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Real-Life Disease Monitoring in Follicular Lymphoma Patients Using Liquid Biopsy Ultra-Deep Sequencing and PET/CT

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Abstract

In the present study, we screened 84 Follicular Lymphoma patients for somatic mutations suitable as liquid biopsy MRD biomarkers using a targeted next-generation sequencing (NGS) panel. We found trackable mutations in 95% of the lymph node samples and 80% of the liquid biopsy baseline samples. Then, we used an ultra-deep sequencing approach with $2 \cdot 10^{-4}$ sensitivity (LiqBio-MRD) to track those mutations on 151 follow-up liquid biopsy samples from 54 treated patients. Positive LiqBio-MRD at first-line therapy correlated with a higher risk of progression both at the interim evaluation (HR_{INT} 11.0, 95% CI 2.10–57.7, $p = 0.005$) and at the end of treatment (HR_{EOT}, HR 19.1, 95% CI 4.10–89.4, $p < 0.001$). Similar results were observed by PET/CT Deauville score, with a median PFS of 19 months vs. NR ($p < 0.001$) at the interim and 13 months vs. NR ($p < 0.001$) at EOT. LiqBio-MRD and PET/CT combined identified the patients that progressed in less than two years with 88% sensitivity and 100% specificity. Our results demonstrate that LiqBio-MRD is a robust and non-invasive approach, complementary to metabolic imaging, for identifying FL patients at high risk of failure during the treatment and should be considered in future response-adapted clinical trials.

Introduction

Follicular lymphoma (FL) is the second most common non-Hodgkin lymphoma in developed countries (1). It is genetically characterized by an upregulation of BCL2 in the progenitor B cell that transforms into a proliferating clone driven by t(14;18) translocations(2). Nowadays, FL is considered an indolent disorder with a relatively favorable course. Long remissions are often achieved with modern day treatments, with median survival rates approaching 20 years (3,4). However, 15–20% of patients are primary refractory or progress during the first two years after first-line therapy (POD24). These patients present a poor outcome, with 5-year overall survival (OS) probabilities between 38% and 50% (5,6).

The disease is characterized by a remitting, relapsing clinical course with progressive shortening of response duration after treatment. Moreover, high-grade transformation (HT) to aggressive lymphoma occurs in around 3% of the patients per year (7–10). Global research efforts have been aimed at identifying patients with a high risk of progression and transformation to optimize the duration of treatment response and to ease suffering and morbidity. Many clinical, molecular, pathological, and imaging biomarkers have been described and used to stratify/group FL patients into several risk categories at diagnosis (11–19). However, most of these tools remain inaccessible in daily practice and have not been adequately tested to select the best therapy.

For this reason, as the understanding of prognosis is crucial, the ultimate goal in a disease such as FL should be to develop tools and approaches to guide therapy. Current studies of risk-adapted therapy based on minimal residual disease (MRD) evaluation remain to be investigational in FL (20). At the molecular level, several studies have shown that MRD assessment is predictive of outcome. Most of these studies have focused on evaluating the BCL2/IgH rearrangements, quantified by polymerase chain reaction (PCR) in bone marrow or peripheral blood. The levels of circulating cell-free DNA (cfDNA) fragments have also demonstrated predictive value (21,22). However, unlike in diffuse large B cell lymphoma (DLBCL), in FL, there are no studies evaluating MRD based on tumoral cfDNA detection (23,24).

In contrast, PET/CT using the 5-point scale Deauville criteria (D5PS) is well established, regardless of the treatment used (25,26), to predict the outcome and attain complete metabolic response. Nevertheless, the use of PET/CT alone is hampered by its limited sensitivity and specificity, and the interpretation of the results is highly dependent on the evaluating radiologist (27,28). Moreover, there is little information on interim PET/CT (26) and its combination with MRD for prognostic assessment (20,29), and there are no studies that have used liquid biopsy NGS methods. Therefore, in this study, we aim to analyze the response

to therapy in FL patients using ultra-deep sequencing of cfDNA and the D5PS scale PET/CT to identify, early on, those patients who have a high risk of relapse in less than 24 months (POD24).

Materials/Subjects and Methods

Patient Cohort and Study Design

This study was designed as a prospective observational study. The cohort included 84 newly diagnosed, recurrent or transformed FL patients recruited from the routine clinical practice at the Hospital 12 de Octubre (H12O) in Madrid, Spain, and the Hospital Universitario de Toledo, Spain. Of the initial 84 patients, 58 were under first-line treatment, 15 received savage therapy and 11 did not receive treatment (Supplemental figure S1). The median follow-up for the patients in first-line therapy was 20.5 months (rank 3-65.5), and 31 (rank 5-43.5) months for patients in savage treatment. One patient (FL5), suffered from a transformation 10 months after the first-line therapy started. After transformation, the patient was included as a transformed case (FL5t). Informed written consent of all patients was obtained according to the Declaration of Helsinki. The study inclusion criteria were; histological confirmation and the availability of enough biological material in sequential samples. Treatment was started according to the *Groupe d'Etude des Lymphomes Folliculaires* criteria (30), and imaging examinations were performed as ordinary clinical practice. Responsible physicians decided on the treatment regimen according to institutional and international guidelines. In all patients, DNA from lymph node biopsies and cfDNA was obtained before the treatment was started. Somatic mutations of these samples were selected as disease biomarkers for liquid biopsy MRD (LiqBio-MRD) analysis at follow-up time-points. The following biological materials were analyzed: DNA from formalin-fixed paraffin-embedded (FFPE) lymph node biopsies (n = 75) and cfDNA (n = 44) before treatment start, and follow-up cfDNA samples during chemo-immunotherapy courses (n = 151).

