Molecular measurable residual disease by immunoglobulin gene rearrangements on circulating tumor DNA predicts outcome in diffuse large B-cell lymphoma

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Abstract

In diffuse large B-cell lymphoma (DLBCL) treatment response relies on imaging. We investigated the potential value of molecular measurable residual disease (MRD) on circulating tumor DNA (ctDNA) to predict the outcome of 73 DLBCL patients. At baseline, next-generation sequencing was used to detect clonal immunoglobulin (IG) gene rearrangements on tumor biopsies (N=57) and ctDNA (N=73). MRD monitoring was applied by tracking the IG clones in ctDNA samples collected during treatment (interim) and at the end of treatment (EOT). MRD results were correlated with clinical data and radiologic disease assessment. Before treatment, clonal IG were found in 91.2% (52/57) of tumor biopsies and in 93.2% (68/73) of ctDNA samples. In paired samples, the same clonotype was found in 69.2% (36/52) of cases. At the interim analysis, ctDNA MRD was negative in 32 of 45 evaluable patients and positive in 13 of 45, correlating significantly with progression-free survival (PFS) (78.1% MRD^- *vs*. 30.8% MRD^+; $P<0.0001$) after a median follow-up of 40 months. Moreover, ctDNA MRD could stratify prognosis of 27 patients in partial response ($P=0.018$). At EOT, ctDNA MRD was negative in 37 of 47 patients and positive in ten of 47 (PFS 83.8% MRD- *vs*. 0% MRD^+; $P<0.0001$). All MRD^+ patients in complete metabolic response relapsed ($P<0.0001$). At multivariate analysis, MRD at EOT independently predicted PFS and overall survival. Monitoring IG-based ctDNA MRD during and after treatment predicts DLBCL patients' outcome. This non-invasive method should be implemented in risk-adapted clinical trials and validated as a treatment decision-making tool.

Introduction

Diffuse large B-cell lymphoma (DLBCL), the most frequent non-Hodgkin lymphoma (NHL) in the Western world, is a heterogeneous disease with genetic diversity and variable outcomes.[1-4] It arises from a mature clonal B-cell population that exhibits clonal immunoglobulin (IG) gene rearrangements. The current standard to monitor DLBCL response to therapy relies on the macro-estimation of tumor reduction by computed tomography (CT) or positron emission tomography/CT (PET/CT) scans, that can show suboptimal specificity.[5] In the last few years, the therapeutic scenario of DLBCL has changed, especially in the relapsed/refractory (R/R) setting with the introduction of novel targeted agents, namely bispecific monoclonal antibodies and cellular-based immunotherapies such as the chimeric antigen receptor (CAR) T-cell therapy, leading to higher rates of complete responses and longer survival.[6-8] Consequently, the possibility of monitoring measurable residual disease (MRD) has become more relevant to further personalize treatment. One major obstacle is the absence of circulating neoplastic cells. The development of robust techniques, including next-generation sequencing (NGS), together with the discovery of circulating tumor DNA (ctDNA), shed into the bloodstream by tumor cells undergoing apoptosis, have opened the door to leukemia-like research in NHL.[9-13] Some research groups have investigated the relationship between baseline levels of ctDNA and conventional markers of tumor burden and its role as a prognostic biomarker.[14-18] More importantly, ctDNA has been used as a non-inva-

sive tool to track recurrently mutated genes in DLBCL, allowing capture of the mutational landscape beyond the intra-tumoral heterogeneity (i.e., liquid biopsy), to monitor the molecular disease during and after treatment, and to evaluate clonal evolution.[9-13,16-18]

Disease-specific clonal rearrangement of the IG genes can be identified on the diagnostic tumor tissue using a single NGS assay that utilizes universal primers for all possible rearrangements. The specific clonotype of each patient can subsequently be tracked for disease monitoring.[19-22]

The aim of this study was to evaluate if ctDNA MRD monitoring based on IG gene rearrangements may improve DLBCL treatment response assessment and outcome prediction. The results obtained indicate that ctDNA monitoring may indeed be proposed as a decision-making tool to guide lymphoma treatment in the future.

Methods

Patients and samples

We performed a multicenter retrospective study on a cohort of 73 consecutive newly diagnosed DLBCL patients (41 from Rome and 32 from Novara), enrolled between September 2015 and March 2021. Inclusion criteria were: i) age ≥18 years; ii) histological diagnosis of DLBCL (including DLBCL not otherwise specified [NOS]; transformed indolent B-cell lymphoma; high-grade B-cell lymphoma with rearrangements of *MYC* and *BCL2* and/or *BCL6*; excluding primary mediastinal B-cell lymphoma); iii) previously untreated patients eligible for curative treatment. Patients were all treated according to guidelines in force at the time of diagnosis, with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) or R-CHOP-like regimens.[23,24] The following biological materials were collected: diagnostic formalin-fixed paraffin embedded (FFPE) tumor biopsies (for genomic DNA, gDNA) and 30 mL of peripheral blood (PB) for ctDNA before the start of treatment, throughout the first three cycles of therapy (cycle 2 day 1, C2D1, for the Novara cohort, and cycle 4 day 1, C4D1, for the Rome cohort), and at the end of treatment (EOT). Early (mid-treatment) and final (EOT) disease response were assessed by CT and PET/CT, respectively. Interim CT scans were performed between three to four cycles and interpreted according to Cheson's criteria.[5] Response to treatment was defined according to the Lugano criteria.[25] Relapse within 12 months from treatment initiation was considered early relapse.

Patients provided written informed consent, the study respected the principles of the Declaration of Helsinki and was approved by the ethical committee of AOU Policlinico Umberto I, Rome (Prot n. 877/19 rif. CE 5629, AIRC 21198). The endpoints of the study were: i) to test the applicability of NGS for IG heavy (IGH) and κ light (IGK) chain gene rearrangements in FFPE diagnostic tumor biopsies and ctDNA extracted from PB; ii) to explore if tracking clonal IGH/IGK rearrangements by NGS can be a non-invasive method to study MRD in longitudinal ctDNA samples during and after treatment; iii) to study the correlation of IG-based ctDNA MRD with clinical data and radiologic disease assessment of early (CT) and final response (PET/CT).

