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ORIGINAL PAPER

Haematological Malignancy – Clinical

# Liquid biopsy for molecular characterization of diffuse large B-cell lymphoma and early assessment of minimal residual disease

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## Summary

Circulating tumour DNA (ctDNA) allows genotyping and minimal residual disease (MRD) detection in lymphomas. Using a next-generation sequencing (NGS) approach (EuroClonality-NDC), we evaluated the clinical and prognostic value of ctDNA in a series of R-CHOP-treated diffuse large B-cell lymphoma (DLBCL) patients at baseline ($n = 68$) and after two cycles ($n = 59$), monitored by metabolic imaging (positron emission tomography combined with computed tomography [PET/CT]). A molecular marker was identified in 61/68 (90%) ctDNA samples at diagnosis. Pretreatment high ctDNA levels significantly correlated with elevated lactate dehydrogenase, advanced stage, high-risk International Prognostic Index and a trend to shorter 2-year progression-free survival (PFS). Valuable NGS data after two cycles of treatment were obtained in 44 cases, and 38 achieved major molecular response (MMR; 2.5-log drop in ctDNA). PFS curves displayed statistically significant differences among those achieving MMR versus those not achieving MMR (2-year PFS of 76% vs. 0%, $p < 0.001$). Similarly, more than 66% reduction in ΔSUVmax by PET/CT identified two subgroups with different prognosis (2-year PFS of 83% vs. 38%; $p < 0.001$). Combining both approaches MMR and ΔSUVmax reduction, a better stratification was observed (2-year PFS of 84% vs. 17% vs. 0%, $p < 0.001$). EuroClonality-NDC panel allows the detection of a molecular marker in the ctDNA in 90% of DLBCL. ctDNA

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reduction at two cycles and its combination with interim PET results improve patient prognosis stratification.

**KEYWORDS**
liquid biopsy , minimal residual disease, molecular haematology, non-hodgkin lymphoma

## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL), the most common subtype of non-Hodgkin lymphoma, is an aggressive and biologically heterogeneous disease.[1] Therefore, it is essential to identify clinical parameters and biomarkers that could help to improve DLBCL patients' risk stratification. Conventional methods for risk stratification and personalized treatment including the International Prognostic Index (IPI),[2] cell of origin,[3] and other molecular subsets[4,5] have some limitations. Progress in molecular characterization may greatly help not only to understand the heterogeneity of this group of lymphomas but also to redefine prognostic categories in order to pave the way towards personalized medicine. However, molecular characterization mostly relies on the availability of invasive tumour biopsies. Liquid biopsies may overcome this barrier by providing a non-invasive collection, availability at any time during the disease course and monitoring of biomarker dynamics with less heterogeneity issues than tumour tissue testing.

The analysis of ctDNA may be utilized for both genotyping and minimal residual disease (MRD) detection.[6] At diagnosis, ctDNA is a potential prognostic biomarker in patients with DLBCL useful for non-invasive detection of tumour-specific mutations and molecular subtyping.[7,8] In addition, baseline ctDNA levels correlate not only with standard clinical indices, serum lactate dehydrogenase (LDH), metabolic tumour volume (MTV), Ann Arbor stage and IPI but also with survival outcomes in patients with DLBCL.[7,9–11]

Nowadays, after treatment with first-line regimens, usually R-CHOP-like, a significant number of patients do not achieve a complete response (CR), and an additional subset of patients ultimately relapse after CR due to the persistence of tumour cells below the limit of detection of traditional image-based approaches. Positron emission tomography (PET) combined with computed tomography (CT) (PET/CT) is considered the gold standard for response assessment and follow-up.[12] Moreover, interim PET/CT after two cycles of treatment is considered a potential biomarker to predict treatment response and possibly also long-term outcome.[13] However, the method is expensive, associated with radiation exposure and is not exempt from false-positive/negative results that could lead to inappropriate decision making.[14] Thus, more sensitive, and less harmful approaches would be more convenient. In this respect, liquid biopsy based on ctDNA detection by next-generation sequencing (NGS) is a very promising tool. To date, several studies have employed ctDNA as a real-time non-invasive approach to monitor the therapeutic response and disease outcome.[15] Quantitative changes in ctDNA at an early stage of therapy have been found to have prognostic value, establishing optimal thresholds of ctDNA reduction after immunochemotherapy to predict survival.[9,16,17]

Based on this background, in the present study we investigated the usefulness of liquid biopsy employing a capture-based NGS approach targeting single-nucleotide variations (SNVs) and structural variants (SVs), in a real-life series of DLBCL patients homogeneously treated in our hospital.

## MATERIALS AND METHODS

### Patients and study design

Eligible patients had histologically confirmed DLBCL according to the 4th WHO classification of lymphoid malignancies[1] and were enrolled before receiving first-line treatment at the University Hospital of Salamanca (HUSA). Only patients selected for curative treatment based on anthracycline-containing immunochemotherapy were eligible. The study was approved by the local Institutional Review Board, and written informed consent, following the Declaration of Helsinki, was obtained from all patients before sample collection.

The main objectives were to assess (1) the ability of ctDNA based assessments at diagnosis to reflect the molecular characteristics present in the paired tumour sample and (2) the prognostic impact of ctDNA-based MRD study after two cycles of treatment. Progression-free survival (PFS) was defined as the time from the start of therapy until lymphoma progression, relapse or death from any cause. Overall survival (OS) was defined as the time from diagnosis until death from any cause. Responses were assessed by the PET/CT imaging following the Lugano criteria.[12]

### Sample collection and processing

Twenty millilitres of blood was collected in EDTA tubes at the HUSA and centrifuged twice (10 min at 1500 *g* followed by 15 min 16 000 g) within 2 h for plasma isolation. The plasma was aliquoted and stored at −80°C. The QIAamp Circulating Nucleic Acid Kit™ (Qiagen, Hilden, Germany) was used for cell-free DNA (cfDNA) extraction from plasma samples. Input volume was 5 mL when available. cfDNA was quantified with the Qubit 2.0 Fluorometer and high sensitivity dsDNA assay kit (Life Technologies, Carlsbad, CA, USA). The quality of cfDNA samples was analysed with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The samples were considered acceptable

for further studies when a peak around 165 bp was detected without the presence of genomic DNA (gDNA) contamination and at least 30 ng was available for NGS studies (cfDNA minimum criteria).

Tumour gDNA was isolated from fresh or formalin-fixed paraffin-embedded (FFPE) tissue biopsy. From fresh tissue, gDNA was extracted with proteinase K followed by salt and ethanol precipitation and was stored at −20°C. From FFPE tissue, gDNA was extracted using QiAmp DNA FFPE tissue kit™ (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Germline gDNA was isolated from post-treatment peripheral blood mononuclear cells (PBMC) to discard potential clonal haematopoiesis of indeterminate potential (CHIP) variants.

