staining with the monoclonal antibody (MoAb) B-ly 7 and B-Ly7 (CD103). In all patients, MRD was found to range from 0.1 to 7.5% (median 0.65%). At a median follow-up of 29 months (median 19.3), nine patients remained in CR, while two relapsed 22 and 27 months from the end of 2-CdA therapy. MFC analysis of HCL cells was also performed in BM aspirates and PB using MoAbs, Leu-12 (CD19) and LeuM5 (CD11c) double-staining. In five of 10 cases, no hairy cells could be detected in the BM aspirates. In addition, no hairy cells were detectable in PB in six partly-different cases; however, hairy cells were identified in BM biopsy by B-ly 7 immunostaining (ranging form 0.1 to 7.5%). In other studies on patients treated with 2-CdA, by IHC was used to evaluate MRD in BM biopsies with the B-lineage antibodies L26 and MB2 (26, 52). In addition, BM core biopsies from 34 patients with HCL were studied before and three months after 2-CdA treatment, based on L26 (CD20) and MB2, and a T-lineage antibody, UCHL-1. Five of the 24 (21%) patients in hematologic CR were found to demonstrate MRD. Among 19 patients evaluated at one year, only one additional patient was found to be positive by immunostaining alone (26). In a longer observation, BM biopsies from 39 patients in CR after a single course of 2-CdA were evaluated by IHC with anti-CD45RO, anti-CD20 and DBA44 staining (25). Patients with detected MRD had a higher probability for disease progression than those without MRD (P=0.016) indicating that IHC evaluation of MRD has prognostic value (40).

Mhawech-Fauceglia et al. evaluated the correlation between the level of MRD and clinical outcome in patients treated with 0.14 mg/kg 2-CdA in subcutaneous bolus injections for five days (42). Conventional histologic examination and IHC were performed on sections of BM stained with CD45, CD20, DBA.44, and CD3 MoAbs in 17 patients with a median follow-up of 55.4 months. The patients were divided into three groups based on MRD level. Group 1 (seven patients) had MRD levels below 1%, and the patients remained in CR throughout the follow-up. In group 2 (six patients) MRD levels ranged from 1% to 5%; of these, three patients remained in CR at 77.9, 63.8, and 108.0 months. Group 3 (four patients) had MRD level above 5%; three patients in this group relapsed at 11.3, 12.1, and 29.6 months. This study further confirms that quantitative assessment of MRD has prognostic value and can predict dsease relapse. Ellison et al. determined MRD in HCL patients with CR using immunohistochemical staining for L26 and DBA.44 in BM biopsies (52). The study evaluated 154 BM biopsies from 42 patients between three months and 25 months after treatment with 2-CdA. Using this method, 91% of the biopsies were found to include DBA.44-positive cells, while 48% samples indicated HCL cells based on morphologic evaluation. Importantly, similar results were obtained over the 25-month follow up. This

TABLE 1 Clinical studies with MRD evaluation in patients with HCL treated with purine nucleoside analogs.

Study regimen	Phase of the study/ Disease status	Number of patients	Response (OR/CR)	Method of MRD evaluation	MRD negativity	PFS	References
2-CdA 0.1 mg/kg/d by continuous i.v. for 7 days	Phase 2/15 previously untreated	33, 31 evaluable.	100%/77%	IHC with MoAb B-ly 7 in L26 and MB2, and UCHL-1	19 from 24 in CR (80%)	NR	Tallman et al., 1992, Hakimian et al., 1993 (31, 40)
2-CdA 0.07 mg/kg/d for 7 days	Retrospective/ relapsed	14	100%/78%	IHC with MoAb B-ly 7	0%	After median follow-up 19.3m 9 pts in CR and 2 relapsed (at 22 and 27 m)	Konwalinka et al., 1995 (41)
2-CdA 0.14 mg/kg s.c. x 5 days	Retrospective	17		IHC with CD45, CD20, DBA.44, and CD3	Gr 1: MRD <1% - 7 pts; Gr 2: MRD 1% to 5% - 6 pts, Gr 3: MRD>5% - 4 pts	Gr 1- all in CR at 55.4 mfollow-up;Gr 2 – 3 pts in CR after 77.9, 63.8, and 108.0 months;GR 3 -3pts relapsed	Mhawech-Fauceglia 2006 (42)
2-CdA 0.085 to 0.1 mg/kg per day x 7 days	Retrospective/ untreated	19 with long CR selected from 358	100%/100%	FC for CD103, CD11c, and CD25) on BM aspirates in 17 or IGH-PCR	47%	Median time from 2-CdA, 16 years	Sigal et al., 2010 (43)
2-CdA (5.6 mg/m^2 for 5 days) followed by R 375 mg/m^2	Phase 2/ untreated 11, relapsed 2	13	100%/100%	IGH-PCR assay and FC	FC negative in 22/ 28 (79%)pts and PCR in 19/27 (70%) after R,	Median response duration 9 m (4-16 m).	Ravandi et al., 2006 (44)
R 375 mg/m^2/wk x 4 after pretreatment with 2-CdA	Phase 2/ pretreated with 2-CdA	8 (2 CR, 4 PR, 2 no response)	100%%/100%%	IGH-PCR	100% 1 yr after the end of R treatment.	NA	Cervetti et al., 2004 (45)
2-CdA (5.6 mg/m^2 for 5 days) followed by R 375 mg/m^2	Phase 2/ untreated	36 (5 with HCLv)	100%/100%	IGH-PCR assay and FC in PB and BM	76% and64%	Mefian not been reached (range,1+-63+ m	Ravandi et al., 2011 (36)
2-CdA (5.6 mg/m^2 for 5 days) followed by R 375 mg/m^2	Phase 2/ untreated 59, relapsed 14, HCLv 7	80	CR untreated100%, relapsed 100% and 86%	Multiparameter FC at the time of response evaluation	94%	5-Year FFS: untreated 95%, relapsed 100% and HCLv 64%,	Chihara et al., 2016 (16)
2-CdA 0.15 mg/kg i.v./d x days 1-5 + R 375 mg/m^{2}concurrent vs delayed	Phase 2/ untreated	68	100%/100%% vs 100%/88%	FC in PB/BM and BM immunohistochemistry.	97 vs 24	Median ?94 vs 12	Chihara et al., 2020 (17)
Pentostatin 4 mg/m^{2}every 2weeks until CR	Phase 1/ relapsed	23	100%/100%	FC in PB and BM frozen sections using a panel of antibodies, CD11c, CD25, CD103 and HC2,	57% in BM,96% in PB	Median 59 m	Matutes et al., 1997 (46)
Pentostatin 4 mg/m^2 every 2 weeks for 5 to 25 (median 13) courses	Phase 3/ untreated	27	100%	Immunohistochemistry with CD20 and DBA.44 antbodes	7/27 (26%)	4/7 patients (57%) with MRD after DCF relapsed at 18, 21, 44, and 59 m after CR ad 0/20 without MRD	Tallman et al., 1999 (40)
Bendamustine 70 or 90 mg/ m^2 for 2 days + R 375 mg/ m^2 days 1 and 15 plus for for six cycles at 4-week intervals.	Phase 2/ relapsed	12	100%/50% for 70 mg/m^2 vs 67% for 90 mg/ m^2	FC in PB/BM and BM immunohistochemistry with L26, MB2, and UCHL-1 antibodies.	67% of CRs for 70 mg/m^2 vs 100% of CRs for 90 mg/m^2	31 months for patients in CR	Burotto et al., 2013 (47)

2-CdA, 2-chlorodeoxyadenosine, cladribine; BM, bone marrow; CR, complete response; DCF, deoxycoformycin, pentostatin;FC, flow cytometry; HCL, hairy cell leukemia; HCLv, HCL variant; IGH-PCR, immunoglobulin heavy chain gene rearrangements by consensus-primer polymerase chain reaction; ICH, immunohistochemistry; MoAb, monoclonal antibody; MRD, minimal residual disease; NA, not available; NR, not reported; PB, peripheral blood; OR, overall response; PCR, polymerase-chain-reaction; RFS, relapse-free survival; RQ-PCR, quantitative PCR.

TABLE 2 Clinical studies with MRD evaluation in patients with HCL treated with novel agents.

Study regimen	Phase of the study/ Disease status	Number of patients	Response (OR/CR)	Method of MRD evaluation	MRD negativity	PFS	References
BL22 3 to 50 microg/Kg every other day x 3 doses.	Phase 1/ relapsed	31	80%/60%	FC and consensus primers PCR	94% by FC and 100% by PCR	36 months for patients in CR	Kreitman et al., 2005 (48)
BL22	Phase 1 &2/ relapsed	10	60%//60%	FC and patient specific RQ-PCR	10% by RQ-PCR	NR	Arons et al., 2006 (38)
Moxe 32 - 50-μg/kg every other day for 3 doses in 4-week cycles	Phase 1 and extension/ relapsed	33	88%/%64	FC in BM aspirate	33%	62.8m in MRD-negative and 12.0 in MRD-positive patients	Kreitman et al., 2012, 2018 (17, 48)
Moxe 40-μg/kg every other day for 3 doses in 4-week cycles	Phase 3/ relapsed	80	75%/41%	FC in PB/BM and BM immunohistochemistry	34%	Median 71.7 months.	Kreitman et al., 2018, 2021 (19, 49)
Vemurafenib 960 mg bid x 16 -18 weeks	Phase 2/ relapsed	54	100%/38%	Immunohistochemistry	0	1-Year PFS - 73%; median RFS 9 months	Tiacci et al., 2015 (19)
Vemurafenib 960 mg, twice daily for 8 weeks + R 375 mg/m^2 for 8 doses over 18 weeks	Phase 2/ relapsed	30	100%/87%	Allele-specific DNA PCR for *BRAF* V600E (sensitivity, ≥0.05% mutant copies)	65%	3-year PFS – 78%;	Tiacci et al., 2021 (50)
Vemurafenib 960 mg bid + Obinutuzumab 1000mg IV on days 1, 8, and 15 of m 2, and day 1 of m3 and 4.	Phase 1/ untreated	9	100%/100%	Digital PCR for BRAFV600E	100%	9.7 m ongoing	Park et al., 2019 (51)
Dabrafenib 150 mg bid for 12 weeks.	Phase 2/ relapsed	10	80%30%	Immunohistochemistry	0	7-60.5m	Tiacci et al.2021 (19)
Dabrafenib 150 mg bid + Trametinib 2 mg/daay until unacceptable toxicity or progression	Phase 2/ relapsed, refractory	43	78%/49%	FC in PB and BM aspirates	15%	1-Year PFS - 98%	Kreitman et al., 2018 (51)
Ibrutinib 420 mg or 840 mg/day until unacceptable toxicity or progression	Phase 2/ relapsed	37	73%/19%	FC in PB and BM aspirates and and BM immunohistochemistry	3 (8%)	3-Year PFS - 73%	Rogers et al., 2021 (24)

2-CdA, 2-Chlorodeoxyadenosine, cladribine; BM, bone marrow; CR, complete response; DCF, deoxycoformycin, pentostatin; FC, flow cytometry; IGH-PCR, immunoglobulin heavy chain gene rearrangements by consensus-primer polymerase chain reaction; ICH, immunohistochemistry; MoAb, monoclonal antibody; MRD, minimal residual disease; NR, not reported; PB, peripheral blood; OR, overall response; PCR, polymerase-chain-reaction; RFS, relapse-free survival; RQ-PCR, quantitative PCR; dabrafenib (150 mg twice daily) and trametinib (2 mg once daily) until unacceptable toxicity, disease progression, or death.

study indicated that immunomorphological analysis is a more sensitive technique for detecting HCL cells than morphology alone.

A study of 358 patients at the Scripps Clinic database by Sigal et al. identified 19 patients with residual MRD in long-lasting continuous hematologic CR after a single 7-day course of 2-CdA based on evaluable BM tissue specimens (43). Of this group, MRD was evaluated by multiparameter FC analysis based on CD103, CD11c and CD25 from the BM aspirates of 17 patients. Nine of the 19 (47%) patients had no evidence of MRD, seven (37%) had MRD and three (16%) had morphologic evidence of HCL.

3.1.2 Cladribine plus rituximab

Cladribine combined with rituximab is more effective than 2-CdA alone in eliminating MRD in classic HCL and HCLv (16, 17, 44). Rawandi et al. treated 13 patients (two relapsed and 11 previously untreated) with 5.6 mg/m^2 2-CdA i.v. for five days, followed by eight weekly doses of rituximab (375 mg/m^2) (44). All patients obtained a CR. MRD was assessed in PB and BM by immunoglobulin heavy chain (IgH) PCR assay using framework-1, -2, and -3 primer and FC assay with a four-color panel of antibodies. MFC confirmed MRD in 11 patients one month after 2-CdA therapy; however, negative MRD was observed in 12 of 13 patients after rituximab treatment. PCR assay confirmed MRD in five of 11 evaluable patients one month after 2-CdA therapy, and this became negative in 11 of 12 evaluable patients after rituximab. No patients have relapsed, with a median follow-up of 14 months (range, 6-16 months).

A subsequent study based on 31 patients with classic HCL and five with HCL variant (HCLv) evaluated a regimen comprising 5.6 mg/m^2 2-CdA for five days, followed one month later with 375 mg/m^2 rituximab once a week for eight weeks (36). MRD was evaluated in BM after the end of rituximab

treatment. Complete MFC and PCR. In most patients, MRD was also assessed by consensus primer PCR. MRD evaluated by MFC in BM was positive in 22 (85%) of 26 patients one month after treatment with 2-CdA, and this value became negative in 22 (79%) of 28 patients following treatment with rituximab. Consensus primer PCR testing identified positive MRD in 13 (54%) of 24 evaluable patients after treatment with 2-CdA, but negative MRD in 19 (70%) of 27 evaluable patients after completion of rituximab treatment. MFC evaluation failed to detect MRD in most patients over a longer follow–up. It was found that PB and BM demonstrated similar results for residual HCL in 23 (82%) patients, including 10 positive and 13 negative results. In the remaining five patients MRD was positive in the BM and negative in PB. These results may indicate that PB is less sensitive for MRD assessment than BM.

In a phase 2 study, Chihara et al. evaluated the efficacy of 2-CdA followed by rituximab in 59 patients with untreated HCL, 14 with relapsed HCL and seven with HCL variant (HCLv) (16, 17). Cladribine was given at a dose of 5.6 mg/m^2 daily for five days, followed by 375 mg/m^2 rituximab once weekly for eight weeks, one month after 2-CdA administration. MRD was evaluated by MFC at the time of response evaluation. The CR rate was 100% in patients with untreated and relapsed HCL and 86% in those with HCLv. Failure-free survival (FFS) at five years for each group was 95%, 100% and 64%, respectively. Negative MRD after treatment was achieved in 94% of the patients. Only 11 (14%) previously-untreated patients demonstrated MRD-negative disease after 2-CdA alone. However, no patients with relapsed disease or HCLv achieved negative MRD after 2-CdA monotherapy. Importantly, in most patients, positive MRD during the follow up did not result in clinical relapse.

Cervetti et al. analyzed the eradication of MRD with four cycles of rituximab in 10 HCL patients after pretreatment with 2-CdA (45). After treatment with 2-CdA, two patients were in CR, six in partial response (PR) and two without response. Median time from the end of 2-CdA treatment to rituximab infusion was 5.7 months. Rituximab was given at a dose of 375 mg/m^2/week for four doses. Two months after the end of anti-CD20 therapy, all evaluated patients were in hematological CR. PCR with two consensus primers was used for MRD evaluation. Rituximab increased the percentage of molecular remission to 100% one year after the end of treatment. All patients but one showed MRD levels lower than those found before rituximab treatment. Recently, Chihara et al. presented the results of a long-term randomized study evaluating the effectiveness of combined rituximab and 2-CdA therapy in the elimination of MRD (17). Previously untreated patients with classic HCL were randomized to 2-CdA at a dose 0.15 mg/kg for five days, with eight concurrent weekly doses of 375 mg/m^2 rituximab from day 1 (CDAR), or delayed rituximab started at least six months after detection of MRD. MRD was evaluated in PB or BM using FC, and BM immunohistochemistry. Six months after treatment, CR rates were 100% for CDAR versus 88% for 2-CdA monotherapy (P =0.11). In addition, MRD negativity rates were 97% versus 24% in BM (P <.0001) and 100% versus 50% in PB (P < 0.0001). At eight years median follow-up, undetectable MRD in CDAR group was 94% versus 12% in the delayed rituximab arm. However, 12 patients in the delayed rituximab arm were MRD negative at the end of rituximab administration were restaged between 6 and 104 (median, 78) months later. These results confirm that combined 2-CdA and rituximab therapy demonstrates high activity in achieving long-lasting MRD elimination in previously-untreated HCL patients, and is more effective than delayed rituximab use after 2-cdA monotherapy.

3.2 Deoxycoformycin

Matutes et al. investigated MRD in 23 classic HCL patients in CR after treatment with deoxycoformycin (DCF, pentostatin) (46). MRD was detected in PB and BM by immunophenotyping based on a panel of four antibodies specific for HCL cells: CD11c, CD25, CD103 and HC2. MRD was detected in 10 of 23 patients (43%) including seven in BM, one in PB and two in both BM and PB. However, the MRD-positive and MRD-negative patients demonstrated similar disease-free survival (DFS) (P=0.8). Unlike some other studies, relapse could not be predicted by MRD results; this could be due to the sensitivity of the method used in the study.

Tallman et al. evaluated MRD in 39 HCL patients treated with 2-CdA and 27 patients treated with DCF (40). The patients treated with 2-CdA received one course of treatment at a dose of 0.1 mg/kg/day for seven days by continuous i.v. infusion. The patients treated with 4 mg/m^2 DCF every two weeks received from 5 to 25 (median 13) courses. All patients were in hematologic CR (Table 1). The criteria for MRD used in this study comprised a lack of HCL cells by routine morphology of PB and BM core sections, the presence of CD20- or DBA.44-positive cells equal to or higher than the number of CD45RO-positive cells, and the detection of 50% of CD20- or DBA.44-positive cells morphologically consistent with HCL cells. Seven of 27 patients (26%) treated with DCF demonstrated MRD compared with five of the 39 (13%) treated with 2-CdA. Among the patients without detected MRD, no relapses were noted in the DCF group, and only three relapses were noted out of 34 (9%) in the 2-CdA group. In total, six of the 12 patients (50%) with detected MRD and three of 54 patients (6%) without detected MRD relapsed. In contrast to the Matutes study above (46), positive MRD was associated with a higher risk of relapse: the estimated 4-year relapse-free survival (RFS) was 55% for patients with MRD and 88% for patients without MRD (P= 0.0023).

3.3 Bendamustin

Bendamustine is an alkylating agent active in the treatment of lymphoid malignancies. It is also effective for the treatment of

classic HCL and HCLv, when used in combination with rituximab (BR) (47, 53). This treatment was evaluated in 12 relapsed or refractory HCL patients (Table 1) (47). The patients received rituximab 375 mg/m^2 on days 1 and 15 and bendamustine 70 mg/m^2 or 90 mg/m^2 on days 1 and 2, for six cycles every four weeks for six cycles. Overall response rate was 100% for 70 mg/m^2 and 90 mg/m^2 bendamustine, with three (50%) and four (67%) CRs in the respective groups. MRD was not detected in 67% and 100% of CRs, respectively, and all six patients without MRD were in CR from 30 to 35 months of observations. MRD was confirmed in BM biopsy by IHC with L26, MB2, and UCHL-1 antibodies.

3.4 Immunotoxins

Anti-CD-22 immunotoxins, especially BL22 and moxetumomab pasudotox (Moxe), have been extensively investigated in relapsed/refractory HCL (48, 49, 54–57). BL22 is a recombinant immunotoxin containing a truncated form of the bacterial toxin Pseudomonas exotoxin A (PE38) attached to an Fv fragment of an anti-CD22 monoclonal antibody RFB4 (55). In a phase 1 study, BL22 was evaluated in 31 patients with PNA-resistant HCL (Table 2); of these, CRs were obtained in 19 (61%) (48, 58). Of the 19 patients achieving CR with BL22, only two were confirmed to demonstrate MRD by MFC, and none were found positive by PCR. In a phase 2 study performed in 36 patients, the OR rate was 72% and CR rate 47% (Table 2) (48, 56, 57). Most patients achieving CR to BL22 did not indicate MRD by either PCR or FC. MRD was then evaluated in 10 patients from the phase 1 and phase 2 studies, taken before or after BL22 treatment using MFC and patient-specific RQ-PCR (38). RQ-PCR was positive in all 62 (100%) MFC-positive samples from 10 patients and in 20 of 22 (91%) MFC-negative samples from six patients. Moreover, the level of MRD quantified by RQ-PCR correlated with disease status and response to treatment.

Subsequently, Kreitman et al. reported the discovery of the second-generation immunotoxin Moxetumomab pasudotox (Moxe (49, 58, 59). Moxetumomab pasudotox is a recombinant immunotoxin that binds to CD22-expressing cells, followed by internalization of the drug-CD22 complex (55). The drug was active and well tolerated in phase 1 and 3 studies performed in relapsed/refractory patients with HCL. In addition, Moxe can eliminate MRD in a significant number of patients, translated into greater CR duration (49, 57, 59). In the phase-1/2 study, Kreitman et al. analyzed the significance of MRD eradication with Moxe in 33 HCL patients, including 12 from the phase 1 study and 22 from the extension cohort, receiving 50-μg/kg Moxe every other day for three doses in four-week cycles (Table 2) (49). MRD was detected by 8-color multiparametric approach on a 3-laser FACSCanto II based on cells coexpressing CD19, CD20, CD22, bright CD11c and monoclonal light chains. Among the 33 analyzed patients, the OR rate was 88% including 64% CR. CR duration was longer in the MRD-negative patients: median CR was 13.5 months in nine MRD-positive CRs and 42.1 months in 11 MRD-negative CRs ($P < 0.001$). In a phase 3 trial 80 patients were treated with Moxe, given at a dose of 40 μg/kg by intravenous (i.v.) infusion on days 1, 3, and 5 of a 28-day cycle (20). Treatment was continued for up to six cycles, or until CR with MRD negativity, disease progression or unacceptable toxicity. MRD was assessed by quantitative MFC analysis of PB or BM aspiration, and by IHC on BM biopsy. At a median follow-up of 24.6 months, overall CR was 41%, with 36% demonstrating durable CR with hematologic response longer than 180 days, and 33% CR longer than 360 days. Twenty-seven (82%) patients with CR (34% of all patients) were MRD-negative. Longer median duration of hematologic remission was noted in MRD-negative patients than in MRD-positive patients (62.8 m vs 12.0 m, respectively).

3.5 BRAF inhibitors

The BRAF kinase inhibitors vemurafenib and dabrafenib are effective drugs in patients with refractory and recurrent HCL, either when used in monotherapy, or in combination with CD20 antibodies or MEK inhibitors (Table 2) (8). In a phase-2 single-arm multicenter study performed in Italy and US, vemurafenib was given as a single drug, 960 mg twice daily for a median of 16 - 18 weeks (21). Overall response rates were 96% (25/26) after a median of 8 weeks in the Italian study and 100% after a median of 12 weeks (24/24) in the US study. Complete response rates were 34.6% (9/26) and 41.7% (10/24), respectively. However, MRD was detected in all patients with CR at the end of treatment, evaluated by IHC. Moreover, the median relapse-free survival (RFS) was only nine months after treatment discontinuation. Deeper remissions were obtained when vemurafenib was combined with rituximab (60, 61).

In a phase 2 trial performed in 30 patients with refractory or relapsed HCL, vemurafenib was administered at a dose of 960 mg, twice daily for eight weeks, in combination with rituximab (375 mg/m^2) for eight doses in 18 weeks (60). MRD was detected in PB and BM aspirates by means of allele-specific DNA PCR for *BRAF* V600E with a sensitivity ≥0.05% mutant copies. The primary end point was CR at the end of planned treatment, which was achieved in 26 patients (87%). Moreover, undetectable MRD was achieved in 17 (65%) of the 26 patients in CR. MRD negativity correlated with longer survival without relapse.

In another phase 2 study, vemurafenib was combined with obinutuzumab in previously-untreated HCL patients (50). Vemurafenib was given at a dose of 960 mg twice per day for four months and obinutuzumab at 1000 mg.iv. on days 1, 8 and 15 of month 2, and day 1 of month 3 and 4. MRD negativity was detected by BRAFV600E using highly-sensitive digital PCR. A total of 11 patients have been enrolled, of whom nine have completed treatment. Seven patients achieved MRD negative CR and two patients PR at the end of treatment. However, both

patients with PR at month 4 converted to MRD negative CR by month 7 and 10. All patients remained in remission with a median follow-up of 9.7 months.

Another BRAF inhibitor, dabrafenib, was evaluated in a pilot phase 2 study in relapsed/refractory patients (22). Ten patients, including two previously treated with vemurafenib, received dabrafenib at a dose of 150 mg twice daily for eight weeks. If no CR was obtained after eight weeks, patients received an additional four-week course. Eight patients (80%) responded, including three with CR (30%) and five with PR (50%). However, all patients with CR had detectable MRD by immunohistochemistry in the BM biopsy. The duration of response in patients with CR was 15.5, 14 and 60.5 months. Moreover, of the patients in PR, one in five had 42-month survival on dabrafenib. The combination of BRAF inhibitor dabrafenib and MEK inhibitor trametinib hence appears even more effective than dabrafenib alone in V600E-mutated HCL (22, 51, 62).

In a phase 2, open-label trial, 43 eligible patients with refractory HCL received a combination of dabrafenib and trametinib (51). Minimal residual disease status was detected by flow cytometry in both PB and BM aspirates. At the time of data evaluation, 35 patients (81%) remained on treatment. Among 41 patients, 32 (78%) responded, including 20 (49%) with CR. Six (15%) patients in CR had no detectable MRD while 14 (34%) CR were MRD positive. Twelve (29%) patients obtained a PR. At the data cut-off; 16 (50%) responses had lasted 18 months or longer and no patients had experienced a relapse.

3.6 Ibrutinib

B-cell receptor (BCR) signaling is involved in HCL pathogenesis (62). In preclinical studies, Bruton's tyrosine kinase (BTK) inhibitor ibrutinib inhibited survival, proliferation and B cell receptor signaling in HCL cells (63). Recently, ibrutinib was evaluated in a phase 2 study in 28 patients with classic HCL and nine patients with HCL-v (24). Ibrutinib was administered at a dose of 420 mg daily in 24 patients and 840 mg daily in 13 patients, until HCL progression or unacceptable toxicity (22). MRD was assessed in each patient based on FC in the PB and BM. and IHC examination using specific markers for HCL in the BM. Response was 24% at 32 weeks, and 36% at 48 weeks. The OR rate was 54% at any time since starting ibrutinib, including seven patients with CR, 13 patients with PR and 10 patients with stable disease (SD). MRD was not detected in three patients. The response rates were similar in patients with classic HCL and HCL-v. The estimated 36-month progression-free survival (PFS) was 73% and the estimated 36-month overall survival (OS) was 85%. However, MRD was not evaluated in this study.

4 MRD in HCL variant

In 2008, the World Health Organization (WHO) distinguished a new variant of hairy cell leukemia (HCLv). It was subsequently included as a provisional entity within the spectrum of splenic B-cell lymphomas/leukemia, unclassifiable (64). In the 5th edition of the WHO Classification of Haematolymphoid Tumours, the new entity splenic B-cell lymphoma/leukaemia with prominent nucleoli (SBLPN) replaces the previous term HCL variant (65). The World Health Organization reported 810 new cases of HCLv each year in the United States (3).

HCL-v is characterized by leukocytosis with lymphocytosis, cytopenias without monocytopenia, and lymphoid cells of relatively large size with prominent nucleoli. A critical aspect of HCL-v diagnosis is an atypical HCL immunophenotype without CD25 expression, and lack of *BRAF* V600E mutation (66, 67). Leukemic cells strongly express pan-B-cell markers, including CD19, CD20, CD22 and FMC7. Surface immunoglobulin expression is strong, with CD5 and CD23 usually negative. In contrast to classic HCL, CD25 and CD123 are negative but CD11c is always positive and CD103 is positive in 2/3 of HCL-v cases. Moreover, in HCL-v, Annexin A1 expression is negative. Some patients have activating mutations in *MAP2K1*, a gene that encodes MEK1, a downstream component of the BRAF-MEK-ERK signaling cascade. While there is no genetic mutation diagnostic of HCL-v, genetic profiling efforts have identified potential therapeutic targets, such as *MAP2K1, KDM6A, CREBBP, ARID1A, CCND3, U2AF1* and *KMT2C*.