Experimental procedure

FFPE diagnostic tissue from lymph node biopsies or from other extra nodal tissue excisions was available in 57 patients. In the remaining 16 cases, the diagnostic biopsy was not available. All samples were sectioned in 3-4 µm thick sections. gDNA was extracted from FFPE tissue with the automated Maxwell RSC DNA FFPE Kit (Promega, Madison, WI), according to the manufacturer's instructions. DNA amounts were assessed using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA). The Specimen Control Size Ladder master mix (Invivoscribe Inc, San Diego, CA) was then used to ensure that the quantity and quality of DNA was adequate. A standardized approach was used to extract cell-free DNA (cfDNA) from the plasma of 73 patients (*Online Supplementary Appendix*).[26] Plasma samples collected from seven healthy donors were also analyzed (*Online Supplementary Appendix*).

The LymphoTrack IGH framework region (FR) 1/2/3 and IGK assay panels (Invivoscribe) were used to analyze the diagnostic FFPE and plasma samples aiming at detecting clonotypic rearrangements and at identifying the DNA sequence specific for each clonal gene rearrangement. Initially, the samples were analyzed for IGH rearrangements. Cases without a detectable clonal sequence through IGH-targeted testing were subsequently tested for IGK rearrangements. IGK was not routinely tested in plasma due to the greater reliability of the IGH marker compared to IGK for MRD monitoring.[27] It was only tested in the 16 cases for which a biopsy was unavailable and in case of a clonality discordance between tumor biopsy and plasma.

Identification of clonality followed a three-step workflow: i) polymerase chain reaction (PCR) amplification; ii) NGS; and iii) bioinformatics analysis (LymphoTrack Dx MiSeq software v2.4.3). Multiplexed libraries were sequenced using 500-bp paired-end runs on a MiSeq sequencer (Illumina, San Diego, CA), aiming at achieving 1 million reads per sample. The lymphoma-derived sequences were then used as target to assess MRD on ctDNA.[26,28] A more detailed description of the methods used for MRD detection can be found in the *Online Supplementary Appendix*. In case of clonal marker discordancy between plasma and tumor biopsy, the sequencing data from both compartments were examined using a diverse bioinformatic analysis to determine if the detected clonal sequences were present in matched tissues at lower burdens than the clonal definition.

Statistical analysis

Univariate analysis was performed to assess normal distri-

bution of characteristics between the two centers. Pearson correlation Student *t* test and Mann-Whitney were used to compare continuous variables, and a χ^2 test was performed to correlate ctDNA clonotype identification with categorical variables.

Survival probabilities were estimated using the Kaplan-Meier method; the log-rank test was used to determine the significance of the difference between Kaplan-Meier curves. Two survival endpoints were considered: progression-free survival (PFS), where an event was defined as progression or relapse from diagnosis, and overall survival (OS), where an event was defined as death resulting from any cause from diagnosis. Regression analysis of multiple covariates was conducted using the Cox proportional hazard model. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were reported. All *P* values were two-tailed. Statistical analyses were carried out using SPSS Statistics v.25.0 (IBM Corp., Armonk, NY).

Results

Patients' population

A series of 73 consecutive untreated DLBCL patients were enrolled and followed after treatment with R-CHOP/R-CHOP-like regimens. Overall, two patients showed refractory disease during treatment, 18 had a relapse after a median of 20.5 months from the start of treatment (range, 7.5-53). In 14 patients, relapse occurred within 24 months and in 6 within 12 months from the start of treatment (early relapse). Two patients (2.7%) had a central nervous system (CNS) involvement at the time of systemic relapse after 8 and 21 months from the start of treatment, respectively. All relapses were documented through imaging and clinical assessment, in this series no patient underwent a new biopsy.

After a median follow-up of 40 months (range, 1-72), OS was 76.6% (17/73) and PFS 72.6% (20/73).

Thirteen of the 20 R/R patients (65%) died of disease progression. Four deaths were observed in the disease-free group of patients (COVID-19 1, stroke 1, second malignancy 1 and comorbidities 1). At univariate analysis, the following risk factors were confirmed as clinical predictors of PFS: ECOG PS (*P*=0.05), IPI score (*P*=0.024) and stage (*P*=0.011). Patients' characteristics are listed in Table 1A, patients' outcome in Table 1B.

Immunoglobulin analysis of tumor biopsy

The FFPE tumor biopsy available in 57 of 73 DLBCL patients was subjected to IG-NGS with the IGH (FR1/2/3) and IGK assay panels. At least one dominant tumor-specific clonotype was identified in 91.2% of cases (52/57). Forty-one patients (71.9%) had clonal sequences detectable by IGH primers and 11 (19.3%) by IGK primers. In the remaining five cases (8.8%), both IGH and IGK clonality assessment showed polyclonal patterns and an index clone was not identified; in these cases with undetectable clonality, DNA was suboptimal in terms of quantity and quality (highly fragmented).

Of the 52 patients with a tumor-specific clonotype, most (65%, 34/52) were successfully characterized by one assay (1 for IGH-FR1, 12 for IGH-FR2, 10 for IGH-FR3 and 11 for IGK), 13 (25%) were clonal for two assays (12 for IGH-FR2/3 and 1 for IGH-FR1/2) and five (10%) were clonal for all three IGH assays (FR1/2/3).

Of these 52 cases, 43 (82.7%, 43/52) showed a unique clonal sequence, six (11.5%) showed two unrelated clonal sequences with different IGH and IGK V-J gene segment usage, while three cases (5.8%) showed two clonal sequences with identical IGH V-J segment usage but differing by ≥2 nucleotide substitutions, with different CDR3 regions. Clonal detection was independent from biopsy site and extra nodal disease involvement at PET/CT.

Pre-treatment circulating tumor DNA immunoglobulin analysis

Basal plasma samples were collected in close temporal proximity of the tumor biopsy, before starting treatment. The cfDNA of the 73 DLBCL patients was subjected to IG-NGS with IGH (FR2/FR3) and IGK assay panels. The median plasma cfDNA concentration was 10 ng/mL (range, 1.89-211) and ctDNA clonality was detected in 68 of 73 patients (93.2%). Most plasmatic ctDNA (75%, 51/68) were clonal by one assay (20 for IGH-FR2, 20 for IGH-FR3 and 11 for IGK), 14 (21%, 14/68) were clonal by two assays (13 for IGH-FR2/3 and 1 for IGH-FR3 and IGK) and three (4%, 3/68) were clonal by three assays (IGH-FR2/3 and IGK).