Interphase fluorescence in situ hybridization (FISH) analysis was carried out in lymphadenopathy to detect *BCL2*, *MYC* and *BCL6* translocations, using specific probes supplied by Abbott Molecular following the manufacturer's specifications.[18] Additionally, a probe for detecting *IGL* translocations (CytoCell) was used in one case.

## Capture panel design, library generation and capture-targeted NGS

The EuroClonality-NDC assay, a targeted capture NGS assay intended for the integrated detection of SVs (including both clonotypic immunoglobulin (IG) and T-cell receptor (TCR) rearrangements and translocations), SNVs and copy number alterations (CNAs), was employed.[19] The term IG rearrangement refers to any clonal rearrangement detected at IG heavy chain (IGH), and/or light chain at kappa (IGK) or lambda (IGL) locus. Library preparation for cfDNA was performed using the KAPA HyperPrep Kit™ (Roche Sequencing Solutions, Pleasanton, CA, US) with a minimum input of 30 ng of cfDNA. For gDNA at least 100 ng and the KAPA HyperPlus Kit (Roche Sequencing Solutions) were employed. Hybridization of the libraries to the EuroClonality-NDC enrichment probes was performed using the KAPA HyperCapture Reagent Kit™ and KAPA HyperCapture Bead kit™ (Roche Sequencing Solutions). The total probe size of the EuroClonality-NDC assay is 344 Kb. For gDNA, pooled libraries were sequenced on the NextSeq 500 (Illumina, Cambridge, UK) using a 75 bp paired-end strategy performed on a 150-cycle NextSeq 500/550 Mid Output Kit (Illumina, Cambridge, UK). For ctDNA, pooled libraries were sequenced on the NovaSeq 6000 (Illumina, Cambridge, UK) using a 100 bp paired-end strategy performed on a S1 200 cycle flow cell (Illumina, Cambridge, UK). Sequencing data have been deposited at the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega/) under accession number EGAS50000000215.

## Bioinformatics analyses for variant calling

The EuroClonality-NDC-developed ARResT/Interrogate software tool was employed for fragment deduplication, generation of contig sequence, false-positive filtering and establishment of a threshold to assign IG/TCR clonality.[20,21] Specific pipelines for the detection of SVs (IG rearrangements and translocations) and SNVs are detailed in the EuroClonality-NDC panel original article.[19] CNA analysis was not performed in this study. Minimum criteria for NGS acceptance on cfDNA samples and gDNA included a median unique on target reads >1000× or >500× respectively.

Variants causing non-synonymous changes, including modifications of splice sites were considered. For the exclusion of common SNPs with allele frequencies >1%, the Genome Aggregation Database (gnomAD) was used. Plausible germline variants were excluded by comparison with NGS data obtained from further ctDNA follow-up (FU) samples, in which somatic variant frequency varied or disappeared while patient-specific germline variants remained comparable, and with variant allele frequency (VAF) ranging from 40% to 60%. In those cases, without a paired sample, variants with VAF ranging from 40% to 60% were not further considered. All variants were confirmed by visual inspection using the Integrative Genomics Viewer software. We established a threshold of 0.5% of VAF for ctDNA and 4% for gDNA as the lower detection limit for the present study.

## Detection of pretreatment and on-treatment ctDNA

ctDNA concentrations were expressed in haploid genome equivalents per mL of plasma (hGE/mL). This value is calculated by multiplying the concentration of cfDNA (pg/mL estimated by Qubit, Thermo Fisher Scientific) by the mean VAF of the detected SNVs and dividing by 3.3, as previously described.[7] This value was then expressed as a base-10 logarithm (Log hGE/mL).

MRD analysis in FU samples was based on the detection of the molecular markers previously identified at diagnosis (tumour informed approach) with at least five altered reads. Serial measurement of ctDNA during the treatment was normalized to basal levels at diagnosis and expressed as a log-fold change. We employed a previously reported threshold, 2.5-log drop in ctDNA after two cycles of treatment,[9] to define patients achieving MMR (major molecular response) also defined in our work as a quantitative molecular response (Q-MR).

SVs were also considered for MRD detection in a qualitative approach. Thus, a patient was considered not to achieve a qualitative molecular response (qualitative-MR) when any SV previously seen at diagnosis (translocation and/or IG rearrangement) with at least five altered reads was detected in the FU sample.

Sensitivity of the panel was established based on a theoretical estimation. Thus, based on the minimum input of 30 ng (or 9000 hGE) for NGS studies and the threshold for MRD based on the detection of at least five altered reads, the sensitivity would be close to 0.05%. However, in an attempt to be more conservative and trying to minimize background errors, we established 0.5% as the limit for MRD studies.

## PET/CT scan analysis

All patients underwent [$^{18}$F] fluorodeoxyglucose PET/CT scan before the onset of first-line treatment (PET0), according to standard procedures.[22] The studies were performed in a Siemens Biograph mCT scanner accredited by the European Association of Nuclear Medicine. Interim PET was planned in all treated patients after two cycles of treatment (PET2), allowing us to assess early response using the five-point Deauville Score (5-DS)[12] and the maximal standardized uptake value reduction ($\Delta SUV_{max}$) calculation as previously reported.[23] Briefly, to assess the $\Delta SUV_{max}$, the hottest tumour in any region or organ on PET2 was used for comparison, even if its location differed from the initial hottest tumour in PET0 (ΔSUVmaxPET0-2). A cut-off value of 66% was used to separate good from bad responders.[23] A PET/CT was also performed at the end of treatment and the response was assessed according to the Lugano recommendations.[12]

## Statistical analysis

PFS and OS were determined by the Kaplan–Meier analysis and compared by log-rank test. The comparison between two groups was conducted using a two-tailed Mann–Whitney test. Significances were calculated by Fisher's exact test between two categorical variables. Statistical analysis and plots were generated using SPSS Statistics 21 (IBM).

# RESULTS

## Patient characteristics, response to first-line treatment and overall sample flow

A prospective series of 68 DLBCL patients were enrolled from April 2017 to May 2020. Patients' clinical and biological features are included in Table 1. After two cycles of first-line treatment, response was evaluated by PET/CT in 63/68 cases, with 28 patients achieving CR, 33 partial response (PR) and 2 progressive disease (PD). According to ΔSUVmaxPET0-2, 51 (81%) patients had a reduction >66%. At the end of treatment, one patient had died and two had not been evaluated, thus 46 patients achieved CR, 9 PR and 10 had progressive disease. Median follow-up of the series was 3 years (range 0.2–4.8). Estimated 2-year PFS and OS were 76% and 87% respectively.