DNA extraction

Lymph node DNA was extracted with a Qiamp gDNA FFPE kit (Qiagen, Hilden, Germany) using two to four sections from 5 to 10 microns, cut from the original paraffin block. Then, the genomic DNA (gDNA) was eluted in 35 µL ATE buffer and quantified using the Qubit BR kit (Thermo Fisher Scientific, Waltham, Massachusett, USA). For cfDNA extraction, 10 to 20 mL peripheral blood was collected in EDTA tubes and processed in less than four hours at the H12O. Samples from Toledo were collected in Roche Cell-Free DNA collection Tubes (Roche Diagnostic, Basel. Switzerland) and sent to the H12O, where plasma separation and cfDNA purification were centralized. There were no differences in cfDNA quantity or quality observed between EDTA and Streck collection tubes. The plasma was separated with two centrifugation steps at 1600 g and 4500g, and stored at -80 *°C* until further use. The purification of cfDNA was performed with a Qiamp Circulating Nucleic Acid kit (Qiagen) and quantified using a Qubit HS kit (Thermo Fisher Scientific). Fragment size and genomic DNA contamination were quantified using a Bioanalyzer 2100 fragment analysis system (Agilent, Santa Clara, California. USA).

Baseline Genotyping and LiqBio-MRD Biomarker Selection

The lymph node gDNA and plasma cfDNA baseline samples were screened for mutations with a short-length Ampliseq Custom Panel (Thermo-Fisher). The panel, established as a routinary diagnosis tool at the H12O, was designed to cover all coding regions of 56 lymphoma-specific genes in the FFPE samples (Supplemental Table S1). Samples were sequenced with an average coverage of 2,150x on an Ion S5 System platform (Life Technologies, Thermo Fisher Scientific). Variant annotation was performed using the default annotate variants single sample workflow from the Ion Reporter software (version 5.18.2.0). Mutations were called when presented more than nine mutated reads and a Variant Allele Frequency

(VAF) above $2 \cdot 10^{-2}$ (2%). In the case of FFPE samples, deamination-related base changes were reduced by filtering out C>T / G>A changes with a VAF below $2 \cdot 10^{-1}$ (20%) and a transformed *p*-value greater than -2, unless previously described as a somatic aberration in FL (COSMIC database). To select best MRD biomarkers for each patient, we first excluded all potential SNPs, germinal variants and missense variants of unknown significance (VUS) using COSMIC, CLinVar, and dbSNP databases. Then we categorize the remaining somatic mutations based on the number of cancer patients affected and prioritized the ones more frequently involved in lymphoma until we have up to 5-10 markers per patient when possible. (Supplemental Table S2).

LiqBio-MRD Methodology and Bioinformatic Pipeline

A median of 16.14 ng per mL of plasma (range 2.63-489 ng/mL) was obtained, being the mean amount of cfDNA from the initial 10–20 mL of peripheral blood (PB) of 73.6 ng (range 15.75–1,935 ng). All samples with a gDNA/cfDNA ratio greater than one, calculated using the bioanalyzer electropherogram, were excluded. The minimum quantity of cfDNA targeted for sequencing was 15ng. Considering that a genome equivalent (GE) has a mass of 3pg (31), 15ng is enough to screen 5,000GE and therefore achieve a sensitivity of $2 \cdot 10^{-4}$ VAF (0.02%).

An amplicon-multiplexed mini-panel was defined for every patient to detect the selected MRD biomarkers identified at diagnosis. The mini-panel included molecular-tagged primer pairs (6-mer tags) to amplify every selected mutation in three biological replicates defined as P1, P2, and P3. (Supplemental Table S3 and Figure S2). Then, the three independent tagged post-PCR products were combined in a single tube to continue with sample preparation. Final libraries were sequenced on the Ion S5 System platform (Life Technologies, Thermo Fisher Scientific Inc.) with an estimated depth of 500,000x per amplicon, as previously described (32,33). Despite the dilution curves of 53 genetic variants indicated a potential VAF

sensibility below 10^{-4} (Supplemental figure S3), all potential MRD biomarkers were screened with the same pipeline in triplicates of three gDNA samples obtained from healthy control donors. This permitted to define the LOD for every MRD biomarker (mean VAF plus three standard deviations) and exclude all the potential MRD markers that presented a LOD above 10^{-4} (Supplemental Figure S4).

The FASTQ files produced after sequencing were automatically demultiplexed to separate the reads from the different amplicons and triplicates. To reduce the false positive rate, a strict bioinformatic pipeline was programmed in Python and R to eliminate low-quality reads. The specific wild-type and mutated sequences were generated for each genetic position. These sequences, obtained from the corresponding demultiplexed output file, cover the affected locus with 15 bp upstream and downstream. Only the reads that perfectly match these sequences were considered to calculate the VAF for each triplicate. The noise effects arising from PCR and sequencing were controlled by identifying and removing triplicates that overpassed the mean VAF plus one standard deviation (SD). Finally, the corrected VAF was compared with the LOD calculated for each mutation independently, using three triplicates of three healthy donors as previously explained. The LOD was computed as the mean VAF in control samples plus three times the SD. Every MRD biomarker with a corrected mean VAF below the LOD was automatically eliminated. (Supplemental Table S3) The final LiqBio-MRD value was defined by the mutation with the highest VAF at the sampling time-point, as shown in Supplemental Figure S5.