The clonal IG rearrangements identified in ctDNA at diagnosis were then matched with lymphoma-specific clonotype identified in the paired tumor biopsy of the 52 patients. Identical IG markers were found in 36 of 52 cases (69.2%). In 11 of 52 cases (21.2%) a different clonal marker was found in the plasma compared to the tumor biopsy and in five of 52 (9.6%) cases no clonality was detected in the cfDNA (*Online Supplementary Table S1*). Among the 11 discordant cases, nine (82%) had IGH rearrangements and only two (18%) IGK rearrangements. No case showed double IGH-IGK rearrangements. Moreover, the clonal sequences detected in ctDNA were absent in the matched tumor tissue, even at lower burden, and *vice versa*. cfDNA concentrations at baseline were measured and correlated to clinical outcome. The analysis revealed that a cfDNA concentration ≥0.7 ng/μL was predictive of a worse outcome (ROC curve). Specifically, in the 73 patients analyzed, those with cfDNA levels ≥0.7 ng/μL had a median PFS of 63.8 months compared to 88.5 months for patients with lower cfDNA levels (*P*=0.022). When examining the subset of 57 patients (biopsy sample available), the median PFS was 57.5 months *versus* 82.4 months (*P*=0.038). Additional details are available in the *Online Supplementary Appendix*.

Table 1A. Patients' characteristics.

Characteristics	Rome cohort N=41	Novara cohort N=32	Entire cohort N=73	*P*
Sex				
Male	24 (58.5)	14 (43.8)	38 (52.1)	0.2
Female	17 (41.5)	18 (56.3)	35 (47.9)	
Diagnosis				
DLBCL, NOS	27 (65.9)	22 (68.8)	49 (67.1)	0.9
HGBCL with rearrangements*	5 (12.2)	5 (15.7)	10 (13.7)	
Transformations of iBCL	5 (12.2)	3 (9.4)	8 (11.0)	
HGBCL, NOS	4 (9.8)	2 (6.3)	6 (8.2)	
Age in years				
Median (range)	63.9 (26.2-78.6)	69 (19-85)	65.19 (19-85)	0.12
Age in years				
<65	23 (56.1)	12 (37.5)	35 (47.9)	0.11
≥65	18 (43.9)	20 (62.5)	38 (52.1)	
Extra nodal disease				
No	19 (46.3)	8 (25.0)	27 (37.0)	0.07
Yes	22 (53.7)	24 (75.0)	46 (63.0)	
B symptoms				
No	33 (80.5)	25 (78.1)	58 (79.5)	0.8
Yes	8 (19.5)	7 (21.9)	15 (20.5)	
Elevated LDH				
Yes	18 (43.9)	20 (62.5)	38 (52.1)	0.11
No	23 (56.1)	12 (37.5)	35 (47.9)	
ECOG PS				
0-1	35 (85.4)	29 (90.6)	64 (87.7)	0.49
2-4	6 (14.6)	3 (9.4)	9 (12.3)	
IPI risk				
Low	18 (43.9)	10 (31.3)	28 (38.4)	0.68
Low-intermediate	7 (17.1)	7 (21.9)	14 (19.2)	
High-intermediate	6 (14.6)	7 (21.9)	13 (17.8)	
High	10 (24.4)	8 (25.0)	18 (24.7)	
Stage				
I-II	19 (46.3)	13 (40.6)	32 (43.8)	0.62
III-IV	22 (53.7)	19 (59.4)	41 (56.2)	
Treatment plan				
R-CHOP-21	34 (82.9)	23 (71.9)	57 (78.1)	0.42
Lower intensity than R-CHOP-21°	2 (4.9)	4 (12.5)	6 (8.2)	
Higher intensity than R-CHOP-21#	5 (12.2)	5 (15.6)	10 (13.7)	
Follow-up in months				
Median (range)	39.02 (5-63.07)	42.5 (1.47-72.3)	39.1 (1.47-72.3)	0.78
Clonal marker on tumor biopsy				
No	2 (6.5)	3 (11.5)	5 (8.8)	0.49
Yes	29 (93.5)	23 (88.5)	52 (91.2)	
Clonal marker on ctDNA				
No	3 (7.3)	2 (6.3)	5 (6.8)	0.97
Different from tumor biopsy	6 (14.6)	5 (15.6)	11 (15.1)	
Yes	32 (78.0)	25 (78.1)	57 (78.1)	

Data is presented as N (%) unless otherwise indicated. *Rearrangements of MYC and/or BCL2 and/or BCL6. °R-CHOP-like regimen with lower intensity: R-mini-CHOP/COMP. #Higher intensity: R-CHOP14-CODOX/M-IVAC. Thirteen patients received radiotherapy (plasma samples were collected at the end of chemoimmunotherapy, before radiotherapy). DLBCL: diffuse large B-cell lymphoma; NOS: not otherwise specified; iBCL: indolent B-cell lymphoma; HGBCL: high grade B-cell lymphoma; LDH: lactate dehydrogenase; ECOG PS: Eastern Cooperative Oncology Group performance status; IPI: international prognostic index; R-CHOP: rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone; ctDNA: circulating tumor DNA.

Table 1B. Patients' outcome.