Peripheral blood samples at diagnosis were processed for plasma isolation, cfDNA extraction and NGS studies. We obtained evaluable NGS data in the cfDNA compartment in 68 cases. Paired germline gDNA was analysed in 25 cases harbouring potential CHIP variants. In addition, paired tumour samples from the diagnostic lymphadenopathy were collected in 21 cases (>20% infiltrating tumour cells) for NGS analysis, providing direct molecular comparisons in 19 cases in the two compartments (there were three cases with no molecular marker in the ctDNA). Overall, after baseline NGS studies,

**TABLE 1** Clinical and biological characteristics of patients.

| **Characteristics, *n* (%)** | |
|---|---|
| Median age years (range) | 65 (34–85) |
| Age >60 years | 42 (62) |
| Male | 41 (60) |
| LDH > ULN | 38/63 (60) |
| Beta-2 microglobulin > ULN | 38/63 (60) |
| ECOG ≥2 | 10/66 (15) |
| Bulky disease (>7 cm) | 30/68 (44) |
| Ann Arbor stage, *N* (%) | |
| I | 7 (10) |
| II | 8 (12) |
| III | 16 (24) |
| IV | 36 (54) |
| B symptoms | 19/46 (41) |
| IPI score | |
| Low (0–1) | 15/62 (24) |
| Low intermediate (2) | 16/62 (26) |
| High intermediate (3) | 18/62 (29) |
| High risk (4–5) | 13/62 (21) |
| Cell of origin (Hans' algorithm) | |
| GCB | 21 (31) |
| Non-GCB | 42 (62) |
| Not available/unclassified | 5 (7) |
| Translocations by FISH | |
| *IGH::BCL2* | 6 (9) |
| *IGH::BCL6* | 20 (29.5) |
| *IGH::MYC* | 5 (7) |
| No translocation | 19 (28) |
| Not available | 18 (26.5) |
| Special profiles | |
| Triple-hit | 1 (1.5) |
| Double-hit (*MYC* + *BCL6*) | 3 (4.4) |
| Double-hit (*MYC* + *BCL2*) | 1 (1.5) |
| Treatment response after two cylces | |
| CR | 28 (42) |
| PR | 33 (48) |
| PD | 2 (3) |
| Not evaluated | 5 (7) |
| Treatment response after six cylces | |
| CR | 46 (67.6) |
| PR | 9 (13.2) |
| PD | 10 (14.8) |
| Not evaluated/early death | 3 (4.4) |
| SUV max, median (range) | 25 [5.1–56.7] |
| ΔSUVmaxPET0-2 | |
| ≤66% | 12/63 (19) |
| >66% | 51/63 (81) |

Abbreviations: CR, complete response; FISH, fluorescence in situ hybridization; GCB, germinal centre B-cell like; IPI, International Prognostic Index; LDH, lactate dehydrogenase; ULN, upper limit of normal; PD, progressive disease; PR, partial response; ΔSUVmaxPET0-2, SUVmax variation measured at diagnosis and two cycles after treatment.

64 patients were selected for MRD studies based on the identification of at least one molecular marker susceptible to be followed for MRD studies. Finally, we obtained a valid result for MRD studies in ctDNA in 50/64 cases (Figure 1).

## Detection of genetic alterations at diagnosis in ctDNA

Median concentration of cfDNA at diagnosis estimated by qubit was 50.1 ng/mL plasma (range: 5.3–494) and 30.2 ng/mL plasma (range: 5.5–420) after two cycles. The EuroClonality-NDC assay was able to identify a molecular marker in 61 out of 68 (90%) ctDNA samples at diagnosis (Figure 1).

A total of 340 SNVs were identified in 54 cases (mean per case 5.9 variants; range [1–23]). Median allele fraction detected in ctDNA was 11% (range [2%–90%]). SNVs were distributed among 50 genes, being the most frequently mutated genes (more than 10% of cases): *KMT2D* (48%), *TP53* (28%), *SOCS1* (26%), *CREBBP* (22%), *HIST1H1E* (20%), *BCL2* (19%), *EZH2* (17%), *TNFAIP3* (17%), *NOTCH2* (15%), *ARID1A* (13%), *BTG1* (13%) and *TET2* (11%) (Figure 2). The complete list of SNVs is detailed in Table S1.

Concerning SVs, in 24 cases only an IG rearrangement was found, in 10 cases only a translocation, whereas in 23 cases both of them were identified. Clonal rearrangements were identified in 47 patients, 12 cases showed only *IGH* rearrangements, 22 only *IGK* and 13 cases both. No rearrangement to the *IGL* locus was detected. Thus, the *IGH* and *IGK* rearrangement detection rates were 25/47 (53%) and 35/47 (74%) respectively. Translocations were detected in 33 ctDNA cases: *BCL6* ($n = 18$), *BCL2* ($n = 14$) and one triple-hit lymphoma. *BCL6* gene was usually translocated to *IGH* ($n = 13$), to *IGK* ($n = 1$) or *IGL* ($n = 2$) and other genes allocated in different chromosomes: chr1 *RCC1* ($n = 1$), chr4 *RHOH* ($n = 1$) and chr6 *HIST1H2BK* ($n = 1$). On the other hand, *BCL2* gene always translocated to the *IGH* region. A translocation involving the *MYC* gene was only identified in the triple-hit lymphoma case (Table S2; Figure S1).

## Validation of genetic alterations in tumour biopsies

In order to confirm the accuracy of cfDNA genotyping, validation studies in paired tumour sample was performed. Finally, there were 19 paired cases with evaluable NGS data in both ctDNA and lymphadenopathy diagnostic samples. In the two remaining cases, no molecular markers were detected in the ctDNA sample.