PET/CT Imaging

The PET/CT scans were performed with a General Electric Discovery MI (GEDMI) Scanner or a Siemens Biograph 6 Scanner. PET/CT and CT images were acquired in the same session after injection of 2.5-3 MBq/kg ^{18}F-FDG (fluorodeoxyglucose) for the GEDMI Scanner and 4-5 MBq/kg ^{18}F-FDG for the Siemens scanner. All follow-ups were performed in the GEDMI scanner. CT scans obtained with a low-dose protocol

were used for attenuation correction of the PET/CT images. Interim and EOT ^{18}FDG-PET/CT scans were visually assessed according to the D5PS, with ^{18}FDG uptake of any residual lesion, using mediastinal blood pool and liver uptake as reference settings. PET/CT was considered to be positive when the Deauville's score was four or five, and Deauville's scores from one to three were classified as PET/CT negative.

In first-line therapy, PET/CT was performed before starting the treatment, after four cycles (*n* = 40), and at the end of treatment (EOT, *n* = 54). After finishing induction, patients were closely monitored with a physical examination and routine laboratory tests. A new scan was performed only when new symptoms or laboratory changes were detected. PET/CT was generally performed at mid-induction (*n* = 7) and EOT (*n* = 14) for patients treated in other lines. The exact time-points are listed in Supplemental Table S4.

Statistical Analysis

The Kruskal–Wallis test was used to determine statistically significant differences in the obtained samples' MRD values among the PET/CT-related categories. Then, a post hoc Dunn test was conducted to identify the statistically significant pairwise comparisons and the corresponding *p*-values. The tests were both performed using Python, the prior with the Python package SciPy (version 1.6.2) and the latter with the Python package scikit-posthocs (version 0.6.7). The Pearson correlation coefficient was used to assess the linear relationship between the different variables under study. Univariable Cox proportional hazards regression models and Kaplan–Meier survival analysis were performed to test statistical associations between genetic and imaging findings and survival outcomes. Statistical calculations were conducted using SPSS 22.0 (IBM SPSS Inc, Chicago). *P*-values of ≤0.05 were considered to be significant.

Results

Patients' Characteristics and Predictive Features

A total of 84 FL patients were included in the study (Supplemental figure S1). The median age was 63 years (35–90 years), and 58% of the patients were female. Eleven cases did not require treatment, 85% of the patients presented low histological grade (1–2), and 79% had advanced (III–IV) Ann Arbor stage (Table 1). Regarding treated cases (*n* = 73), 58 patients received first-line treatment (39 RCHOP, 10 R-bendamustine, 4 rituximab monotherapy, and 5 radiotherapy). The other 15 patients were treated with salvage therapy after a previous relapse (Supplemental Table S4). More important prognostic indexes in FL were analyzed. High-risk patients defined by FLIPI, FLIPI2, m7-FLIPI, and PRIMA PI did not show shorter PFS. Bulky disease, the presence of symptoms B, and a lymphocyte-to-monocyte ratio (LMR) of <2.5 were the only variables associated with a higher risk of relapse ($p < 0.05$). After a median follow-up of 22 months (rank 3-61.5), 19 patients relapsed after a median of 19 months (16 patients with grade I, II, or 3A and 3 patients transformed). Eight cases died after a median of 29 months (five patients had a low histological grade, and three patients were transformed). The causes of death were lymphoma in four cases (three transformed), infection (*n* = 2), and secondary neoplasia (*n* = 1). Regarding the subgroup of patients treated in first-line therapy (n=58) 15 patients relapsed after a median of 12.6 months (3.9-44.9).

According to the interim evaluation of response in patients treated with first-line therapy (n=40), 14 patients (35%) reached partial response (PR), and 26 complete response (CR; 65%). At the end of induction treatment (n=54), 40 patients reached CR (74%), eight patients PR (15%), one stable disease (SD; 2%) and five progression disease (PD; 9%). In the relapse setting at mid-induction (n=7), two patients reached CR (29%), and five PR (71%). At the end of treatment (n=14), ten patients achieved CR (72%), one PR (7%) and three PD (21%).

Baseline Genotyping on cfDNA Complements Lymph Node Screening in Follicular Lymphoma

Baseline genotyping with the targeted NGS panel was performed in 75 lymph node samples and 44 cfDNA plasma samples. In the lymph node samples, 510 mutations were detected with an average of 6.8 somatic mutations per patient (range 0–31) and a mean VAF of 0.31 (range 0.026–1.0). In the cfDNA plasma samples, 144 mutations were detected (average 3.3, range 0–11) with a mean VAF of 0.22 (range 0.025–0.857). Only 4 of 75 (5%) lymph node samples did not present any alteration suitable for MRD monitoring. This number increased to 20% (9 of 44) when only baseline cfDNA was considered. However, somatic mutations were detected in the cfDNA fraction for six of the eight patients without lymph node samples available.

As previously described (12), the most frequently mutated genes were *KMT2D*, *CREBBP*, *BCL2*, *TNFRSF14,* and *EZH2* (Figure 1A). Although the samples from transformed patients had a similar genetic profile, an increase of *TP53* mutations was observed in this subcohort (3/10, 33% vs. 7/73, 9%, Supplemental Figure S7). Within the 36 cases with available paired lymph node and plasma samples, 88 somatic mutations were identified in both fractions, 33 somatic mutations were only detectable in liquid biopsy, and 160 somatic mutations were only in the lymph node (Supplemental Table S2, Supplemental Figure S6).