Characteristics	Rome cohort	Novara cohort	Entire cohort	*P*
Response at interim (Cheson Criteria) CR PR SD/PD	 19 (47.5) 20 (50.0) 1 (2.5)	 7 (25.0) 19 (67.9) 2 (7.1)	 26 (38.2) 39 (57.4) 3 (4.4)	0.14
MRD at interim time points Negative Positive	 29 (72.5) 11 (27.5)	 14 (77.8) 4 (22.2)	 43 (74.1) 15 (25.9)	0.67
MRD at EOT Negative Positive	 31 (79.5) 8 (20.5)	 23 (88.5) 3 (11.5)	 54 (83.1) 11 (16.9)	0.34
Response at EOT by PET/CT CR PR/SD PD	 35 (85.4) 4 (9.8) 2 (4.9)	 29 (90.6) 1 (3.1) 2 (6.3)	 64 (87.7) 5 (6.8) 4 (5.5)	0.56
CR CR No CR	 35 (85.4) 6 (14.6)	 29 (90.6) 3 (9.4)	 64 (87.7) 9 (12.3)	0.49
R/R disease No progressive disease R/R after first line	 28 (68.3) 13 (31.7)	 25 (78.1) 7 (21.9)	 53 (72.6) 20 (27.4)	0.35
Status at follow-up Alive Dead	 30 (73.2) 11 (26.8)	 26 (81.3) 6 (18.8)	 56 (76.7) 17 (23.3)	0.41

Data is presented as N (%) unless otherwise indicated. Full count is not available for all variables due to lack of data. CR: complete response; PR: partial response; SD/PD: stable/progressive disease; MRD: minimal residual disease; EOT: end of treatment; PET/CT: positron emission tomography/computed tomography; R/R: relapsed/refractory.

Correlation of baseline circulating tumor DNA clonotype with clinical variables

The clonotype detection on ctDNA was correlated with pre-treatment clinical characteristics including histology, stage, lactate dehydrogenase (LDH), international prognostic index (IPI), B symptoms, extra nodal disease, and Eastern Cooperative Oncology Group Performance Status (ECOG PS). No statistically significant differences were found between ctDNA clonotype detection and histology, B symptoms, ECOG PS. In contrast, we recorded a significant association of clonality detection with IPI (intermediate/high) (*P*=0.02), elevated LDH (*P*=0.01, and stage (III/IV) (*P*=0.05). We found only a trend with the presence of extra nodal disease (*P*=0.11).

The five patients with no clonal marker detectable on ctDNA had favorable characteristics such as localized stage (80%, 4/5), low-risk IPI (100%, 5/5), absence of B symptoms (100%, 5/5), normal LDH (100%, 5/5) and absence of extra nodal disease (80%, 4/5). All are alive and in complete response (CR).

The 11 patients with a different clonal marker found on ctDNA compared to the tumor biopsy also had favorable characteristics, such as absence of B symptoms (100%, 11/11), ECOG PS 0-1 (100%, 11/11), normal LDH (72.7%, 8/11), low-risk/low-intermediate IPI (90.9%, 10/11). Among them, no relapse was observed. Additional details are available in the *Online Supplementary Appendix* and *Online Supplementary Table S2*.

Longitudinal monitoring of circulating tumor DNA measurable residual disease

The association between MRD and clinical data was assessed in 52 patients. Patients with discordant clonotype (11 cases) or absence of clonality on plasma (5 cases) or on biopsy (5 cases) were excluded (*Online Supplementary Figure S1*).

Longitudinal MRD analysis was performed on ctDNA samples during chemotherapy and at EOT. A total of 92 longitudinal plasma samples were studied: 45 at interim with two different time points (15 at C2D1, 30 at C4D1) and 47 at EOT, according to material availability.

The median plasma cfDNA concentration in the interim and EOT samples was 9.2 ng/mL (range, 0.99-131) and 5.6 ng/mL (range, 1.54-65.2), respectively.

Circulating tumor DNA measurable residual disease during treatment

During treatment, ctDNA MRD analysis was possible in 45 patients: in 71.1% (32/45) the basal tumor clonotype disappeared and in 28.9% (13/45) it persisted. Interim MRD correlated significantly with PFS at the last follow-up, being

78.1% for MRD⁻ *versus* 30.8% for MRD⁺ patients (*P*<0.0001), with an estimated median PFS of not reached (NR) *versus* 20.7 months (OR=1.97; range, 1.1-3.5) (Figure 1A). The predictive power of interim MRD was confirmed at both time points (C2D1 *P*=0.001, C4D1 *P*=0.037). Additional details are available in the *Online Supplementary Figure S2A, B*.

Overall, among the 13 MRD⁺ patients, nine (69.2%) progressed clinically, after 4.5-52 months from diagnosis, with one being refractory and four (30.8%) experiencing an early relapse. By contrast, only seven of the 32 MRD⁻ patients (21.9%) progressed after 16.6-48.5 months from diagnosis; none within 12 months.

Interim CT was available for 44 of 45 patients: 34.1% patients obtained a CR, 61.4% a partial response (PR) and 4.5% a stable/progressive disease (SD/PD).

Overall, the concordance between ctDNA MRD and radiological disease detected by interim CT was 52.3% (CR/MRD⁻ or PR-SD-PD/MRD⁺). Interim ctDNA MRD could stratify the prognosis of the 27 patients in PR, as shown in Figure 1B, with a PFS of 78.9% for MRD⁻ patients *versus* 37.5% for MRD⁺ patients (*P*=0.018). Only four PR patients showed undetectable ctDNA and subsequently relapsed, with an estimated median PFS of 57 months (range, 32-61).

Circulating tumor DNA measurable residual disease at end of treatment

At EOT, ctDNA MRD analysis was performed in 47 patients: 78.7% (37/47) were MRD⁻ and 21.3% (10/47) were MRD⁺, with a PFS of 83.8% for MRD⁻ patients *versus* 0% for MRD⁺ patients (*P*<0.0001) (Figure 2A). All ten MRD⁺ patients relapsed, three (30%) within the first 12 months from diagnosis and four (40%) between 12 and 24 months. By contrast, only six of 37 MRD⁻ patients (16.2%) progressed after 19.5-48.5 months from diagnosis; none within 12 months.

Overall, plasma ctDNA evaluation had no false positives at EOT, with a positive predictive value of 100%; indicating that all patients with a positive ctDNA test experienced disease progression. Conversely, the negative predictive value was 84%, reflecting that only 16% of patients with a negative ctDNA test experienced disease progression.

According to PET/CT disease assessment of final response, 85.1% patients (40/47) achieved a CR, 10.6% (5/47) had a PR/SD and 4.3% (2/47) showed a PD. A complete metabolic response at EOT was confirmed as a PFS predictor at univariate analysis (*P*=0.001). Overall, the concordance between ctDNA MRD and PET/CT was 72.3% (CR/MRD⁻ or PR-SD-PD/MRD⁺).