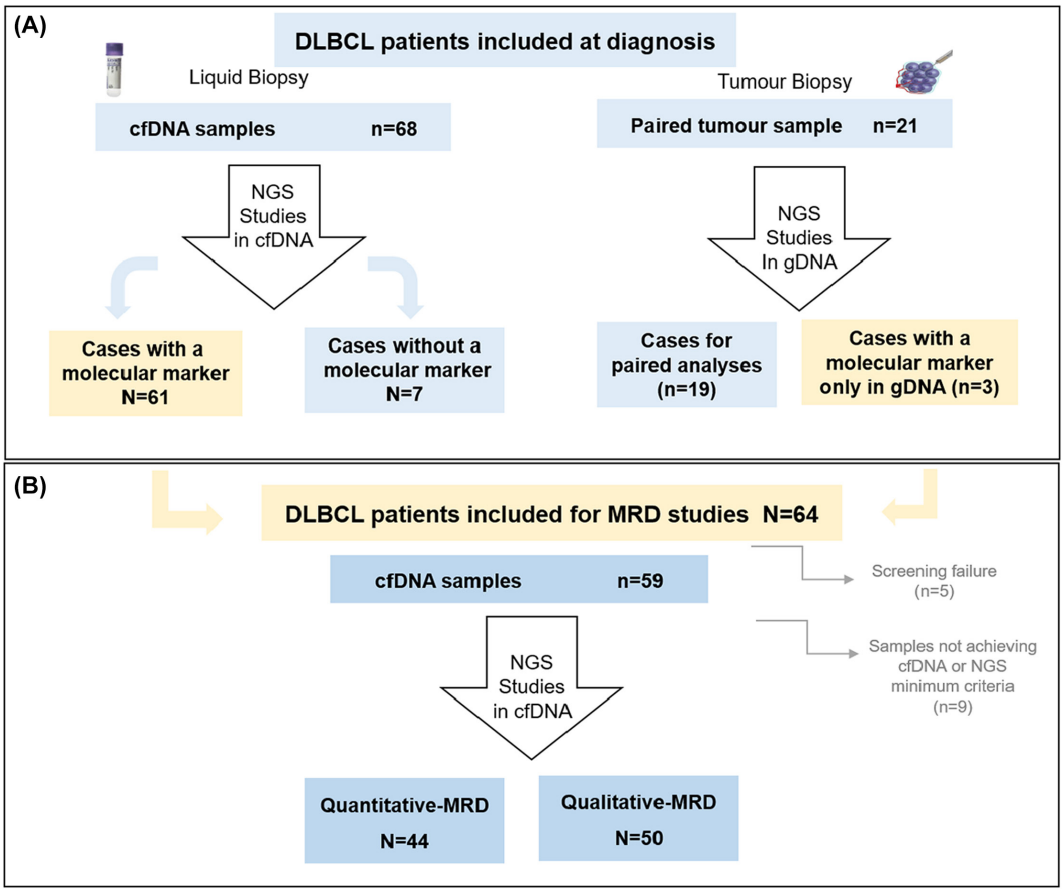



**FIGURE 1** Flowchart of samples at diagnosis for marker identification and paired tumour comparison (A) and for minimal residual disease (MRD) assessment (B). cfDNA, cell-free DNA; DLBCL, diffuse large B-cell lymphoma; gDNA, genomic DNA; NGS, next-generation sequencing. [Colour figure can be viewed at wileyonlinelibrary.com]


**FIGURE 2** Mutational profile identified in the circulating tumour DNA of diffuse large B-cell lymphoma patients ($N = 55$) at diagnosis by next-generation sequencing. [Colour figure can be viewed at wileyonlinelibrary.com]

Regarding SNVs, we identified 202 SNVs in the tumour biopsy, with 144 (73%) also detected in the paired ctDNA samples and 38 (19%) exclusively in the former one. On the contrary, there were only 16 SNVs (8%) that were solely detected in the ctDNA (Figure S2). On comparing the VAF of variants exclusively detected in the tumour biopsy (median 10%; range [4%–55%]) versus those variants simultaneously detected in both compartments (median 24%; range [4%–75%]), statistically significant differences were observed ($p < 0.05$). Thus, for a more refined comparison, we selected only those variants present in the lymphadenopathy with a VAF >15% (internally established threshold) ($n = 132$). In this setting, only 16 (12%) remained exclusively detected in the tumour biopsy, whereas 116 (88%) were simultaneously detected in both compartments (sensitivity: 88%).

Regarding the detection of SVs in both compartments, we achieved a concordant result in 18/19 (95%) and 16/19 (84%) for translocations and rearrangement detection respectively. These discrepancies corresponded to three patients where the SVs were detected in the tumour biopsy but were not seen in the ctDNA sample (data not shown).

FISH studies were carried out to validate some of the translocations detected by NGS (ctDNA and tumour biopsy) in the same tumour biopsy (Table S3). Concordance between both techniques was observed in 15/19 (79%) of the cases. The four discrepant cases corresponded to three translocations involving *BCL6* and one affecting *BCL2* which were detected by NGS but not by FISH (*BCL6* to *IGLJ1*, *IGLJ3* and *IGHG3sw* and *BCL2* to DH6-13 region in *IGH*). Additional FISH studies with probes for *BCL6* and *IGL* were performed in the leftover sample from Case 8. The results showed a *BCL6* microdeletion and an *IGL* translocation, which suggests the presence of a *BCL6::IGL* translocation. There was also a minor discrepancy in Case 3 where the *BCL6* translocation was not detected in the ctDNA sample, probably due to a low sensitivity.

## Prognostic value of ctDNA at diagnosis

The median amount of ctDNA was 2.9 log hGE/mL [1.9–4.7]. Several cut-off points ranging from 2.0 to 3.5 log hGE/mL were tested to establish the threshold for pretreatment ctDNA that best separated patients for PFS. Finally, the previously reported[9] 2.5 log hGE/mL of ctDNA at diagnosis was also selected in our series to classify patients in two groups. Then, to assess the prognostic value of ctDNA at diagnosis, we first compared the levels of ctDNA with standard clinical indices. Those patients with higher levels of ctDNA displayed elevated LDH ($p < 0.001$), advanced Ann Arbor stages [(III, IV) vs. (I, II) $p = 0.028$] and high-risk IPI [(3,4,5) vs. (0,1,2) $p = 0.021$] (Figure 3).

We also explored the impact of ctDNA levels at diagnosis on patient outcome. Patients with high levels of ctDNA displayed a trend to shorter PFS than those with low levels (80%