PNA treatment yields unsatisfactory results for HCL-v treatment (66, 67). However, greater effectiveness has been reported for the combination of rituximab and 2-CdA. Kreitman et al. treated 10 patients with 0.15 mg/kg 2-CDA on days 1–5, with eight weekly standard doses of rituximab (68). Nine patients (90%) achieved CR, compared with three out of 39 (8%) treated with 2-CDA alone. In eight patients, MRD negativity was achieved. The median duration of response to 2-CDA + rituximab was longer than that seen for first-line 2-CDA alone (72 months vs not reached, $P = 0{\cdot}004$). Positive MRD was noted during the follow up, but this did not result in any clinically-relevant relapse. Visentin et al. report effective treatment of three previously-untreated elderly patients with combined bendamustine and rituximab (53). All patients achieved a CR with no evidence of MRD, indicated by the absence of leukemic cells according to post-therapy immunohistochemical (CD20 and CD22) staging and flow-cytometry marrow examination. All three patients were in CR after a median follow-up of 19 months.

Another treatment regimen active in HCL-v is moxetumomab pasudotox (19, 49, 59). In a phase 1 and phase 3 study including six patients with HCL-v, MRD was independently evaluated using immunohistochemical staining for the HCL/B

cell antigens CD20, CD79a, Annexin A1,DBA.44, and PAX-5 and by flow cytometric analysis of peripheral blood and/or bone marrow aspirate, according to each site' s procedures. Although most of the patients responded to the treatment, no separate details exist for the subgroup with HCL-v. Future treatment for HCL-v may include targeted therapies such as ibrutinib, trametinib, binimetinib and venetoclax, and potentially anti-CD22 chimeric antigen receptor T cell therapy (CART) (69–72). Recently ibrutinib was evaluated in 37 patients, including 28 with classic HCL and nine with HCL-v (24). The HCL and HCL-v patients demonstrated similar response rates and estimated 36-month PFS and OS scores; however, the study was not designed to evaluate difference between both diseases. Ibrutinib is currently not approved by the FDA in HCL and HCL-V. Patients with HCL-v do not have a BRAF mutation and cannot be treated with BRAF inhibitors.

5 Practical considerations and perspectives

In most studies performed in HCL, investigators were able to predict relapse in patients with hematologic CR and a positive MRD test. In addition, eradicating MRD leads to a better outcome, longer PFS or OS or even recovery (17, 25). However, some studies indicate MRD was positive in most patients treated with 2-CdA with very long follow-up (median 16 years) (43). The practical value of MRD monitoring currently remains unclear, as does the value of a positive test for MRD as a predictor of clinical relapse. In some studies, patients with positive MRD after treatment with 2-CdA can survive even 16-18 years without clinical relapse (43). In addition, some patients treated with 2-CdA remain MRD negative for a considerable time, and can be considered as cured. In the future, MRD evaluation can be useful in deciding whether to continue treatment to achieve deeper response, to prolong CR duration or even cure. The introduction of novel drugs, such as immunotoxins and BRAF inhibitors, or novel combination regimens, such as immunochemotherapy, can be used to eliminate persistent MRD in some patients and decrease the risk of relapse (26). MRD monitoring may also be a useful indicator of the efficacy of novel drugs, as it allows shorter follow-up than standard criteria like PFS or CR duration, as with CLL. Currently, MRD is easily detectable by MFC and molecular techniques, provided the right technical methods are applied. Moreover, in hematologic malignancies, MRD detection is currently managed by internationally-applied external quality assessment/proficiency testing schemes, which confirms that it has clinical utility besides controlled trials. Recent studies with novel drugs have demonstrated that CR can be achieved with undetectable MRD in increase time of response. Several assays can be used to detect MRD in HCL patients; however, while IHC analysis of BM specimens used to be more popular, more recent guidelines recommend the use of MFC and PCR methods for detecting the mutant *BRAF* V600E gene or consensus primers for *IGH* (26, 44, 60).

Currently, MFC and allele-specific PCR analysis for mutant *BRAF* are recommended for detecting MRD in HCL (73–75). For patients treated with anti-CD20 monoclonal antibodies, MRD should be evaluated with other antibodies, such as the use of other B-cell marker (CD79a) or HCL-specific markers (eg, VE1) for IHC staining (1, 44). Recently, the ISCCA protocol for standardized prospective monitoring of patients treated with anti-CD20 therapies has been developed (73). MFC and quantitative or digital PCR are significantly more sensitive than IHC, and these tests should be recommended in the future studies and clinical practice. While MFC can achieve a sensitivity below 1/100,000 ($1x10^{-5}$), investigated cells (75), molecular methods can achieve 10^{-6}. MFC can be also used for the detection of MRD in a BM aspirate or PB. However, consensus needs to be reached regarding minimal level of detection in MRD in HCL, i.e. from 0.1% to 0.001%. The optimal sample type used for MRD detection is BM, however PB is also sometimes used.

Bone marrow core biopsy offers an alternative sample but immunohistochemical methods have limitations and are difficult to quantify. MFC appears to be a more sensitive technique for detecting MRD than IHC. Molecular tests, such as real-time quantitative PCR for unique immunoglobulin heavy chain (IgH) gene rearrangements with consensus V primers, demonstrates sensitivity ranging from $1x10^{-4}$ to $1x\ 10^{-5}$, with even greater values being noted for PCR techniques with clone specificity for *BRAF* V600E ($1\ x\ 10^{-6}$). Clone-specific PCR (spPCR) is able to detect one HCL cell among 10^6 normal cells, and appears most appropriate for use in patients negative for MRD by MFC. At present, standardization for MRD detection is unachievable due to lack of standards and different platforms, reagents and processing methods.

No study has determined the optimal timing of the MRD evaluation in HCL patients. It seems rational to assess MRD when evaluating the response to treatment. The Consensus guidelines recommend that after 2-CdA therapy, a BM biopsy should be performed for four to six months after drug administration, or later if response is delayed and continuing improvement observed (1). In patients treated with DCF, the BM biopsy should be performed after optimal clinical response, including normalization of PB parameters. A similar approach seems to be rational for novel agents, active in HCL. There is a need to standardize MRD assessment in HCL, as has been the case in other hematologic malignancies, including chronic myeloid leukemia, acute lymphoblastic leukemia and chronic lymphocytic leukemia.

An expert panel should reach a consensus regarding the minimal level of HCL cell detection, optimal time point for MRD measurement, optimal type of samples used for MRD detection and detection (BM, PB) and optimal methods used for MRD

evaluation. Currently it is not possible to standardize the methods used in MRD detection in HCL due to lack of standards and the wide range of platforms, reagents and processing methods currently used in different centers. Although harmonization is possible using different platforms, reagents and processing methods, it is difficult in the case of rare diseases. The Hairy Cell Leukemia Consortium is the most suitable platform for working on a definition of MRD, and establishing the optimal time point, tissue type and methods to measure MRD in HCL.

6 Conclusions

Measurable residual disease is defined as the lowest level of HCL cells that can be detected accurately and reproducibly using validated methods. MRD negativity is associated with high rates of durable complete response and long-term outcome may be improved by clearing MRD in patients with advanced HCL. However, long-term observation is needed to confirm the clinical benefit of MRD-negative CR after front-line treatment. Methods used for MRD detection include MFC, IHC and molecular tests. In HCL, discrepancies commonly exist between MRD results in blood aspirate and core biopsy. Bone marrow core biopsy offers an alternative sample, but immunohistochemical methods have limitations and are difficult to quantify. In addition, any MRD detection program should incorporate quality assurance that can confirm the ability of participating laboratories to accurately and reproducibly detect MRD. Available data on the role of MRD in the management of patients with HCL are not unambiguous and at present, MRD monitoring in HCL cannot be recommended in clinical practice. In the coming years, MRD assessment should be standardized asis the case in other hematologic malignancies, including acute lymphoblastic leukemia and chronic lymphocytic leukaemia (CLL). The Hairy Cell Leukemia Consortium has recently created a platform to work on a definition for MRD, and to establish the optimal time point, tissue type and methods for measuring MRD in HCL. Their opinion on the value of MRD monitoring in HCL patients is expected soon.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Obinutuzumab plus chlorambucil versus ibrutinib in previously untreated chronic lymphocytic leukemia patients without TP53 disruptions: A real-life CLL campus study

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One of the main issues in the treatment of patients with chronic lymphocytic leukemia (CLL) deals with the choice between continuous or fixed-duration therapy. Continuous ibrutinib (IB), the first-in-class BTK inhibitor, and obinutuzumab-chlorambucil (G-CHL) are commonly used therapies for elderly and/or comorbid patients. No head-to-head comparison has been carried out. Within the Italian campus CLL network, we performed a retrospective study on CLL patients without TP53 disruption treated with IB or G-CHL as first-line therapy. Patients in the G-CHL arm had a higher CIRS

score and the worst renal function. The overall response rates between the G-CHL and IB arms were similar, but more complete remissions (CRs) were achieved with G-CHL (p = 0.0029). After a median follow-up of 30 months, the progression-free survival (PFS, p = 0.0061) and time to next treatment (TTNT, p = 0.0043), but not overall survival (OS, p = 0.6642), were better with IB than with G-CHL. Similar results were found after propensity score matching and multivariate analysis. While PFS and TTNT were longer with IB than with G-CHL in IGHV unmutated patients (p = 0.0190 and 0.0137), they were superimposable for IGHV mutated patients (p = 0.1900 and 0.1380). In the G-CHL arm, the depth of response (79% *vs*. 68% *vs*. 38% for CR, PR and SD/PD; $p < 0.0001$) and measurable residual disease (MRD) influenced PFS (78% *vs*. 53% for undetectable MRD *vs*. detectable MRD, p = 0.0203). Hematological toxicities were common in the G-CHL arm, while IB was associated with higher costs. Although continuous IB provides better disease control in CLL, IGHV mutated patients and those achieving an undetectable MRD show a marked clinical and economic benefit from a fixed-duration obinutuzumab-based treatment.

KEYWORDS

obinutizumab, ibrutinib, treatment-naive, MRD, economic impact

Introduction

The treatment landscape of chronic lymphocytic leukemia (CLL) has significantly changed in the last few years thanks to the discovery of targeted drugs directed against pivotal kinases, such as BTK [ibrutinib (IB), acalabrutinib, zanubrutinib, and pirtobrutinib] or PI3K (idelalisib and duvelisib), anti-apoptotic protein, such as BCL2 (venetoclax), and new monoclonal antibodies targeting CD19 (tafasitamab) or CD20 (1–3). Among the latter, obinutuzumab (G), a glycoengineered type II humanized anti-CD20 monoclonal antibody, displays increased direct cell death, B-cell depletion, FcγRIIIa binding, and antibody-dependent cell-mediated cytotoxicity, and it has a lower capacity to re-localize CD20 into lipid rafts upon binding and to decrease complement-dependent cytotoxicity (4, 5).

The current frontline therapy of CLL patients can be either a continuous BTK inhibitor or a fixed-duration G-based therapy (1). Choosing between the two approaches remains a challenge, since a continuous treatment might offer better disease control for some aggressive subsets of patients, balanced however by an increased rate of long-term adverse events (AEs) and costs for the health system (6–9). On the other hand, a fixed G-based therapy is administered for a short period, allowing the achievement of deep remission, which is likely to be less prone to the development of resistant clones, but requires an outpatient clinic admission (10–12).

There is no head-to-head comparison between IB and G-chlorambucil (G-CHL) both in clinical trials and in real-life studies. A cross-trial comparison between Resonate-II and CLL11 suggests that overall IB seems to be better than G-CHL (13) in terms of survival analysis and safety profile during the first 6 months of treatment (grade 3 events, 50% *vs*. 71%). Furthermore, there are only a few retrospective studies that have assessed the efficacy of G-CHL and measurable residual disease (MRD) in the real-life setting (14–18).

In this study, we performed a retrospective study within the Italian Campus CLL network comparing the efficacy, MRD rates, safety, and economic cost of G-CHL *vs*. IB in treatment-naive CLL patients. We found that IB provides better disease control in most cases, but those patients who were IGHV mutated (M-IGHV) patients and who achieved an undetectable MRD (uMRD) showed a sustained clinical and economic benefit from a fixed-duration G-CHL-based therapy.

Methods

Study design

This is a retrospective study aimed at collecting and analyzing data of CLL patients treated outside of clinical trials with frontline IB or G-CHL from their reimbursement in Italy

up to December 2021. Inclusion criteria were (i) diagnosis of CLL and the need for treatment according to the iwCLL 2018 guidelines (19) and (ii) patients unfit for fludarabine-based therapy (as evaluated by the treating physician). Exclusion criteria were (i) unable to sign the informed consent, (ii) relapsed/refractory patients, and (iii) ECOG >3.

Patients received IB 420 mg daily until progression or unacceptable toxicity, while G was administered at 100 mg on day 1, 900 mg on day 2, and 1,000 mg on days 8 and 15 of the first cycle, then at 1,000 mg of day 1 of cycles 2–6. CHL was used at the dose of 0.5 mg/kg every 2 weeks or according to local policies.

Efficacy and survival analyses were focused in patients without TP53 abnormalities (including FISH 17p13 deletion and/or TP53 mutation). The primary endpoint was progression-free survival (PFS) with G-CHL *vs.* IB. Secondary endpoints were overall response rate (ORR), which included complete remission (CR) and partial remission with/without lymphocytosis (PR-L and PR), time to next treatment (TTNT), overall survival (OS), subgroup analyses, locally performed flow cytometry to assess measurable residual disease (MRD), AEs, and economic impact of treatments.

In order to compare the costs of the drugs, we used the ex-factory prices in Italy in 2021: €16.47 for CHL (os, 2 mg each pill, 25 pills in each box), €2,828.63 for G (ev, 1 bottle, 1,000 mg), and €7,299.59 for IB (os, 140 mg each pill, 90 pills in each box). Costs of outpatient visits (€14.50), emergency room accesses, and days of hospitalization (€530/day) were counted based on the regional prices of reimbursement. Costs of other concomitant therapies were not included.

Biological markers and MRD analysis

Cytogenetics by FISH (20, 21), TP53 mutation (22), and IGHV mutational status (23, 24) were performed in all recruited patients in local accredited laboratories, and their protocols are summarized in the supplementary materials. An IGHV gene sequence homology ≥98% was considered as unmutated (U-IGHV), as opposed to mutated (M-IGHV) (25). For MRD assessed by flow cytometry, mononuclear cells were marked according to the ERIC protocol (26) or its update. Briefly, 1,000,000–2,000,000 events were acquired for each sample and analyzed by Infinicyt™. MRD was considered undetectable when $<10^{-4}$ (uMRD), as opposed to detectable MRD (dMRD) (27). MRD was not performed in patients with progressive disease (PD) at response assessment.

Statistical analysis

Categorical variables were compared by the Chi-square test or the Fisher exact test, when appropriate. Continuous variables were compared using the Mann–Whitney test. PFS was calculated as start time of treatment to disease relapse or death (event) or last known follow-up (censured). TTNT was calculated according to the start time of G-CHL or IB to the beginning of a new line of therapy (event) or last known follow-up (censured). OS was calculated starting from the start of CLL treatment to death for any cause or last known follow-up. Survival analyses were performed by the Kaplan–Meier method, and the Log-rank test was used to compare survival curves between groups. The prognostic impact for the outcome variables was investigated by univariate and multiple Cox regression analysis. In Cox models, data were expressed as hazard ratios (HRs) and 95% confidence intervals (CIs). All covariates as well as all variables significantly unbalanced between the two study arms were jointly introduced into the same multiple Cox regression model (6). A propensity score matching analysis (1:1) with and without resampling was also carried out with a 0.2 caliper width. A p-value < 0.05 was considered as statistically significant. Correction for multiple comparison was also applied when indicated.

Results

Patients

We collected data of 284 patients from 16 Italian hematological centers within the Italian CLL campus network; 104 patients received G-CHL as frontline treatment and 180 patients were treated with IB. As shown in the consort plot, we excluded 101 patients due to the presence TP53 abnormalities: 1 subject in the G-CHL arm, and 100 patients in the IB arm [the latter has been previously published (28)]. For the final analysis, we included patients without TP53 abnormalities: 103 patients treated with G-CHL and 80 patients treated with IB (Figure 1A).

Patients' characteristics are summarized in Table 1. Patients belonging to the two arms were balanced (i.e., p-values > 0.05) for age (74.7 years *vs.* 69.2 years), male gender (66% *vs.* 53%), advanced Rai stage (59% *vs.* 46%), increased β2-microglobulin levels (both 54%), and 11q22–23 deletion by FISH (11% *vs.* 16%). We observed that more patients treated with G-CHL were octogenarian (20% *vs.* 5%, $p = 0.0038$), were comorbid (median CIRS 6 *vs.* 4, $p = 0.0009$), and had an impaired kidney function (67% *vs.* 48%, $p = 0.0061$). In addition, a higher rate of U-IGHV patients received IB as frontline treatment compared to G-CHL (74% *vs.* 55%, $p = 0.0087$).

Eighty-three percent of patients received all the eight scheduled doses of G and chlorambucil; treatment was reduced or discontinued by 35% of patients. Forty-four percent of patients decreased the dose of IB and 79% were still under IB treatment at the last follow-up.

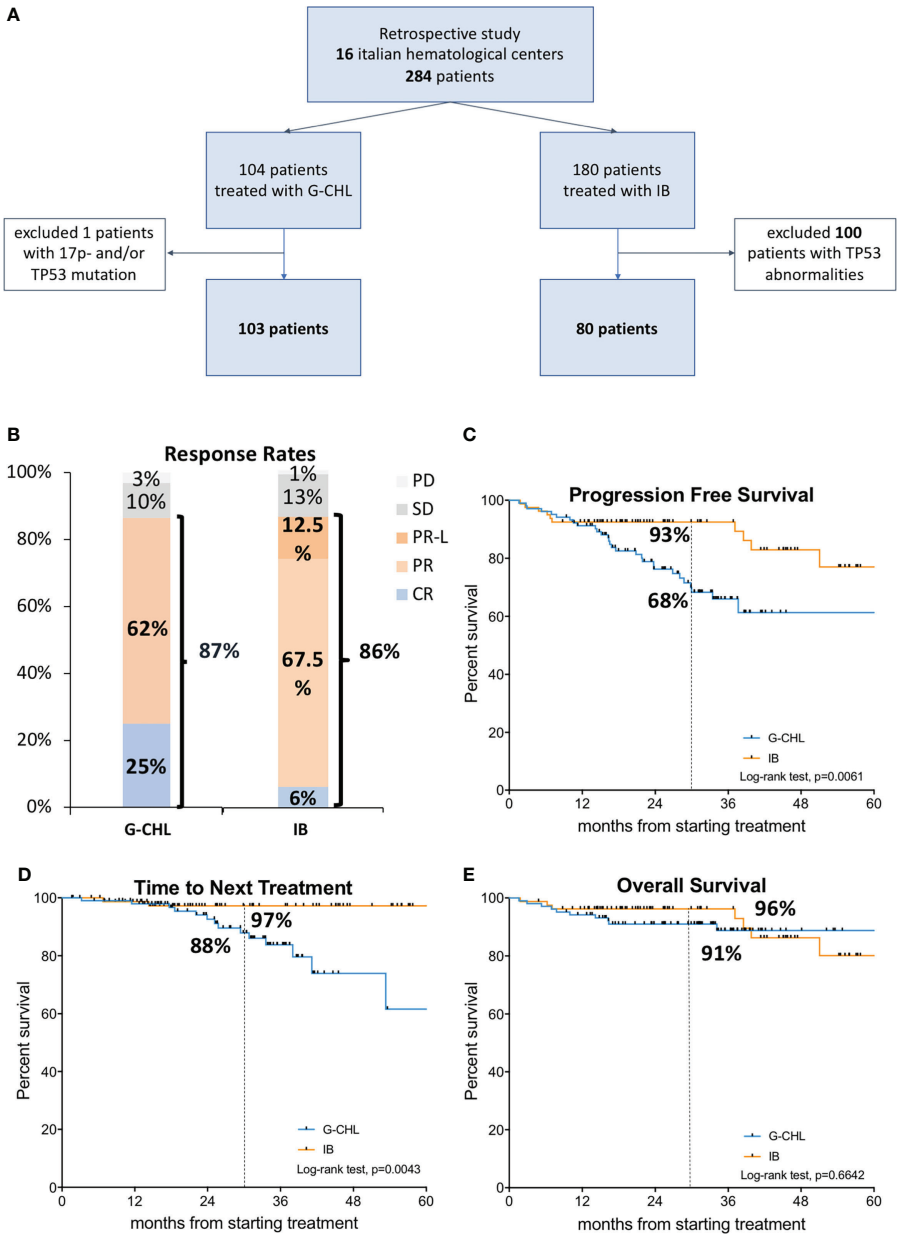

FIGURE 1
Study design and treatment efficacy. In the upper panel **(A)**, the consort plot of the study is shown. Among the 284 patients recruited within 16 Italian hematological centers, 101 were excluded due to the presence of TP53 abnormalities, including deletion of 17p13 (17p-) and/or TP53 mutation. In the middle panel, on the left **(B)**, the response rates plot for G-CHL (n = 103) and IB treatments are shown, and on the right **(C)**, the Kaplan–Meier curve of progression-free survival of G-CHL (n = 103) *vs.* IB (n = 80) is displayed. In the lower panel, on the left **(D)**, the Kaplan–Meier curves of time to next treatment is shown, and on the right **(E)**, that of overall survival of G-CHL (n = 103) *vs.* IB. (n = 80) is displayed.

Efficacy

After 9 months of treatments (i.e., 2–3 months after the end of the G-CHL), the overall response rate (ORR) according to iwCLL criteria was 87% for G-CHL and 86% for IB (Figure 1B). Despite a similar ORR, a higher rate of patients treated with G-CHL achieved a CR (Table 1, 25% *vs.* 6%, p = 0.0029) according to the iwCLL criteria (i.e., normalized complete blood count, negative CLL residue in the bone marrow, and lymph node size <1.5 cm). As expected, in the IB arm, there was a higher rate of PR/PR-L (Table 1, 62% *vs.* 80%). Variables associated with the achievement of a CR were M-IGHV (p = 0.0093), creatinine clearance (p = 0.0271), and G-CHL therapy (p = 0.006) (Table S1).

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TABLE 1 Characteristics of recruited patients.

	G-CHL n = 103	IBRUTINIB n = 80	p-values
Age (median ± sd, years)	74.7 ± 6.6	69.2 ± 6.9	0.1064
≥80 years (%)	20 (20%)	4 (5%)	**0.0038**
Male/Female (%)	68 (66%)/35 (34%)	42 (53%)/38 (47%)	0.0935
Median CIRS (range)	6 (2-18)	4 (0-12)	**0.0009**
Median creatinine cl. ± sd (ml/min)	61.2 ± 17.5	66.7 ± 14.0	**0.0011**
Creatinine cl. < 70 ml/min (%)	69 (67%)	38 (48%)	**0.0061**
Rai stage III–IV (%)	62 (59%)	37 (46%)	0.0743
β2-microglobulin >3.5 mg/L (%)	53 (54%)	34 (54%)	>0.9999
IGHV status U/M (%)	56 (55%)/47 (45%)	59 (74%)/21 (26%)	**0.0087**
FISH del11q- (%)	11 (11%)	13 (16%)	0.5417
Overall Response Rate (ORR)	90 (87%)	69 (86%)	
CR	26 (25%)	5 (6%)	**0.0029**
PR/PR-L	64 (62%)	64 (80%)	
SD/PD	13 (13%)	11 (14%)	

CIRS, cumulative illness rating scale; creatinine cl., creatinine clearance; IGHV status U/M, unmutated/mutated; CR, complete remission; PR, partial remission; PR-L, partial remission with lymphocytosis; SD, stable disease; PD, progressive disease; sd, standard deviation.
Bold values means statistically significant variables.

Survival analysis

After a median follow-up of 30 months, 24 patients have relapsed in the G-CHL arm and 3 patients have relapsed in the IB arm; 17 patients required a subsequent treatment in the G-CHL arm (14 BTK inhibitors and 3 venetoclax ± rituximab) and 2 patients in the IB arm (both venetoclax ± rituximab); 10 patients died (4 due to sepsis, 2 due to pneumonia, 2 due to CLL, and 2 due to cardiovascular events) in the G-CHL arm *vs.* 8 in the IB arm (3 due to cardiovascular events, 1 due to RS, 1 due to pneumonia, 1 due to sepsis, 1 due to lung cancer, and 1 due to unknown cause). None developed a Richter syndrome transformation with G-CHL, but 1 did in the IB arm.

Overall, IB was associated with better PFS and TTNT but not with OS compared to G-CHL (Figures 1C–E). The 30-month PFS was 68% and 98%, and the estimated 5-year PFS was 61% and 82% for G-CHL and IB, respectively (p = 0.0061). Patients who received IB as frontline therapy had a 2.5-fold lower risk of disease progression or death than patients in the G-CHL arm (HR 2.58, 95% CI 1.38–4.84) (Figure 1C).

The 30-month TNTT was 88% and 97%, and the estimated 5-year TTNT was 61% *vs.* 97% for G-CHL and IB, respectively (p = 0.0043). IB was associated with a sixfold decrease in the need of a second line of treatment (HR 6.07, 95% CI 2.39–10.44) (Figure 1D).

The 30-month OS was 91% and 96% for G-CHL and IB, respectively (p = 0.6642), without a significant difference at 5 years (88% *vs.* 86%) (Figure 1E).

Given that somatic hypermutation of the IGHV gene is one of the most important prognostic and predictive markers in CLL (24, 25, 29, 30), we assessed the impact of the IGHV mutational status in our patients. In U-IGHV patients, the 30-month PFS and TTNT were 72% *vs.* 90% (p = 0.0199, HR 2.58, 95% CI 1.19–5.57) and 82% *vs.* 96% (p = 0.0137, HR 5.38, 95% CI 1.73–11.69) for G-CHL and IB, respectively (Figures 2A, B). The median PFS was reached by G-CHL-treated U-IGHV patients at 37.7 months, while it was not reached by patients treated with IB. In M-IGHV patients, the 30-month PFS and TTNT were 82% *vs.* 96% (p = 0.1900, HR 2.54, 95% CI 0.83–7.84) and 94% *vs.* 100% (p = 0.1380, HR 3.93, 95% CI 0.93–13.64) for G-CHL and IB, respectively (Figures 2C, D).

Impact of depth of response and MRD in the G-CHL arm

Subsequently, we analyzed the impact of depth of clinical response and MRD on the survival of patients in the G-CHL arm. According to iwCLL response rates, the median PFS was not reached for patients in PR and CR, but it was only 11.2 months for patients who did not respond to G-CHL therapy (i.e., classified as SD or PD) (p < 0.001). The 30-month PFS was 79%, 68%, and 38% for patients who achieved CR, PR, and SD/PD, respectively (Figure 2E). TTNT was not impacted by the type of response rate (Figure S1A). Conversely, patients with SD/PD had a shorter OS (median OS, 34.1 months), while it was superimposable for patients who achieved a CR or PR (30-month OS, 95.7% *vs.* 94.9% *vs.* 61.5%, p < 0.0001, Figure S1B).