A first subanalysis was performed in the 40 patients achieving a complete metabolic response by PET/CT; eight (20%) were ctDNA MRD⁺ and all relapsed (0% *vs*. 87.5%, *P*<0.0001), two experiencing an early relapse (Figure 2B). Only one PR patient was MRD⁺ by PET/CT and experienced a relapse.

A second subanalysis was performed to assess the impact of the kinetic of the detectable IG marker between interim time points and EOT in 42 patients in which MRD analysis was performed both during and after treatment: 61.9% (26/42) were double MRD⁻ and 11.5% (3/26) experienced a relapse, 23.8% (10/42) were MRD⁺ at EOT and all relapsed

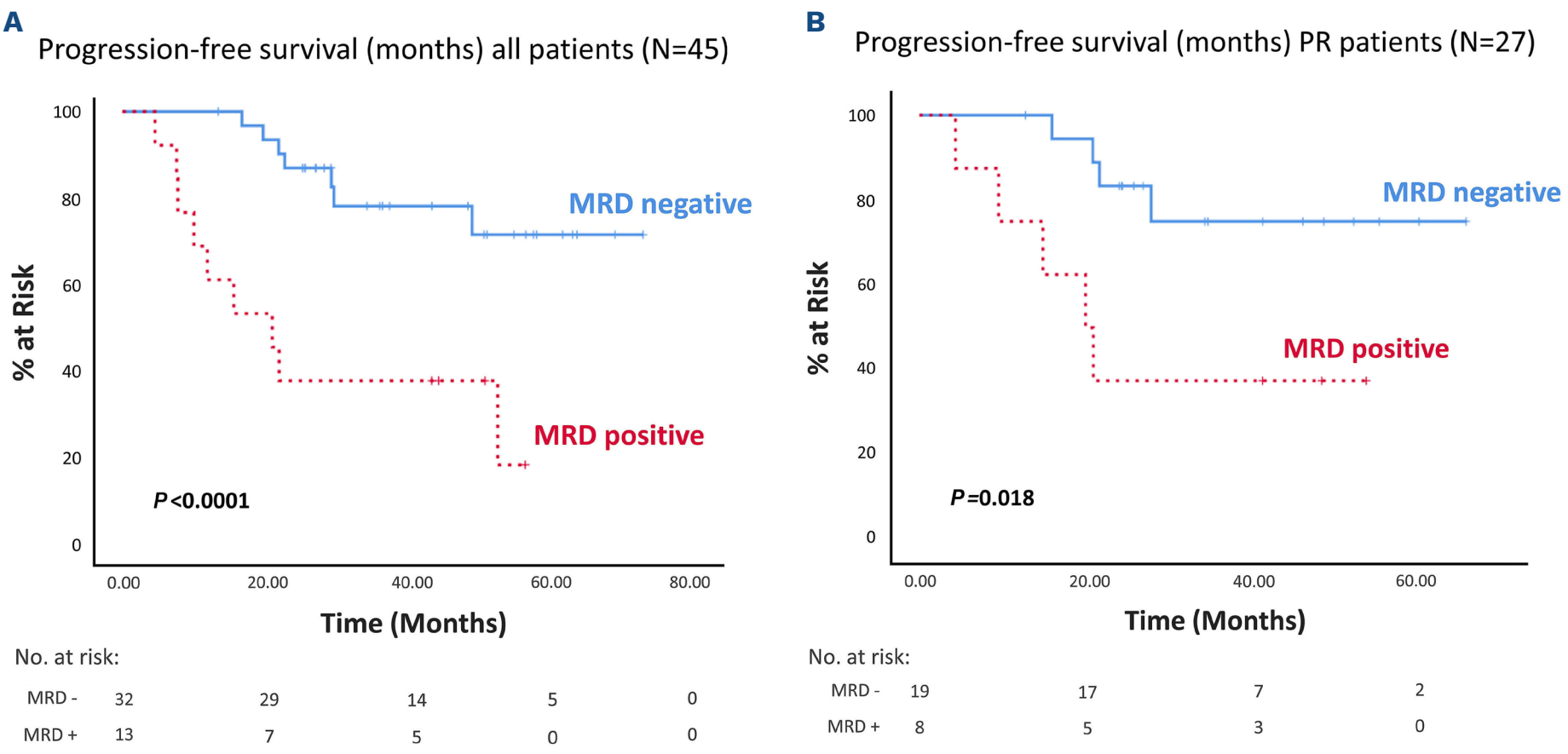


Figure 1. Prognostic value of molecular measurable residual disease on circulating tumor DNA during treatment. (A) Kaplan-Meier estimates of progression-free survival (PFS) from diagnosis for patients evaluated at interim time points stratified by measurable residual disease (MRD) positivity or negativity are shown. (B) Kaplan-Meier estimates show the PFS of patients in partial response at the interim computed tomography scan stratified by circulating tumor DNA MRD status.