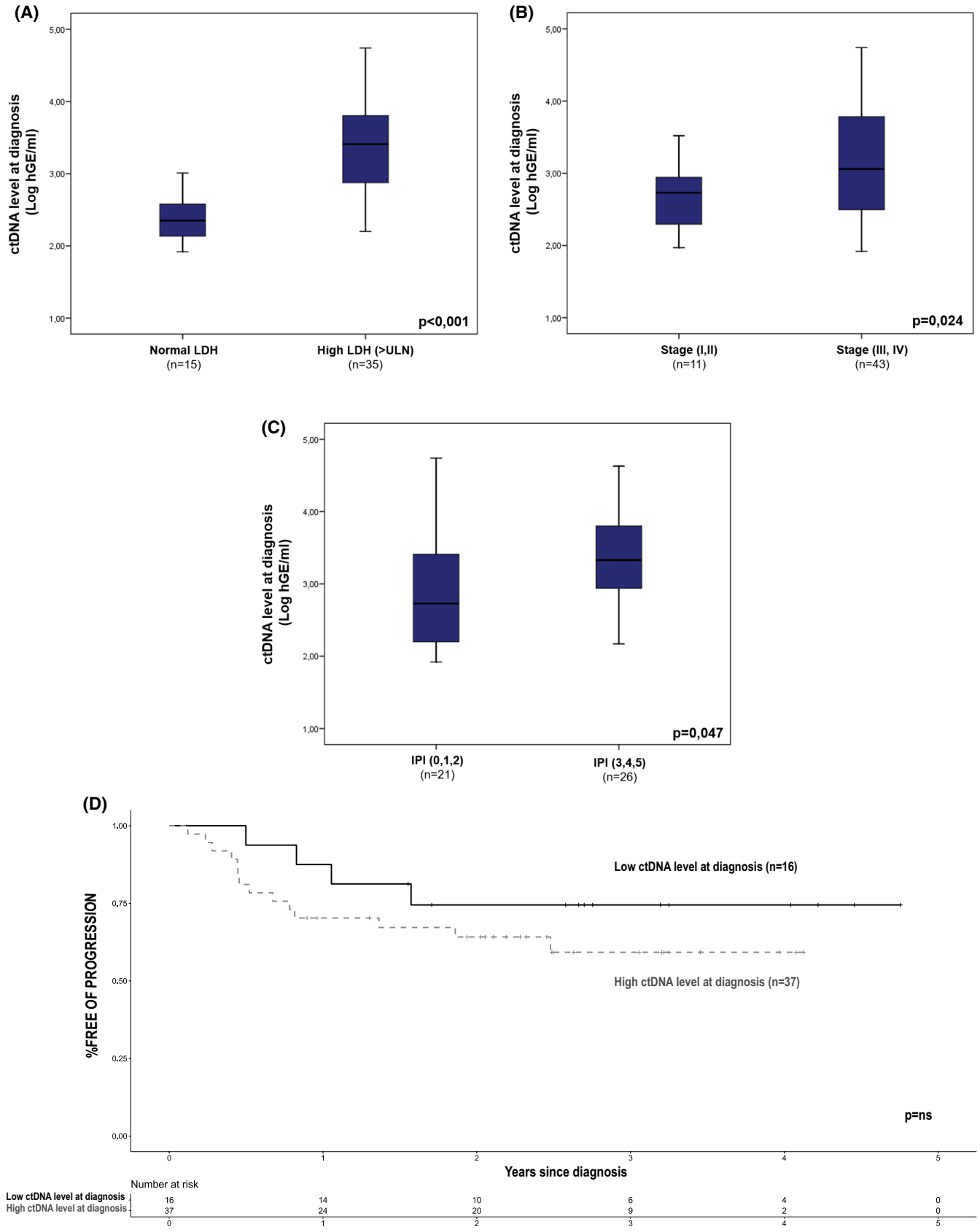



**FIGURE 3** Correlation between ctDNA level at diagnosis (Log hGE/mL) represented in the *Y*-axis and LDH level (low vs. high (>ULN)) (A), Ann Arbor stage (I, II vs. III, IV) (B) and IPI (0,1,2) vs. (3,4,5) (C) in the *X*-axis. (D) Progression-free survival Kaplan–Meier plot based on the ctDNA level at diagnosis. ctDNA, circulating tumour DNA; IPI, International Prognostic Index; LDH, lactate dehydrogenase; ULN, upper limit of normal. [Colour figure can be viewed at wileyonlinelibrary.com]

vs. 62% at 2 years; $p$ = NS) (Figure 3). No significant differences were observed regarding CR rates after first-line treatment.

## Impact of MRD by NGS-based ctDNA and PET/CT

MRD studies after two cycles of treatment were performed in those patients with a tumour molecular marker identified at diagnosis ($n$ = 64) either from the cfDNA sample ($n$ = 61) or from tumour biopsy ($n$ = 3) (Figure 1). There were five cases with a screening failure (no sample collected), therefore 59 FU plasma samples were processed. Nine cases did not achieve cfDNA minimum criteria ((i) less than 30 ng input or (ii) cfDNA contaminated by gDNA). We obtained valuable NGS data for molecular response assessment in 44 cases (Q-MR equivalent to MMR) and in 50 cases when SVs were also considered (qualitative-MR) (Figure 1). The six additional cases corresponded to three cases studied from tumour biopsy sample (no molecular markers were identified

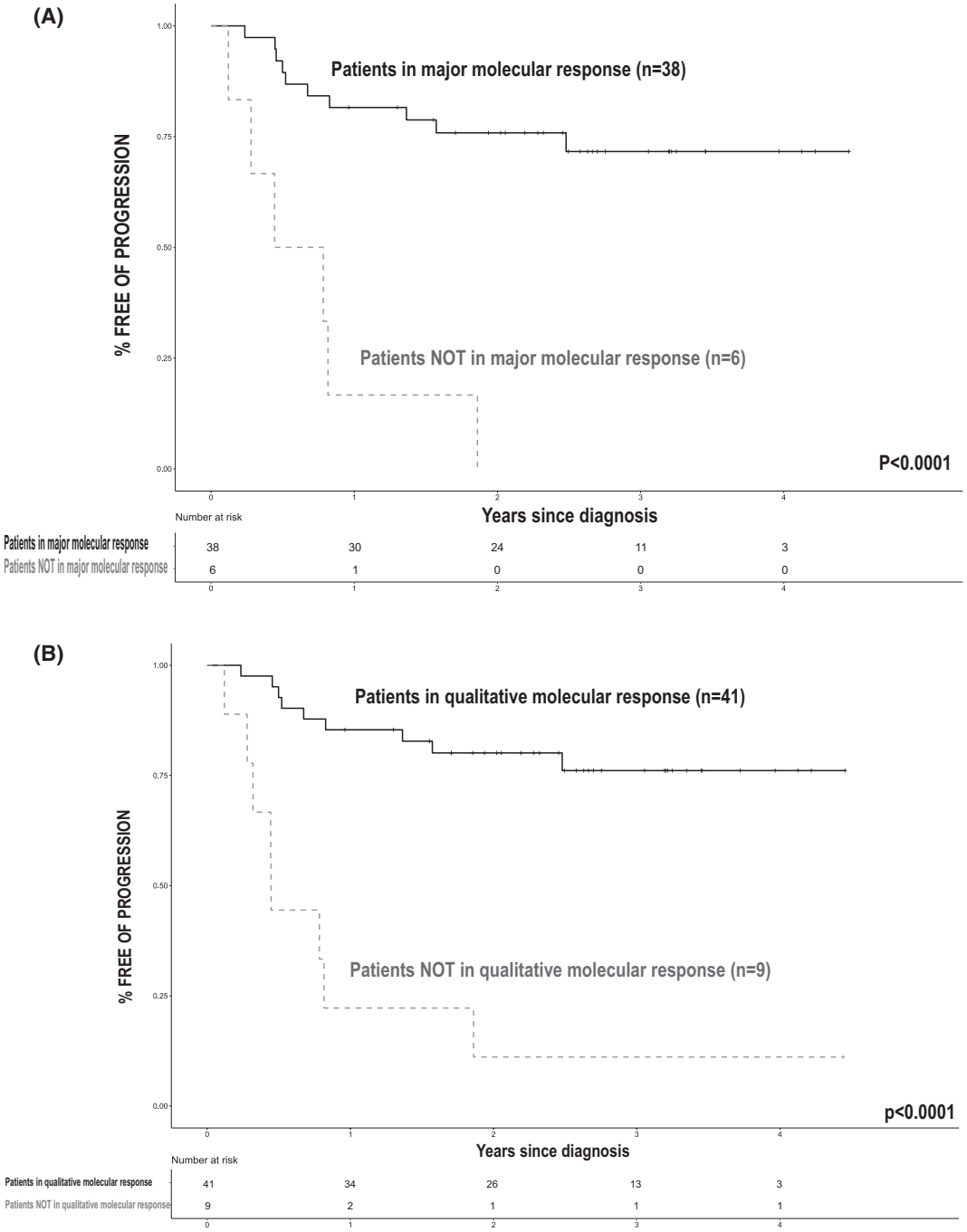



**FIGURE 4** Progression-free survival curves according to the molecular response achieved by a (A) quantitative approach ($n$ = 44) and (B) qualitative approach ($n$ = 50).

in the ctDNA) and three cases without SNVs, thus Log hGE/mL reduction could not be calculated.