Clinical Impact of Disease Monitoring by LiqBio-MRD

The dynamics of the baseline mutations were analyzed on 151 cfDNA follow-up samples from 54 of 73 patients that received treatment (Figure 1B). Additionally, sequential samples of eleven untreated ”watch and wait” patients were screened. On average, 3.6 somatic mutations per patient (range 1–12) were selected as MRD biomarkers. Nine mutations were excluded from further analyses as they presented a LOD above $1 \cdot 10^{-4}$. These mutations mainly affected insertions or deletions of one base (Supplemental Figure S4). On the other hand the elimination of outlier triplicates permitted to identify and correct nine

false positives follow-up samples. To compare PET/CT and liquid biopsy results, we defined four groups based on the PET/CT data available when the liquid biopsy sample was collected. The first group (complete response, CR) included follow-ups with a Deauville score of 1, 2, or 3 in nodal or extranodal sites with or without a residual mass. The Non-progressive disease group included partial response with a Deauville score 4, or 5 with reduced uptake compared with baseline and residual mass(es). The Progressive disease includes a Deauville score 5 in any lesion with an increase in the intensity of FDG uptake from baseline. The Non-treatment group had samples before treatment started or from untreated patients. (Figure 1C) The LiqBio-MRD values were significantly lower in the CR and Non-progressive disease groups compared to the progressive group ($p < 0.001$ and *p=0.019,* respectively). However, of 53 samples included in the CR group, four were LiqBio-MRD positive. Of note, all samples in ”watch and wait” group (eight samples from eight patients) were positive by the LiqBio-MRD test.

The 54 treated patients with sequential samples available presented an average of three liquid biopsy samples (rank 1–8) after treatment started. Of them, 44 patients were under first-line treatment. To calculate the clinical impact of the LiqBio-MRD test in first-line patients, we defined three different timeframe groups: Early follow-up ($n = 23$) included cfDNA samples from cycles I and II; the interim group ($n = 23$) included samples obtained in cycles III and IV; the final or EOT group included 32 cfDNA samples obtained in cycle VI or the first sample available under maintenance (Figure 2, left). Positive LiqBio-MRD values in the early group did not increase the risk of progression (HR_{EARLY} 2.3, 95%, CI 0.44–11.8, $p = 0.310$). This tendency changed at interim monitoring (HR_{INT} 11.0, 95% CI 2.10–57.7, $p = 0.005$). The differences between LiqBio-MRD positive and negative cases were even more pronounced at EOT (HR_{EOT}, HR 19.1, 95% CI 4.10–89.4, $p < 0.001$), Figure 2, right). The results of the entire cohort, including relapsed patients, are shown in Supplemental Figure S8.

Interim Monitoring by PET/CT of Previously Untreated Patients Predicts Progression

PET/CT D5PS tests also showed prognostic value at interim evaluation. PET/CT positive cases had a $mPFS_{INT}$ of 19 months vs. NR ($p < 0.001$**,** Figure 3A). Comparable results were observed at EOT ($mPFS_{EOT}$ PET/CT 13 months vs. NR, $p < 0.001$, Figure 3B), and when relapsed patients were included (Supplemental Figure S8). Of note, the distribution of cases according to interim and EOT TPs for PET/CT and LiqBio-MRD presented a concordance of 76% (Kappa = 0.401).

The Combination of LiqBio-MRD and PET/CT Identifies POD24 Patients

Next, we studied the 41 first-line patients with data available on both PET/CT and LiqBio-MRD. The last TP (interim or EOT) with data for both tests was considered for this analysis. Twenty-six patients were negative by both techniques, seven were positive, and eight presented discordant results. Considering only concordant results (*n*=33), the combination of both tests showed a sensitivity (SE) of 88% and a specificity (SP) of 100%, with a PPV of 100% and NPV of 96%. Strikingly, all positive patients by both tests had a 2-year PFS below 24 months (mPFS of 7 months for +/+ vs. NA for -/- cases, $p < 0.001$, Figure 4). Moreover, the only case (FL25) that progressed with a negative result by both tests, was positive in a sequential cfDNA sample obtained at maintenance, five months before progression. Regarding the eight cases with discordant results (20%), six patients were incorrectly classified by PET/CT, and only two patients were incorrectly classified by LiqBio-MRD (Figure 4). The results including relapsed patients are shown in Supplemental Figure S9.

Other approaches for disease monitoring were also tested. Flow cytometry data was only available for eleven patients with bone marrow infiltration. Two patients were positive at follow-up and nine patients were negative at follow-up, concurring at 100% with PET/CT and LiqBio-MRD. The BCL2/IgH rearrangements were screened in 33 patients with peripheral blood samples available. Rearrangements

were only detected at baseline diagnosis for ten of the patients, being all negative in follow-up samples. Two of these cases were positive by PET/CT and LiqBioMRD, four cases presented discordant results, and three cases were negative by both tests.

Dynamics of Somatic Mutations during the Follow-up of FL Patients

As indicated above, only one patient (FL25) had a negative result by PET/CT and LiqBio-MRD at interim and EOT and eventually progressed. Three somatic mutations affecting *KMT2D*, *CREBBP,* and *ARID1A* were found in this case. Of interest, this patient was MRD negative in three TPs obtained during the first year but had a positive LiqBio-MRD sample obtained 15 months after the start of treatment. An additional positive liquid biopsy was received three months later, and PET/CT was performed, confirming progression but with a low tumor burden (Figure 5A). The patient continued maintenance therapy, achieving a complete response a few months later.

The opposite dynamics were observed in patient FL5. This patient did not respond to RCHOP (DS5) and received R-bendamustine as second-line therapy. The interim PET/CT showed a poor response (DS5), and the biopsy confirmed the transformation to high-grade lymphoma. After failure of rescue treatment with R-GEMOX-dexamethasone and R-polatuzumab bendamustine, the patient received radiotherapy where a reduction in the main clone was observed (Figure 5B). Of note, the clone detected in cfDNA disappeared under first-line therapy.

Patient FL30 presented two mutations detected only in lymph node and two more only detected in cfDNA. In follow-up samples, the four mutations were undetectable. In the PET/CT evaluation, a residue was observed in the scans, complicating the interpretation of the imaging results (Figure 5C). This patient is still in CR after two years of follow-up.