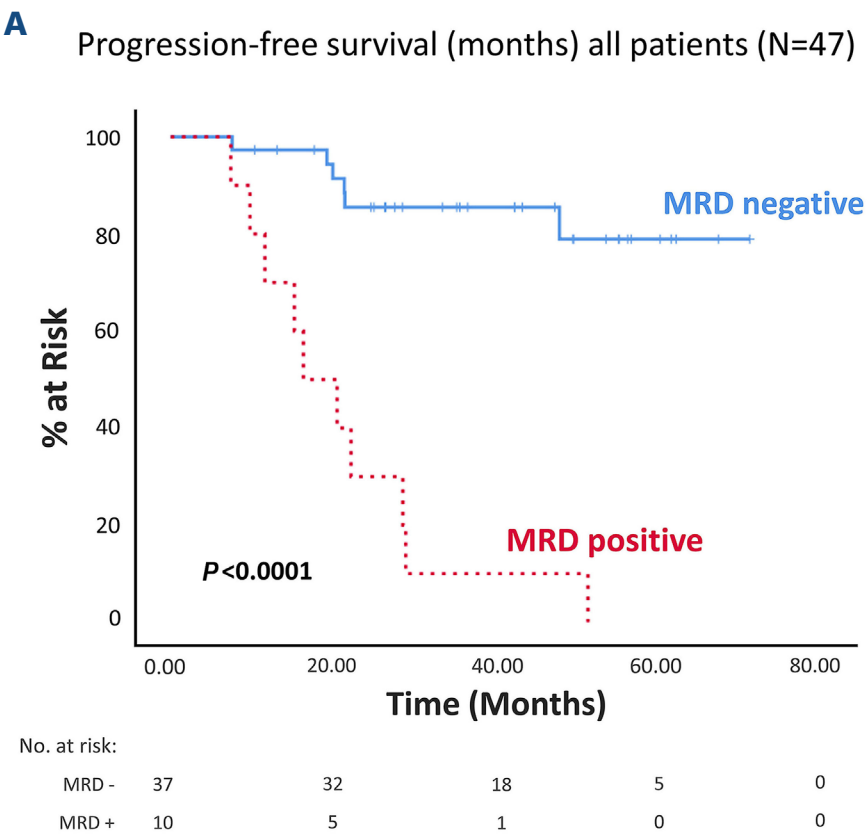
irrespective of the interim MRD status; 14.3% (6/42) were MRD$^+$ at interim evaluation and MRD$^-$ at EOT, and 33.3% (2/6) relapsed (P<0.0001; Figure 2C). In particular, six patients had double MRD$^+$ at interim/EOT: three relapsed within 12 months (7.5, 9.9, 11.7, respectively), one at 15.4 months, one at 20.7 months and one at 52 months.

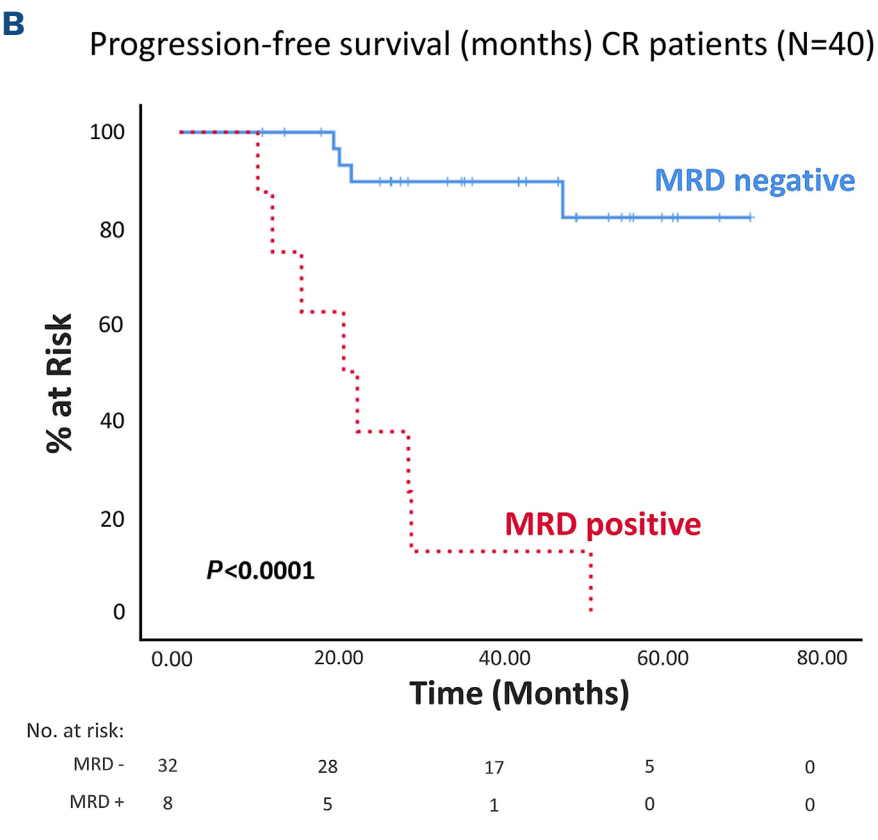
Multivariate analysis

Cox regression multivariate analysis (MVA) was performed combining all significant risk factors for PFS, which included: response at interim CT, response at EOT PET/CT, ctDNA MRD during treatment and ctDNA MRD at EOT. Interim MRD (HR=4.2; 95% CI: 1.3-13.4), metabolic response at EOT (HR=4.76; 95% CI: 1.3-17.3), and EOT MRD (HR=24.5; 95% CI: 5.9-100.8) were confirmed as independent prognostic factors, with the latter having the highest HR on PFS (P<0.0001).

Overall survival analysis

At univariate analysis, ECOG PS 2-4 (P=0.05), response at interim CT (P=0.01), response at EOT PET/CT (P=0.05), ctDNA MRD during treatment (P=0.001) and ctDNA MRD at EOT (P<0.0001) were significant risk factors for OS and included in MVA. Interim MRD$^-$ patients presented at the