Based on the 2.5-log drop in ctDNA, 38 cases achieved MMR and 6 cases did not. PFS curves displayed statistically significant differences among those achieving MMR versus those not achieving MMR (2-year PFS of 76% vs. 0%, $p < 0.001$; Figure 4A). Upon considering SVs for the assessment of qualitative MR, we were able to include six more cases; thus, patients were further classified in two groups and those achieving a qualitative MR displayed statistically significant better prognosis in PFS (80% vs. 11% at 2 year, $p < 0.001$; Figure 4B).

As regards interim imaging studies, achievement of CR by PET/CT and ΔSUVmaxPET0-2 >66% identified a subgroup of patients with statistically significant better prognosis regarding both PFS and OS (Table 2).

Multivariate analysis of PFS by Cox regression identified MMR and ΔSUVmaxPET0-2 >66% as the two variables with an independent association with the PFS (Table 2). Based on these results, we combined both variables and classified patients into three groups: patients achieving MMR and ΔSUVmaxPET0-2 >66% (MMR(+)/ΔSUV(+) $n = 32$), mixed pattern (MMR(+)/ΔSUV(−) or MMR(−)/ΔSUV(+) $n = 6$) and those patients achieving none of them (MMR(−)/ΔSUV(−) $n = 5$). These groups displayed statistically significant differences in PFS (84% vs. 17% vs. 0% at 2 year, $p < 0.001$; Figure 5).

Longitudinal analysis of the plasma samples collected during treatment and at relapse together with PET/CT results has been represented in Figure 6 as an illustrative example. In this case, SNVs (5) and SVs (4 IGK rearrangements and a BCL6 translocation) were identified at diagnosis in ctDNA as molecular markers useful for MRD follow-up. A quick drop in the level of hGE/mL was observed after two cycles of treatment; however, ctDNA levels were still detectable (SNVs and IGK rearrangement) and PET/CT scan also displayed active disease. Then, a slow increase in both, ctDNA level by liquid biopsy and the SUV max by PET/CT scans, was observed while tumour biopsy remained negative. One month later, relapse was confirmed by tumour biopsy and a significant increase in the ctDNA level as well as in the SUV max by PET/CT was observed.

## DISCUSSION

In this study, we have assessed the utility of ctDNA profiling by targeted high-throughput NGS for tumour genotyping at diagnosis and surveillance of MRD in a series of DLBCL homogeneously treated in our institution.

**TABLE 2** Prognostic value of traditional clinical–biological parameters, PET/CT after two cycles and liquid biopsy related variables.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| **Progression-free survival** | ***n*** | **% free of progression at two years** | ***p*-Value** | ***n*** | **HR (95% CI)** | ***p*-Value** |
| LDH (Low vs. >ULN) | 62 | (79% vs. 66%) | ns | | | |
| ECOG (0, 1 vs. ≥2) | 65 | (71% vs. 75%) | ns | | | |
| Age (<60 vs. ≥60) | 66 | (66% vs. 75%) | ns | | | |
| Extra-nodal involvement (none vs. ≥1) | 58 | (80% vs. 74%) | ns | | | |
| Ann Arbor stage (I–II vs. III–IV) | 66 | (79% vs. 69%) | ns | | | |
| IPI (0,1,2) vs. (3,4,5) | 62 | (72% vs. 67%) | ns | | | |
| Pretreatment ctDNA (low vs. high) | 53 | (74% vs. 64%) | ns | | | |
| Major molecular response (MMR vs. not MMR) | 44 | (76% vs. 0%) | <0.001 | 43 | 0.193 [0.058–0.639] | 0.007 |
| PET2 (CR vs. no CR) | 63 | (100% vs. 47%) | <0.001 | 43 | - | - |
| ΔSUVmaxPET0-2 (66% or lower) | 63 | (83% vs. 38%) | <0.001 | 43 | 0.233 [0.07–0.776] | 0.018 |
| **Overall survival** | ***n*** | **% alive at 2-year** | ***p*-Value** | ***n*** | **HR (95% CI)** | ***p*-Value** |
| LDH (Low vs. >ULN) | 62 | (87% vs. 83%) | ns | | | |
| ECOG (0, 1 vs. ≥2) | 66 | (85% vs. 80%) | ns | | | |
| Age (<60 vs. ≥60) | 67 | (80% vs. 87%) | ns | | | |
| Extra-nodal involvement (none vs. ≥1) | 58 | (100% vs. 82%) | ns | | | |
| Ann Arbor stage (I–II vs. III–IV) | 66 | (86% vs. 84%) | ns | | | |
| IPI (0,1,2) vs. (3,4,5) | 62 | (86% vs. 84%) | ns | | | |
| Pretreatment ctDNA (low vs. high) | 49 | (85% vs. 79%) | ns | | | |
| Major molecular response (MMR vs. not MMR) | 44 | (84% vs. 66%) | ns | | | |
| PET2 (CR vs. no CR) | 63 | (100% vs. 71%) | 0.003 | 62 | - | ns |
| ΔSUVmaxPET0-2 (66% or lower) | 63 | (92% vs. 50%) | <0.001 | 62 | 0.131 (0.37–0.465) | 0.002 |

Abbreviations: CR, complete response; ctDNA, circulating tumour DNA; IPI, International Prognostic Index; LDH, lactate dehydrogenase; PET/CT, positron emission tomography combined with computed tomography; ULN, upper limit of normal; ΔSUVmaxPET0-2, SUVmax variation measured at diagnosis and two cycles after treatment.