Eighty-seven (75%) patients of the G-CHL arm were studied locally for MRD by flow cytometry in the peripheral blood. No patient with PD was studied for MRD. Considering all the 103 patients treated with G-CHL at disease evaluation (i.e., month

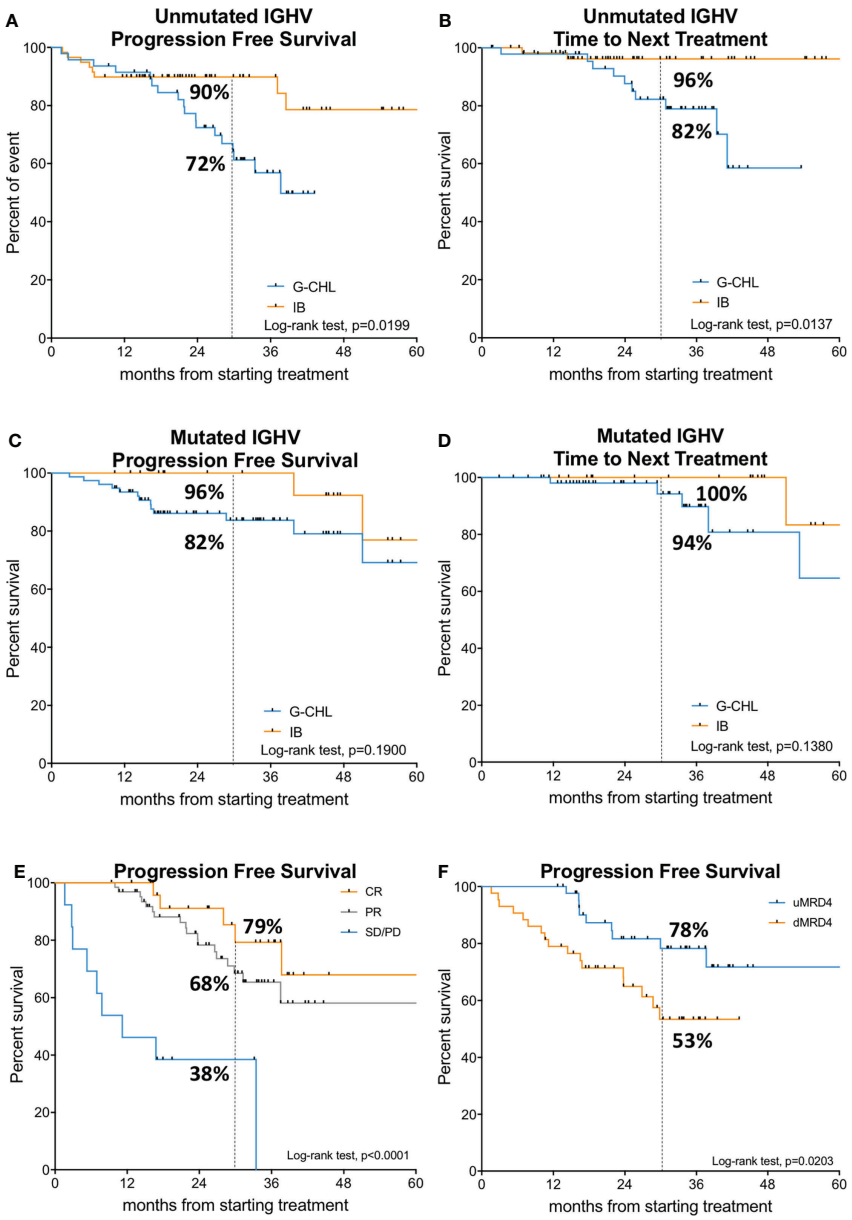

FIGURE 2
Survival analysis based on IGHV mutational status and deep of response. In the upper and middle panels, the Kaplan–Meier curves of progression-free survival and time to next treatment of IGHV unmutated (**A, B**); G-CHL, *n* = 56; IB, *n* = 59) and mutated patients (**C, D**); G-CHL, *n* = 47; IB, *n* = 21) are shown. In the lower panel, the Kaplan–Meier curves of progression-free survival of the G-CHL arm (*n* = 103) according to the iwCLL response rate on the left **(E)** and MRD (measurable residual disease) response on the right (*n* = 87) **(F)** are displayed.

+8 or +9), 43% of patients were able to achieve a uMRD in the peripheral blood, 43% had a dMRD, and 16% were not assessed (Figure S1A). Forty-nine patients were assessed for MRD in the bone marrow, 10% achieved uMRD, 38% had detectable MRD, and 52% were not studied. Ten patients (20%) had uMRD both in the peripheral blood and in the bone marrow, 8 (16%) had uMRD in the peripheral blood but a dMRD in the bone marrow, and 31 (63%) had a dMRD both in the peripheral blood and in the bone marrow. The concordance rate between peripheral blood and bone marrow assessment was 83%. Variables associated with uMRD in the peripheral blood were an M-IGHV status ($p = 0.0219$) and creatinine clearance ($p = 0.0311$).

The 30-month PFS was significantly higher for patients achieving uMRD4, which was 78% *vs.* 53% for uMRD patients and dMRD patients, respectively ($p = 0.0203$) (Figure 2F). The median PFS was not reached. Patients with dMRD at the end of

the G-CHL treatment had a 2.5-fold greater risk of progression than those with uMRD (HR 2.49, 95% CI 1.15–5.43).

TTNT was also influenced by the MRD response with an estimated median TTNT of 43.2 months for patients with dMRD, while it was not reached for those with uMRD (Figure S2D). The 30-month TTNT was 96.7% *vs.* 74.2% for uMRD and dMRD patients ($p = 0.0211$) (Figure S1G). Patients with dMRD were at threefold greater risk of starting a new treatment than those with uMRD (HR 3.4, 95% CI 1.19–9.92).

Adjusted and propensity score matched analysis

An unadjusted Cox analysis performed joining all the patients of both arms ($n = 183$ patients) showed that IB was significantly more effective than G-CHL in decreasing the risk of disease progression (HR 0.37, $p = 0.0078$) or next line of therapy (HR 0.14, $p = 0.0086$) in treatment-naïve patients with CLL (Table S2). To minimize the confounding effect, we adjusted the relationship between treatment arms (IB *vs.* G-CHL), PFS, and TTNT for all the variables skewed between arms (Table 1), as well as for all variables significantly associated with PFS and TTNT in the Cox univariate analysis (Table S2). After introducing these covariates into a multiple Cox regression model, the protective effect of IB *vs.* G-CHL in terms of risk of disease progression (HR 0.32, 95% CI 0.13–0.81, $p = 0.0163$) or next treatment (HR 0.12, 95% CI 0.03–0.61, $p = 0.0102$) was confirmed independently of potential confounders (Table S3).

Given the relevant differences of comorbidities and IGHV status between G-CHL and IB arms, we also performed a propensity score matched analysis (1:1). New arms were created, either with ($n = 79$) or without ($n = 50$) replacement balancing differences among treatment groups (Tables S4, S5). Even after this matched analysis, PFS and TTNT, but not OS, were longer in the IB arm than in the G-CHL arm (Figures S2A, B).

Safety and economic analysis

Overall, patients treated with G-CHL had more AEs than those receiving IB (2.98 *vs.* 1.68 AE/month of treatment/person), and less ambulatory outpatient visits (RR 0.17, 95% CI 0.15–0.20) and hospitalizations (RR 0.42, 95% CI 0.17–1.10). However, only the number of outpatient visits was statistically significant.

Ninety-eight percent of patients received premedication (paracetamol 1 g iv, anti-H1 iv, and methyl-prednisolone iv) before G infusion. Infusion-related reactions (IRRs) were recorded in 36.9% of patients, the majority being grade 1 or grade 2 and only 4.9% being grade 3. Given the retrospective nature of the study, we focus only on severe (grade ≥3) AEs. The most relevant G ≥ 3 AEs were neutropenia (35% *vs.* 9%, $p < 0.0001$), infections (13% *vs.* 16%, $p = 0.3188$), thrombocytopenia (12% *vs.* 1%, $p = 0.0004$), anemia (6% *vs.* 0%, $p = 0.0002$), and atrial fibrillation (2% *vs.* 9%, $p = 0.0813$) for G-CHL and IB, respectively. No tumor lysis syndrome occurred.

An economic analysis was carried out on 92 patients, 69 patients from the G-CHL arm and 23 patients from the IB arm. The characteristics of the economic cohort is reported in Table S6. As shown in Figure S2C, IB was associated with higher monthly costs, mainly related to the costs of the drug rather than the management of AEs. The mean total monthly cost per patient was €1,545 with G-CHL and €5,587 with IB, resulting in a mean savings per month of €4,074 (95% CI 3,267–4,881). This difference is mainly due to the savings in first-line drug cost (€1,029 *vs.* €5,297) and slightly to the decrease in hospitalization and/or outpatient visits (€95 *vs.* €290) (Figure S2C).

Discussion

We gathered data from 183 CLL patients without TP53 abnormalities who were treated with continuous IB or with 6 months of G-CHL therapy as first-line therapy in the real-life setting. We found that (i) a remarkable number of patients were able to achieve a uMRD with G-CHL, and (ii) PFS and TTNT, but not OS, were better with IB than with G-CHL. The similar OS is likely due to the fact that all patients received targeted therapies with either a BTK or a BCL2 inhibitor as second-line therapy.

Furthermore, recent studies found that a high number of comorbidities, assessed by the CIRS score, have a detrimental impact of target therapies' efficacy (31–33). In our study, despite a relevant number of comorbid patients, they showed a remarkable outcome with G-CHL.

The IGHV mutational status is one of the most important prognostic and predicted markers in CLL, being able to identify patients who might benefit most from a fixed-duration therapy (6, 10, 30, 34). When PFS and TTNT curves were stratified for the IGHV status, we found that IB improvement was significant only for the U-IGHV patients. Conversely, among M-IGHV patients after a median follow-up of 30 months, the PFS and TTNT curves of the G-CHL and IB almost overlapped, thus suggesting that fixed-duration therapy might be a key strategy in M-IGHV CLL patients in clinical practice.

G-CHL treatment was approved based on the results of the CLL11 trial, where G-CHL was compared with rituximab-CHL and CHL alone (35). The median age was 73 years (range, 39–90 years); 61% were U-IGHV, 8% harbored a del17p-, and 16% harbored a del11q-. All patients had a CIRS score >6 and/or a creatinine clearance <70 ml/min. G-CHL led to a better PFS, TTNT, and OS than the other arms. A uMRD in the peripheral blood at the end of treatment was significantly more common in patients receiving G-CHL compared to those who received

rituximab-CHL (35.8% *vs*. 3.3%, $p < 0.001$). Patients with uMRD had a median PFS of 56.4 months compared to 23.9 months for patients categorized as MRD intermediate (MRD events between 10^{-4} and 10^{-2}) and 13.9 months for dMRD patients ($p < 0.001$). MRD response was also significantly associated with a better OS (35). In our study data, we excluded patients with TP53 abnormalities (deletion or mutation) and fewer patients harbored U-IGHV and/or del11q- by FISH. The presence of fewer patients with unfavorable markers in our study might explain the higher uMRD rate (43% *vs*. 35.8%) and the longer PFS. Furthermore, G premedication significantly decreased IRR (66% in the CLL11 trial *vs*. 36.9% in our study, G3 21% in the CLL1 trial *vs*. 4.9% in our study).

Since CHL is a weak partner, G has been combined with continuous IB [iLLUMINATE trial (36)], continuous acalabrutinib [ELEVATE TN (37)], or the 12-month venetoclax [CLL14 (10)] and compared with G-CHL. In all these trials, the combination of G plus an oral targeted drug led to higher uMRD rates, particularly for G-venetoclax, and sustained longer PFS than G-CHL. Remarkably, IRRs were lower when G was given in combination with BTK inhibitors (36, 37).

Recently, G-CHL has been compared with the fixed-duration oral therapy IB-venetoclax (38). The GLOW trial included patients ≥65 years old or those with CIRS score ≥6 or creatinine clearance <70 ml/min. The uMRD rate in the bone marrow by next-generation sequencing was significantly higher for IB-venetoclax than for G-CHL (56% *vs*. 21%, $p < 0.001$), which led to a significantly longer PFS. The improvement in PFS with IB-venetoclax was consistent across patients ≥65 years and/or with a CIRS ≥ 6.

A041202 is a phase 3 clinical trial comparing IB ± rituximab with another chemoimmunotherapy schedule used in elderly patients, i.e., bendamustine-rituximab (BR) (39). With a median follow-up of 55 months, the median PFS was 44 months with BR and was not reached in the IB arms. An economic analysis showed that costs (associated with protocol-specified resource use) were significantly higher for patients receiving IB ± rituximab (mean $189,335 or $219,908; $p < 0.0001$) compared to BR (mean $51,345), driven by the higher costs for IB (40). Quality-adjusted life years were also similar between arms. In line with our data, IB provides better disease control in patients with del11q by FISH and U-IGHV, counteracted by a much higher cost of the drug. IB plus rituximab was also tested against FCR in CLL patients aged ≤70 years in the E1912 trial (41). With a median follow-up of 5.8 years, the median PFS was superior for IB-rituximab ($p < 0.001$). Notably, only in the E1912 trial did IB-rituximab improve not only PFS compared to FCR in patients with IGHV mutated and unmutated gene (HR 0.27, $p < 0.001$) but also OS (HR 0.47, $p = 0.018$).

The main limitation of our study is its retrospective structure and the sample size. To minimize selection and attrition biases as well as imprecise reporting of data inherent to observational studies, we asked the treating physician to report all CLL patients treated frontline with G-CHL. We analyzed the reported data, excluded cases with TP53 abnormalities, and performed computerized manual consistency checks on each case report form. Furthermore, given the differences in the clinical characteristics of patients (Table 1), particularly age and comorbidities, we applied a propensity score matched analysis with ($n = 79$) and without ($n = 50$) replacement balancing (Tables S4, S5). The small size of the samples affects the conclusions of the study. In addition, the median follow-up of 30 months does not allow us to reach conclusions about the OS.

The Italian CLL campus experience with G-CHL confirms the effectiveness of this treatment, particularly for M-IGHV patients capable of reaching a CR or a uMRD. Although MRD assessment is still not recommended by current guidelines, an increasing number of centers utilize this analysis (42). Continuous treatment with IB provides longer remission in elderly CLL patients unfit for fludarabine-based therapy (31). However, it is noteworthy that some patients can achieve long-term disease control with a less expensive fixed-duration obinutuzumab-based therapy, which may represent an option for first-line treatment in countries with economic constraints (8, 9).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Azienda Ospedale Università Paadova. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AV designed the study, performed statistical analysis, visited patients, and wrote the article. GC, AF, CV, AS, FC, PS, MG, GR, FQ, VM, AG, MM, LS, GR, SP, FA, and ACe provided intellectual inputs and visited patients. FM, ACu, RF, SM, MC, LL, PG, and LT visited patients, provided intellectual inputs, and reviewed the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AV received honoraria from Janssen, Abbvie, CSL Behring, and Italfarmaco. LT received research funding from Gilead, Roche, Janssen, and Takeda, and is on the advisory board for Roche, Takeda, Abbvie, and AstraZeneca. GR received research funding from Gilead. FM is on the advisory board for Janssen, Takeda, and Abbvie. ACu is on the advisory board and speaker bureau for Roche, Abbvie, Gilead, and Janssen. RF is on the advisory board or speaker bureau for Incyte, Amgen, AstraZeneca, Janssen, Gilead, and Novartis. LL received honoraria from Abbvie, Janssen, Astra Zeneca, and Beigene. FQ plays an advisor role for AstraZeneca and Janssen, is a speaker for Janssen, and is a consultant for Sandoz. LS received honoraria from AbbVie, AstraZeneca, and Janssen.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1033413/full#supplementary-material

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"Friends and foes" of multiple myeloma measurable/minimal residual disease evaluation by next generation flow

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Next Generation Flow (NGF) represents a gold standard for the evaluation of Minimal Residual Disease (MRD) in Multiple Myeloma (MM) patients at any stage of treatment. Although the assessment of MRD is still not universally employed in clinical practice, numerous studies have demonstrated the strength of MRD as a reliable predictor of long-term outcome, and its potential to supersede the prognostic value of CR. The possibility to acquire millions of events, in combination with the use of standard reagents and a good expertise in the analysis of rare populations, led to high chance of success and a sensitivity of 10^{-6} that is superimposable to the one of Next Generation Sequencing molecular techniques. Some minor bias, correlated to the protocols applied, to the quality of samples and to the high heterogeneity of plasma cells phenotype, may be overcome using standard protocols and having at disposition personnel expertise for MRD analysis. With the use of NGF we can today enter a new phase of the quantification of residual disease, switching from the definition of "minimal" residual disease to "measurable" residual disease. This review takes account of the principle "friends and foes" of Myeloma "Measurable" Residual Disease evaluation by NGF, to give insights into the potentiality of this technique. The optimization of the quality of BM samples and the analytic expertise that permits to discriminate properly the rare pathologic clones, are the keys for obtaining results with a high clinical value that could be of great impact and relevance in the future.

KEYWORDS

multiple myeloma, minimal residual disease, next generation flow, complete remission, progression free survival

Introduction

Multiple Myeloma (MM) is a Plasma Cells (PCs) malignancy characterized by the uncontrolled proliferation of pathologic PCs in the Bone Marrow (BM) (1). These cells secrete a monoclonal nonfunctional immunoglobulin (M protein) whose accumulation causes the typical clinical symptoms of the disease, such as hypercalcemia, renal impairment, anemia, and bone lesions (i.e., CRAB criteria) (2–4). MM median age at presentation is above 70 years, and its incidence has increased in the last 25 years, representing today 1-2% of all cancers and about 10% of hematological diseases (5). In recent years, the introduction of new drugs has improved Progression Free Survival (PFS) and Overall Survival (OS) of MM patients (from a median of 3–4 y to a median of 8–9 y) (6, 7). These drugs comprise the immunomodulatory (IMIDs) Thalidomide, Lenalidomide, and Pomalidomide; the Proteasome Inhibitors (PIs) Bortezomib, Carfilzomib, Ixazomib; the Monoclonal Antibodies (MoA) Daratumumab, Elotuzumab, Isatuximab, Belantamab (8). They can be used alone or combined in triplet or quadruplet, leading to exceptional responses that can reach 90% of the treated patients (9), and they are useful to treat also aggressive conditions, such as extramedullary disease (10–12). Moreover, strategies such as consolidation therapy and maintenance after Autologous Stem Cell Transplantation (ASCT) contribute to further improvement of PFS and OS (13–15).

However, in some cases MM patients may still relapse or develop resistance to treatment regimens, leading to the necessity of better and higher-sensitive techniques to monitor Minimal Residual Disease (MRD) and discriminate patients at risk for relapsing. Indeed, the achievement of MRD negativity has superseded the conventional Complete Response (CR) and has been showed as a surrogate endpoint for Progression Free Survival and Overall Survival (16). Clinicians need to deal with MRD assessment in routine clinical practice, and its use in taking therapeutic decisions surely represents one of the most challenging but fascinating issues to be addressed in the next years (17). The evident survival progress and better quality of life of MM patients, associated with higher chances to reach and maintain deep responses, pave the way to the hope that Myeloma could not be anymore an "incurable disease" (18). In this context, Next-Generation Sequencing (NGS) and Multicolor Flow Cytometry (MFC) are currently the best techniques available to monitor MM patients and evaluate MRD with sensitivity up to 10^{-6} (19–22).

NGS vs NGF

The molecular techniques use the clonal Immunoglobulin (Ig) gene rearrangement as target for the detection of MM MRD levels. The Allele-specific Oligonucleotide Polymerase Chain Reaction (ASO-PCR) and digital PCR (dPCR) have been widely substituted by Next Generation Sequencing (NGS), whose high sensitivity permits to obtain optimal MRD results. However, the feasibility of this approach is limited by high costs, long turnaround time, and required specific expertise (23). Multicolor Flow Cytometry (MCF), on the other hand, is efficient in detecting and quantifying normal vs. pathologic PCs by looking at both markers present on the surface of cells or in the cytoplasm. PCs are characterized by the expression at high level of two main markers, CD38 and CD138; however, MM PCs may be recognized because they could express markers such as CD56, CD28, CD200 and CD117, and, compared with normal PCs, generally are $CD45^{-}$low, $CD19^{-}$, $CD27^{-}$, and $CD81^{-}$.All together, these markers, in addition to the clonal restriction of MM PCs to just one of two immunoglobulin light chains, κ or λ, contribute to easily discriminating normal from clonal MM PCs (24). Older conventional flow cytometric assays are now replaced by advanced assays that permit to simultaneously assess more than eight markers; the great step forward has been made with the introduction of Next Generation Flow (NGF), the high-standardized approach, developed by Flores-Montero et al. (25) which permits, by acquiring $\geqslant 10^{7}$ cells, to reach a sensitivity that is indeed superimposable to NGS, but with shorter turnaround time and a substantial costs reduction. Although different combinations of antibodies have been tested, using in-house cocktails, i.e., 10-color (26, 27) or 8-color single-tube (28, 29), the protocol developed by the EuroFlow™ Consortium, has been validated for MRD definition in several studies (30, 31). This protocol, based on the use of two single eight-color tubes containing the markers for MM PCs recognition and combined with the use of specific Standard Operating Procedures (SOPs) that could guarantee the best results in terms of MRD evaluation, has become the gold standard in use in the majority of laboratories. Table 1 summarizes the characteristics of NGS vs. NGF techniques for MM MRD measurement. The choice of NGS and/or NGF for MM MRD evaluation nowadays just depends on the availability of the laboratory (23, 32, 33), and a hybrid approach, that permits to simultaneously assess MRD by looking at both molecular and cellular characteristics of myeloma clones, could be of great help when appliable (34, 35).

Depth and timing of MRD

The International Myeloma Working Group (IMWG) defined response criteria in which MRD negativity cut-off was set at 10^{-5} detected either by NGS or NGF. Together with the bone marrow search for monoclonal plasma cells also whole-body imaging such as PET-CT is important to exclude bone focal lesions that could be a disease "reservoir" for relapse. Many

TABLE 1 NGS vs. NGF characteristics for MM MRD measurement.

	ADVANTAGES	DISADVANTAGES	COMMON FEATURES
NGF	99% Applicability 2-3 h turnaround time Not require diagnostic sample Intrinsic hemodilution evaluation Gives cells characteristics Wide Availability High Reproducibility Harmonization Lower costs	Requires $2x10^7$ cells Requires fresh samples Does not give molecular characteristics	Qualitative analysis Sensitivity at 10^{-6}
NGS	Requires $2\text{-}3x10^6$ cells Does not require fresh samples	Lower Applicability 90% Long turnaround time (7 days) Requires diagnostic sample No hemodilution evaluation No cell characteristics Limited Availability High costs	Qualitative analysis Sensitivity at 10^{-6}

trials are now trying to increase MRD sensitivity to 10^{-6} which seems to be a better predictor of PFS (30, 36). Timing of MRD testing is also important and should be at treatment cessation 3 months after autologous stem cell transplant and every 6 months thereafter, at least for 2 years if negativity is achieved. This systematic evaluation could reveal a sustained MRD negativity status that is crucial for long term remission (37).

Advantages of using NGF

Next Generation Flow has many advantages: it is applicable to almost 100% of MM cases, it is very fast, requiring just 2–3 h of processing, and it does not require a diagnostic sample (25). Having at disposition the analysis of the myeloma clone at diagnosis helps defining a pre-treatment panel that could be used as a reference for MRD monitoring (38), as it happens for other leukemias in the so-called Leukemia Associated Immunophenotype (LAIP) approach (39–41). However, it doesn't overcome the possibility of clonal evolution or the presence of additional subclones that could be minimally represented or be absent at diagnosis, leading to the necessity of considering also a Different from Normal (DfN) approach (42–44). Moreover, in many cases the diagnostic sample is not available for MM patients. In order to obtain the proper Limit Of Detection (LOD), calculated as 50 clonal PCs among 10^7 nucleated cells, and Limit of Quantification (LOQ), calculated as 20 clonal PCs among 10^7 nucleated cells, NGF is done by acquiring at least 10 million of events per tube (13, 14); in this way, NGF permits to obtain a high sensitivity of 10^{-6} that is comparable and superimposable to NGS assays (18). The two 8-color pretitred tubes, containing the markers for the recognition of plasma cells, are constructed to perform a sequential gating based on the recognition of the backbone markers (CD45, CD19, CD38, CD138) and the expression of the additional markers that could be aberrantly present on MM PCs surface (CD56, B2, CD117, CD81, CD27, CD28). Moreover, the discrimination of pathologic PCs over the normal counterpart is done by taking into consideration Ig light chains restriction. Figure 1 shows an example of analysis performed by using a BD Facs Lyric cytometer.

The flow-cytometric assays need to be performed following the Standard Operating Procedures (SOPs), that have been designed by EuroFlow™ to provide full technical standardization and best results for MRD evaluation (25, 45–47); these procedures are applied in order to harmonize reagents, fluorochromes panels, sample processing procedure, platforms used and data analysis (24, 48). Once acquisition of data has been completed by cytometer, subsequent analytic steps are nowadays performed using softwares that permits to merge the data from different analyses and compare the expression of all the markers tested at the different steps of treatment, correlating results with that of MRD data contained in databases. In particular, the Infinicyt™ software developed by EuroFlow™ contains representative flow cytometry data sets from normal healthy BM samples, processed in different standardized centers. These databases are at disposition for the analysis of Acute Leukemias, Chronic Lymphoproliferative Disorders, Primary Immunodeficiencies and Plasma Cells Dyscrasias, and allows for an automated analysis of the complete BM sample, considering both normal and pathologic populations; in this way, the software provides a photograph of the whole immune profile, giving information that may be of great interest and relevant for prognosis of patients and that permit to be confident about MRD results.

MM MRD evaluation is largely performed on Bone Marrow (BM) samples; indeed, BM aspirates are still the gold standard patients' samples for prognostication and genetic characterization. However, they also represent a limitation due to the aggressiveness of the procedure, to the impossibility, with a single BM aspirate, to reflect the complex MM heterogeneity (15, 49), and to the risk of assessing bad quality BM samples that

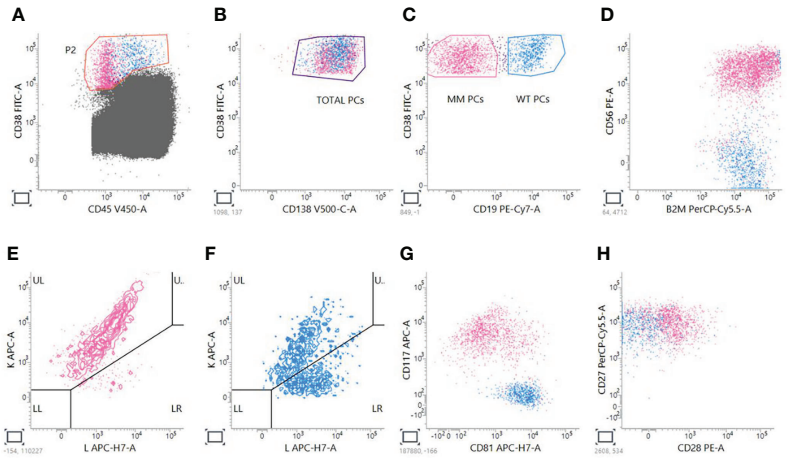

FIGURE 1
Analysis of MM MRD evaluation by NGF. A sequential gating strategy is used; first of all, PCs are gated on CD38 versus CD45 plot **(A)**; then, $CD38^+CD138^+$ total PCs are taken **(B)**, and two gates are created to distinguish MM $CD38^+CD19^-$ PCs from Wild Type (WT) $CD38^+CD19^+$ PCs **(C)**. For $PCST^{TM}$ tube, we look at expression of CD56 **(D)** and presence of restriction to just one of the two light chains for MM PCs **(E)** compared to WT polyclonal PCs **(F)**. For tube PCD^{TM}, we look also at the expression of CD117 and CD81 **(G)**, CD28 and CD27 **(H)**.

might not be representative of the real degree of infiltration of the disease. For this reason, recently the same MRD analysis has been tested also on peripheral blood to look at the percentage of Circulating Tumor Cells (CTCs) that could give an idea of patient's responses to therapy (50, 51). Different studies have already demonstrated the reliability of evaluating CTCs level in MM patients at diagnosis (52) or during different treatment regimens. Detection and isolation of circulating tumor cells (CTCs) is still a developing field in many cancers (53); in case of myeloma patients, basing on the available literature, it's a process that requires around 3-14 mL of blood to obtain $\geqslant 10^7$ cells per sample necessary to maintain NGF high sensitivity (54, 55), and offer a promising and minimally invasive alternative for tumor assessment, genetic characterization and extramedullary dissemination study of MM patients (56, 57). Flow-cytometry permits to detect CTCs easily, contributing in this way to understanding the pathogenesis of MM and to enlighten mechanisms of this disease that could be useful to clarify how other similar tumor develop and disseminate in the human body (57, 58).

Bias of using NGF

Flow-cytometric analysis must be performed taking also in account some minor bias that could, if not considered, reduce the reliability of Multiple Myeloma MRD evaluations (24). First, often there is a high difference in terms of bone marrow cellularity and percentage of plasma cells observed by cytological analysis compared to flow-cytometry methods. This apparent inconsistency is due firstly to the fragility of plasma cells themselves, with a pool of plasma cells loss during laboratory manipulation; secondly, the lower PC count obtained by flow-cytometry may be related to a possible hemodilution of the BM samples, with the risk to underestimate the percentage of pathologic PCs (59). Different methods have been recommended to accurately evaluate the degree of hemodilution. These methods are based on an automated lymphocyte count, PB contamination indices that takes account of PC percentages, $CD34^+$ cells, and $CD10^+$ neutrophils (60), or numbers of CD16 bright neutrophils (61). In the case of flow cytometric analysis, NGF can also provide the qualitative assessment of patient samples by allowing for analysis of normal B-cell compartments and non-PC BM cells, such as mast cells or RBCs, which can give us a quite accurate estimation of the hemodilution of analyzed BM samples. Moreover, the good clinical practice of sparing the first aspirated sample from the iliac crest for flow-cytometric assays, could reduce the risk of performing MRD evaluation from low quality samples (62, 63).

The other major pitfall in MM MRD evaluation by NGF is correlated to the high heterogeneity of MM plasma cells phenotype, and to the possibility of a "shift" of plasma cells phenotype depending on the therapy that patients have been exposed (64, 65).. It has been widely demonstrated that patients starting treatment with immunomodulatory drugs may experience a change on plasma cells phenotype, and the use of drugs such as Daratumumab, that could mask the CD38 overexpressed molecule, can make even more difficult MM clones recognition (66). This last problem has been overcome by introducing CD38 multiepitope antibodies that permit, by binding to sites that are different from the one occupied by the drug, to still recognize MM PCs even during treatment with

Anti-CD38 monoclonal antibodies (67). Moreover, the availability of analytic softwares like Infinicyt™ provides the possibility to analyze automatically MRD data and compare individual results to the set of data stored into database, increasing the accuracy and precision of the evaluation, and helping operators in those situations in which a manual gating could miss minor phenotypic alterations that could be related to resistance mechanisms or type of treatment (68). In combination, when possible, with NGS analysis, this approach could theoretically give the possibility to monitor adequately 100% of myeloma patients.