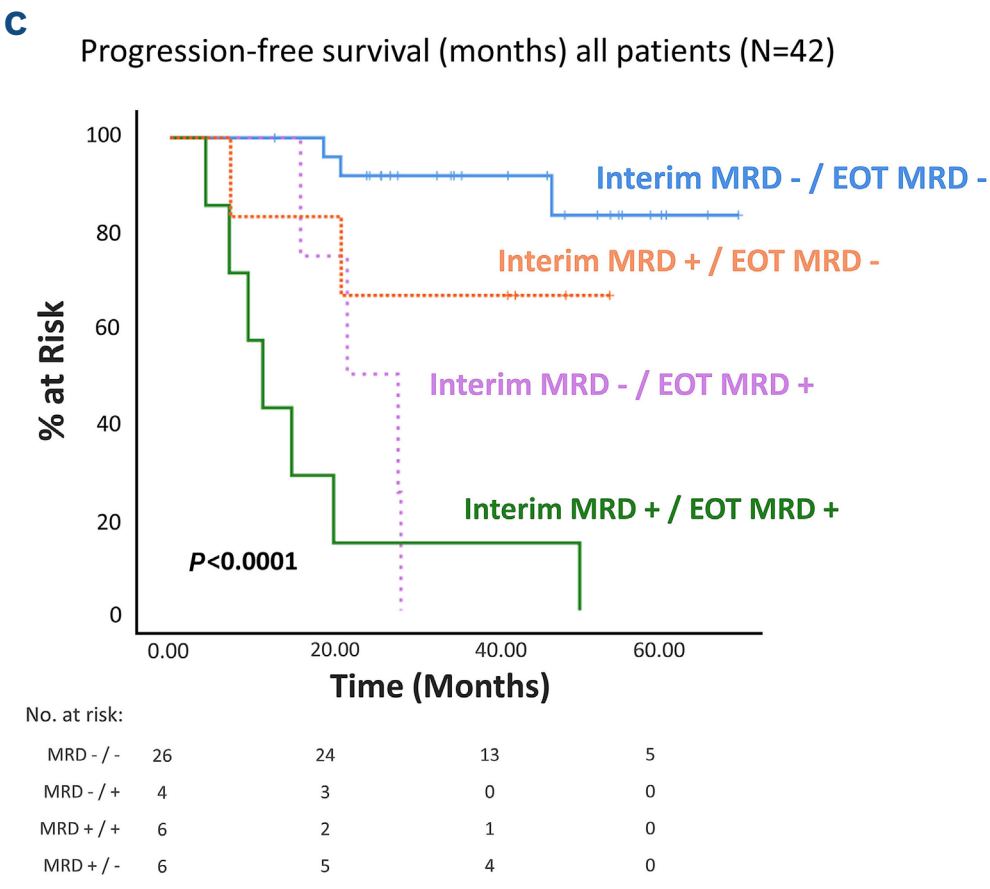


Figure 2. Prognostic value of molecular measurable residual disease on circulating tumor DNA at the end of treatment. (A) Kaplan-Meier estimates of progression-free survival (PFS) from diagnosis for patients evaluated at end of treatment (EOT) stratified by measurable residual disease (MRD) status (positive or negative) are shown. (B) Kaplan-Meier estimates show the impact of MRD status on PFS in patients achieving final complete metabolic response (CR) according to positron emission tomography/computed tomography. (C) Kaplan-Meier estimates show the PFS of patients based on the combination of MRD status during and after treatment. Patients are categorized into 4 groups: double negative MRD (-/-), double positive MRD (+/+), MRD positive during treatment and MRD negative at EOT (+/-), MRD negative during treatment and MRD positive at EOT (-/+).

last update an OS of 84.4%, compared to 38.5% for MRD$^+$ patients (P<0.0001). MRD at EOT significantly predicted a worse OS in MRD$^+$ patients (20%), compared to 86.5% for MRD$^-$ patients (P<0.0001). The estimated median OS was 32 months for MRD$^+$ patients at interim time points and 30 months for those MRD$^+$ at EOT, and not reached for the MRD$^-$ patients at both time points. We performed a multivariate analysis that confirmed the predictive power of these parameters. MRD positivity at interim (HR=16.7; 95% CI: 1.7-160.1; P=0.014) and at EOT (HR=4.9; 95% CI: 1.1-20.6; P=0.02) and high ECOG PS (HR=14.9; 95% CI: 1.5-149.5; P=0.02) independently predicted a shorter OS.

Discussion

Our retrospective observational study shows that in DLBCL patients the detection of a clonal marker and MRD analysis can be effectively applied to the plasma compartment (ctDNA) through an IG-based NGS approach. This method has been tested and validated on gDNA in other neoplasms, demonstrating high sensitivity and specificity for initial clonal characterization and disease monitoring compared to conventional methods.[29-32] At baseline, analysis of the IG gene rearrangements on the FFPE tissue biopsy and on pre-treatment plasma samples allowed to identify a clonal marker in 91.2% and 93.2% of patients, respectively. The utilization of all assay panels (FR1/2/3 and IGK) increased the detection rate of clonality in both compartments. Paired analysis of tumor tissue and plasma sample was possible in 52 cases showing consistent IG clonality in 36 (69.2%). In five cases, no clonality was found in ctDNA, suggesting limited disease spread, while a different clonal rearrangement was identified in 11 cases. Both subgroups had favorable clinical characteristics and outcome, indicating a potential clinical predictive value of the discordance. In five cases, a clonal marker was solely detected in the plasma compartment, and two of five patients relapsed; however, the absence of clonality in the tumor biopsy was attributed to low quality DNA, precluding further considerations. Consequently, these 21 cases were excluded from the MRD outcome analysis, the primary endpoint of this study. Overall, the total amount of cfDNA at baseline was predictive of PFS.

PET/CT is the recommended imaging strategy for treatment response assessment,[5,6] with the most accurate prognostic classification achieved by the EOT PET/CT.[33] Interim PET/CT conducted after two cycles of treatment has been explored for the early identification of chemotherapy-refractory as well as good-risk patients, but it is not currently used in the clinical practice.[34,35] It is also known that imaging techniques are limited by low sensitivity and/or specificity.[33-35] Our findings show that MRD analysis on ctDNA could overcome many of these limitations and refine the imaging-based evaluation of response, although nowadays a biopsy-proven relapse remains the gold standard.

MRD positivity at interim time points emerged as an independent prognostic factor especially in patients fulfilling Cheson's criteria of PR at interim CT (C2D1 P<0.0001, C4D1 P=0.037), indicating that an early MRD clearance during treatment is associated with a significantly favorable prognosis. These results align with other studies demonstrating that a decrease in ctDNA levels at early time points predicts a favorable outcome.[17-20]

At EOT, both MRD and PET/CT were independent predictors of PFS in multivariate analysis, but ctDNA MRD exhibited a higher predictive power (HR=24.5 for positive MRD *vs*. HR=4.76 for positive PET). MRD positivity predicted relapse in 67% of patients achieving a complete metabolic response, underscoring its relevance as a complement to PET/CT, with high specificity.

An increasing number of studies have explored MRD monitoring in DLBCL using IG-NGS or targeted-gene mutation approaches, all of which provided insights into patients' outcomes.[13] The first two studies based on IG ctDNA monitoring suggested that patients with undetectable IG rearrangement on ctDNA after two cycles of therapy had a longer PFS compared to ctDNA positive patients.[19,20] More recently, IG ctDNA MRD positivity in the apheretic stem cell collection of R/R DLBCL patients undergoing autologous transplantation was predictive of both PFS and OS.[21] Also in patients treated with CAR T cells IG-MRD on ctDNA was predictive of clinical outcome,[22] and could help to clarify false PET-positive signals in the early assessment after infusion.[36] The latter studies[21,22,36] used the clonoSEQ IG-MRD NGS assay, commercially available and approved in the US to test bone marrow/PB in patients with multiple myeloma (MM), B-cell acute lymphoblastic leukemia (B-ALL) and chronic lymphocytic leukemia (CLL).