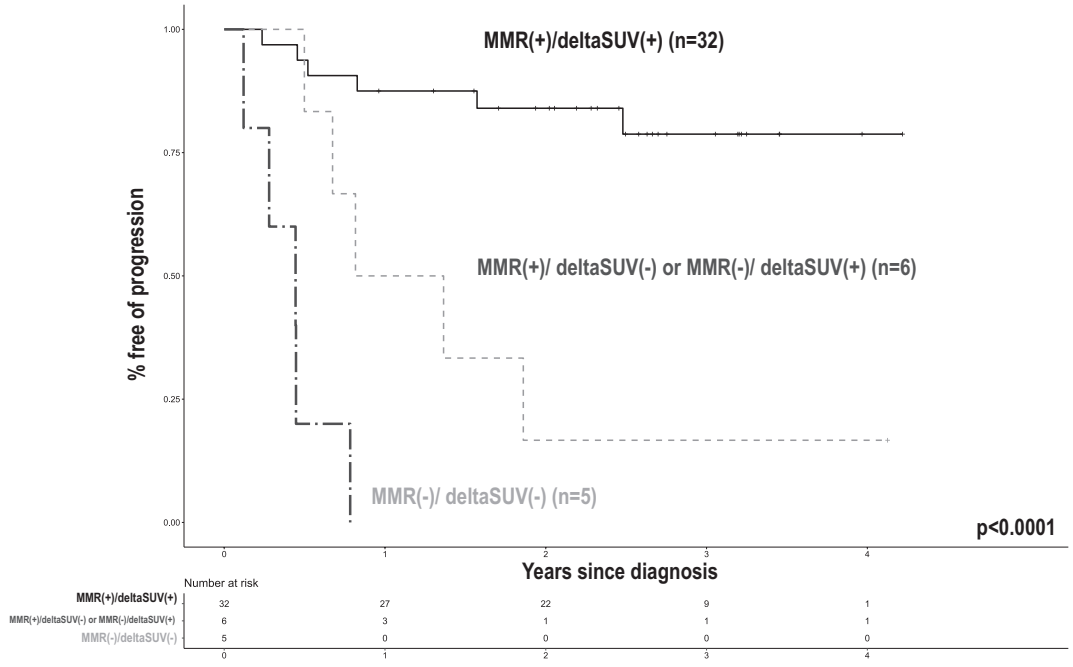
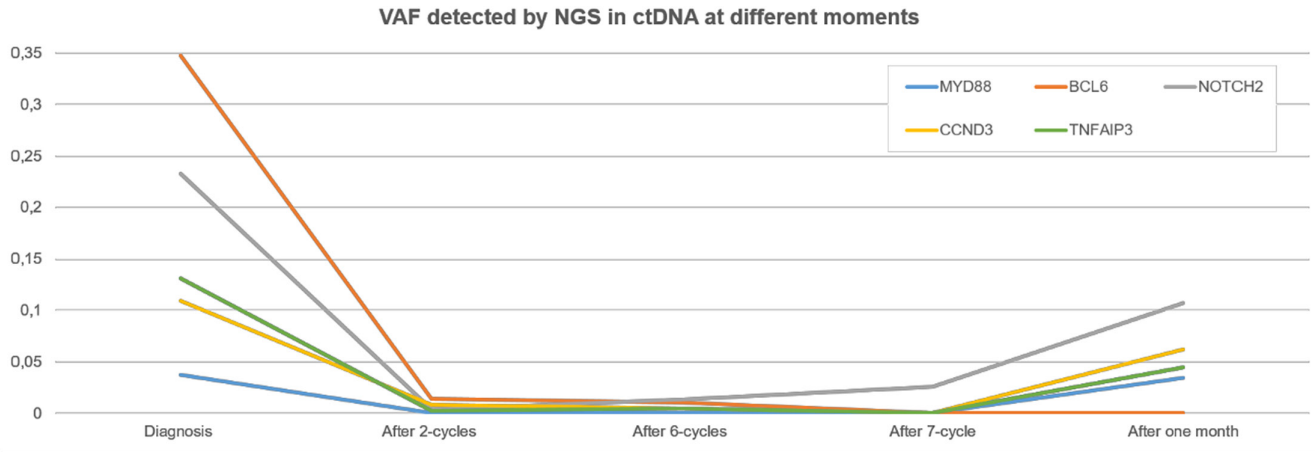



**FIGURE 5** Progression-free survival plot based on minimal residual disease detection by liquid biopsy and ΔSUVmaxPET0-2 combined ($N = 45$).




| | Diagnosis | After 2-cycles | After 6-cycles | After 7-cycles | After 1 month |
|---|---|---|---|---|---|
| **Time schedule** | November 2017 | December 2017 | April-2018 | July-2018 | September-2018 |
| **PET/CT scan (Deauville score)** | | PR (4) | PR vs diagnosis Progression vs. PET2 | Progression (5) | Progression (5) |
| **SUV (max)** | 28,6 | 5,1 | 23,6 | 26,2 | 29,6 |
| **Biopsy** | | - | - | Negative | Positive |
| **hGE/ml** | 1750 | 45 | 72 | 98 | 103 |
| **Structural variants** | IGH/BCL6 IGK (4) | IGK(1) | IGK(4) | IGK (4) | IGH/BCl6 IGK(4) |

**FIGURE 6** Illustrative example of baseline and follow-up by NGS in ctDNA and paired PET/CT scan results. At baseline, NGS in ctDNA identified SNVs (5) and SVs (one translocation and four clonal rearrangements) providing several molecular markers suitable for minimal residual disease detection. A sharp decrease in the VAF, hGE/mL but also in the SUVmax estimated by PET/CT was observed after two cycles of treatment. Also, detection of four out the five SVs was lost in the ctDNA. Close monitoring after six and seven cycles evidenced a progression by image-based and NGS methods; however, this progression was not confirmed by tumour biopsy. Finally, 1 month later progression was confirmed by another tumour biopsy, PET/CT and liquid biopsy, the former one showing an important increase the VAF of the SNVs and the SVs previously identified at diagnosis. ctDNA, circulating tumour DNA; NGS, next-generation sequencing; PET/CT, positron emission tomography combined with computed tomography; SNV, single-nucleotide variation; SV, structural variant; VAF, variant allele frequency. [Colour figure can be viewed at wileyonlinelibrary.com]

The EuroClonality-NDC panel, a robust tool for simultaneous detection of B-cell clonality, translocations and sequence variants, has been validated in gDNA samples.[19] Our data show that this assay is able to identify a molecular marker (either SNV, SV or both) in ctDNA at diagnosis in 90% (61 of 68) cases. This finding is consistent with previous publications, in which the rate of detection varied from 63%[24] to 98%[9]; however, this range should be considered with caution due to differences in the design and number of genes included, sequencing strategy and the resulting coverage depth in each particular study.

Regarding the mutational profile observed in the ctDNA, genes more frequently altered were *KMT2D*, *TP53*, *SOCS1*, *CREBBP*, *BCL2* and *HIST1H1E*, similar to other data previously published in the ctDNA[8] and in the tumour biopsy from DLBCL patients.[4,5] Clonal *IGH* and *IGK* rearrangements were detected in 47 cases, being *IGK* more frequently detected than *IGH* (74% vs. 53%) probably due to higher rate of somatic hypermutation in *IGH* genes in DLBCL patients.[25] Similar detection rate of these rearrangements has been reported, which confirms *IGK* rearrangement as an interesting target for MRD follow-up as previously published.[10]

Nowadays, histopathological assessment of tumour tissue obtained from invasive surgical procedures is the gold standard for DLBCL diagnosis, depiction of genetic landscapes and subtype classification, leaving liquid biopsy profiling a complementary tool. However, one essential issue is that liquid biopsy robustly mirrors the molecular profile observed in the tumour biopsy. Several studies have shown that the concordance between tissue-based and ctDNA-based genotyping is usually greater than 70%.[7,8,26] In our hands, clonal driver SNVs present in the tumour tissue were consistently detected in the plasma counterpart, whereas minor SNVs (VAF <15%) were more prone to be lost. In addition, there were some mutations that were only detected in the ctDNA, confirming that ctDNA profiling can capture spatial tumour heterogeneity. On the other hand, SVs achieved a high concordance in both settings, with only minor discrepancies (one translocation and two *IGH* rearrangements) not seen in ctDNA that could probably be due to lack of sensitivity.