Finally, given the peculiarity of flow-cytometry analysis a high personnel expertise is essential in order to obtain reliable results, especially in demanding cases in which anti-CD38 therapy, presence of different pathologic clones or presence of normal PCs, together with low MRD burden, could lead to bias (69). Reducing the subjectivity in data analysis requires the work of experienced laboratories, that are constantly monitored and trained, and whose results could be assessed and tested by external quality assurance programs and interlaboratory comparisons.

Discussion and future perspectives

Since MRD detection is now strongly recommended although not mandatory for guiding clinical treatment decisions, the possibility to employ NGF to test the depth and duration of response in Multiple Myeloma patients represents a great advantage and a great promise for the management of this disease. Additionally, NGF is an easy and low-cost technique and therefore is widely used nowadays for the analysis of MM MRD. The optimization of the quality of BM samples and the analytic expertise, that permits to discriminate properly the rare pathologic clones, are the main keys for obtaining results with a high clinical value that could be of great use in the future. The minor "foes" associated with the application of this technique could be easily overcome and do not reduce the value of using NGF for measurable residual disease of MM patients. With the use of NGF we can today enter a new phase of the quantification of residual disease, switching from the definition of "minimal" residual disease to "measurable" residual disease, and have the chance to accurately monitor MM patients and be able to early recognize those achieving long deep responses that may, in the future, be considered "cured" from the disease. Finally, the possibility to employ NGF for analysis of Circulating Tumor Cells (CTCs) represents a promising and minimally invasive alternative for tumor assessment and may enlighten mechanisms of disease dissemination that could be of great interest also applied to other cancers.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Measurable residual disease in chronic lymphocytic leukemia

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Measurable residual disease (MRD) is defined as the presence of residual cancer cells after treatment in patients with clinically undetectable disease, who would otherwise be considered in complete remission. It is a highly sensitive parameter which indicates the disease burden and predicts survival in this setting of patients. In recent years, MRD has gained a role in many hematological malignancies as a surrogate endpoint for clinical trials: undetectable MRD has been correlated to longer progression free survival (PFS) and overall survival (OS). New drugs and combinations have been developed with the aim to achieve MRD negativity, which would indicate favorable prognosis. Different methods to measure MRD have also been devised, which include flow cytometry, polymerase chain reaction (PCR) and next generation sequencing (NGS), with different sensitivity and accuracy in evaluating deep remission after treatment. In this review, we will analyze the current recommendations for the detection of MRD, with particular focus on its role in Chronic Lymphocytic Leukemia (CLL), as well as the different detection methods. Moreover, we will discuss the results of clinical trials and the role of MRD in new therapeutic schemes with inhibitors and monoclonal antibodies. MRD is not currently used in the clinical practice to evaluate response to treatment, due to technical and economical limitations, but it's gaining more and more interest in trials settings, especially since the introduction of venetoclax. The use of MRD in trials will likely be followed by a broader practical application in the future. The aim of this work is to provide a reader-friendly summary of the state of art in the field, as MRD will soon become an accessible tool to evaluate our patients, predict their survival and guide physician's therapeutic choices and preferences.

KEYWORDS

measurable residual disease, chronic lymphocytic leukemia, flow cytometry, ASO-PCR, next generation sequencing, surrogate endpoint

1 Introduction

The use of Measurable Residual Disease (MRD) is extensive in acute myeloid diseases and other conditions where therapy has a curative objective. Oppositely, it has a controversial role in Chronic Lymphocytic Leukemia (CLL), which has changed over the years. Recently, MRD in CLL has raised interest again, thanks to the advent of target therapies which induce deep molecular response, such as the BCL-2 inhibitor venetoclax.

Undetectable MRD has been defined by the international workshop on CLL (iwCLL) and ERIC as the presence of <1 CLL cell per 10.000 leukocytes (1, 2). Standard staging methods with cytology can detect the presence of one CLL cell in up to a maximum of 100 leukocytes (3), therefore they are much less accurate than MRD in defining the burden of disease at the end of a treatment. According to the iwCLL criteria, we can consider a patient in complete remission (CR) when he/she presents with (i) less than $4x10^9$ lymphocytes/liter, more than $1.5x10^9$ neutrophils/liter, more than $100x10^9$ platelets/liter and more than 11.0 grams/deciliter hemoglobin level in peripheral blood; (ii) absence of lymphadenopathy >1.5 cm and splenomegaly or hepatomegaly at physical examination; (iii) absence of constitutional symptoms (1). Nevertheless, the clinical assessment alone is not considered accurate enough in the era of molecular biology and personalized therapy: thus, the need for a deeper definition of CR is emerging in clinical trials and will likely guide treatment choices in the clinical practice in the next future (4). Starting from the awareness that disease relapse comes from the expansion of any residual clone after therapy, we can easily get to the conclusion that the larger is the number of persistent clones, the earlier will relapse occur. As a matter of fact, even the smallest amount of residual leukemic cells can lead to relapse over time, when allowed to expand in the treatment free interval, if only looking at the clinical outcome of the previous treatment.

Given that clinical parameters correlate and can predict the PFS of those patients, a more powerful tool to predict such outcome is the highly sensitive detection of MRD, which is able to recognize very small amounts of residual clones in both peripheral blood (PB) and bone marrow (BM) (5). Nevertheless, determining MRD is more costly and technically difficult than clinical assessment, which explains why it is not yet recommended by the current guidelines and not routinely used in the clinical practice.

In the era of targeted therapies, monoclonal antibodies and combinations of such, the deepening of the response to treatment measured by MRD is considered an endpoint to establish the superiority of a therapeutic approach over another (6). To note, different treatment platforms obtain different MRD levels. The old chemo-immunotherapy regimen with Fludarabine-Cyclophosphamide-Rituximab (FCR) induces a long-lasting CR, at times accompanied by MRD negativity, which of course represents the most important predictor of survival (4). On the other hand, new targeted therapies obtain a heterogeneous variety of responses. Bruton tyrosine kinase receptor (BTK) inhibitors, including ibrutinib and acalabrutinib, obtain a rapid nodal reduction and increase of the hemoglobin and platelets levels, but are not able to reach MRD at any time, rather they induce partial remission (PR) which needs continuative administration of therapy, until relapse or toxicity, to maintain such response (4). Contrarily, the BCL2 inhibitor venetoclax, in combination with anti-CD20 antibody, has shown durable MRD negativity and a promising long-lasting progression-free survival in relapsed and refractory patients (7). Furthermore, the combination of BTK and BCL2 inhibitors (ibrutinib and venetoclax) achieves even deeper MRD negativity, and it has a favorable prognostic profile in terms of PFS, but it is now available only in few clinical trials (8).

In this review, we will go through all the laboratory methods that allow the definition of MRD with different rates of sensitivity as well as different costs and technical requirements. We will try to summarize the state of art in the detection of MRD on PB rather than on BM. We will also focus on the impact of MRD on both traditional and emerging therapeutic approaches, and its relevance to tailor the treatment based on patients' age, clinical status, and future perspectives.

We strongly believe that MRD has a crucial impact on the definition of personalized therapeutic strategies, as new clinical trials involve the detection of MRD to delineate next steps of patients' management. Therefore, it is important for any clinician to have a clear idea of the meaning of MRD detection from a technical point of view, but more relevantly, as a tool that will possibly be introduced in real life to guide and refine treatment choices.

2 MRD detection methods

Thanks to the technical advances of the last years, different methods to determine the burden of residual disease in CLL patients after treatment are available. At the same time, the lack of standardized guidelines makes the comparison between different clinical trials hard, due to the heterogenicity of techniques used and their sensitivity in detecting persistent clones (2). We will try to display the currently available options according to updated recommendations. Table 1 summarizes the difference between the three methods in terms of sensitivity, target, and standardization.

2.1 Flow cytometry

Multiparametric flow cytometry allows automated phenotyping of cells with fluorescently labelled antibodies (9). Panels of antibodies

TABLE 1 Summary table comparing the sensitivity of the three laboratory methods (flow-cytometry, PCR and NGS) in terms of sensitivity, target, and standardization.

	Flow cytometry	*ASO-PCR*	*NGS*
Sensitivity	MRD5	MRD6	MRD5
Target	CD19, CD20, CD5, CD43, CD79b and CD81	Ig hypervariable region	CDR3 sequence of the Ig
Standardization	ERIC 2016	none	none

linked to different fluorochromes can identify a specific CLL phenotype, characterized by expression of certain surface antigens.

The first attempts to measure minimal disease evaluated the clonality by immunoglobulin light chain (κ or λ) restriction on a CD19/CD5 co-expressing population (10). This approach, virtually applicable to all CLL cases, later demonstrated a low sensitivity and was deemed unsuitable for predicting response status according to later iwCLL/NCI criteria or identifying cases with no detectable MRD (11).

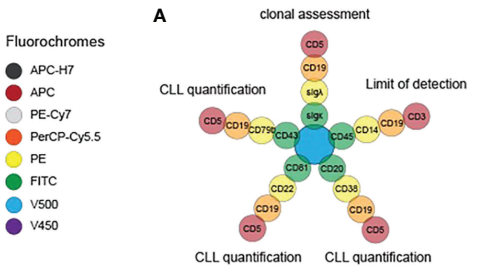
The standardized cytofluorimetric approach for the detection of MRD dates to 2007, but it still gives valuable information to assess the presence of residual cells on PB (2). Cell preparation was performed by a whole-blood lysis method with or without fixatives such as ammonium chloride or FACSLyse, to allow quantitative enumeration of CLL cells. The antibodies used to detect MRD were against CD19, CD5, CD20, CD38, CD22, CD81, CD43, CD79b, combined in four different four-color tubes: one clonality tube (CD19, CD5, surface light chains κ or λ), one limit of detection tube (CD19, CD3, CD45, CD14) and three tubes dedicated to MRD enumeration (2) (Figure 1A).

This first protocol was suitable for the detection of 1 cell in 10.000 lymphocytes in PB within an adequate sample of 1 to 2 million cells, thus a sensitivity of 0.01%/10^{-4}, also termed MRD4 according to Wierda et al (6). Even though this four-colors set of antibodies showed good performance and multiple standardization measures were adopted (2), there was a significant inter-laboratory variability and the MRD determination was still highly operator-dependent. Moreover, this approach needed four/five tubes, which further increased the risk of procedural errors.

With the evolution of flow cytometry instruments, more parameters became readily available and the MRD panel was improved to two 6-color tubes (CD19/CD5/CD20/CD3/CD38/CD79b and CD19/CD5/CD20/CD81/CD22/CD43; Figure 1B) (11). This approach reduced the amount of time and sample required for MRD enumeration and reached the ability to quantitatively detect residual disease in the 0.001–0.01% (MRD4-MRD5) range.

In 2016, the European Research Initiative on CLL (ERIC) further validated a standardized flow cytometry approach to reliably detect CLL clones up to the level of 0.001% (MRD5) on a single tube: this assay includes a core panel of six markers, namely CD19, CD20, CD5, CD43, CD79b and CD81, as summarized in Figure 1C. Although the initial panel was designed with 8 colors, including CD22 and CD3, these markers were ultimately considered not essential, and the latter was deemed informative only if a very high accuracy (<10^{-5}) was necessary (Figure 1C, arrows). This system was designed to work independently from reagents and laboratory equipment (e.g., by processing the ratio of median fluorescence intensity of the positive signal over a negative signal, rather than raw fluorescence intensities), and could be validated locally, in different laboratories, using normal PB. To confirm the reliability of this 6-color, 1-tube method, a parallel analysis of high-throughput sequencing with ClonoSEQ assay was performed and showed good concordance with flow cytometry results at the MRD4 level, which represents the MRD threshold defined by the iwCLL guidelines in 2008 (12). Nevertheless, this method demonstrated to provide good qualitative results up to a detection limit of 1 in a million (10^{-6}) (13). The only significant drawback of this setup is that can be insensitive in presence of atypical phenotypes, therefore the knowledge of the pre-treatment phenotype is advisable (11). An example of a flow cytometry panel for MRD detection can be visualized in Figure 2.

Flow cytometry has the advantage to be a rapid method which works for most of typical CLL cases with very good sensitivity. Moreover, these instruments are widespread in most diagnostic laboratories, and they are operator friendly and easy to run, making this technique the preferred choice of most clinical investigators. On the other hand, the disadvantage is that samples must be processed within 48h and in any case not later than 72h, so fresh blood preparations are needed, and no cell storage can be performed (2, 14).



		4-color, 4/5 tubes	6-colors, 2 tubes	6/8-colors, 1 tube
Sensitivity		MRD4	MRD5	MRD5
MRD4	**Number of cells required**	5 million/tube	2 million/tube	1 million/tube
	Acquisition time*	20 minutes/tube	8 minutes/tube	4 minutes/tube
MRD5	**Number of cells required**	>40 million/tube	10 million/tube	5 million/tube
	Acquisition time*	3 hours/tube	40 minutes/tube	18 minutes/tube
Pretreatment phenotype knowledge		No	No	Preferable (test less sentitive to atypical phenotype)

* at 4500 evt/sec

FIGURE 1
Evolution and requirements for flow cytometry panels for MRD testing in CLL in 2007 **(A)**, 2013 **(B)** and 2016 **(C)**.

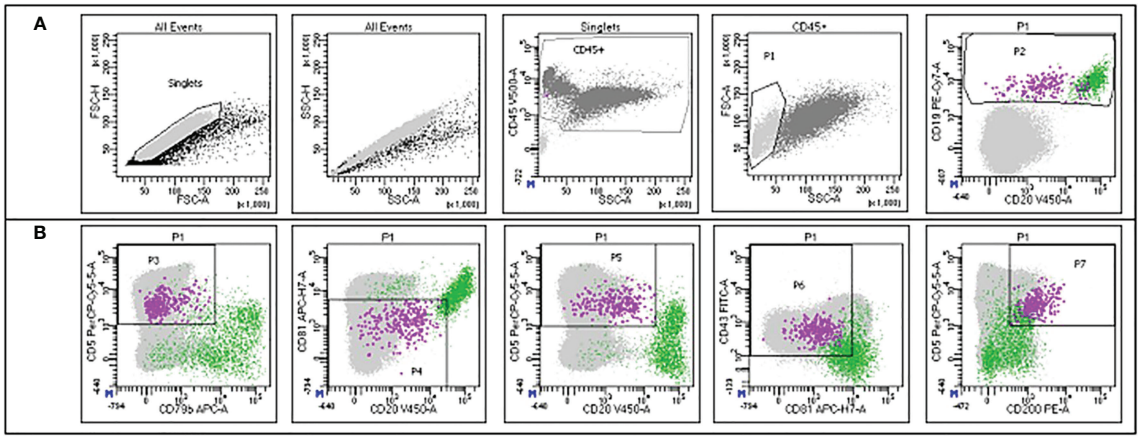


FIGURE 2
Example of the gating strategy employed for MRD detection in the flow cytometry panel. The green dots represent normal B cells, while the violet dots represent CLL cells. The gates are set up hierarchically. **(A)** Singlets are selected on FSC-H/FSC-A; the same population is refined on SSC-H/SSC-A; leukocytes are selected through the CD45 staining on CD45/SSC; mononuclear cells are selected on FSC/SSC (P1) and B cells are selected on CD19/CD20 (P2). **(B)** CLL cells are further characterized according to CD5/CD79b (P3), CD81/CD20 (P4), CD5/CD20 (P5), CD43/CD81 (P6) and CD5/CD200 (P7) expression. Courtesy of Prof. Giovanni D´Arena and Dr. Antonella Aiello.

Over time, several integrations and extensions have been proposed, to increase the sensitivity and limit of detection of the ERIC 6-color panel, also incorporating novel markers such as ROR1, CD200, CD160 (15–17) [also reviewed in D'Arena et al. (18)].

Innovative next-generation flow cytometry methods, capable of recording tens of millions of events and coupled with advanced analysis software, may potentially allow the reach of MRD6 in the next future (19). However, these systems will likely require significant hardware and software capabilities, initially limiting the application of these innovative technologies to few specialized laboratories.

2.2 Polymerase chain reaction-based methods: Allele-specific and digital PCR

Given that CLL is caused by aberrant proliferation of a specific B-cell population, each B-cell clone can be identified on a genetic level based on its uniquely rearranged immunoglobulin (Ig) genes within hypervariable regions. These regions are a unique characteristic of the leukemic cell; therefore, allele specific oligonucleotide (ASO) PCR takes advantage of the patient-specific Ig gene rearrangement to identify CLL clones and detect MRD. In this method, ASO primers matching the hypervariable region of each leukemic cell are used with reverse consensus JH germline primers and a fluorescent hydrolysis probe annealing to a downstream family specific JH region on a real-time thermal cycler. A graphical representation of the procedure can be visualized in Figure 3A, while the output of this procedure is shown in Figure 3B. Calculation of the MRD level is based on comparative analysis between follow-up samples and standard cells in ASO from polyclonal DNA, normalized to albumin PCRs as internal control (20). Guidelines for the interpretation of ASO-PCR results are available and attempt to standardize the results across different laboratories. Application of such guidelines and strict quality control assure the comparability of results obtained from different clinical trials (21).

The sensitivity of this method has been attested between 10^{-4} and 10^{-5}. Moreover, this method can be applied to frozen samples, which can be stored for a long time, and does not require processing of fresh samples (20). On the other hand, this method is intrinsically dependent on the amount of amplified DNA: in this regard, reliable clonotype identification requires that at least 3-5 copies of target DNA should be present within the sample; an adequate amplification would therefore need at least 100 nanograms of DNA for MRD4, 1 microgram for MRD5, 10 micrograms for MRD6. Furthermore, since ASO-PCR is tailored to be patient-specific, a representative pathological sample is required to set-up of the method, for positive controls in subsequent testing, as well as for the standard dilution curve for determining limit of detection and quantitative range in each experimental session; running out of such material would impair the quantitative power of ASO-PCR, which could be used only for qualitative assessment of MRD. Overall, this method can be very powerful but requires an expertise which is not available in any laboratory, thus rendering its use more difficult in the routine clinical practice.

An improvement of PCR-based detection is digital PCR [see also Dogliotti et al (22)], in which single DNA molecules are encapsulated in a confined space and amplified in presence of a fluorescent reporter (intercalating dye or hydrolysis probe); the resulting amplification will theoretically be either positive, if target DNA is present, or negative, hence the term "digital". The main advantage is that this is a quantitative technique, as it does not require a standard curve (hence does not possess a quantitative range) and is independent of reaction efficiency. A sensitivity up to MRD5, and possibly MRD6, is reached when the amplification is selective for known specific alterations, as *BCL2*-IgH translocation in Follicular Lymphoma, or *MYD88* L265P Waldenström Macroglobulinema (23, 24). This is not however the case of CLL, where a single lesion, acting as a tracker, is not present; in this case, patient-specific lesions may be exploited to monitor the disease's trajectory, but the emergence of novel clones without the monitored mutations must be taken into consideration.

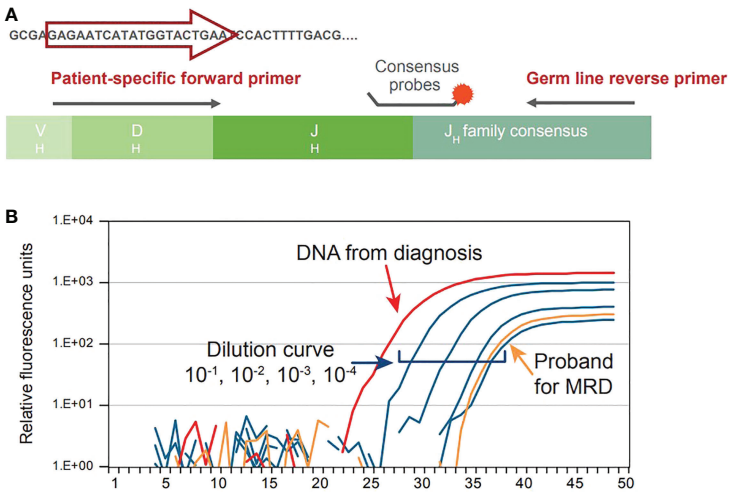


FIGURE 3
Graphical representation of ASO-PCR procedure **(A)** and output **(B)**.

2.3 Next generation sequencing

Ultra-deep next-generation sequencing (NGS) has emerged in recent years as an important diagnostic tool for the quantification of tumor burden, since many patients with undetectable MRD at flow cytometry and/or ASO-PCR, which both have a 10^{-4} sensitivity, as recommended by the iwCLL guidelines, relapse after few years, especially if their disease is characterized by high-risk molecular features such as unmutated IGHV. NGS can identify residual cells by amplification of all VDJ sequences from a single DNA sample (25, 26); the method requires previous knowledge of the specific CDR3 sequence of the immunoglobulin expressed by the pathological clone, which the investigator can later search for in MRD samples. Compared to ASO-PCR, NGS has the advantage that the amplification does not require patient-specific primers but is only dependent on the amount of loaded DNA, for which the MRD5 target (1 microgram of input DNA) is generally achievable (27). Therefore, undetectable MRD by NGS represents nowadays the most reliable predictor of survival in CLL patients. The main drawback is that NGS is not widely available, and economically viable only for centers facing significant volumes of testing; on the other hand, these centers would be equipped with adequate instrumentation and automate most of the analyses, significantly reducing the raw costs of a single test and the handling time and building up the necessary expertise to analyze NGS data efficiently. Therefore, at present, one of the optimal contexts for NGS in MRD evaluation resides within clinical trials, which are likely to centralize the most expensive analyses, thus also guaranteeing some degree of standardization. The absence of highly standardized commercial methods limits the applicability of NGS in the routine clinical practice and this is probably why, for now, it is not mentioned by the iwCLL recommendations (1).

The current landscape of technologies for MRD detection is quickly approaching a steady MRD5 detection limit, and the final choice of method is ultimately dependent on each laboratory's set up. Flow cytometry may be most suited for laboratories with an established cytometry facility, standardized instruments and trained personnel; however, it has a short "vein-to-brain" turnaround time, and the result can be produced within a few hours. Oppositely, ASO-PCR is a method that can be implemented in most molecular laboratories as it does not rely on a particularly advanced equipment if applied through real-time PCR, whereas digital PCR is more limited to specialized centers; NGS is equally, if not more, elective to specialized facilities, however it has the significant advantage that the investigator can search for the CDR3 sequence of the pathological clone directly within the sequencing output, providing higher sensitivity and specificity (28). Overall, these techniques may represent different but complementary tools for a comprehensive MRD detection, providing molecular detection where the cell phenotype may vary (for example CD20 expression after therapy with rituximab) or, vice versa, rely on a stable phenotypic marker in presence of ongoing somatic hypermutation and intra-clonal diversification of IGHV genes which may hamper patient specific CDR3 recognition.

3 MRD detection in peripheral blood vs bone marrow

CLL is characterized by the accumulation of leukemic cells in PB, BM and lymphoid tissues as spleen, liver, and lymph nodes (29). Therefore, the presence of leukemic cells in different tissues claims for clarification of the best candidate samples to determine MRD. MRD status is strongly prognostic for PFS and OS both in PB and BM of CLL patients after treatment (30). Nevertheless, the multi-compartment nature of CLL suggests the possibility of discordant MRD results on different tissues; thus, the sampling site may affect the prognostic ability of this parameter, and the choice depends on many factors such as timing of the sampling and treatment status. In general, it has been demonstrated that concordance between PB and BM MRD status is ~85% at the 10^{-4} threshold (6). For the anti-CD20 monoclonal antibody Rituximab, the concordance lowers to 79%: the sensitivity of MRD detection in BM is higher than that of PB, with added value for predicting prognosis or treatment effects (31). Nevertheless, the collection of BM samples is invasive and

painful for patients, so it cannot be used in routine follow up; therefore, samples from PB are commonly used instead.

4 Clinical significance of MRD in the era of targeted therapies

Some history of CLL treatment may be helpful to understand the role of MRD in the current and future clinical practice. Before 1990, any CLL treatment aimed at palliation: they included alkylating agents as chlorambucil or purine analogues as fludarabine in monotherapy. The advent of combination treatments including both fludarabine and cyclophosphamide improved the survival outcomes and response rates, even if the real revolution happened in 2010 with the introduction of the anti-CD20 monoclonal antibody rituximab, which combined to fludarabine and cyclophosphamide (FCR) gained great results in terms of survival (32). Around 2014, the combination of obinutuzumab and chlorambucil was devised for elderly and frail patients (33). In the same year, the anti-BTK inhibitor ibrutinib (34) opened the era to targeted therapies, followed later by the anti-BCL2 inhibitor venetoclax (35). The advances of CLL treatment led to improvement of the long-term outcomes in terms of survival and depth of response, which is why nowadays MRD became a valuable instrument in the post-treatment evaluation of patients (36). MRD can have a role as surrogate primary endpoint in clinical trials, since it is an accurate indicator of treatment efficacy which predicts PFS (36). On the other hand, it can be used as a determinant of future treatment choices since patients who do not achieve MRD-negativity after treatment can benefit from further treatment or new molecules to achieve a deeper remission and prolong PFS (5, 37). The timing of MRD assessment can vary depending on the duration of the treatment and on the use of continuous or fixed time regiments, for which MRD is usually measured at the end of the treatment. Figure 4 summarizes the role of MRD in the clinical practice.

4.1 MRD assessment after chemo-immunotherapy

The evaluation of MRD with the recommended sensitivity of 10^{-4} can predict survival of naïve patients undergoing first line treatment with chemo-immunotherapy. Several studies investigated the results in term of MRD negativity after different combination therapies, all proving that MRD is an independent predictor of survival (30).

Lamanna et al. investigated the prevalence of MRD negativity in patients treated with sequential fludarabine, high dose cyclophosphamide and rituximab as first line, and found 56% prevalence of MRD negativity by flow cytometry and 33% by PCR in PB (38).

The German group established the addition of rituximab to fludarabine and cyclophosphamide in 2010, through the CLL8 trial which achieved great results compared to the past (32). Subsequently, Boettcher et al. analyzed the clinical significance of flow cytometric MRD between the arms of the CLL8 trial, quantifying MRD in both PB and BM and categorizing patients into low/undetectable ($<10^{-4}$), intermediate (10^{-4}-10^{-2}) and high ($>10^{-2}$) level of MRD detected on PB. PFS was 68.7% for the low MRD group and 40.5% for the intermediate and high MRD groups. The results of this analysis showed that the level of MRD after FCR is predictive of both OS and PFS, which validates the use of MRD as a marker to assess the efficacy of such treatment (39).

The German group also investigated the prevalence of MRD-negativity in previously untreated patients undergoing treatment with bendamustine and rituximab (BR): 57.8% of them obtained MRD negativity below 10^{-4} in PB, which was associated to longer event-free survival compared to patients who did not achieve such deep remissions (40).

The CLL11 trial by Goede et al. investigated the prevalence of MRD negativity evaluated by PCR on PB, which was 37.7%, and BM, which was 19.5%. Also, in this case, MRD negativity was predictive of improved event-free survival (33).

4.2 MRD assessment after BTK inhibitors

The long-lasting experience with chemo-immunotherapy led to the awareness of the importance of MRD in CLL as predictor of treatment outcome and survival. The introduction of ibrutinib has opened the way to target therapies, initially as second line in relapsing CLL (34) and then as first line in previously untreated CLL patients (41). Its biological mechanism of action involves binding of the ibrutinib molecule to the ATP active site of the BTK which blocks the constitutionally activated BCR signaling involved in cells survival and expansion (42). Despite the encouraging results in terms of survival, ibrutinib is characterized by the maintenance of MRD positivity on the long term, thus requiring continuous therapy until either progression of the disease or toxicity of the drug. After its introduction, given the excellent clinical response but poor result in terms of MRD negativity, the role of MRD as predictor of survival was questioned (36). According to Ahn et al., MRD negativity was achieved only by 10.2% of both treatment naïve and relapsed/refractory patients after 5 years of continuous ibrutinib administration, but this result was surprisingly correlated to great outcomes in terms of PFS (74.4% of patients) and OS (85.3% of patients). The CR rate was 37.5% in the low MRD group and 21.3% in the high MRD group, but PFS was not statistically different between the 2 groups. Therefore, when it came to treatment with ibrutinib, MRD was not predictive of poor event-free survival in patients treated with monotherapy (43).