Although a solid biopsy was unavailable for patient FL31, six somatic mutations were detected in the baseline liquid biopsy sample. In follow-up cfDNA samples, despite an initial reduction during the first two cycles, a rapid increase in the disease burden after cycle III was observed. The patient progressed in cycle VI and died under rescue therapy only nine months from the start of the treatment (Figure 5D). The dynamics of all the treated patients with follow-up samples available are shown in Supplemental Figure S9.

Discussion

This prospective study evaluates, for the first time, the usefulness of liquid biopsy MRD by ultra-deep sequencing in combination with D5PS PET/CT to identify, early on, those FL patients with a high risk of relapse in less than 24 months (POD24). Our approach is based on the use of somatic mutations as disease biomarkers, as we previously described for acute myeloid leukemia (32). First, we screened baseline lymph node and plasma samples to identify patient specific biomarkers. The genetic profile of our cohort mimics the one previously described by Pastore et al. (12) but with an expected increase of *TP53* alterations in transformed cases (34) (Supplemental Figure S1). Although the custom DNA panel was initially designed for FFPE samples, the small amplicon size permitted the detection of lymphoma-specific mutations in baseline liquid biopsies (Figure 1A). In our study, limited by the follow-up and the small and heterogeneous number of subjects, the only clinically relevant prognostic factor for PFS in newly diagnosed patients (n = 84) was the low lymphocyte-to-monocyte ratio (19). We did not find prognostic differences by applying IPI, FLIPI, FLIPI2, m7-FLIPI, or PRIMA IPI. However, somatic mutations suitable for LiqBio-MRD monitoring were found in 95% of patients with lymph node samples and 80% of patients with liquid biopsy samples available. Our results indicate that follicular lymphoma gene driver mutations are detectable in liquid biopsy. Still, more sensitive approaches are needed. In any case, the number of patients with potential

MRD biomarkers considerably improves the applicability of MRD assessment compared to other described techniques, such as PCR of the IGH/BCL2 translocation (20,29). Although PCR-positive is predictive of lower PFS, a considerable number of patients are t(14;18) negative (60%). Moreover, FL is a predominantly nodal disease, and the absence of t(14;18) in bone marrow does not adequately reflect the response status (41). Further, t(14;18) can also be found in healthy individuals at low level.

In this study, LiqBio-MRD was evaluated in the plasma of 54 patients that received treatment (43 first-line therapy). The analysis, performed in 151 follow-up cfDNA samples allowed us to define patient-specific disease dynamics (Figure 1B, Supplemental Figure S7) and their correlation with the clinical outcome (Figure 1C). To the best of our knowledge, there are no studies in FL evaluating early MRD assessed by liquid biopsy. Conversely, there are already some studies in DLBCL (24,35,36) and Hodgkin lymphoma (37) where it is known that levels often change rapidly after the initiation of therapy. Following this hypothesis in FL, we performed an early LiqBio-MRD evaluation (Cycle 2) on 23 patients. Although a trend to shorter PFS was observed in MRD-positive cases, 6 of 13 cases eventually became negative at later time points (Figure 2), suggesting that FL presents a different dynamic than DLBCL (38) and treatment need longer to cleanse the tumor.

Since the Lugano classification, the criteria to determine the quality of treatment response rely on the D5PS PET/CT evaluation (38). Several studies, including large retrospective subanalyses of randomized trials have shown that PET/CT negativity correlates solidly with PFS (25,39). Nevertheless, the use of PET/CT alone is hampered by its limited sensitivity and specificity and the interpretation of the results being highly dependent on the evaluating radiologist (27,40). Consistent with previous reports (23), from the 64 patients with available PET/CT scans at WOT (50 patients in first-line therapy), 70% of the patients reached a CR.

On the other hand, the LiqBio-MRD test showed an extraordinary capacity to identify patients at risk of progression after only four cycles of RCHOP (Figure 3A). Comparable results were observed by interim PET/CT (Figure 3B). This result suggests that LiqBio-MRD or PET/CT interim evaluation should be considered in future clinical trial settings. Regarding EOT evaluation, both tests segregated high-risk patients (Figure 3C, D). Although similar results have already been shown in FL patients using PCR-based MRD techniques, these approaches were hampered by a lower applicability (20,29,41–43).

PET/CT has only been used in combination with PCR in a small exploratory analysis and MRD refined the predictive power of PET/CT (29). However, no combination of PET/CT and MRD strategies has been reported using NGS liquid biopsy techniques. In our study, the combination of both methods identified 100% of the POD24 patients. As shown in Figure 4, patients with concordant results (*n* = 28) were almost perfectly segregated. These results remained after excluding transformed cases (Supplemental Figure S8). The only double negative patient (FL25, Figure 5A) that progressed presented an increase in tumor burden detectable by LiqBio-MRD five months before progression in a sample obtained within maintenance. This suggests that sequential monitoring with a minimally invasive test such as LiqBio-MRD may be essential to identify POD24 cases and anticipate patients' relapses. Another interesting case, FL5, illustrates how LiqBio-MRD may have other possible applications (Figure 5B). In this chemo-refractory patient, radiotherapy had an abscopal effect, confirmed by the rapid descend of ctDNA not otherwise explicated (44).