On the other hand, MRD monitoring by targeted-gene mutations in DLBCL was first evaluated by applying a CAPP-sequencing approach.[16,17] More recently, a CAPP-sequencing MRD monitoring on ctDNA has been used in the large Polarix trial, comparing R-CHOP *versus* polatuzumab-R-CHP at C5D1 and EOT.[37] Patients with undetectable MRD at both C5D1 and EOT showed a prolonged 3-year PFS and OS, irrespective of the treatment arm. Similar to our findings, ctDNA MRD refined the prognostic significance of the complete metabolic response at the EOT PET, but only in the experimental arm and not in the R-CHOP arm. A novel PhasED-sequencing approach has been proposed to overcome the low sensitivity of CAPP-sequencing.[18,38] In Europe, the EuroClonality NGS DNA Capture (EuroClonality-NDC) assay, released to detect clonal Ig and/or T-cell receptor gene rearrangements, translocations, copy-number alterations, and somatic mutations in lymphoproliferative disorders,[39] was applied to plasmatic ctDNA in DLBCL[40] with results mostly in agreement with ours. A baseline molecular marker (either a single nucleotide variant [SNV] and/or translocation and/or clonal IG gene rearrangement)

was identified from the plasma in 90% of 68 patients. As for other studies, pre-treatment ctDNA levels were significantly associated with known clinical parameters of tumor burden (LDH, stage and IPI).[10,17,22,40] In 19 cases with paired sequencing of lymphoma tissue, 19% of variants were only detected in tissue and 8% of SNV were solely detected in ctDNA, mirroring, as in our study, the sensitivity limitation of ctDNA assay on the one hand and the theoretical advantage of ctDNA to capture spatial heterogeneity on the other hand. IG rearrangements resulted concordant between tissue and plasma in 84% of 19 cases. Both a quantitative 'major molecular response' (MMR), defined as a 2.5-log reduction in ctDNA after two treatment cycles, and a 'qualitative MMR', defined as the undetectable structural variants from plasma post-cycle two, were predictive of a prolonged PFS. Integrating MMR with interim PET refined PFS prediction. At variance from ours, in this paper[40] no EOT MRD evaluation was performed.

Finally, the concept of a dynamic measure of a given patient's risk throughout the course of the disease, based on serially collected longitudinal data, has gained increasing relevance for a superior outcome prediction.[41] Indeed, the Continuous Individualized Risk Index (CIRI) score is updated dynamically as additional information becomes available, such as MRD measure by ctDNA or interim imaging studies. The two-point MRD measure suggested by our paper and by others[37] supports the need of a dynamic evaluation of patients' prognosis.

Our results add to the literature testing in a real-life context of DLBCL another IG-NGS assay on ctDNA. Testing ctDNA is a feasible non-invasive and dynamic method that can be used as often as necessary to detect subclinical disease in longitudinally monitored tumor-related clones. We obtained the expected amount of cfDNA reported in the literature, with no samples excluded from the MRD analysis for insufficient quantity. However, the need of diagnostic tumor tissue still remains.

In the clinical setting, the possibility of MRD on ctDNA in identifying false negative CT or PET/CT results at interim time points and EOT, respectively, opens the scenario of pre-emptive immunotherapy strategies. Moreover, the early identification of poor responders to first-line therapy would enable the use of second-line CAR T cells in patients with better clinical conditions and lower disease burden.

This study has some limitations: the relatively limited sample size, the lack of paired tumor tissue screening for all patients, the lack of an absolute quantification of MRD. Thus, more work needs to be done to optimize the proposed methodology and confirm its potential in larger series of patients. Moreover, comparative analyses are required across different ctDNA-MRD approaches in order to identify the most suitable for clinical purposes. However, the detected IG clone in ctDNA proved positive at EOT in all R/R patients and negative in most of non-relapsing patients, indicating a homogenous clinical behavior of MRD^+ and MRD^- patients at EOT. This implies that despite the relatively low sensitivity, IG clone detection is clinically relevant and reflects the persistence of the disease.

The broad application of this and other testing modality in the routine practice is hindered by the lack of standardized guidelines. Indeed, despite the data provided in the literature in the last decade, we still face the absence of an established and standardized tool to monitor MRD in DLBCL on ctDNA, due to the uncertain balance between the optimal sensitivity and specificity and the lack of clinical and inter-laboratory validation of the different proposed approaches. Given the clinical relevance of this topic, the field is open to larger studies and coordinated efforts that aim at finally moving MRD monitoring in risk-adapted clinical trials and in the clinical practice of DLBCL patients.[42,43]

Disclosures

IDG received honoraria for participation in advisory board and educational events from AstraZeneca, Janssen, Roche, and Takeda. ADR received honoraria for participation in advisory board and educational events from Janssen, Roche, Takeda, AbbVie, Gilead/Kite, Incyte, and Lilly. RM received honoraria for participation in advisory board from BeiGene, AbbVie, and Johnson & Johnson. GG received honoraria for participation in advisory board and/or speakers bureaus from AbbVie, AstraZeneca, BeiGene, Hikma, Incyte, Johnson & Johnson, Lilly, and Roche. MM received research support from Alexion; and received honoraria for participation in advisory board and/or speakers bureaus from Roche, Gilead, Novartis, Takeda, Incyte, Janssen, BMS, BeiGene, and Lilly. The remaining authors have no conflicts of interest to disclose.

Contributions

IDG, ADR and RF conceived and designed the study. RM, DT, MA, LP, ADR and MM provided study materials of patients. RS, GMA, IDS and VB performed the NGS-IG screening and MRD analysis. TB managed the clinical database. RS and GMA performed bioinformatic analysis. RS, GMA, IDS, ADR, GG, MM, AG, RF and IDG contributed to data interpretation. RS, GMA and IDG wrote the manuscript. All authors contributed to manuscript revision and its final approval.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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