The combination of ibrutinib and rituximab did not obtain better results compared to monotherapy in terms of MRD negativity tested in PB at 12 months (8.3% vs 59.2% in patients treated with FCR): nevertheless, PFS was 65% and OS was 83%, lower with ibrutinib and rituximab than with chemo-immunotherapy, so again MRD was not predictive of lower event-free survival (44).

The ILLUMINATE trial investigated the efficacy of the combination of ibrutinib and obinutuzumab compared to chlorambucil and obinutuzumab. This study obtained the best result in terms of MRD negativity for ibrutinib, which was 30% in PB (vs 20% in the Chl-Obinu group) and 20% in BM (vs 17% in the Chl-Obinu group).

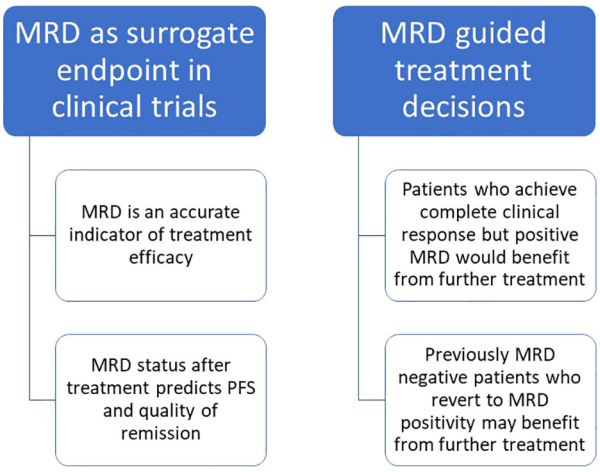


FIGURE 4
Role of MRD in the clinical practice and potential use in routine management of CLL patients.

Finally, the HELIOS trial investigated the combination of ibrutinib with BR compared to BR alone. The rate of MRD negativity given by the combination of ibrutinib and BR was 26.3% (45), so also in this case it was significantly higher than for ibrutinib monotherapy and combination of ibrutinib and rituximab.

Figure 5 summarizes the rate of MRD negativity obtained by ibrutinib monotherapy (43), ibrutinib combined with anti CD20 monoclonal antibodies (44, 46) or with BR (45).

4.3 MRD assessment after BCL2 inhibitors

The anti-BCL2 inhibitor venetoclax, on the other hand, is characterized by higher rates of MRD negativity, also in this case defined as less than 10^{-4}, in CLL. A pooled analysis of patients enrolled in different clinical trials, which we will report below, showed an overall 42% of confirmed undetectable MRD in either PB, BM or both. The median time to obtain MRD negativity was 18 months and 90% of patients obtained MRD negativity within 24 months, while no patient obtained MRD negativity after 24 months without dose escalation. Deletion of chromosome 17p correlated, as expected, to a lower probability to obtain MRD negativity and consequently to a higher rate of relapse. Of those who did not obtain MRD negativity, 78% patients developed progressive disease at a median time of 19 months, confirming that, also upon treatment with venetoclax, MRD is a strong predictor of event-free survival (47).

The efficacy of venetoclax monotherapy has been investigated in studies which included patients with heterogeneous chromosome 17p deletion and *TP53* status as well as previous exposure to BTK inhibitors (48). The M13-982 study investigated CLL patients with relapsed/refractory (R/R) disease and with 17p deletion, a small number of whom had previously received BTK inhibitors: 20% of the enrolled patients obtained MRD negativity in PB (35). The M14-032 study included CLL patients who previously failed treatment with BTK inhibitors, regardless of their mutation status: 42% of the enrolled patients obtained MRD negativity in PB. Moreover, 71% of patients who progressed after treatment with BTK inhibitors responded to venetoclax (49).

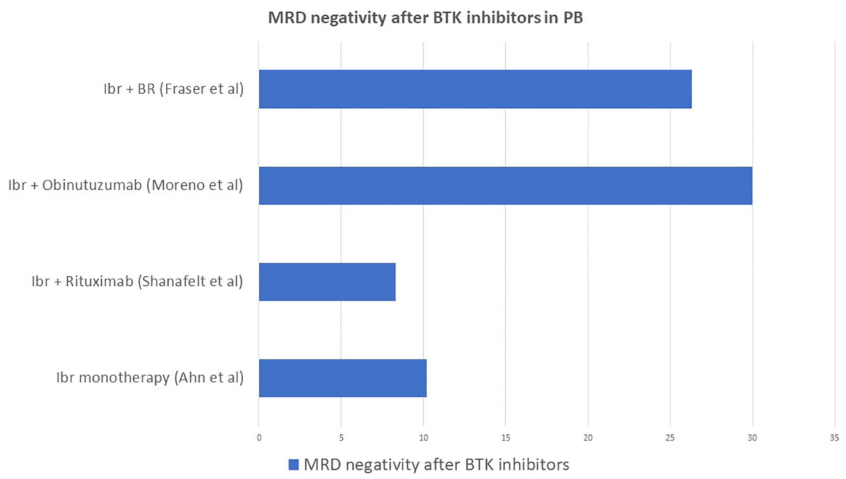


FIGURE 5
Rate of MRD negativity obtained by ibrutinib monotherapy, ibrutinib combined with anti CD20 monoclonal antibodies or with BR.

The combination of venetoclax and rituximab as fixed therapy for 24 months in relapsed/refractory CLL has been investigated by the MURANO trial, which compared it to the traditional chemo-immunotherapy with bendamustine and rituximab. This study showed an encouraging high rate of MRD negativity in PB at 9 months for the venetoclax and rituximab group (62.4%) compared to the BR counterpart (13.3%), which strictly correlated with a longer event-free survival (84.9% vs 34.8% at 2 years). Within the venetoclax-rituximab arm, patients with undetectable MRD achieved 85% PFS, while those with detectable MRD 65% (50).

The CLL14 trial investigated the efficacy of venetoclax and obinutzumab, as fixed therapy for 12 months, against chlorambucil and obinutuzumab in first line. This study reported a 76% of MRD negativity at end of treatment in the group who received venetoclax and obinutuzumab, which correlated to a longer PFS compared to the Chl-Obi group, with a 0.31 hazard ratio (CI 0.22-0.44) (51).

The CLL13 trial investigated the outcome of four different therapeutic schemes: CIT with FCR or BR, venetoclax plus rituximab, venetoclax plus obinutuzumab and venetoclax plus obinutuzumab and ibrutinib in first line for fit patients. This study reported 52% MRD negativity for FCR or BR, 57% for venetoclax plus rituximab, 86.5% for venetoclax plus obinutuzumab and 92.2% for venetoclax plus obinutuzumab and ibrutinib (52).

Figure 6 summarizes the best rates of MRD negativity obtained by venetoclax monotherapy (35, 49), venetoclax combined with anti CD20 monoclonal antibodies (50, 51) and with ibrutinib (52) in different clinical trials.

4.4 New combinations and future perspectives

The development of target therapies for CLL and the spread of rapidly available MRD detection methods opened the way to a wider MRD use in clinical practice. New trials aim at more and more personalized therapies, where the treatment strategies can be designed based on patients' epidemiological characteristics, molecular biology of the disease and MRD detection at end of treatment. The CAPTIVATE trial moves in this direction: it is a multicenter randomized phase II trial which studies the combination of ibrutinib and venetoclax in two cohorts, the MRD-guided and the fixed-duration cohort. For the sake of our topic, we will describe the study design for the MRD guided cohort: during the pre-randomization phase, patients received ibrutinib monotherapy for three cycles followed by ibrutinib and venetoclax for 12 cycles. At the end of the 12 cycles, MRD was tested, and patients were divided into MRD negative cohort, which was randomized to ibrutinib continuation or placebo, and MRD positive cohort, which was randomized to ibrutinib monotherapy or continuation of ibrutinib and venetoclax. At the end of the pre-randomization phase, 75% of patients obtained MRD negativity in PB and 68% in BM. After the randomized phase, in the MRD negative cohort, undetectable MRD in PB went from 100% to 84% for patients who received placebo and from 100% to 77% for patients who received ibrutinib. In the MRD positive cohort, undetectable MRD remained 45% for patients who received ibrutinib monotherapy while it went from 50% to 69% for those who received the combination of ibrutinib and venetoclax (53). Such encouraging results support the preclinical evidence of a synergistic effect of ibrutinib and venetoclax, which target the BTK and Bcl2 receptors at the same time resulting in higher cytotoxicity, and consequently deeper molecular response, compared to the two drugs alone (54). Comparable results in terms of MRD were also confirmed in the cohort treated with the fixed duration regimen, with achievement of deep molecular response which correlated to a favorable PFS (55). Figure 7 summarizes the study design and results in terms of MRD negativity.

The importance of this trial is to open the way to a more and more tailored approach which address patient's needs depending on the depth of remission they obtain, beyond the clinical characteristics. Other than the effectiveness of the two drugs and their combination, it is important to consider how this trial emphasizes the role of MRD in guiding physician's choices on treatment management, which may reflect an upcoming practical application of MRD in the clinical practice.

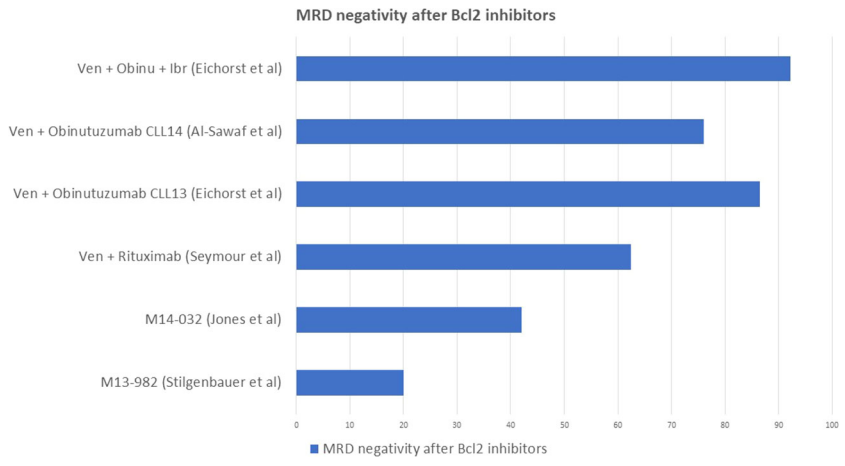


FIGURE 6
Rate of MRD negativity obtained by venetoclax monotherapy and venetoclax combined with anti CD20 monoclonal antibodies.

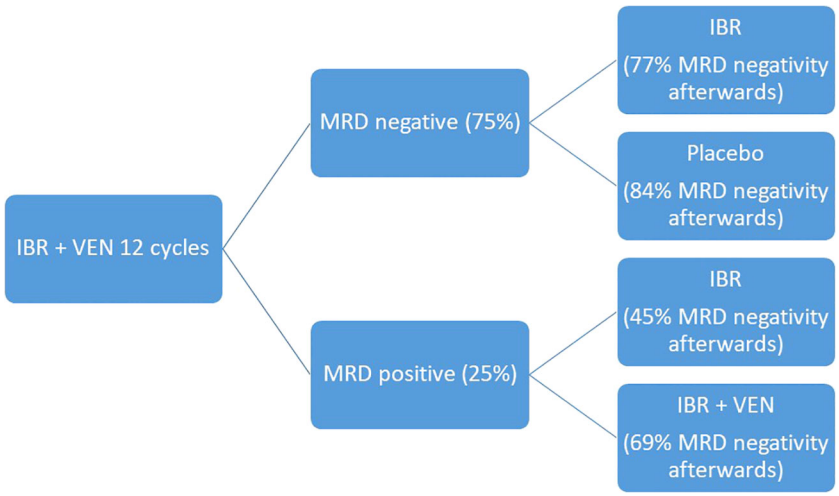


FIGURE 7
CAPTIVATE study design and results in terms of MRD negativity.

5 Conclusion

Measurable residual disease has always gained interest in the field of hematology, but its importance is increasing steadily thanks to the advances in the treatment of CLL. Nevertheless, MRD does not currently have a defined role in the clinical practice. The three main laboratory methods used to detect MRD include flow cytometry (2), ASO-PCR (20) and NGS (26), and they all have a role in clinical trials- The cytometric method standardized by the ERIC guidelines (2, 11) appears to be the most accessible, in terms of feasibility and costs, in the clinical practice.

The development of chemo-immunotherapy combination platforms (FCR, BR, Chl-Obinu) back in the days, shed light on the possibility to deepen the molecular response of CLL and obtain a long-lasting event free survival (39). Surprisingly, the advent of ibrutinib discouraged the use of MRD as a surrogate endpoint for PFS, as long-lasting partial responses were obtained with continuative ibrutinib treatment regardless of persistent MRD negativity (36). Venetoclax monotherapy or in combination with anti-CD20 monoclonal antibodies restored the key role of MRD in clinical trials and validated its correlation to event free survival in patients treated with the BCL2 inhibitor (35, 47–51). Furthermore, the newest combination of ibrutinib and venetoclax, which already showed a synergistic effect in pre-clinical models, obtained even deeper molecular response, and explored the use of MRD itself to determine further steps of patients' management (53–55).

In conclusion, MRD has a valuable role in defining remission at a more profound level compared to clinical assessment alone, and it can help guiding treatment choices to obtain a more durable event free survival, which is a turning point for such a chronic and incurable condition. For this reason, even if at present MRD is not part of routine evaluation of patients at the end of treatment in the clinical setting, it may gain a role in the next years and it may even be included in new guidelines, as part of the recommended steps to establish patients' response and prognosis.

Author contributions

GB and FP wrote the paper. VG, FP and GD'A provided the cytofluorimetric background and the related figures. VG and FP provided the ASO-PCR and NGS background. FP, II, AF, FA revised and corrected the manuscript. LL supervised and coordinated the work. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Immunophenotypic measurable residual disease monitoring in adult acute lymphoblastic leukemia patients undergoing allogeneic hematopoietic stem cell transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) offers a survival benefit to adult patients affected by acute lymphoblastic leukemia (ALL). However, to avoid an overt disease relapse, patients with pre or post transplant persistence or occurrence of measurable residual disease (MRD) may require cellular or pharmacological interventions with eventual side effects. While the significance of multiparametric flow cytometry (MFC) in the guidance of ALL treatment in both adult and pediatric patients is undebated, fewer data are available regarding the impact of MRD monitoring, as assessed by MFC analysis, in the allo-HSCT settings. Aim of this article is to summarize and discuss currently available information on the role of MFC detection of MRD in adult ALL patients undergoing allo-HSCT. The significance of MFC-based MRD according to sensitivity level, timing, and in relation to molecular techniques of MRD and chimerism assessment will be also discussed.

KEYWORDS

measurable residual disease, multiparameter flow cytometry, acute lymphoblastic leukemia, allogeneic hematopoietic stem cell transplantation, adult patients

Introduction

In acute leukemia of either lymphoid or myeloid lineage, measurable residual disease (MRD) is defined as the presence of residual malignant cells in bone marrow (BM) or peripheral blood (PB) of patients who achieved morphologic complete remission (CR) after treatment interventions (1). The methods currently available for MRD detection are multiparameter flow cytometry (MFC), and/or molecular biology techniques including real-time quantitative polymerase chain reaction (RQ-PCR), digital droplet PCR, and next-generation sequencing (NGS) (2). Importantly, the different sensitivity limits of these techniques, ranging from $1x10^{-4}$ (MFC) to $1x10^{-6}$ (NGS), and the occurrence of disease relapse in otherwise MRD negative

patients, have recently prompted the replacement of the adjective "minimal" with that of "measurable" in reference to residual disease (3). For an in-depth review of molecular techniques of MRD analysis the reader is referred to recent reviews (2, 4).

In acute lymphoblastic leukemia (ALL), the most used techniques for MRD monitoring are MFC, which relies on the identification of aberrantly expressed antigens by leukemic cells, and RQ-PCR analysis, which detects rearranged immunoglobulin (Ig)/T-cell receptor (TCR) genes, or recurrent gene fusions such as BCR-ABL1 in chromosome Philadelphia (Ph) positive patients (1–3). Although both MFC and RQ-PCR are applicable to most ALL cases (i.e., 90% and 90-95%, respectively) the two techniques differ in terms of sensitivity, with RQ-PCR being generally more sensitive than MFC (i.e., $1x10^{-4}$ to $1x10^{-5}$ *vs* $1x10^{-4}$) (4, 5). Nonetheless, MFC is widely used in many countries, including United States (6) where the consensus from North American experts recommends using RQ-PCR over MFC for Ph-positive ALL patients only (7). On the contrary, European countries use more frequently standardized RQ-PCR for MRD testing (5). Accordingly, a recent survey on 95 European Society for Bone Marrow Transplantation (EBMT)-affiliated centers has reported that in Europe ALL MRD monitoring is mainly performed by RQ-PCR, either alone or in conjunction with MFC (8).

Over the years MRD monitoring has been introduced in clinical trials and disease-specific guidelines as measure of treatment efficacy and predictor of relapse, thus informing response-adapted therapies in pediatric (9) and adult ALL patients (10, 11). Although there is no consensus on which sensitivity threshold should be reached to define MRD positivity, it is now generally accepted to use methods detecting at least 1 leukemic cell out of 10,000 nucleated cells ($\geq 1x10^{-4}$) (7).

Despite rigorous indications regarding MRD monitoring (by either MFC or RQ-PCR) throughout induction and consolidation therapies (10, 11), limited information is available about MRD assessment in adult ALL patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), which in turn is a potentially life-saving treatment for selected patients with high-risk features or MRD positivity following induction and consolidation (11, 12).

Based on these premises, aim of this review is to explore the role of MFC MRD monitoring in adult ALL patients undergoing allo-HSCT, highlighting both advantages and pitfalls of the MFC technique even in relation to RQ-PCR. Eventual correlations with analysis aimed at evaluating patient chimerism status throughout immune reconstitution will be also discussed.

MFC MRD monitoring in ALL patients: Technical issues

MFC rapidly analyzes single cells or particles as they flow past single or multiple lasers while suspended in a buffered salt-based solution. Each particle or cell is analyzed for visible light scatter and one or multiple fluorescence parameters detected as a result of emission by fluorochrome-conjugated specific monoclonal antibodies against surface, cytoplasm or nuclear antigens that are differently expressed by leukemic *vs* normal cells (13, 14). A key feature of MFC, which remains an indispensable tool for the immunophenotypic characterization of leukemic cells at diagnosis, is the capability to distinguish cellular subpopulation *via* multiparametric assessment of quantitative differences in antigen expression on single cells, and to enumerate the relative size of the resulting subpopulation (14). Importantly, the possibility to discriminate and enumerate different subpopulations within complex mixtures of cells such as BM, PB, or cerebral fluid has made MFC a highly suitable technique for MRD detection and quantification (14). ALL is a heterogeneous malignancy that originates from B- and T-lineage lymphoid precursors and is driven by a spectrum of genetic aberrations including mutations, chromosome translocations and aneuploidy in genes involved in the development of lymphoid cells and regulation of cell cycle progression (15). The most common markers used to identify leukemic B cells and to differentiate them from normal progenitor B cells (hematogones) are CD10, CD19, CD34, CD38, and CD45. In B-ALL, CD10 and CD45 usually show abnormally low levels, although, in some cases, CD10 expression is higher, which helps in the distinction from hematogones, or absent (16). Further markers include CD58, that is usually overexpressed in ALL cases (17), and antigens associated with genetic lesions such as CD123 (hyperdiploidy), CD66c (hyperdiploidy and BCR/ABL), NG2 (MLL-rearrangements), CRLF2 (CRLF2-rearrangements), and lack of CD44 positivity (TEL/AML1 and B-ALL with MYC-translocation) (18). Worthy of note, MRD analysis in B-ALL patients treated with CD19-targeted therapies may require an alternative gating strategy without the use of CD19 as B-cell-specific marker (19). As for T-ALL the most common markers used to identify leukemic blasts include the down-modulation of surface CD3 expression and the cytoplasmic CD3 positivity, with the expression of terminal deoxynucleotidyl transferase (TdT) and CD34 suggesting an immature T-lymphoid process (16). The positive expression or variations in intensity of CD2, CD4, CD5, CD7, and CD8 levels are frequently used as a gating strategy for MRD (16). CD1a can show a positive or negative expression and may be a useful target for MRD evaluation (16).

MFC MRD can be tracked by two methods of analysis: i) through the identification of the immunophenotypic pattern of leukemic cells at the diagnosis (i.e., leukemia-associated immunophenotype/LAIP) that can be followed over time; ii) by discriminating the differences between the immunophenotype of leukemia cells in the MRD sample compared to normal B-lymphoid progenitors (i.e., hematogones) or normal T-lymphoid progenitors (i.e., thymocytes), through a "different from normal" (DFN) approach (20, 21). As both LAIP- and DFN-methods present potential pittfalls due respectively to immunophenotypic shifts of leukemic blasts and post-chemotherapy changes of hematopoiesis, it is generally suggested to maximize the accuracy of MRD analysis through a comprehensive integrated LAIP-based DFN approach (21–23). The latter define a set of aberrancies including (a) the abnormal expression of antigens not typically expressed by the particular cell type, (b) the over/under-expression of normally expressed antigens, and/or (c) the asynchronous expression of normally expressed antigens (21).

As previously stated, MFC has generally a lower sensitivity than molecular biology techniques, however the use of standardized protocols allows to reach a similar sensitivity to RQ-PCR provided the acquisition of an adequate number of cells (preferably more of 4×10^6) from a first-aspirate, fresh, viable sample (24). MFC MRD monitoring has some advantages over other methods. These include the rapidity of execution, the relatively low cost, the ability to quantify

antigens for targeting agents, and the possibility to analyze samples without knowing the immunophenotypic characteristics of leukemic cells at diagnosis, which is an added value for referral transplant centers (7, 20). Disadvantages include the risk of false negative results due to immunophenotypic shifts throughout treatment, the difficulty to discriminate leukemic B cells from hematogones in a regenerating/reconstituting BM, the large dependence of the analysis on the operator skill, and lack of standardization (7, 20). With the latter regard, protocols aimed at standardizing MFC analysis among laboratories in terms of harmonization and alignment of the technical aspects are currently ongoing (24, 25).

MFC MRD monitoring in adult ALL treatment

MFC MRD monitoring has been demonstrated as a valuable tool for assessment of response to treatment and prognostic evaluation not only in pediatric (26, 27) but even in adult ALL patients after induction and consolidation. For instance, in a retrospective study analyzing 323 adult patients affected by B-ALL and monitored by MFC (4-6 color panel, sensitivity 10^{-4}), Ravandi and colleagues found that a negative post induction MRD status was associated with a significantly higher disease-free survival (DFS) according to multivariable analysis (28). In a prospective multicenter trial monitoring 179 adolescent and adult high-risk Ph-negative ALL patients by MFC MRD (4 color panel, sensitivity 5 x 10^{-4}), undetectable levels of early post consolidation MRD were associated to a quite favorable prognosis even in the absence of allo-HSCT (29). In a multicenter series of 1487 pediatric and adult patients affected by B-cell precursor (BCP) ALL, positive MFC MRD (6 color panel, sensitivity 10^{-4}) on days 15, 29 and 79 was significantly associated with hazard of relapse in multivariable analysis (30). Finally, according to a very recent report on 134 Ph negative pediatric and adult B-ALL patients, integrated dynamic MFC MRD assessed on days 14, 25 and 45 (8 color panel, sensitivity 10^{-4}) was an independent factor for overall survival (OS) at multivariate analysis, also defining risk-classification criteria leading to effective allo-HSCT in high-risk, but not in low and intermediate risk patients (31). Concerning T-ALL, a multicenter study regarding 274 pediatric and adult patients showed that a negative MFC MRD assessment (6 color panel, sensitivity 6 x 10^{-5}), on day 15 might be useful for an early and accurate identification of patients with a very low risk of relapse (32). Similarly, a retrospective study on 94 adult patients affected by T-ALL showed that MFC MRD (6-8 color, sensitivity 10^{-4}) positivity at the end of induction was an independent prognostic factor for cumulative incidence risk, relapse-free survival, and OS (33).

MFC MRD monitoring in adult ALL patients undergoing allo-HSCT: MRD matters

Adult ALL remains an aggressive disease. In fact, despite dose-intensification strategies leading to high response rate to induction chemotherapy, and the availability of highly active targeted immunotherapies for resistant or relapsed disease (34), only 30-40% of adult ALL patients will achieve long-term remission (35). In this scenario allo-HSCT still represents an effective therapeutic treatment and is currently part of adult ALL standard clinical care (36, 37). However, a significant percentage (~40%) of patients will relapse after allo-HSCT, while other (15 to 26%) will die due to non-relapse mortality (NRM) (37–39) despite recent advances in transplant management (38, 39). In keeping with these premises, in young adult and adult patients allo-HSCT is currently part of post-consolidative therapy in case of high-risk features such as Ph-positivity, Ph-like disease, and persistent MRD as assessed by either MFC or RQ-PCR (11, 12). In MRD regard, prospective and retrospective multicenter studies have demonstrated that allo-HSCT improves the outcome of adult ALL patients who are MRD positive after induction (33, 40) or consolidation therapy (41, 42).

Although less explored, MRD testing has been shown to have a prognostic significance even with respect to allo-HSCT outcome. For instance, a retrospective EBMT registry study on 2780 adult ALL patients undergoing myeloablative allo-HSCT in first complete remission (CR) and evaluated by MFC and/or RQ-PCR techniques (threshold $>10^{-4}$) demonstrated by multivariate analysis that MRD positivity at transplant was a significant independent factor for lower OS, leukemia free survival (LFS), and for higher relapse incidence (RI) (43). Similar data have emerged from a recent meta-analysis on 21 published reports according with a positive MFC or RQ-PCR MRD at allo-HSCT is associated with lower OS, event free survival (EFS) and relapse-free survival (RFS) (44). Overall, this evidence underlines the leading role played by MRD regarding the best timing of allo-HSCT, mostly in light of the availability of new drugs such as inotuzumab ozogamicin and blinatumumab, potentially able to obtain pre-transplant MRD clearance with mechanisms of action different from chemotherapy (45). Interestingly, a deep MRD negativity may also question the advisability of allo-HSCT. In fact, a recent trial assessing MRD with a high sensitivity (limit of detection $0.2x10^{-6}$) and standardized technology (2 tube 8 color MFC panels for BCP-ALL and T-ALL, respectively) (24) has shown that adults with high-risk features, Ph^- ALL, and deep MRD clearance after induction and early consolidation have favorable outcomes without allo-HSCT (46).

A few studies have specifically analyzed the role of MFC MRD monitoring in ALL prior to and following allo-HSCT. As shown in Table 1 (33, 47–55), data related to adult patients mostly derive from retrospective and heterogeneous series, sometimes including children, and using different sensitivity levels [10^{-3} to 10^{-5}].

Pre allo-HSCT MFC MRD

Eight (89%) out of 9 studies including a total of 1180 patients showed a predictive role of positive pre allo-HSCT MRD towards DFS/LFS (33, 47–51, 54, 55), cumulative incidence of relapse (CIR) or risk (33, 48, 50, 55), and OS (33, 49, 50, 54, 55), while only 1 study did not find any impact on transplant outcome (53) (Table 1). Similar data were observed in the pediatric setting. In fact, according to a retrospective study on 64 children with ALL, low (10^{-4} to $<10^{-3}$) and high ($\geq10^{-3}$) pre allo-HSCT MFC MRD levels were predictive of a

proportionally increasing 5-year CIR (56). Similarly, in a retrospective study on 36 children, MFC MRD levels ≥ 10^{-4} were associated to a higher CIR (57). According to a prospective study on 105 children, patients with MFC MRD ≥ 10^{-3} had a higher CIR than subjects with MRD < 10^{-3} or negative (58). Finally, in a retrospective study on 69 children evaluated by either MFC or RQ-PCR, a positive pre transplant MRD was associated to a higher CIR (59).

Post allo-HSCT MFC MRD

As reported in Table 1, post allo-HSCT MFC MRD was evaluated only in 6 series including adults, and accounting approximatively for 355 ALL patients. According to all studies a positive MRD was associated to a reduced DFS/RFS (33, 52, 53), OS (33, 50, 52), time to relapse (47, 49), and to a higher CIR or risk (33, 50, 52, 53). Few data on post allo-HSCT

TABLE 1 Impact of pre and/or post allo-HSCT MFC MRD according to ALL series published in the last 20 years and including adult patients.