Our study has several limitations, including the limited number of patients, the heterogenous treatment administered, and the absence of available tests in all the time-points. However, several factors permitted the development of a LiqBio-MRD test with extraordinary performance. First, the MRD amplicons were designed shorter than 120bp and all MRD samples screened presented at least 15ng of cfDNA to guaranty the amplification of enough tumor cfDNA molecules. More importantly, the use of triplicates and the

definition of LOD in healthy control donors permitted to identify and correct the false positive values induced by PCR and sequencing errors or the variant intrinsic noise due to the genetic context. Although it seems mandatory to perform larger studies to confirm this preliminary data, our results demonstrate, for the first time, that NGS-based MRD quantification is feasible in liquid biopsies from FL. The achievement of negative ctDNA after treatment and in an interim analysis enhances prognostic information on the patients´ outcomes, both in first-line therapy and at relapse. PET/CT and LiqBio-MRD can synergistically contribute to predicting progression and POD24 with high sensitivity and specificity. Additionally, this test better reflects intra-patient tumor heterogeneity (45,46) and could be used to detect drug resistance, high-risk transformation and guide and monitor treatment.

In conclusion, LiqBio-MRD monitoring in FL represents a promising option, complementary to metabolic imaging, to identify patients at high risk of failure early on during treatment and is a useful approach to response-adapted precision therapy to be considered in clinical trials.

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Author Contributions

AJU, MP, IR, MG, RA, JML and SB designed the research. JC, RS, LR, AJ and MR performed the experiments. AM, YR, SD and JMR, CW, PT and SB defined the bioinformatic pipeline and performed sequencing data analysis. AJU, MP, GF, AR, CB, LPN, CG, MM, LFC, MC, TB, MG, PS and RS provided patient samples and clinical data. All authors analyzed and interpreted the data. AJU, MP, AM and SB wrote the manuscript which was approved by all authors.

Competing Interests

RA, JML and SB are equity shareholders of Altum Sequencing Co. LFC received honoraria and received research funding from Roche, Novartis, Astra Zeneca, Janssen, BMS, Pfizer and Incyte. The remaining authors declare no competing financial interests.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figure 1. Baseline genotyping and potential of the liquid biopsy MRD test (LiqBio-MRD) to monitor disease progression: (**A**) Oncoplot of the baseline genotyping of 75 lymph node solid biopsy samples (red) and 44 plasma liquid biopsy samples (yellow). Patients are represented in the X-axis, genes in the Y-axis; (**B**) Dynamics of circulating free DNA (cfDNA) quantified by LiqBio-MRD in 55 Follicular Lymphoma (FL) patients. The LiqBio-MRD values for each follow-up datapoint (Y-axis) are plotted against the month from treatment start (X-axis), patients that progressed are represented in red, complete and partial responses are represented in blue. (**C**) Correlation of Liqbio-MRD and PET/CT. The complete response (CR) included follow-ups with a Deauville score of 1, 2, or 3 in nodal or extranodal sites with or without a residual mass. The Non-progressive disease (excluding CR) group included partial response cases with Deauville score of 4, or 5 with reduced uptake compared with baseline and residual mass(es). The progressive disease includes a Deauville score 4 or 5* in any lesion with an increase in the intensity of FDG uptake from baseline.

Figure 2. Clinical impact of early monitoring by LiqBio-MRD: (**left**) Swimmer plot of the different follow-up time-points screened for all patients under first-line treatment included in the survival analysis. Red boxes represent time-points with positive Minimal Residual Disease (MRD) value. Blue boxes, samples with negative MRD value. The samples used in the survival analysis at Early (E, 1-2 RCHOP cycles), Interim (I, 3-4 RCHOP cycles) and Final (F) or end of treatment (EOT, 5-6 RCHOP cycles) time-points are indicated for

each patient. (**Right**) Kaplan–Mayer curves of the impact of LiqBio-MRD monitoring at the different time-points. These analyses were performed with all the patients under first-line treatment.

Figure 3. Interim monitoring by PET/CT predicts progression in first-line treated patients. Kaplan–Meier curves showing the impact of interim(**A**) and end of treatment (EOT) (**B**) monitoring by PET/CT scan in patients under first-line therapy.

Figure 4. Clinical Impact of the LiqBio-MRD and PET/CT combination in first-line therapy. Kaplan–Meier curves show the capacity to identify patients that progress during the first two years after first-line therapy (POD24) when both tests are combined. An analysis was performed using the last time-point with both determinations available (interim or EOT).

Figure 5. Examples of the disease dynamics monitored by LiqBio-MRD. The left panel represents the baseline genotyping of lymph nodes (SolBio) and plasma cfDNA (LiqBio) samples after applying the low sensitive targeted panel (sensitivity $2{\cdot}10^{-2}$). The panel in the middle represents the variant allele frequency (VAF) values of the different mutations obtained by the ultrasensitive LiqBio-MRD test (sensitivity $2{\cdot}10^{-4}$). The right panel represents the limit of detection (LOD; mean + three standard deviations) defined in healthy control datapoints for every tracked mutation. Mutations with LOD above $1{\cdot}10^{-4}$, represented with dotted lines, were not used for MRD value calculation. RCHOP: cyclophosphamide/doxorubicin/ prednisone/rituximab/vincristine. R-Benda: rituximab/bendamustine. Pola: polatuzumab. GEMOX: gemcitabine/oxaplatin. Dexa: Dexamethasone.

Table 1. Patient characteristics.