Another interesting finding regarding the detection of translocations was the overall concordance rate of 79% between NGS (performed in ctDNA and tumour biopsy) as compared to benchmark FISH studies. Three discrepant cases corresponded to *BCL6* translocation to *IGL* and *IGH* switch regions not routinely assessed by our FISH screening, confirming the utility of the NGS panel to detect novel translocations not detected by FISH.

At this moment, clinical relevance of tumour genotyping by ctDNA is increasing, mainly to overcome limitations of tissue biopsy, or for certain situations in which tumour tissue is inaccessible or cannot be obtained repeatedly over the course of the disease. In line with previous statement, several groups have investigated the relationship between ctDNA level at diagnosis and conventional markers of tumour burden and its role as a prognostic biomarker in DLBCL. Based on our results, we confirmed that pretreatment ctDNA level is significantly associated with well-described clinical parameters of tumour burden (serum LDH, Ann Arbor stage and IPI), as previously reported in other series.[7,9,27] Interestingly, this correlation has been turned into a poor prognostic effect, since ctDNA level stratified patients in two groups with different prognosis.[7,9,11,26,28] In our series, patients with high levels displayed a trend to shorter PFS than those with low levels, although the difference did not reach statistical significance, probably due to the small sample size. Similarly, the lack of statistically significant prognostic value of IPI was surprising and discrepant with the published literature; it could be explained by the small sample size and the few events that have occurred so far.

Nowadays, PET/CT is frequently used for interim response assessment; however, these results are inconsistent with the final outcome in a significant proportion of patients.[30,31] Consequently, there is a growing interest in identifying prognostic and predictive factors beyond interim PET. In this sense, ctDNA assessment might help overcome these limitations and improve patient risk stratification. Indeed, the quantitative assessment of on-treatment ctDNA has shown to be highly prognostic in various DLBCL series. Some studies define two landmarks for the assessment of 'molecular response' in DLBCL during treatment, early molecular response (EMR) and MMR.[9] To shed light in this scenario of response assessment during treatment, we have evaluated the prognostic impact of two parameters based in functional imaging: traditional PET/CT assessment of response and ΔSUVmaxPET0-2 (66% reduction or not) together with NGS-based achievement of MMR (2.5-log ctDNA drop). As regards PFS, the three parameters were able to classify patients in different prognosis groups; however, only the achievement of ΔSUVmaxPET0-2 >66% and MMR by NGS maintained the statistical significance in the multivariate analyses. Moreover the combination of MMR and ΔSUVmaxPET0-2 was able to stratify PFS in a very similar way as described (combination of interim PET/CT and molecular response assessment).[9] Patients with favourable results for both MMR and ΔSUVmaxPET0-2 >66% had excellent outcomes. In contrast, the combination of a ΔSUVmaxPET0-2 <66% and no MMR identified a group of patients at extremely high risk for treatment failure. This trend was also maintained in the OS, suggesting that the combination of these two parameters provide a robust prognostic classification of DLBCL patients. It is also worthy to mention that it will be very interesting to analyse larger series of patients to find out potential differences among the two groups classified as mixed pattern (MMR(+)/ΔSUV(–) and MMR(–)/ΔSUV(+)) to weight the prognostic influence of each one.

Our study is limited by its small sample size, which could justify that well recognized prognostic factors (IPI) was not statistically significant in the PFS and OS curves. In addition, in seven cases we were not able to identify a molecular marker at diagnosis probably due to limited sensitivity of the panel. Thus, additional larger studies implementing error suppression methods with unique molecular identifiers and new technologies such as PhasED-Seq[31] to deepen the sensitivity would be necessary to confirm these results. For those prospective

multicentre studies within a clinical setting, rather than the present research scenario, several preanalytical conditions need to be considered in order to achieve consistent data: collection of plasma in cfDNA collection tubes (i.e. Streck) would be essential to prevent genomic contamination of the plasma, processing of at least 5 mL of plasma to obtain enough cfDNA and the analysis of paired non-tumour DNA to discard CHIP and germline variants.

In summary, ctDNA was useful for estimating the tumour burden and to detect the molecular alterations present in the tumour in our prospective cohort of patients with DLBCL. Thus, providing useful markers to be followed during the treatment in almost all cases. ctDNA dynamic reduction is a very valuable tool to identify patients responding to treatment, and its combination with ΔSUVmaxPET0-2 delivers a promising prognostic parameter that allows the identification of patients with high risk of treatment failure, this particular combination would finally provide decisive information to tailor therapeutic approaches in a near future.

## AUTHOR CONTRIBUTIONS

Conceptualization, methodology, and investigation: MA, JPS, MG-Á, LGD, CJ, AM and MCC. NGS studies on central, large-scale facility: JG and JPS. Pathological studies: OB. Image based-studies: LGD, PT and CM. Patient selection and inclusion: LGD, FJD, MJP, SF, AC and NCG. Writing—original draft preparation: MA and MES. Writing—review and editing: MDC, RG-S, MG, PT, DG, AMG-S and MES. Supervision: AMG-S, RG-S and MES. Funding acquisition: PT, AMG-S, MDC, MG, RG-S and MES. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

Dr Marcos Gonzalez Díaz served on the advisory board and received lecture fees from Janssen, Roche, Gilead and AbbVie. Dr Alejandro Martin received honoraria from Roche, BMS/Celgene, Janssen, Gilead/Kite, Takeda, Eusa Pharma and Novartis; consulting fees from Roche, BMS/Celgene, Kyowa Kirin, Clinigen, Eusa Pharma, Novartis, Gilead/Kite, Incyte, Lilly, Takeda, ADC Therapeutics America, Miltenyi, Ideogen and AbbVie; research funding from Janssen; travel and accommodation expenses from Gilead/Kite, Janssen, Roche and BMS/Celgene. Dr David González has received honoraria, consultancy and/or research funding from Roche, AstraZeneca, Novartis, Incyte, Eli Lilly, Amgen, Janssen and Illumina and is cofounder and Director of Univ8 Genomics Ltd.

## DATA AVAILABILITY STATEMENT

Raw data were generated at The Patrick G Johnston Centre for Cancer Research Queen's University Belfast, Belfast. These data have been deposited at the European Genome-Phenome Archive (http://www.ebi.ac.uk/ega/) under the accession number EGAS50000000215.

## ETHICS STATEMENT

Ethics committee of the Area de Salud de Salamanca (Comité de Ética de la Investigación con medicamentos del área de salud de Salamanca) approved the execution of this Project (ID of the approval: CEIm PI 2020 11 626).

## PATIENT CONSENT STATEMENT

All patients were informed of the sample collection before signing the informed consent form.

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## SUPPORTING INFORMATION

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