Ref. Study type (years)	Pts n ALL type	Age (range)	Condition donor	Colors source cells/tube	Sensitivity	Pre allo-HSCT status		MRD$^+$ pts outcome°	Post allo-HSCT status		MRD$^+$ pts outcome°
						CR	MRD$^+$		CR	MRD$^+$	
(47) Retrospective 1999-2001	40 B Ph$^-$ 23 B Ph$^+$ 7 T 10	18 (3-49)	MAC 100% MD 77.5%	4 MC 5x10^5	3x10^{-4} to 1x10^{-3}	24* 100%	6/24 25%	↓ 2-yr DFS	40/40 100%	11/40 27.5%	Increasing MRD levels anticipated relapse
(48) Retrospective 2004-2010	86 B Ph$^-$ 49 B Ph$^+$ 27 T 10	20.5 (1-63)	MAC 79% MD NA	4-8 WB 1x10^5	1x10^{-4} to 1x10^{-3}	86 100%	10/86 11.6%	↑ 2-yr RI ↓ 3-yr DFS	NA	NA	NA
(49) Retrospective 1999-2010	102 B Ph$^-$ 55 B Ph$^+$ 23 T 24	NA <14, 46%	MAC 100% MD 38.2%	4 MC 2-5x10^5	1x10^{-5}	102 100%	30/102 29.4%	↓ OS ↓ LFS ↓ EFS	NA	NA	↑ TTR in MRD$^+$ pts (MRD level dependent)
(50) Retrospective 2006-2011	160 B 134 T 24 Biph 2	24.6 (0.6-61.8)	MAC 100% MD 32%	7 WB NA	1x10^{-4}	153 95,6%	59/153 38.6%	↑ 3-yr CIR ↓ 3-yr OS ↓ 3-yr RFS	144/153 94%	NA	↑ CIR ↓ OS
(51) Retrospective 2000-2015	102 T 102	31 (2-72)	MAC 77% MD 42%	NA BM 2x10^5	1x10^{-3}	84 100%	18/84 21.4%	↓ PFS	NA	NA	NA
(52) Retrospective 2011-2016	155 B Ph$^+$ 155	31 (4-63)	MAC 100% MD 31%	8 WB NA	1x10^{-5}	155 100%	33/155 21.3%	NA	155/155 100%	NA	Day 30 ↑RI Day 60 ↑RI, ↓DFS, ↓OS Day 90 ↑RI, ↓DFS
(53) Retrospective 2009-2016	133 T 133	22 (1-74)	NA	7-8 WB 2x10^6	1x10^{-4} to 1x10^{-3}	74§ NA	NA	NS	NA	22	↑ 4-yr CIR ↓ RFS
(54) Retrospective 2010-2016	139 B Ph$^+$ 54 B Ph$^-$ 85	30 (14-76)	MAC NA MD 42.7%	8 BM NA	1x10^{-4}	74 NA	46/74 62%	↓ OS ↓DFS	NA	NA	NA
(55) Retrospective 2011-2016	543 B Ph$^-$ 284 B Ph$^+$130 T 129	24 (2-59)	MAC 100% Haplo 100%	8 WB 7.5x10^5	3x10^{-4} to 1x10^{-3}	543 100%	119/543 21.9%	↑ 6-mo RI ↓ 6-mo LFS ↓ 6-mo OS	NA	NA	NA
(33) Retrospective 2014-2019	115 T 115	27.5 (16-73)	MAC NA	6-8 BM NA	1x10^{-4}	99/115 86.1 %	94/115 96.9%	↑ 2-yr CIR ↓ 2-yr RFS ↓ OS	NA	NA	↑ 2-yr CIR ↓ 2-yr RFS ↓ OS

Pts, Patients; n, Number; Condition, Conditioning Regimen; MRD, Measurable Residual Disease; CR, Morphological Complete Remission; MAC, Myeloablative Conditioning; MD, Matched Related Donor; MC, Mononuclear Cells; DFS, Disease Free Survival; NA, Not Available; WB, Whole Blood; RI, Relapse Incidence; Biph, Biphenotypic Acute Leukemia; CIR, Cumulative Incidence of Relapse; OS, Overall Survival; EFS, Event Free Survival; RFS, Relapse Free Survival; LFS, Leukemia Free Survival; EFS, Event Free Survival; TTR, Time to Relapse; HAPLO, Haploidentical Donor; mo, Months. NS, Not Significant.

° Post allo-HSCT outcome.

* Patients with pre allo-HSCT MRD assessment.

§ Patients undergoing allo-HSCT.

MFC MRD monitoring are available in the pediatric setting. A multinational study on 616 pediatric and young adult ALL patients evaluating pre and post allo-HSCT MRD levels by either MFC or RQ-PCR, showed by univariate analysis that low ($<10^{-4}$) to very high ($\geq10^{-3}$) post-transplant MRD levels were associated to a progressively higher relapse hazard (60). Moreover, patients undergoing allo-HSCT with detectable MRD and showing high or very high post transplant MRD had increasingly higher chances of relapse according to Cox regression model (60).

Dynamic peri-transplant MFC MRD

Interestingly, recent evidences support the usefulness of dynamic peri-transplant (i.e., serial pre and post allo-HSCT) MFC MRD monitoring. For instance, a retrospective study on 271 T-ALL adult and pediatric patients has recently shown that dynamic peri-transplant MFC MRD monitoring could be better in discriminating the risk of relapse than single time point pre or post allo-HSCT assessments (61). Similarly, in a pediatric series of 166 ALL patients undergoing haploidentical unmanipulated transplant and dynamic peri-transplant MFC MRD assessments, increasing MRD levels were associated to lower LFS and OS, and higher CIR (62).

Overall, regardless technical differences and the relatively low series number, the studies summarized in Table 1 indicate that in adult ALL patients undergoing allo-HSCT MFC can be a reliable MRD assessment technique. Moreover, studies in adult and pediatric patients indicate that MFC may have an increasing predictivity depending on MRD positivity levels (47, 49, 60) and/or peri-transplant trend (61, 62). Unfortunately, no data are available regarding the predictive impact of post over pre allo-HSCT MFC MRD monitoring. However, in a large multicenter study including 616 children, post transplant MRD (evaluated by either MFC or RQ-PCR) resulted more predictive than pre transplant MRD with respect to allo-HSCT outcome (60).

The paucity of studies on MFC MRD monitoring in adult (and even pediatric) ALL patients prior to and after allo-HSCT is somehow surprising considering the wide use of MFC MRD assessment of the same patients while undergoing induction and consolidation therapies (10, 11, 63). Worthy of note, several authors have recently shown the feasibility and predictive significance of MFC MRD positivity prior and/or following allo-HSCT even in adult AML patients (64–66). For instance, in a series of 279 patients receiving myeloablative conditioning in first or second CR, a positive MFC (10 color panel, sensitivity $\leq10^{-3}$) MRD prior to allo-HSCT was associated with inferior OS and higher risk of relapse in a multivariable analysis (65). Furthermore, in a study on 810 adult AML patients who underwent MFC MRD monitoring before and 20 to 40 days after allografting, peri-allo-HSCT MRD dynamics improved accuracy of risk over pre- and post-allo-HSCT assessment across conditioning intensities (66).

MFC versus RQ-PCR MRD monitoring in allo-HSCT

Previous data from ALL studies have shown that MFC and RQ-PCR amplification of antigen-receptor genes yield remarkably similar measurements if MRD is present at a $\geq 10^{-4}$ level (67). Although most information on RQ-PCR MRD monitoring in adult allo-HSCT setting derives from a limited number of studies, often focused on Ph positive patients (Table 2), based on our literature revision the prognostic significance of MFC and RQ-PCR towards allo-HSCT outcome seems quite comparable. Accordingly, 5 (71.4%) out of 7 studies including 2267 patients evidenced a predictive role of detectable RQ-PCR MRD levels towards DFS/RFS (70, 72), CIR (68, 73), and OS (68, 70, 72, 73) (Table 2). The significance of post allo-HSCT RQ-PCR MRD was evaluated by 4 studies on more than 612 patients, all evidencing the impact of RQ-PCR MRD monitoring towards DFS/RFS (52, 70, 71), CIR (52, 68, 71) and OS (52, 70, 71) (Table 2). Of note, similar data were observed in the pediatric setting (74–76).

MRD and chimerism monitoring after allo-HSCT

Chimerism analysis, the investigation of the genotype origin of post-allografting hematopoiesis, has been historically considered a well-established method for monitoring the outcome of allo-HSCT in terms of engraftment and eventual risk of relapse (77). About chimerism the term "complete donor chimerism" refers to a hematopoiesis that is fully genetically derived from donor, whereas the term "mixed chimerism" refers to a hematopoiesis with genetic origins from both donor and patient (78). Over the years, several methods for chimerism analysis have been progressively introduced in clinics, including assessing short tandem repeats (STR), fluorescent PCR, RQ-PCR of single nucleotide polimorphism, and fluorescence *in situ* hybridization in gender-mismatched allo-HSCT (77). Chimerism can be defined on several levels, but PB and BM are the most frequently used sources. Notably, the degree of chimerism can be analyzed in these tissues without any further manipulation (i.e., overall chimerism) or within certain cellular fractions, such as T cells, B cells, CD34+ or myeloid cells (i.e., subset chimerism) (78). Currently, there is no general agreement on the preferred source/subpopulation of assessment (79, 80), which in turn is dependent on the technique used.

The American Society for Transplantation and Cellular Therapy recommends chimerism evaluations at specific time points during the first year post allo-HSCT (e.g., days +30, +90, +180, and +365) and whenever required according to disease characteristics (81), while the EMBT generally suggests serial and quantitative analysis of chimerism given the short time interval between mixed chimerism detection and relapse (82). Chimerism is in fact a dynamic process, and patients with increasing levels of recipient chimerism have been traditionally retained at risk of relapse and therefore treated with preemptive immune therapy (i.e., immunosuppressive drug tapering, DLI) (79, 83).

Little data are available on the clinical impact of chimerism with respect to MRD monitoring as determined by MFC and/or molecular biology techniques (83, 84). A retrospective study analyzing 101 adult allo-HSCT ALL patients undergoing chimerism monitoring by multiplex STR assay (sensitivity 10^{-2}), showed that an increasing mixed chimerism in CD34+ BM cells was an independent negative prognostic factor for OS and relapse in multivariable analysis (84). However, in a subgroup of 22 patients undergoing RQ-PCR MRD monitoring, MRD assessment was much more sensitive (86%) and

TABLE 2 Impact of pre and/or post allo-HSCT RQ-PCR MRD according to ALL series published in the last 20 years and including adult patients.

Ref. Study type (years)	Pts n ALL type	Age (range)	Condition donor	Transcript	Sensitivity	Pre allo-HSCT status		MRD$^+$ patient outcome°	Post allo-HSCT status		MRD$^+$ patient outcome°
						CR	MRD$^+$		CR	MRD$^+$	
(68) Retrospective 1996-2006	43 B 37 T 6	30 (18-36)	MAC 95.3% MD 55.8%	BCR/ABL MLL/AF4 IgH/TCR	NA	43 100%	31/43 72.1%	↓ 3-yr OS ↑ 3-yr CIR	36/36 100%	16/36 44.4%	↑ 3-yr CIR
(69) Prospective 1999-2010	65 B Ph$^+$ 65	43.2 (18-62)	MAC 83.1% MD 47.7%	BCR/ABL	NA	65 100%	41/65 63.1%	NS 5-yr OS NS 5-yr DFS ↑ 5-yr CIR	NA	NA	MRD$^+$ pts underwent TKI ± DLI
(52) Retrospective 2011-2016	155 B Ph$^+$ 155	31 (4-63)	MAC 100% MD 31.6%	BCR/ABL	NA	155 100%	91/155 58.7%	NS	155/155 100%	NA	Day 30 ↑RI, ↓DFS Day 60 NS Day 90 ↑RI, ↓DFS, ↓OS
(70) Retrospective 2005-2016	441 B Ph$^+$ 441	44 (18-70)	MAC 82% MD 36%	BCR/ABL	1x10^{-4}	404 92%	257/404 64%	↓ 5-yr OS ↓ 5-yr DFS	421	119/421 28%	↓ OS ↓ DFS
(71) Retrospective 2004-2018	94 B Ph$^-$ 39 B Ph$^+$ 37 T 18	43.4 (20-68)	MAC 53.3% MD 30.9%	IgH/TCR BCR/ABL IZKF1 del other	≥1x10^{-4}	68 72.3%	28/68 41.2%	NS	NA	23/NA	↑3-yr CIR ↓3-yr RFS ↓3-yr OS
(72) Retrospective 2002-2017	1625 B Ph$^+$-1625	48 (16-71)	MAC ~70% MD NA	BCR/ABL	≥1x10^{-5}	1523* 93.7% 102** 6.3%	412/1523 27% 41/102 40%	↓4-yr OS ↓4-yr DFS ↓4-yr OS ↓4-yr DFS	NA	NA	NA
(73) Prospective 1999-2013	542 B Ph$^-$ 316 T 204 Other 16	32 (15-55)	MAC ~80% MD 32%	IgH/TCR	1x10^{-4}	130 NA	47/130 30% 16/130 10%	↑ RI ↓ 5-yr OS	NA	NA	NA

Pts, Patients; n, Number; Condition, Conditioning Regimen; MRD, Measurable Residual Disease; CR, Morphological Complete Remission; MAC, Myeloablative Conditioning; MD, Matched Related Donor; OS, Overall Survival; CIR, Cumulative Incidence of Relapse; RI, Relapse Incidence; DFS, Disease Free Survival; NA, Not Available; NS, Not Significant, RFS, Relapse Free Survival; mo, Months.
° Post allo-HSCT outcome.
* CR1, ** CR2.

specific (95%) than chimerism (84). In a retrospective study regarding a small series of adult patients affected by AML and ALL, MFC (6 color panel) and RQ-PCR (WT-1) showed a moderate concordance with chimerism analysis (assessed by STR-PCR), suggesting the usefulness of MRD monitoring over chimerism in stratifying patients with respect to relapse risk (85). Recently, Pincez and colleagues have demonstrated in a pediatric series of 72 patients, mostly affected by AML and ALL, that an increasing mixed chimerism (assessed by STR-PCR) was never the first evidence of relapsing leukemia, that in turn was detected by more sensitive techniques of MRD analysis (i.e., RQ-PCR and only partially MFC with a sensitivity ranging from 2 to 10 x 10^{-4}) (86). Interestingly, Semchenkova and colleagues have recently demonstrated that in doubtful MRD positive cases, RQ-PCR chimerism testing in questionable MRD+ sorted cells can be useful for approval or disapproval of MRD presence (87).

In the absence of large studies, clear indications about assessment schedules, and due to the lack of reference methods among the increasing number of different strategies of chimerism analysis, it is difficult to establish the role of MRD and hence, MFC MRD monitoring, with respect to chimerism. Therefore, any comparison between chimerism and post allo-HSCT MRD monitoring should consider the sensitivities and specificities of the techniques available in each center. As shown in Table 3, most of the techniques currently used for MRD evaluation (88–98) including MFC (88–90) display a higher sensitivity than the majority of chimerism detection methods.

Concluding remarks

Allo-HSCT is a complex therapeutic procedure whose outcome depends on several patient-, disease- and transplant-related cofactors. Although the prognostic role of pre-transplant MRD (as assessed by either MFC or RQ-PCR) is generally accepted (44, 99), few data are available on the post-transplant setting, which is characterized by a delicate balance between the graft-versus-leukemia effects, that in turn depend on graft-versus-host disease (GVHD) prophylaxis, occurrence and treatment, and the eventual residual disease. Moreover, no definite guidelines regarding MRD time-point assessments or levels for preemptive interventions are currently available.

TABLE 3 MRD and chimerism assessment techniques according to sensitivity and preferable source of analysis.

	Technique	Sensitivity						Source
MRD		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
	MFC (4 colors)[88,89,90]			x	x			BM, PB
	MFC (6-8 colors)[88,89,90]				x			BM, PB
	MFC (≥ 8 colors)[88,89]				x		x	BM, PB
	RQ-PCR[88,89,90,91,92,93]				x	x		PB, BM
	ddPCR[88,92]				x	x		PB, BM
	NGS[88,92]				x		x	PB, BM
CHIMERISM	VNTR[95,97]		x					PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	RFLP[96]	x						PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	X/Y FISH[97]			x		x		PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	STR-PCR[94,95,96,97]		x					PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	RQ-PCR[94,95,96,97]		x	x				PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	ddPCR[94,96]		x	x				PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]
	NGS[96,97]		x		x			PB, BM, PB sorted lymphoid and myeloid cells [94,95,98]

MFC, multiparameter flow cytometry; BM, bone marrow; PB, peripheral blood; RQ-PCR, real-time quantitative PCR; ddPCR, digital droplet PCR; NGS, next generation sequencing; VNTR, variable number of tandem repeats; RFLP, restriction fragment length polymorphism; FISH, fluorescent in situ hybridization; STR-PCR, short tandem repeats-PCR. X values indicate the sensitivity of each technique according to the reference column.

In agreement with previous literature analysis (44, 99), 89% of the studies here retrieved reported a negative impact of pre allo-HSCT MFC MRD on the post-transplant outcome of adult ALL patients (Table 1). Although the extent to which the intensity of conditioning may affect MRD clearance remains debated (1), patients from most of these series underwent myeloablative regimens that resulted ineffective. Importantly, newly available drugs such inotuzumab ozogamicin and blinatumumab are currently used to obtain pre-transplant MRD clearance (45).

The role of MRD monitoring after allo-HSCT has been traditionally poorly explored. In addition to the previous lack of effective relapse-preventing interventions outside immunosuppressive drug tapering and donor lymphocyte infusion (DLI), or tyrosine kinase inhibitors in Ph positive ALL patients (100, 101), this was mainly due to the use of chimerism analysis as MRD surrogate. Nowdays, the availability of potential premptive and therapeutic post allo-HSCT interventions in either pediatric or adult ALL patients (102–107) highlights the need of highly specific and sensitive measures of MRD. However, post-transplant MRD monitoring may be troublesome for referral centers, mostly due to a difficult access to diagnostic samples, whose availability is critical in case of LAIP-based MFC and RQ-PCR Ig/TCR gene techniques (6). MFC can be a valuable tool for post allo-HSCT MRD monitoring as it is fast, applicable to most ALL cases, and somehow independent from diagnostic samples when a DFN approach is used (20, 21). According to our literature revision, all studies specifically addressing the role of post-transplant MFC MRD monitoring reported an adverse outcome for MRD positive patients (Table 1). Yet, transplant clinicians should be aware that the sensitivity and reliability of MFC MRD monitoring is dependent on sample type (BM) and quality (adequate cell number and vitality), provided rigorous technical assumptions (at least 6-8 color panel, acquisition of at least $4x10^6$ cells), standardization, and operator expertise (24, 25). As BM samples from patients with concurrent GVHD or herpetic infections can be inadequate for MFC assessment due to a low cellularity, some transplant centers evaluate MRD by both MFC and molecular methods, though with economic burden (8). In fact, in case of inadequate BM samples, MFC MRD should be interpreted with caution and integrated, if possible, with data obtained by RQ-PCR. Whatever the technique used, an additional issue for transplant physician is the need to combine MRD and chimerism data, as they may give contrasting results based on different sample sources and method sensitivities. Moreover, standards for measurement intervals for MRD and chimerism and definitions of thresholds for initiating therapy are still missing (84).

Overall, many questions remain to be addressed regarding MFC MRD monitoring in adult ALL patients undergoing allo-HSCT, mostly in the post-transplant setting. Although MFC can be a reliable tool for MRD assessment, potentially reaching RQ-PCR sensitivity levels, a close interaction between transplant clinicians and reference laboratory is recommended in order to select the optimal method for MRD evaluation in each patient and to obtain clinically useful data.

Author contributions

CT and AR performed literature search and wrote the manuscript. MK supervised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Bone marrow CD34+ molecular chimerism as an early predictor of relapse after allogeneic stem cell transplantation in patients with acute myeloid leukemia

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Background: Minimal residual disease (MRD) monitoring is an important tool to optimally address post-transplant management of acute myeloid leukemia (AML) patients.

Methods: We retrospectively analyzed the impact of bone marrow CD34+ molecular chimerism and *WT1* on the outcome of a consecutive series of 168 AML patients submitted to allogeneic stem cell transplantation.

Results: The cumulative incidence of relapse (CIR) was significantly lower in patients with donor chimerism on CD34+ cells ≥ 97.5% and *WT1* < 213 copies/ABL x 10^4 both at 1st month (p=0.008 and p<0.001) and at 3rd month (p<0.001 for both). By combining chimerism and *WT1* at 3rd month, 13 patients with chimerism < 97.5% or *WT1* > 213 showed intermediate prognosis. 12 of these patients fell in this category because of molecular chimerism < 97.5% at a time-point in which *WT1* was < 213.

Conclusions: Our results confirm that lineage-specific molecular chimerism and *WT1* after allo-SCT (1st and 3rd month) are useful MRD markers. When considered together at 3rd month, CD34+ molecular chimerism could represent an earlier predictor of relapse compared to *WT1*. Further studies are necessary to confirm this preliminary observation.

KEYWORDS

***WT1*, allogeneic stem cell transplantation, minimal residual disease (MRD), lineage specific molecular chimerism, pre-emptive therapy**

Introduction

Minimal residual disease (MRD) monitoring is crucial for the management of patients with acute myeloid leukemia (AML) (1–3). Two assays are currently available: multiparametric flow cytometry (MFC) on the leukemia associated immunophenotype (LAIP) and quantitative RT-qPCR on genes known to be mutated or over-expressed in a subgroup of AML (e.g. *FlLT3-ITD*, *NPM1* mutation, *CBF*-fusion transcripts, *WT1* gene,…) (4, 5). Each of these two assays is associated with different specificity, sensitivity and accuracy, and, with the exception of RT-qPCR on *NPM1* mutation, no conclusive data are available on the superiority of one test over the other (4, 6). Nevertheless, several studies confirmed the role of MRD monitoring after induction/consolidation, irrespective of the methods used and the threshold adopted, in order to measure the depth of response during the whole treatment program (3, 4). In particular, it has been suggested that MRD monitoring should be considered as a dynamic event, suggesting that AML risk may be refined during the treatment program (3, 4). Focusing on this issue, we reported how bone marrow (BM) LAIP <0.2% and BM-*WT1* < 121 copies/ABLx10^4 after first consolidation were associated with improved outcome; moreover, after 1st intensification cycle, peripheral blood (PB) *WT1* < 16 copies/ABLx10^4 was significantly correlated with a better prognosis (3). The issue of MRD monitoring is a crucial step in the path to cure of AML patients, especially in low-intermediate ELN risk categories, for which firstline allogeneic stem cell transplantation (Allo-SCT) in case of MRD persistence is a mainstay of good clinical practice (1–4).

Moreover, MRD detection before allo-SCT is very important to guide the intensity of transplant conditioning regimen (7–10). Then, the issue of MRD detection and monitoring after allo-SCT is particularly relevant, since early detection of residual disease may allow a pre-emptive treatment approach, including not only the early immunosuppression withdrawal and donor lymphocytes infusions (DLI), but also the introduction of new drugs such as hypomethylating agents (HMA), venetoclax, and tyrosine-kinase inhibitors (11–13). Although several studies have explored this topic, the methods and timepoints for the detection of patients at high risk of relapse are still a matter of debate (1–4). In particular, besides their limitations in terms of sensitivity and specificity, and the lack of prospective, controlled data, both MFC and RT-qPCR on selected gene targets are applicable in no more than 30-40% of the patients after allo-SCT (4). As a consequence, *WT1* has been suggested as a universal marker of MRD monitoring after allo-SCT, as its expression, although with low specificity, is increased in more than 80% of AML at diagnosis (5, 10, 13).

In this scenario, considering that AML arises from the hematopoietic stem cell, and that more than 90% of AML blasts express CD34 antigen, an option to monitor if allo-SCT has been able to cancel autologous hemopoiesis is the assessment of molecular chimerism on CD34+ cells (14–17). Both short tandem repeat analysis and single nucleotide polymorphism analysis by RT-qPCR have been suggested to be potentially useful tools to measure the degree of donor hematopoiesis. Thus, it may be considered as a surrogate marker of MRD, which can be associated with a high probability of disease recurrence (14–17). Several studies have confirmed that lineage-specific molecular chimerism is a reliable marker of MRD and relapse risk (14–17), but the interplay between CD34+ chimerism and other markers of MRD (e.g., leukemic blasts detection with MFC or *WT1*) possibly associated with MRD persistence is poorly understood and under-studied (18, 19).

With this background, we analyzed a cohort of 168 AML patients consecutively allotransplanted in our Institution between December 2015 and January 2022, for whom at least one between BM-CD34+ chimerism or BM-*WT1* level was available at 1 and 3 months after allo-SCT. The primary endpoint of this retrospective analysis on these two tests was to describe their accuracy in measuring the risk of relapse and their interplay in the definition of patients' prognosis.

Patients and methods

From December 2015 to January 2022, a total of 191 AML patients were consecutively submitted to allo-SCT in our Institution. 168 out of these transplants (88%) are included in the present analysis, as they represent a consecutive series for which data on lineage specific molecular chimerism (CD34+) and/or molecular monitoring of *WT1* gene are available at 1st and/or 3rd month after transplant. All patients included in this analysis provided informed consent for data registration in the PROMISE database, in which clinical and biological data are collected. Additional data were extracted from the revision of the clinical charts of each patient, including both the transplant phase and the subsequent follow up. The study was conducted in compliance with current national and European legislation on clinical trials, in accordance with the Declaration of Helsinki and the principles of good clinical practice.

Lineage-specific chimerism and *WT1* monitoring

According to our guidelines, molecular chimerism assessment on BM-CD34+ cells was planned at months 3, 6, 9, 12, 18, and 24 after allo-SCT. From 2020 we implemented another timepoint of assessment at day +30 after allo-SCT.

CD34+ cells were isolated from bone marrow using CD34 MicroBeads human (Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer protocol. Briefly, cells were incubated with 100 µL of CD34 MicroBeads and 100 µL of FcR Blocking Reagent for 30 minutes at 4°C, washed, resuspended in 500 µL buffer and applied onto one-step, semiautomated MACS device, AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of cellular subsets post-separation was determined by FACS analysis (BD FACSCanto^{TM} II) and BD FACSDiva software (BD Biosciences, San Jose, CA). Genomic DNA obtained after CD34+ selection from BM samples was extracted using mini blood kit (QIAGEN, Valencia, CA), following the manufacturer instructions. Validation of the CD34-enrichment was performed comparing the chimerism percentage of CD34+ and chimerism percentage of MNC between groups by 2-sided Student *t* test

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(continuous variables with normal distribution). P<.05 was considered significant. Calculations were conducted in Prism 5 (GraphPad, La Jolla, CA). Comparative statistical analysis showed significant difference (P= .0008) and validation of the method. The AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies Inc., Foster City, CA) containing 15 polymorphic STR (short tandem repeat) loci and the amelogenin marker was used to evaluate chimerism status in patients post transplant (20). Genomic DNA obtained after CD34+ selection (Automacs System -Miltenyi) from bone marrow samples was extracted using mini blood kit (QIAGEN, Valencia, CA), following the manufacturer instructions. Serial dilutions were created by mixing DNA samples with standardized mixed chimeric samples, in a range between 0% and 100%. The level of sensitivity of this test was 2.5%. The data were analyzed by GeneMapper®ID v3.2 software calculating the amount of donor's DNA.

All the patients with AML were evaluated for *WT1* expression level at diagnosis. Focusing on this series, the time-points of *WT1* evaluation on BM were the same as for chimerism and its assessment was performed by Q-PCR (protocol: Ipsogen *WT1* ProfileQuant) according to the ELN method as previously published (21). The cut-off for positive samples, according to the sensitivity of our platform and available literature, was ≥ 213 *WT1* copies/ABL1x10^4 on BM (21).

Statistical analysis

Descriptive statistics was employed for summarizing patients characteristics. Categorical variables were presented as numbers and percentages, continuous variables as median and range, respectively. Chi-Squared or Fisher's Exact test and the Wilcoxon Rank-sum or Kruskal-Wallis tests were used to test differences among subgroups, as appropriate. Median survival with 95% confidence interval (95%CI) was calculated according to reverse Kaplan-Meier method. Overall survival (OS) was measured from the time of transplant to the date of last follow-up or death, cumulative incidence of relapse (CIR), considering non-relapse mortality (NRM) as a competitive event, was carried out according to the Fine-Gray model. Log-rank and Gray tests were employed to verify differences among the different groups. One-month and 3-month landmark analyses were conducted in order to evaluate association between *WT1* (cut-off 213 copies/ABL1x10^4) and donor chimerism (cut-off 97.5%) on subsequent CIR. Sensitivity, specificity and diagnostic accuracy of post-transplant chimerism and *WT1* values in predicting relapse occurrence were also measured. Statistical analysis was performed with EZR (version 1.61), as previously described (22).

Results

Table 1 reports the most important clinical and transplant characteristics of these patients. The median age at transplant was

TABLE 1 Clinical and transplant characteristics of 168 AML patients included in this analysis.