		All series (n=84) *	First-line therapy (n=58)	Other lines of therapy (n=15) **
Age (y), median (rank)		63 (35-90)	65 (37-86)	62 (44-90)
Male gender		35/84 (42%)	27/58 (47%)	4/15 (27%)
Median follow-up since treatment (months)		-	20.5 (3-61.5)	31 (5-45.3)
Ann-Arbor Stage ≥3		66/84 (79%)	46/58 (79%)	14/15 (93%)
Histological grade	**1-2**	71/84 (85%)	51/58 (88%)	11/15 (73%)
	3A	4/84 (5%)	2/58 (3%)	1/15 (7%)
	3B	1/84 (1%)	1/58 (2%)	1/15 (7%
	Transformed	7/84 (9%)	4/58 (7%)	3/15 (20%)
B symptoms		27/84 (32%)	16/58 (28%)	10/15 (66%)
ECOG-PS ≥ 2		9/84 (11%)	5/58 (9%)	4/15 (27%)
Bulky disease		17/84 (20%)	14/58 (24%)	3/15 (20%)
Bone marrow involvement		34/83 (41%)	23/58 (40%)	8/15 (53%)
Haemoglobin < 12 g/dL		12/84 (14%)	8/58 (14%)	4/15 (27%)
Lactate dehydrogenase >UNL		33/84 (39%)	26/58 (45%)	3/15 (20%)
B_2-microglobulin elevated		31/77 (40%)	24/55 (44%)	3/11 (28%)
FLIPI risk	**Low**	16/84 (19%)	9/53 (17%)	5/15 (33%)
	Intermediate	30/84 (36%)	17/53 (32%)	4/15 (27%)
	High	38/84 (45%)	27/53 (51%)	6/15 (40%)
FLIPI2 risk	**Low**	39/71 (55%)	25/55 (43%)	5/10 (50%)
	Intermediate	17/71 (24%)	13/55 (24%)	4/10 (40%)
	High	15/71 (21%)	12/55 (23%)	1/10 (10%)
PRIMA PI	**Low**	39/77 (51%)	25/55 (51%)	4/10 (40%)
	Intermediate	17/77 (22%)	10/55 (18%)	3/10 (30%)
	High	21/77 (27%)	17/55 (31%)	3/10 (30%)
Lymphocyte to monocyte ratio < 2.5		29/84 (34.5%)	21/58 (36%)	5/15 (33%)