	N (%)
Age, yr, median (range)	56.5 (23.8 – 74.1)
Sex	
Male	91 (54.2)
Female	77 (45.8)
Disease status at SCT	
First CR	83 (49.4)
Other disease status	85 (50.6)
Disease Risk Index	
High - Very	66 (39.3)
Low - Intermediate	102 (60.7)
Follow-up, yr, median (range)	1.5 (0.05 – 14.5)
SC source	
PBSC	128 (76.2)
BM	35 (20.8)
UCB	5 (3.0)
Conditioning intensity	
MAC	93 (55.4)
RIC	75 (44.6)
Donor	
Related	55 (32.7)
MUD	77 (45.8)
Haplo	31 (18.5)
UCB	5 (3.0)
CD34+ donor chimerism (1st month)	*Available on 36 pts*
< 97,5% donor	10 (27.8%)
≥ 97,5% donor	26 (72.2%)
BM *WT1* (1st month)	*Available on 45 pts*
< 213 copies/ABLx10^4	11 (24.4%)
≥ 213 copies/ABLx10^4	34 (75.6%)
CD34+ donor chimerism (3rd month)	*Available on 91 pts*
< 97,5% donor	29 (32%)
≥ 97,5% donor	62 (68%)
BM *WT1* (3rd month)	*Available on 120 pts*
< 213 copies/ABLx10^4	99 (82.5%)
≥ 213 copies/ABLx10^4	21 (17.5%)
CD34+ donor chimerism/*WT1* (3rd month)	*Available on 75 pts*
≥ 97,5% donor/< 213 copies/ABLx10^4	53 (71%)

(Continued)

TABLE 1 Continued

	N (%)
< 97,5% donor or ≥ 213 copies/ABLx10^4	13 (17%)
< 97,5% donor and ≥ 213 copies/ABLx10^4	9 (12%)

M, male; F, female; CR, complete remission; DRI, Disease Risk Index; PBSC, peripheral Blood Stem Cells; BM, Bone Marrow; UCB, Umbilical Cord Blood; MAC, Myeloablative Conditioning; RIC, Reduced-Intensity Conditioning; MUD, Matched Unrelated Donor; Haplo, Haploidentical Donor; WT1, Wilm's Tumor gene.

56.5 years (23.8-74.1), and patients were equally distributed between sexes. The disease risk index (DRI) was intermediate/high in two thirds of the cases, and 49.4% of the patients received the transplant in first complete remission (CR) following a myeloablative conditioning in 55.4% of the cases. Peripheral blood stem cells (PBSC) were used in the 76.2% of the cases, and donor was other than a sibling in more than 50% of the transplants (matched unrelated donor in 45.8% and haploidentical in 18.5% of the cases). No significant differences were detected comparing the same characteristics, dividing patients according to the percentage of donor chimerism on CD34+ cells (above or below 97.5%) and *WT1* levels (above or below 213 copies/ABL1x10^4) both at 1st and 3rd month (data not shown).

Overall, the total number of patients for whom *WT1* could be considered for MRD monitoring (at diagnosis > 213) was 125/168 (74%). Molecular chimerism on CD34+ cells and *WT1* at 1st month was available on 36 (21%) and 45 patients (27%), respectively. In 72.2% of the cases (26/36) the percentage of donor CD34+ cells was above 97.5%. Focusing on *WT1*, its level was < 213 copies/ABL1x10^4 in 24.4% of the cases.

Moving to the 3rd month, molecular chimerism on CD34+ cells and *WT1* were available on 99 (53%) and 125 patients (67%), respectively. Donor chimerism ≥ 97.5% was detected in 63 patients (65.6%) and *WT1* levels < 213 copies/ABL1x10^4 in 103 cases (82.4%).

Additional molecular markers of disease persistence during follow up were *FLT3-ITD* (2 cases at 1st month and 4 cases at 3rd month) and *NPM1A* (2 cases at 1st month and 2 cases at 3rd month). All the patients with positive *FlLT3-ITD* MRD had mixed chimerism on CD34+ and *WT1* level above 213 copies/ABL1x10^4, experienced hematological relapse and did not survive. The 2 patients with *NPM1A* positive residual disease showed complete donor chimerism and *WT1* level < 213 copies/ABL1x10^4 and are alive in continuous complete remission at last follow up.

Cumulative incidence of relapse and overall survival

After a median follow up of 4.5 years (range 3,5-5,0), the 1, 3, and 5 years cumulative incidence of relapse (CIR) was 26.9% (95% CI 20.3-34.0), 46.8% (95% CI 38.6-54.4) and 50.8% (95% CI 42.2-58.9), respectively (Figure 1A). This translated into an overall survival (OS) at 1,3 and 5 years of 67.3% (95% CI 59.4-74), 50.9% (95% CI 42.6-58.6), and 43.2% (95% CI 34.8-51.3), respectively (Figure 1B).

At 1st month, both donor chimerism on CD34+ cells ≥ 97.5% and *WT1* levels below 213 copies/ABL1x10^4 significantly correlated with CIR (chimerism: 13% *vs* 70% at 1 year; p=0.008 – Figure 2A; *WT1*: 31.8% *vs* 81.8%; p=0.03 – Figure 2B) and OS (chimerism: 81.8% *vs* 9.5% at 1 year; p<0.001 – Figure 2C; *WT1*: 54.3% *vs* 18.2%; p<0.05 – Figure 2D).

As reported in Figures 3A, D, the results at 3rd month confirmed the predictive value of the two markers on CIR and OS. In particular, the 1 and 2 years CIR for patients with donor chimerism on CD34+ cells ≥ 97.5% *vs* those with donor chimerism < 97.5% was 5.3% and 26% *vs* 61% and 74%, respectively (Figure 3A; p<0.001). This translated into a 1 and 2 years OS of 93% and 72.4% *vs* 44.2 and 25.4%, respectively (Figure 3B; p<0.001). Moving to *WT1* at 3rd month and comparing patients with a level below or above 213 copies/ABL1x10^4, we observed that the CIR at 1 and 2 years was 12.6%

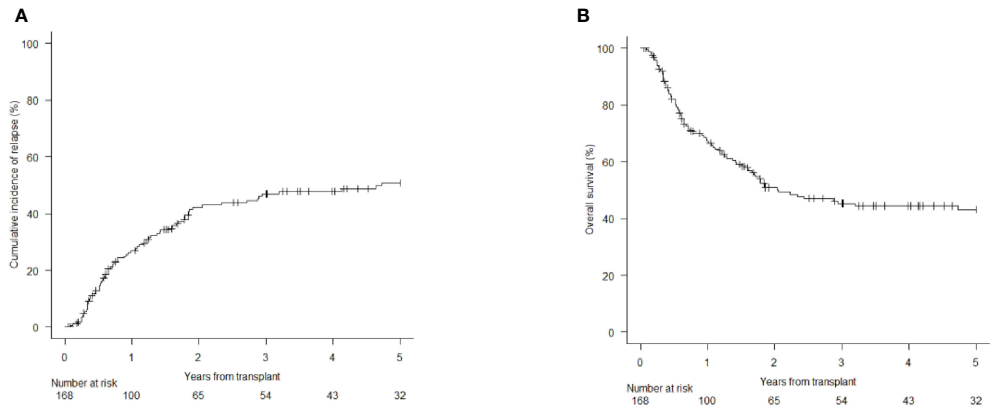

FIGURE 1
Cumulative Incidence of Relapse (CIR) and Overall Survival (OS) of the 168 AML patients included in this analysis. [CIR at 1, 3 and 5 years; 26.9% (95% CI 20.3-34.0), 46.8% (95% CI 38.6-54.4) and 50.8% (95% CI 42.2-58.9) **(A)**; OS at 1, 3 and 5 years: 67.3% (95% CI 59.4-74), 50.9% (95% CI 42.6-58.6) and 43.2% (95% CI 34.8-51.3) **(B)**].

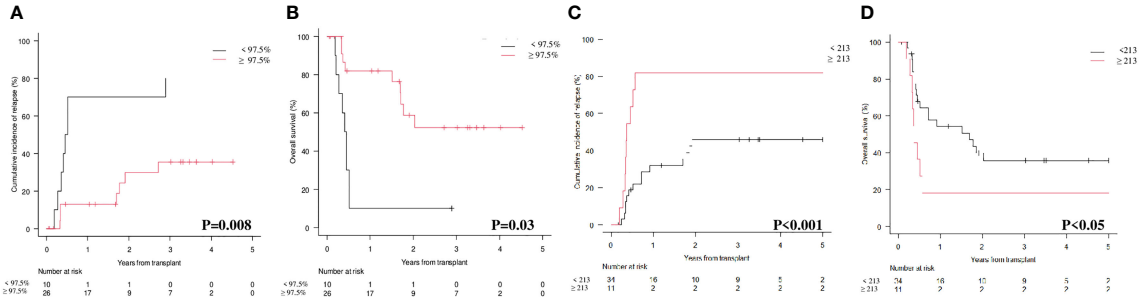

FIGURE 2
Cumulative Incidence of Relapse (CIR) and Overall Survival (OS) according to molecular chimerism on CD34+ cells and *WT1* levels at 1st month. **(A)** CIR at 1 year CD34+≥97.5% vs <97.5% donor: 13% (95% CI 3.3-29.7) vs 70% (95% CI 32.9.89.2). **(B)** OS at 1 year CD34+≥97.5% vs <97.5% donor: 81.8% (95% CI 58.5-92.8) vs 9.5% (95% CI 5-35.8). **(C)** CIR at 1 year *WT1* < 213 copies/ABL1x10^4≥213 copies/ABL1x10^4: 31.8% (95%CI 16.7-48.2) vs 81.8% (95%CI 44.7-95.1). **(D)** OS at 1 year *WT1* < 213 copies/ABL1x10^4≥213 copies/ABL1x10^4: 54.3% (95%CI 35.3-69.9) vs 18.2% (95%CI 2.8-44.2).

and 28.6% *vs* 80.9% and 97.3%, respectively (Figure 3C; p<0.001). As expected, 1 and 2 years OS was 83.3% and 65.8% *vs* 20.3% and 6.7% (Figure 3D; p<0.001).

The sensitivity, specificity, and accuracy of molecular chimerism on CD34+ cells at 3rd month was 53.3% (95% CI 34.3-71.7), 83.7% (95% CI 70.3-92.7), and 72.2% (85% CI 60.9-81.7). For *WT1* at 3rd month we observed a sensitivity of 33.3% (95% CI 17.3-52.8), a specificity of 98% (95% CI 89.1-99.9) and an accuracy of 73.4% (95% CI 62.3-82.7). We then looked at the sensitivity, specificity and accuracy of the combination of donor chimerism and *WT1* levels at 3rd month and we found that they were 53.3% (95% CI 34.3-71.7), 81.6% (95% CI 68.0-91.2) and 70.9% (95% CI 59.6-80.6).

Interestingly, by combining CD34+ donor chimerism </≥ 97.5% and *WT1* </≥ 213 copies/ABL1x10^4, three categories could be identified with significantly different prognosis both on CIR (p<0.001; Figure 3A) and on OS (p<0.001; Figure 3B): (i) donor chimerism ≥ 97.5% and *WT1* < 213 (53 patients) [CIR at 1 year 4.1% (95% CI 0.8-12.4) and OS at 1 year 94.2% (95% CI 83.0-98.1)]; (ii) donor chimerism < 97.5 or *WT1* ≥ 213 (13 patients) [CIR at 1 year 30.7% (95% CI 9.5-55.4) and OS at 1 year 76.9% (95% CI 44.2-91.9)]; (iii) donor chimerism < 97.5% and *WT1* ≥ 213 (9 patients) [CIR at 1 year 100% (95% CI NA) and OS at 1 year 0% (95% CI NA)]. Moreover, 12/13 patients included in the "intermediate" group (donor chimerism < 97.5% or *WT1* ≥ 213) fell in this category because of donor chimerism < 97.5% and only 1 patient because of *WT1* ≥ 213 copies/ABL1x10^4.

Pre-emptive treatment following the detection of CD34+ donor chimerism < 97.5% and/or *WT1* ≥ 200 copies/ABL1x10^4

Overall, 43 and 66 patients had at least one detection of donor CD34+ chimerism < 97.5% and/or *WT1* levels ≥ 213 copies/ABL1x10^4 at 1st and/or 3rd month. Whenever clinically possible (no graft versus host disease and no active infections) these patients were managed with early tapering of immunosuppression. If clinical and hematological conditions were permissive, additional pre-emptive therapy was administered (11 patients). Results in the different subgroups are reported in Supplementary Table 1.

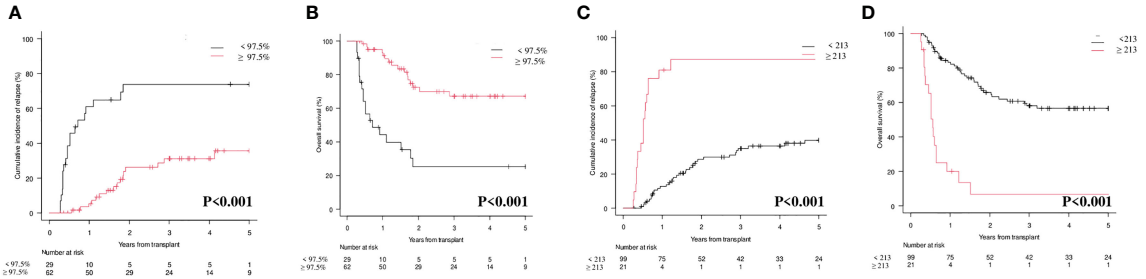

FIGURE 3
Cumulative Incidence of Relapse (CIR) and Overall Survival (OS) according to molecular chimerism on CD34+ cells and *WT1* levels at 3rd month. **(A)** CIR at 1 year CD34+≥97.5% vs <97.5% donor: 5.3% (95% CI 1.4-13.4) vs 61% (95% CI 40.3-76.4). **(B)** OS at 1 year CD34+≥97.5% vs <97.5% donor: 93.1% (95% CI 82.6-97.3) vs 44.2% (95% CI 25-61.9). **(C)** CIR at 1 year *WT1* < 213 copies/ABL1x10^4≥213 copies/ABL1x10^4: 12.6% (95%CI 6.9-20.2) vs 80.9% (95% CI 56.9-92-4). **(D)** OS at 1 year *WT1* < 213 copies/ABL1x10^4≥213 copies/ABL1x10^4: 83.3% (95%CI 74.2-89.4) vs 20.1% (95%CI 6.2-39.5).

Discussion

Minimal Residual Disease (MRD) monitoring is crucial in the management of AML patients, and is a dynamic process during all the treatment plan, including the post-transplant phase (1–17, 23, 24).

Our study clearly shows that both lineage-specific molecular chimerism and *WT1* levels are useful markers for MRD detection and monitoring after allo-SCT in AML, either alone or in combination. At 1st month after allo-SCT, lineage-specific molecular chimerism (Figure 2A; p=0.008) and *WT1* levels (Figure 2C; p<0.001) were significantly correlated with the CIR. This was also confirmed at 3rd month (Figure 3A; p<0.001 and Figure 3B; p<0.001). Interestingly, by combining molecular chimerism and *WT1* at 3rd month, we identified three categories of patients with different prognosis: (i) donor chimerism ≥ 97.5% and *WT1* < 213 (53 patients); (ii) donor chimerism < 97.5 or *WT1* ≥ 213 (13 patients); (iii) donor chimerism < 97.5% and *WT1* ≥ 213 (9 patients). The lowest CIR and the longest OS were observed in patients with donor CD34+ ≥ 97.5% and *WT1* < 213 copies/ABL1x10^4 (Figure 4A; p<0.001 and Figure 4B; p< 0.001). This strongly reinforces the significance of these two tests for MRD monitoring after allo-SCT. Notably, focusing on the intermediate category (donor CD34+ chimerism < 97.5% or *WT1* ≥ 213 copies/ABL1x10^4), we observed that nearly all of these patients (12/13) were included in this group because of mixed donor chimerism, at a timepoint in which *WT1* levels were still within the normal range. This suggests that molecular chimerism may detect persistence of MRD earlier than *WT1*. In other words, once *WT1* gets positive, disease relapse is highly likely to occur in a very short time-frame. Even if numbers are small to be conclusive, we think that these results reinforces the usefulness of both methods for MRD monitoring after allo-SCT and suggests that lineage-specific molecular chimerism may an earlier predictor of relapse than *WT1*. On the other hands, a recently published paper (18) suggests that day +100 MRD positivity is a stronger predictor of relapse after allo-SCT compared to mixed chimerism. Notably, the series published by Klyuchinov and Colleagues includes intermediate-risk AML only, and MRD monitoring was performed with MFC and RT-qPCR on *NPM1A*, of which at least *NPM1A* is extremely disease-specific as a marker of MRD. These two aspects may be responsible for the different results.

We then looked at the use of pre-emptive therapy guided by one or both of the MRD markers (chimerism and/or *WT1*). Pre-emptive treatment was administered in a minority of patients (n=11). As a consequence results are anecdotal and no conclusions can be drawn. Interestingly, the higher response rate (in terms of conversion to full donor chimerism or increase in the percentage of donor CD34+ cells) was observed in patients with mixed chimerism at 3rd months (n=29). In this group, 7 patients (24%) received a pre-emptive approach with either HMA alone or in combination with venetoclax/DLI or DLI alone, 4/7 (57%) patients achieved a response and at the last follow up 9/29 (31%) patients were alive.

The relatively small number of patients included in our analysis may hamper drawing final conclusions. Nevertheless, our study confirms the prognostic value of lineage-specific chimerism at very early timepoints (1st and 3rd month) and suggests that patients at high risk of relapse may show mixed chimerism before positivity of *WT1* as a marker of MRD. The aim of this study was not to compare lineage-specific molecular chimerism and *WT1*, but our results indirectly suggest that chimerism on CD34+ cells could be an earlier predictor of relapse. The small number of cases with available data at day +30 depends on the fact that early assessment of chimerism and MRD monitoring were implemented only from 2020 in our Institution and suggests caution both in results interpretation and conclusion drawing.

Overall, our data are in line with other published papers, highlighting the role of both lineage specific molecular chimerism and *WT1* as markers of MRD after allo-SCT (14–19, 24–28). The issue of the superiority of molecular chimerism on CD34+ cells over

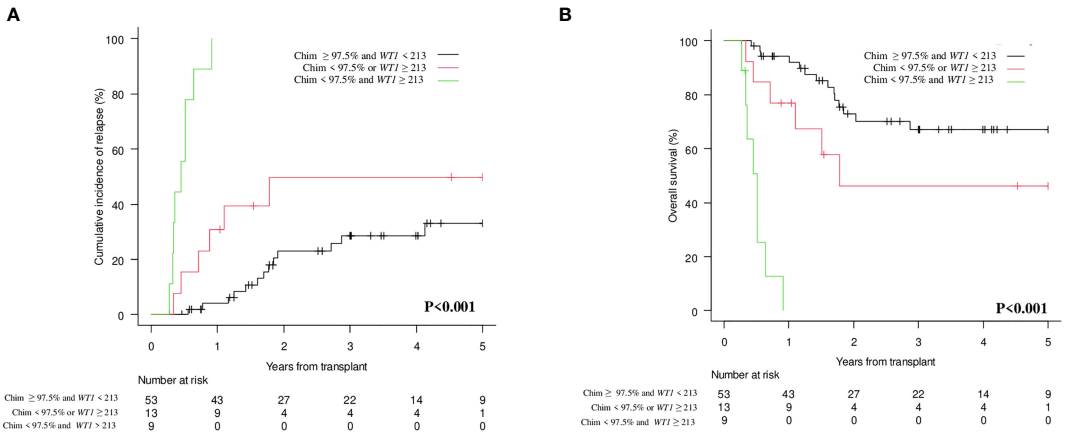

FIGURE 4
Cumulative Incidence of Relapse (CIR) and Overall Survival (OS) according to combination of molecular chimerism on CD34+ cells and *WT1* levels at 3rd month. **(A)** CIR at 1 year CD34+≥97.5% and *WT1* < 213 copies/ABL1x10^4 vs CD34+ <97.5% *WT1* ≥ 213 copies/ABL1x10^4 vs CD34+ <97.5% or *WT1* ≥ 213 copies/ABL1x10^4: 4.1% (95% CI 0.8-12.4) vs 30.7% (95% CI 9.5-55.4) vs 100% (95% CI NA). **(B)** OS at 1 year CD34+≥97.5% and *WT1* < 213 copies/ABL1x10^4 vs CD34+ <97.5% or *WT1* ≥ 213 copies/ABL1x10^4 vs CD34+ <97.5% and *WT1* ≥ 213 copies/ABL1x10^4: 94.2% (95% CI 83.0-98.1) vs 76.9% (95% CI 44.91.9)] vs 0% (95% CI NA).

other methods for leukemia relapse prediction is still unsolved. Some data suggest that *WT1* could be more sensitive than lineage specific molecular chimerism (29) or that the two methods are concordant (30), also when analyzed in specific cellular sub-types, such as CD3 negative mononuclear cells (31). On the other hand, in the study by Rossi and Colleagues a higher concordance between positive results from MFC and *WT1* was detected among patients with mixed rather than complete chimerism (32). Several issues are still open, such as the role of the source used for the detection of both chimerism and MRD. If it is true that PB may be used for MRD monitoring in AML and has some advantages over bone marrow (13), there are no conclusive data regarding this issue when we consider lineage-specific molecular chimerism and different sources are used in the published papers, according to each Center's guideline (15, 26–32). Interestingly, as suggested by Gambacorta and Colleagues, PB may allow a tighter follow up of the patients and may allow higher specificity in case of positive samples. The Authors give an intriguing explanation for this, speculating that BM detects a significant "background noise" possibly related to the aspiration of host stromal cells (15). Moreover, new technologies such as digital PCR (dPCR) or next generation sequencing (NGS) may be a useful tool to increase both the specificity and sensitivity of lineage-specific molecular chimerism. Further prospective studies are thus warranted in order to clarify if lineage-specific molecular chimerism is superior to *WT1* to identify imminent relapse, which time-points are more reliable for an optimal prediction of disease recurrence, if PB should be preferred to BM and if new technologies may increase the power of molecular chimerism for relapse prevention.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MM, NP, ArLa, DR designed the study. EM, VR, EB, SB, FR, DaBo and AlLe collected the data. ArLa, AB, FB, MC performed chimerism analysis. DuBr performed molecular analysis on WT1. MM, NP, AlLe, ArLa, SB, FR analyzed the data. MM, NP, ArLa and DR wrote the Manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1133418/full#supplementary-material

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Research Topic: Measurable Residual Disease in Hematologic Malignancies. Can digital droplet PCR improve measurable residual disease monitoring in chronic lymphoid malignancies?

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Minimal/measurable residual disease (MRD) monitoring is progressively changing the management of hematologic malignancies. The possibility of detecting the persistence/reappearance of disease in patients in apparent clinical remission offers a refined risk stratification and a treatment decision making tool. Several molecular techniques are employed to monitor MRD, from conventional real-time quantitative polymerase chain reaction (RQ-PCR) to next generation sequencing and digital droplet PCR (ddPCR), in different tissues or compartments through the detection of fusion genes, immunoglobulin and T-cell receptor gene rearrangements or disease-specific mutations. RQ-PCR is still the gold standard for MRD analysis despite some limitations. ddPCR, considered the third-generation PCR, yields a direct, absolute, and accurate detection and quantification of low-abundance nucleic acids. In the setting of MRD monitoring it carries the major advantage of not requiring a reference standard curve built with the diagnostic sample dilution and of allowing to reduce the number of samples below the quantitative range. At present, the broad use of ddPCR to monitor MRD in the clinical practice is limited by the lack of international guidelines. Its application within clinical trials is nonetheless progressively growing both in acute lymphoblastic leukemia as well as in chronic lymphocytic leukemia and non-Hodgkin lymphomas. The aim of this review is to summarize the accumulating data on the use of ddPCR for MRD monitoring in chronic lymphoid malignancies and to highlight how this new technique is likely to enter into the clinical practice.

KEYWORDS

digital droplet PCR, measurable residual disease (MRD), non-Hodgkin lymphoma, chronic lymphocytic leukemia, hairy cell leukaemia (HCL)

1 Introduction

Monitoring of measurable/minimal residual disease (MRD) is progressively impacting on the management and outcome of different hematologic malignancies, since it can predict patients' outcome, redefine prognostic risk stratification and response to treatment and in acute leukemias and chronic myeloid leukemia also guide treatment decisions (1–5). Several molecular techniques are employed to monitor MRD, from conventional real-time quantitative polymerase chain reaction (RQ-PCR) (6–9) to next-generation sequencing (NGS) (10–13) and digital droplet PCR (ddPCR) (14–17), through the detection of fusion genes, immunoglobulin (IGH) or T-cell receptor (TCR) gene rearrangements, or disease-specific mutations. They are applied to different tissues or compartments, i.e. bone marrow (BM) and peripheral blood (PB) - for both genomic DNA from circulating neoplastic cells or circulating cell-free DNA (cfDNA) from plasma (18, 19).

RQ-PCR still represents the gold standard for MRD. International guidelines for analysis and reporting have been established by the EuroMRD Consortium (8). Despite the high sensitivity of RQ-PCR, a non-negligible fraction of samples with low-level positivity within the 1 x 10^{-4} to 1 x 10^{-5} range (i.e. 1 tumor cell within 10.000-100.000 normal cells) cannot be precisely quantified according to the EuroMRD guidelines (20). The reason could reside in the lack of reproducibility of the samples at these levels. However, in most cases it is difficult to distinguish the PCR amplification signal of very few residual leukemic cells from the non-specific signal (20). Moreover, MRD quantification by RQ-PCR is based on a standard curve built on the dilution of the diagnostic sample within a pool of healthy donors' DNA.

NGS, widely employed to detect disease-specific mutations with high sensitivity (<1%) when compared to Sanger sequencing (10-20%), can also be employed for target screening and MRD monitoring. It shows the remarkable advantage of a wide applicability (≥95% of cases) and of providing additional information on the whole clonal composition and/or clonal evolution of each neoplasm. The EuroMRD Consortium has recently established the indications to apply NGS for target screening (10–13). However, since NGS sensitivity for MRD detection increases with the increase of DNA input, the issue of the balance between costs and feasibility is still a matter of debate.

ddPCR, considered the third-generation PCR, yields a direct, absolute, and accurate detection and quantification of low-abundance nucleic acids, with documented advantages in the context of MRD quantification (see below). ddPCR is actively investigated in the context of the EuroMRD group. At present, standard operating procedures have been published as a guide for digital analysis in lymphoid malignancies (21).

NGS and ddPCR could also be applied in combination: NGS can be optimized to detect the target sequence of IGH rearrangements, which can be employed to design patient-specific probes to be monitored by ddPCR, which allows to reduce costs, time and efforts compared to NGS monitoring.

At present, the use of ddPCR and NGS to monitor MRD in the clinical practice is limited by the lack of international guidelines. Nevertheless, their application within clinical trials is progressively growing in lymphoid malignancies, such as Philadelphia-positive and -negative acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphomas (NHL) (1–4, 15–17, 20, 22).

The aim of this review is to summarize the accumulating data on the use of ddPCR for MRD monitoring in chronic lymphoid malignancies and to highlight how this new technique can enter into the clinical practice.

2 Technical principles of ddPCR

The ddPCR system is based on the generation of droplets through a water-oil emulsion of the sample. This partitioning process allows to obtain multiple PCR sub-reactions, in which each generated droplet contains single, few or no target sequences (23, 24). PCR partitions are read and counted as negative or positive by thresholding based on their fluorescence amplitude. Based on Poisson' statistics, the number of positive and negative partitions is used to calculate the concentration of the target sequence, which can be a known mutation or a "patient-tailored" sequence (25, 26).

High precision and sensitivity (down to a level of detection of 0.001%) are given by compartmentalization that renders PCR less sensitive to reaction inhibitors, and reduces any template competition, allowing the detection of rare target sequences in a wild-type background (26–29). Assays are evaluated on the basis of specific parameters: Limit of Blank, which is the highest amplitude in which a blank sample stands when it is not containing any target sequence; Limit of Detection, the lowest amplitude at which target amplification can be distinguished from the blank; Limit of Quantification, the lowest concentration at which a target sequence can be quantified (25, 29). However, ddPCR still requires a marker-specific tuning of PCR reactions, i.e. annealing temperature, primer/probes concentration and, for results analysis, a manual positioning of a threshold cycle. In addition, at variance from NGS, ddPCR has technical limitations in the multiplex approach.

In the MRD setting, while RQ-PCR quantification is relative to a standard curve built on the dilution of the diagnostic sample in a pool of DNA from healthy donors, ddPCR MRD evaluation is an absolute quantification that makes unnecessary the standardized dilution curve at each time point of disease monitoring. Adaptability, reproducibility and ease of use are distinctive features of this method, that has spread in the general practice.

3 ddPCR in chronic lymphoid malignancies

With the advent of chemo-immunotherapy and, more recently, with the introduction of new targeted agents in various combinations, the prognosis of CLL and NHLs has considerably changed over the years. Complete responses are increasing in rate and long-lasting over time. However, a consistent proportion of

patients experiences a relapse after achieving a complete remission. Thus, MRD analysis has acquired relevance in the effort of predicting patients' outcome, stratifying more accurately patients into risk categories, redefining the clinical response to treatment, and possibly optimizing treatment strategies also in chronic lymphoid malignancies (2–4, 16).

During the last few years, ddPCR has been investigated for the monitoring *BCL2::IGH* rearrangement in follicular lymphoma (FL), *BCL1::IGH* in mantle cell lymphoma (MCL), *MYD88* mutations in Waldenstrom macroglobulinemia (WM) and *IGH* rearrangements in chronic lymphocytic leukemia (CLL), proving a promising tool to further refine MRD monitoring (Table 1).

3.1 Follicular lymphoma

The genetic hallmark of FL is the *BCL2::IGH* rearrangement, which is a result of the t (14, 18) (q32;q21) translocation which enhances anti-apoptotic activity posing the *BCL2* gene under the transcriptional control of the heavy chain gene enhancer. The rearrangement can occur in the major breakpoint region (*MBR*) or, rarely, in the minor cluster region (*mcr*) (30, 31). It is detectable at diagnosis by conventional PCR in 50-60% of cases with advanced FL both by qualitative and quantitative approach (31–35). This low sensitivity can be explained by the employment of large internal primers which target both chromosomes 14 and 18 in the qualitative reaction and the proximity of breakpoints site to target sequences for RQ-PCR (31). In localized FL, the *BCL2::IGH* rearrangement is found in a lower proportion of cases, especially when staged by PET/CT in comparison with historical series (36).

MRD in FL is of great potential value given the heterogeneous clinical behavior of the disease. Large clinical trials in the last years have tried to validate MRD assessment in FL through BCL2::IGH monitoring (32–35). MRD negativity is predictive of a better progression-free survival (PFS) in all clinical trials conducted in the past two decades, even in relapsed patients, and possibly of a longer survival in studies with a prolonged follow-up (4). Nonetheless, MRD monitoring is to date not included in the recommended guidelines for FL management (37).

The introduction of chemo-immunotherapy with anti-CD20 monoclonal antibodies has allowed an increase in the rates of MRD negativity at the end of induction (EOI) up to 70-80% (rituximab-based) and 90% (obinutuzumab-based), respectively (4). Anti-CD20 maintenance holds and increases the rates of MRD negativity. Recently, the assessment of MRD at earlier time points with respect to EOI has been tested for the first time in the Gallium trial and has proven informative (38, 39).

MRD analysis is also a sensitive tool to refine clinical response assessment in FL. The combination of molecular and metabolic-defined response is a promising and valuable tool to be further explored, as well as the possibility of a MRD-driven modulation of the post-induction therapy in FL (35).

Given this landscape, it is clear which clinical benefit could come from optimizing the use of ddPCR in FL to maximize the sensitivity of *BCL2/IGH* detection. The droplets are analyzed on the basis of FAM fluorescence BCL2/JH-linked and corrected by the unspecific background fluorescence. *BCL2::IGH* can be detected down to 1×10^{-4} *BCL2/JH*-positive cell line (limit of detection).

Drandi et al. (28) compared RQ-PCR to ddPCR in 30 patients with FL, 18 with multiple myeloma (MM) and 21 with MCL. A highly significant level of concordance was observed between qPCR and ddPCR (r = 0.94, P <0.0001; 95% CI, 0.94–0.97), with 189 of 222 samples (85.1%) fully concordant. In the MRD quantification of 26 samples resulting positive not-quantifiable (PNQ) by RQ-PCR, 27% resulted quantifiable and 23% negative when assessed by ddPCR. This experience showed how ddPCR can be a valid option for MRD detection.

Cavalli et al. (27) tested a cohort of 67 patients affected by early-stage FL both in the PB and BM at diagnosis and after radio-immunotherapy. Among 138 samples, the concordance between RQ-PCR and ddPCR was 81.9%, which raised to 97.5% for the subset with quantifiable disease (40/138) (21). Moreover, at baseline ddPCR identified a MBR marker in 8 of 18 (44%) samples that by qualitative nested PCR resulted as MBR−/mcr−. A molecular tumor burden at diagnosis ≥1 x 10^{-5} significantly predicted PFS only when quantified by ddPCR but not by RQ-PCR (36). Again, a higher sensitivity of ddPCR was shown in RQ-PCR PNQ samples (27).

Della Starza et al. (40), through a collaborative effort of four laboratories belonging to the Fondazione Italiana Linfomi (FIL) MRD Network for FL and MCL MRD assessment, demonstrated that there is a proportion of "borderline" samples (31/187, 17%), those resulting alternatively positive and negative by RQ-PCR/qualitative PCR, that challenge the inter-laboratory reproducibility. There was no inter-laboratory discordance when "borderline" samples were tested by ddPCR analysis.

In another experience by Delfau-Larue et al. (41) quantification of circulating *BCL2/IGH*+ cells and cfDNA was retrospectively performed by ddPCR in 133 FL patients. PB was tested for *BCL2::IGH* rearrangement and the *ANKRD30B* gene was used as the reference gene to quantify the cell-free circulating equivalent genome using the PrimePCR ddPCR copy number assay. A significant correlation was found between the total metabolic tumor volume (TMTV) and both circulating tumor cells (CTCs) (P <0.0001) and cfDNA (P <.0001). With a median follow-up of 48-month, the 4-year PFS was lower in patients with TMTV >510 cm^3 (P = 0.0004), CTCs >0.0018 PB cells (P = 0.03), or cfDNA >2550 equivalent-genome/mL (P = 0.04). Total cfDNA levels and TMTV were independent predictors of outcome. In this experience, ddPCR proved to be promising in the evaluation of multiple compartments in FL, including cfDNA (41).

For the first time in the context of a clinical trial, MRD analysis was assessed by ddPCR in the Relevance protocol (42). At the EOI, 98% and 78% of patients achieved a complete molecular response in the PB and BM, respectively. A complete molecular response was reached more frequently with the rituximab + lenalidomide combination (90%) than with rituximab-chemo (77%) (p = 0.022) (42) (Table 1).

Mutations other than *BCL2::IGH* are gaining interest for their prognostic relevance in FL, such as the gain-of-function mutations of the *EZH2* gene. Alcaide et al. (43) optimized a multiplex ddPCR for the detection of 4 *EZH2 Y641* and *STAT6* mutations. This assay accurately determined whether the samples harbored either an

TABLE 1 Experiences reporting on ddPCR and MRD in lymphoproliferative disorders.

Studies comparing ddPCR to RQ-PCR							
Study	Disease	N° of patients (samples)	Rationale	Tissue Timing	Marker	Concordance with RQ-PCR	Major Advantages of ddPCR
Drandi et al.[28]	FL+MM +MCL	30+18+21 (222)	Comparison between RQ-PCR and ddPCR	BM at diagnosis and MRD	*BCL2::IGH IGH*	85%	7 of 26 PNQ samples (26.9%; five MM, one MCL, and one FL) by RQ-PCR were quantified by ddPCR; 6/26 (23.1%) were negative by ddPCR
Cavalli et al.[27]	Early stage FL	67 (138)	Comparison between RQ-PCR and ddPCR	PB+BM at diagnosis and MRD	*BCL2::IGH*	81.9%	8/18 (44.4%) negative at diagnosis were MBR+ by ddPCR Tumor burden at diagnosis correlates with PFS only when quantified by ddPCR
Drandi et al.[29]	MCL	166 (416)	Comparison between RQ-PCR and ddPCR	PB+BM at MRD	*BCL1::IGH IGH*	ICC = 0.79, 95% CI: 0.75-0.83	Among 240 PNQ samples at qPCR, 39% were positive by ddPCR, 49% negative and only 12% remained positive below quantifiable ddPCR limits
Drandi et al.[46]	WM	148 (291)	Reliability of ddPCR to detect *MYD88*^{L265P}	PB+BM ctDNA at diagnosis	*MYD88*^{L265P}	/	122 of 128 (95.3%) BM and 47/66 (71.2%) baseline PB samples scored positive for MYD88L265P. High concordance between ctDNA and BM levels
Della Starza et al.[75]	ALL, CLL, MCL, FL	216 (620)	Comparison between RQ-PCR and ddPCR	PB+BM at diagnosis and MRD	IGH TCR BCL2::IGH	76.4%	Significant reduction of PNQ samples, from 18% to 11% Significant increase of quantifiable MRD, from 29% to 38.4%
Guerrini et al.[81]	HCL, SMZL	47 (141)	Comparison between RQ-PCR and ddPCR	BM+PB at diagnosis and MRD	BRAF V600E	/	Sensitivity of ddPCR is about half a logarithm superior to RQ-PCR Superiority in the identification of MRD+ after treatment
Clinical trials employing ddPCR for MRD monitoring							
Study	Disease	N° of patients	Therapy	Tissue Timing	Marker+ at diagnosis	MRD- at EOI	Clinical impact
Delfau-Larue et al.[42]	Untreated advanced FL	440	Phase 3 Relevance trial. Rituximab plus lenalidomide (R2) vs R-CHOP, both arms were followed by rituximab maintenance	PB+/-BM at diagnosis and MRD	222/440 (50.45%) BCL2::IGH +	MRD- at EOI (week 24): PB 98% and BM 78% R2 arm: MRD- 90% (105/117) R-CHOP arm: MRD- 77% (70/90)	3-Year PFS: 84% for MRD- vs 55% for MRD+ 3-Year PFS: 85% for BM MRD- vs 54% for BM MRD+ MRD+ at EOI: HR 3.3 (1.2-9.2, p=.02) for R-CHOP arm HR 2 (0.6-6.8; p=.27) for R2 arm
Pulsoni et al.[36]	Untreated localized FL Stage I (78%)- Stage II (22%)	67	IFRT (24-30Gy) + 4 weeks of Rituximab in MRD+	PB+ BM at diagnosis and MRD	72% BCL2:: IGH+	MRD- after RT:50% MRD- after R:84% In MRD+ post IFRT: superior PFS in patients treated with R vs untreated with R	84-m PFS: 75% for BCL2/IGH- vs 59% for BCL2/IGH+ by RQ-PCR at baseline (p=.26) 84-m PFS: 90.9% in 11 pts with MRD $<10^{-5}$ vs 38% in 19 pts with MRD$_{=}10^{-5}$ by ddPCR at baseline (p=.015)

It includes studies comparing ddPCR to RQ-PCR for MRD monitoring or clinical trials with ddPCR-based MRD. ddPCR, digital droplet polymerase chain reaction; RQ-PCR, real quantitative polymerase chain reaction; ALL, acute lymphoblastic leukemia; FL, follicular lymphoma; HCL, hairy cell leukemia; MCL, mantle cell lymphoma; MM, multiple myeloma; SMZL, splenic marginal zone lymphoma; WM, Waldenstrom macroglobulinemia; BM, bone marrow; PB, peripheral blood; ctDNA, circulating tumor DNA; PNQ, positive not-quantifiable; MRD, minimal residual disease; IFRT, involved field radiotherapy; ICC, intraclass correlation; EOI, end of induction; PFS, progression-free survival.

EZH2 or a *STAT6* mutation (or both) or whether samples were lacking mutations at both hotspots (43). In a small report, the *EZH2* mutant clone was also detectable in liquid biopsies (44).

These experiences open the way to larger studies to better define the prognostic role of these mutations in FL and if they are suitable markers for MRD.

3.1.1 Other indolent lymphomas

In WM, $MYD88^{L265P}$ is a diagnostic and predictive biomarker of response to ibrutinib (45). Beside allele-specific RQ-PCR, ddPCR has recently proven to be a suitable and sensitive tool for $MYD88^{L265P}$ screening and MRD monitoring (46). Both unsorted BM and PB samples can be reliably tested, as well as circulating tumor DNA (ctDNA), which represents an attractive and less invasive alternative to BM for $MYD88^{L265P}$ detection (46).

$MYD88^{L265P}$ detection in the cerebrospinal fluid (CSF) by ddPCR is also useful to diagnose the Bing-Neel syndrome (47).

Promising results have been preliminarily shown in splenic marginal zone lymphoma (MZL), where MRD has been assessed in the BM and PB by ddPCR employing IGH allele-specific oligonucleotide (ASO) primers in the phase II BRISMA/IELSG36 trial (48).

3.2 Mantle cell lymphoma

MCL is characterized in most cases by a specific t (11, 14)(q13;q32) translocation. It can be detected by FISH in around 70% of MCL at diagnosis and corresponds to the *BCL1::IGH* rearrangement, with BCL1 proliferating activity enhanced by the heavy chain regulatory gene. The most frequent breakpoint is the major translocation cluster (*MTC*) (31, 49, 50). IGH rearrangements are detected by PCR in 80–85% of MCL cases. In at least 10% of cases the detection failure is linked to purely nodal forms without circulating neoplastic cells; *BCL1::IGH* rearrangements are detected by PCR in 30%–40% of such cases, resulting in a proportion of double negative cases ranging from 5 to 10% (51, 52).

The gold standard approach for MRD monitoring relies on *BCL1::IGH* and IGH rearrangements monitored by RQ-PCR, capable of detecting up to 1 clonal cell among 100,000 analyzed (1×10^{-5}) (52–56). Several large studies sustain the predictive role of MRD in MCL (52–56). Among the most recent, the FIL MCL0208 trial compared maintenance with lenalidomide vs. observation after an intensive chemo-immunotherapeutic regimen and autologous stem cell transplant (ASCT) in 300 young MCL patients (54). A molecular marker (*BCL1::JH* and/or *IGH* rearrangements) was found in 83% of patients, and a MRD negativity was achieved in 78% of patients after high-dose chemotherapy and in 79% after ASCT (54). A time-varying kinetic model, combining the MRD status at two or more consecutive time points (post-ASCT, months +6, +12) was conceived. The combination of the MRD status with the MIPI (Mantle Cell Lymphoma International Prognostic) index proved to be an informative tool in predicting relapse and determining time-to-progression (TTP) (54).

The Nordic Lymphoma Group assessed MRD in 183 MCL patients who underwent an ASCT by performing PCR for *BCL1::JH* and *IGH* rearrangements. Shorter progression-free survival (PFS) and overall survival (OS) were demonstrated for patients who were MRD-positive pre- or after-ASCT: median PFS 20 months in the MRD-positive group vs. 142 months for the MRD-negative patients. OS was 75% at 10 years with a median not reached in the MRD-negative group compared to 35 months in the MRD-positive group (55). This association was even stronger in patients who achieved a complete response (CR) (56).

Also in this setting, the pitfalls of RQ-PCR, especially the contamination risk, the presence of disease levels below the quantitative range and the requirement of a standard curve offer the possibility to improve MRD monitoring by the employment of ddPCR (29, 54).

Drandi et al. (29) compared ddPCR with RQ-PCR in MCL evaluated by both molecular markers. Overall, from a total of 166 patients from four prospective MCL clinical trials, 416 MRD samples were tested by ddPCR, with an over-representation (61%) of below the quantitative range cases by RQ-PCR. ddPCR and RQ-PCR gave comparable results in MRD samples with at least a 0.01% positivity. Amongst 240 samples below the quantitative range with duplicate or triplicate analysis, 39% were positive by ddPCR, 49% negative and only 12% remained positive below quantifiable ddPCR limits. In another experience from the same group, patient-specific IGH rearrangements were amplified and directly sequenced from diagnostic DNA determining specific ASO primers tested both in RQ-PCR and ddPCR. Sixty-seven MCL samples (18 BM and 4 PB diagnostic, and 45 follow-up samples) were tested (28). Only 11.9% were discordant between the two methods, 1 major qualitative discordance and 7 minor qualitative discordances (28).

Della Starza et al. (57) reported alternative targets, such as immunoglobulin kappa-deleting-element (IGK-Kde) rearrangements, as suitable for MRD detection in MCL patients by RQ-PCR and ddPCR. *IGK-Kde* rearrangements were found in 76% (28/37) of cases, representing the sole molecular marker in 73% (8/11) of BCL1::IGH double negative cases. MRD RQ-PCR monitoring was possible in 57% (16/28) of cases, showing a 100% concordance with the conventional targets. Also in this setting, ddPCR showed a good concordance with RQ-PCR (19/24; 79%) and it might help to identify false positive/negative results in samples with low level of residual disease (57).

3.3 Diffuse large B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) includes a variety of biologic subtypes and variants. The distinction of the cell of origin, i.e. activated B-cell like (ABC) and germinal center B-like (GCB) DLBCLs, is based on the gene expression profile evaluated using the nanostring technology (58). More recently, mutation-based cluster classifications have been provided by the genomic profiling evaluated by NGS (59, 60).

At variance from FL or MCL, circulating cells in DLBCL are rarely detectable, thus many researchers started to use the plasma as a source of tumor DNA, either by extracting cfDNA or the circulating exosomes (61–64). Liquid biopsy of DLBCL at diagnosis and the identification of lymphoma-associated mutations has opened the way to MRD monitoring also in this disease (61, 64). In addition, testing *IGH* and *IGK* clonality on biopsy samples has shown that up to 83% of DLBCL carry an immunoglobulin molecular marker, which can be monitored on ctDNA by NGS and is associated with prognosis and prediction of relapse (62), also in new therapeutic contexts such as chimeric antigen receptor T (CAR-T) cell therapy (65). In this setting, a NGS based approach could overcome some limitations represented by unproductive IGH rearrangements, the variable and generally low amount of cfDNA extracted from plasma and a relapse with a different clone from the baseline one (19, 62).

So far, the application of ddPCR to DLBCL monitoring has been limited to given conditions. One is the monitoring of specific compartments such as the central nervous system (CNS) through analysis of the CSF (66–68). Bobillo et al. (67) characterized tumor tissue mutations by whole exome sequencing in 19 patients with DLBCL (6 restricted CNS lymphomas, 1 systemic and CNS lymphoma, 12 systemic lymphomas). Then, they tested plasma and CSF with a target specific ddPCR designed for each mutation. ctDNA was detectable at diagnosis in the CSF of all patients with primary CNS lymphoma (PCNSL), but not in patients with systemic lymphoma without CNS involvement. At variance, plasma ctDNA was detected in only 2/6 patients with restricted CNS lymphoma with lower variant allele frequencies than CSF ctDNA. CSF ctDNA resulted more sensitive than flow cytometry in documenting residual CNS disease and in 2 cases ctDNA was detected in the CSF months before the full-blown relapse (67).

Also in the experience of Ferreri et al. (68), CSF proved to be a promising compartment to screen and monitor PCNSL in 36 patients at diagnosis and 27 at relapse. A *MYD88* mutation was detectable in 72% of CSF samples by PCR and IL10 messenger RNA in 88% of newly diagnosed PCNSL, never in controls, showing an 82% biopsies-CSF concordance. The high detection rates of *MYD88* mutations in the CSF in PCNSL both at initial diagnosis and at relapse could be further improved by using ddPCR, thus becoming a potential useful tool in patients with lesions unsuitable for biopsy (68).

Another specific condition is the monitoring of expansion and persistence of CAR-T cells in DLBCL patients after infusion. Cheng et al. (69) demonstrated a consistent concordance between flow-cytometry and ddPCR in monitoring anti-CD19 CAR-T cells both *in vitro* and *in vivo*. Similar findings were reported by Monfrini et al. (70) who tested 42 patients (33 DLBCL, 8 primary mediastinal B-cell lymphomas and 1 MCL) treated with commercial anti-CD19 CAR-T cells. A unique ddPCR primer-probe assay was developed to quantify CAR vectors on genomic DNA. CAR-T cells were significantly higher in patients obtaining a CR at 10 days (mean 146 vs 18 CAR+ cells/μl, $p < 0.05$) with major magnitude of expansion at 30 days (mean area under the curve (AUC) 0-30) = 1431.2 vs 584.3; $p < 0.05$). These data were independent from the product employed. ddPCR showed a significant correlation with flow cytometry ($r=0.95$, $p < 0.0001$ by Pearson correlation) with the advantage of detecting residual CAR-T cells in samples with limited cellularity and/or cryopreserved (bag-leftovers, cryopreserved BM, biopsies, cfDNA) (70). Different assays have been developed for commercial CAR-T cell monitoring. Badbaran et al. (71) designed a single CAR primer/probe combination by sequencing the CAR construct from the lentiviral tisa-cel and axi-cel vectors and designed primers and Black hole quencher (BHQ) probes complementary to the sequences achieving excellent specificity with a detection limit sensitivity of one single CAR copy, corresponding to a sensitivity of approximately 1 in 5000 cells (0.02%) for 100 ng genomic DNA (71).

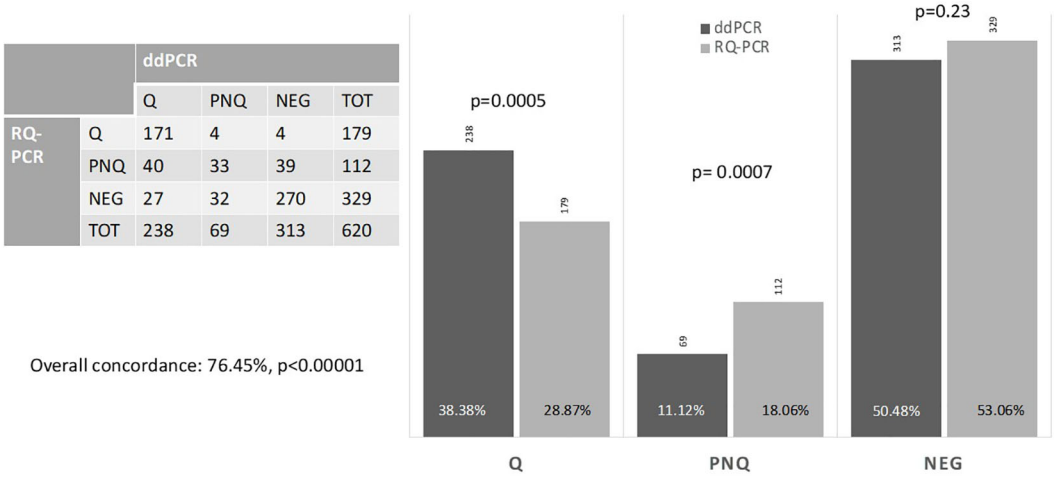
3.4 Chronic lymphocytic leukemia

Among indolent B-cell malignancies, CLL is the most frequent. The therapeutic landscape of this disease has markedly changed by the availability of targeted drug combinations and the increasing rate of deep CR. MRD monitoring in this context is acquiring progressively increasing importance (2, 72, 73). Standard MRD assessment is based on flow cytometry and on RQ-PCR with IGH ASO primers (73). NGS has also been recently employed as a promising tool that can produce reliable and accurate results in this scenario (74). Data on ddPCR MRD monitoring in CLL are scanty. Our group has conducted a comparative study of ddPCR and RQ-PCR in more than 600 baseline and MRD samples from different lymphoid malignancies, including 128 CLL samples (Figure 1) . In all disease entities investigated, a high correlation of the methods was found (76.5%) with most discordances recorded in samples with low RQ-PCR MRD levels, in which ddPCR was able to identify a quantifiable disease more reliably than RQ-PCR (75). In this experience, the advantage of this technique in diminishing the number of PNQ patients was evident (75).

Some experiences have been reported on the monitoring of mutations by ddPCR in CLL. Frazzi et al. (76) tested *TP53* exons 5-6-7 by ddPCR in 47 patients both for mutation and copy number variation. The AUCs for the assays were between 0.91 and 0.98, indicating very high sensitivities and specificities for the deletion assessment with this technique. Concordance between FISH and ddPCR was high for both non-deleted and deleted patients (93.1% and 90.0% respectively). A multiplex approach has been suggested by this experience (76).

Minervini et al. (77) validated a ddPCR based assay for c.7541-7542delCT NOTCH1 mutation. A NOTCH1 mutation was detected in a proportion of CLL cases (53.4%) higher than expected. In follow-up samples, ddPCR showed a statistically significant reduction of the NOTCH1 mutated allelic burden when measured after treatment (median fractional abundance (FA) 11.67% vs 0.09%, respectively, $p = 0.01$) (77). Hoofd et al. (78) validated a highly sensitive and quantitative ddPCR assay for the NOTCH1 delCT mutation (c.7541_7542delCT). The mutation was detected at allele frequencies as low as 0.024% in 166 CLL tested samples; 25% of unselected cases and 55% of trisomy 12 cases were positive. Association of NOTCH1 delCT and trisomy of chromosome 12 was associated to shorter overall survival (78).

In another experience from our group, mutations and deletions of *BIRC3* were tested by ddPCR in a cohort of 134 CLL with del(11q).



		ddPCR			
		Q	PNQ	NEG	TOT
RQ-PCR	Q	171	4	4	179
	PNQ	40	33	39	112
	NEG	27	32	270	329
	TOT	238	69	313	620

FIGURE 1
MRD comparison between ddPCR and RQ-PCR. At our Center, we evaluated 216 patients (113 ALL, 47 CLL, 48 FL, 8 MCL) at diagnosis and during the post-treatment follow-up, reaching a total number of 620 evaluations performed by both RQ-PCR and ddPCR, and distributed as follows: 326 ALL, 128 CLL, 142 FL, 24 MCL. The figure shows the overall concordance of the two methods and differences in defining a sample as Quantifiable (Q), Negative (NEG) or Positive Not-Quantifiable (PNQ) for MRD.

BIRC3 deletion was identified in 105/134 11q- patients (78%) and mutations occurred in 10/134 cases (7.5%), all *BIRC3* deleted, resulting in a biallelic disruption of the gene associated with a poor prognosis. *BIRC3* deletions were identified when carried by 10% of cells (79).

3.5 Other chronic lymphoproliferative disorders of B or T-cell lineage

In hairy cell leukemia (HCL), a ddPCR approach has been tested for the molecular detection and monitoring of $BRAF^{V600E}$mutation (80–82). ddPCR was retrospectively compared to RQ-PCR in 47 patients (29 HCL and 18 splenic MZL) for the detection of $BRAF^{V600E}$. The sensitivity of ddPCR was about half a logarithm superior to that of RQ-PCR (5×10^{-5} vs. 2.5×10^{-4}), with comparable specificity (81). In terms of MRD monitoring, at the end of treatment, among patients in CR, 33% were still MRD-positive by ddPCR versus 28% by RQ-PCR. In another experience, the $BRAF^{V600E}$ mutational burden has been tested in 35 HCL patients on PB and BM at diagnosis, at the time of response assessment and at relapse (82). Mean values were 12.2%, 0.02% and 16.5% respectively for PB and 23.5%, 0.26% and 13.9% for BM. In 4 out of 6 patients evaluated at response $BRAF^{V600E}$ was negative in the PB, whilst among patients with long-lasting CR after one course of cladribine the mean $BRAF^{V600E}$ was 0.05% in 4 cases and negative in 10. These preliminary results suggest that ddPCR may allow to assess the active tumor burden in HCL at different stages of the disease, to refine the response assessment and possibly to identify patients "cured" of their disease.

Limited experience is available regarding the employment of ddPCR in chronic T-cell lymphoproliferative disorders. Tanzima Nuhat et al. (83) reported a good performance of ddPCR in the screening of G17V RHOA mutations in a cohort of 67 patients with peripheral T-cell lymphomas (PTCL), 40 angioimmunoblastic and 27 PTCL-not otherwise specified (NOS), with diagnostic purposes. The ddPCR was compared to NGS: G17V RHOA mutation was detected in 27 of 67 (40.3%) patients by NGS and in 31 of 67 (46.3%) by ddPCR (83). Additionally, variant allele frequencies were highly concordant between the methods (P <.001) (83). Thus, for point mutation detection, ddPCR has a higher sensitivity that NGS, but its targeted nature has to be taken into account, since the whole spectrum of mutations can be missed. In the setting of anaplastic large cell lymphoma, ddPCR seems to be feasible for disease detection and MRD monitoring through ALK fusion transcripts (84, 85).

4 Conclusions

Based on the growing body of evidence, ddPCR may be considered as an alternative tool for molecular MRD assessment in lymphoid malignancies. Over the past 5 years, many groups have tested ddPCR for MRD evaluation and several technical advantages have been reported. The main clinical advantage provided by ddPCR is the absolute quantification of the disease, avoiding the need of the diagnostic sample dilution to build the reference standard curve, and the decrease in the number of PNQ samples, that represent a primary unmet need in the clinical practice where treatment decisions are based on MRD monitoring.

Although no guidelines for ddPCR MRD analysis and interpretation have so far been defined, a major standardization effort is underway within ESLHO (European Scientific Foundation for Laboratory Hemato Oncology) through the EuroMRD Consortium (www.euromrd.org) for its future application.

The value of ddPCR for MRD analysis needs to be conclusively documented in the context of prospective clinical trials. This will allow to define whether it could contribute to a further improvement of patients' management and outcome in different hematological malignancies.

Author contributions

GA, IDG, and IDS conceived and wrote the paper; MCa, RS, MCo, VB, and MT involved in MRD analysis in acute and chronic lymphoid malignancies; AG and RF revised and conceived the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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