* Eleven patients in "watch and wait" policy at the time of analysis

** Eight patients with two previous therapy lines and six with three or more (see supplementary table 4)

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649 **Figures**

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Figure 1

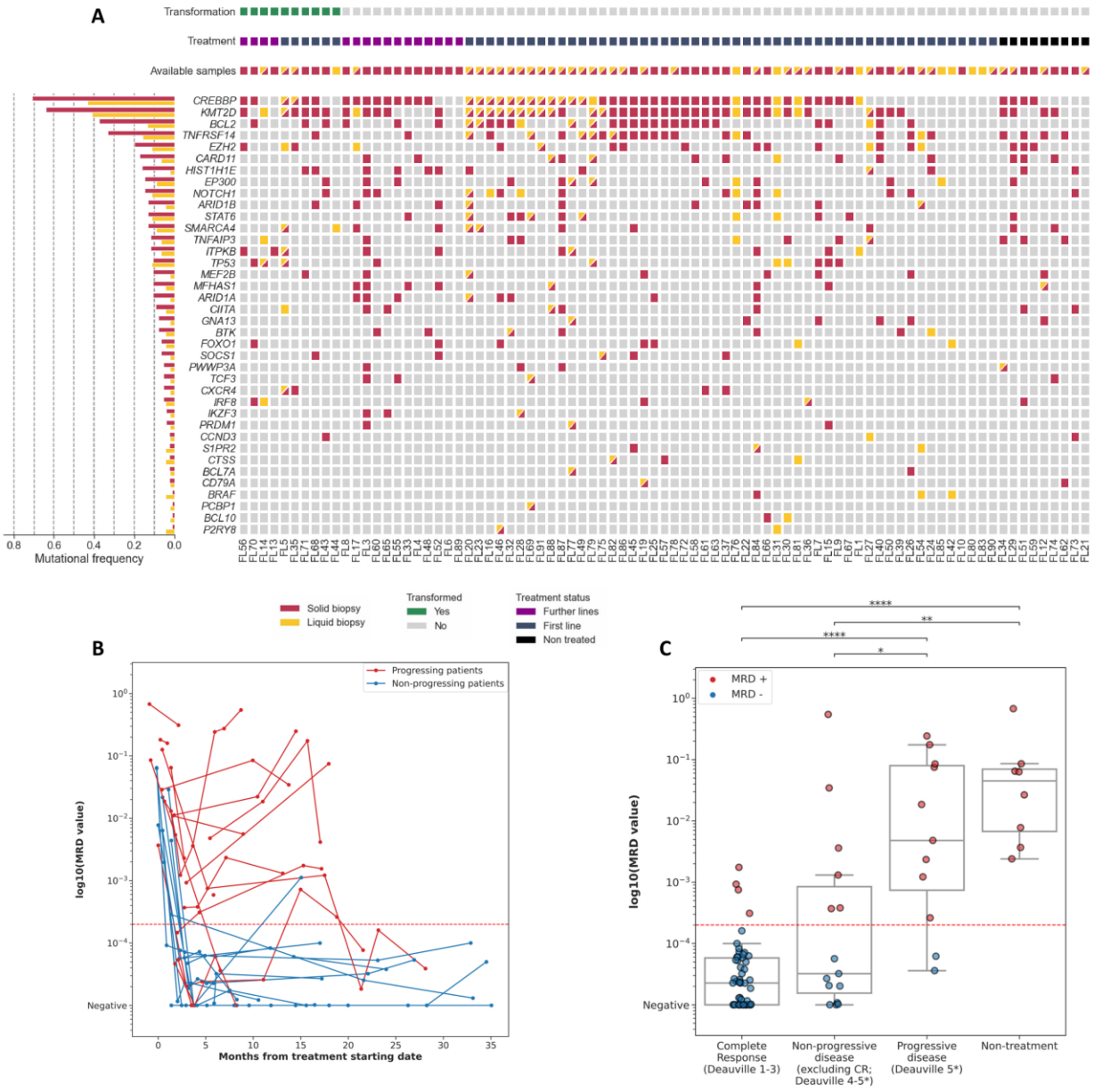

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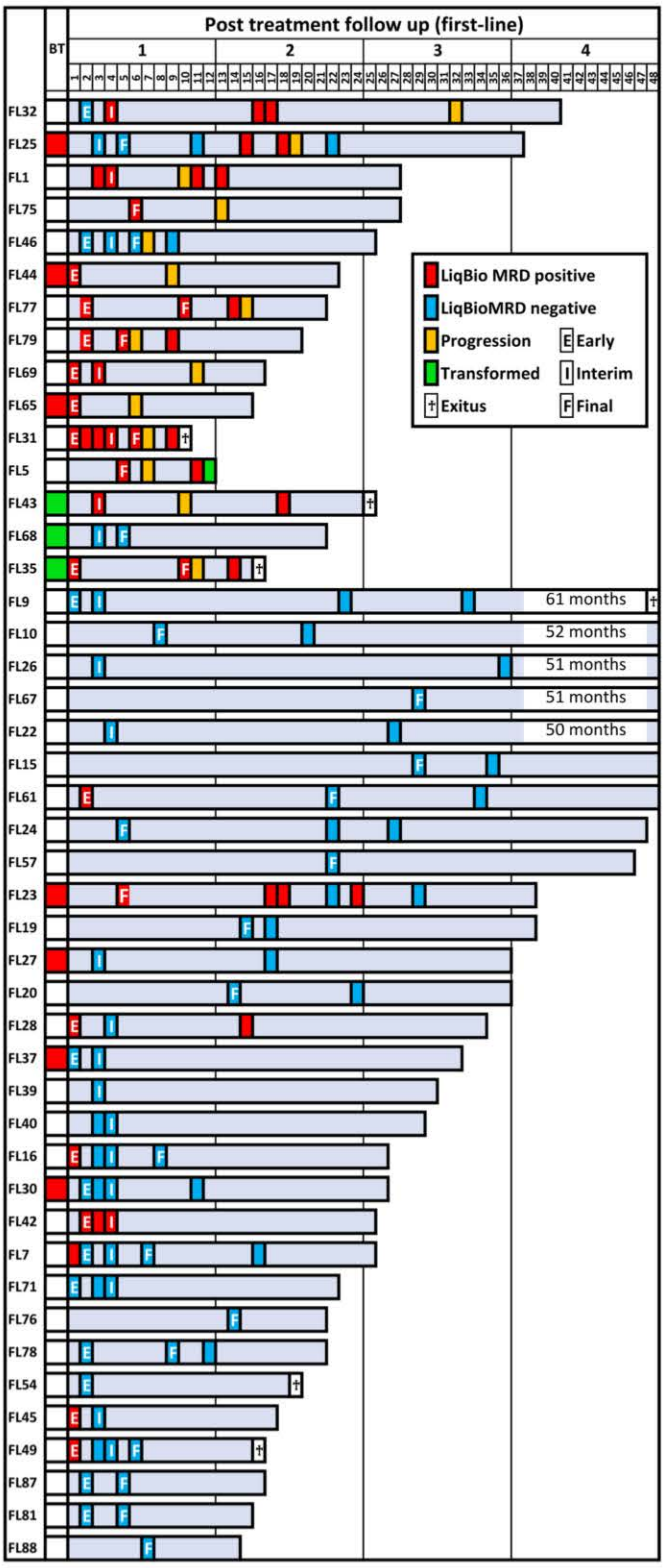
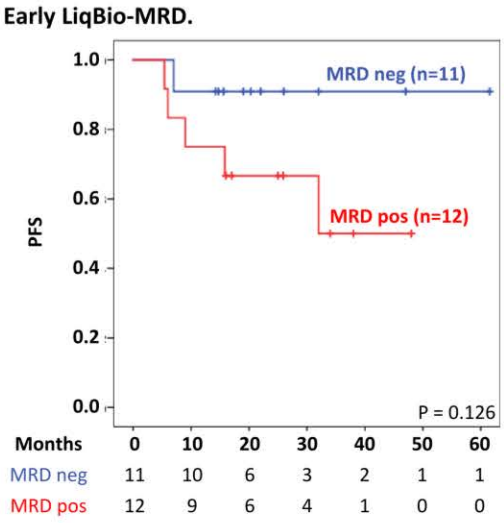
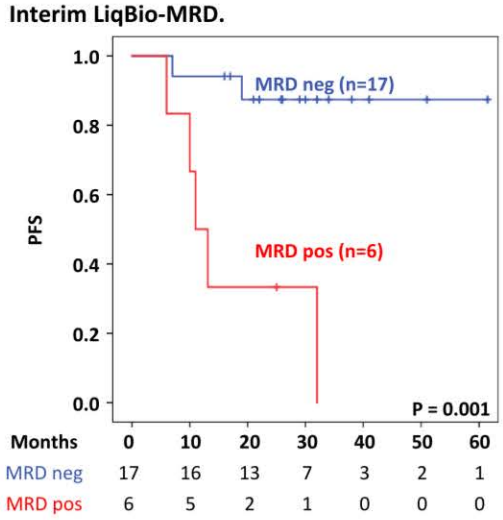
Figure 2


Post treatment follow up (first-line)
BT
1
2
3
4
LiqBio MRD positive
LiqBioMRD negative
Progression
Transformed
Exitus
E Early
I Interim
F Final
61 months
52 months
51 months
51 months
50 months


Early LiqBio-MRD.
MRD neg (n=11)
MRD pos (n=12)
PFS
P = 0.126
Months 0 10 20 30 40 50 60
MRD neg 11 10 6 3 2 1 1
MRD pos 12 9 6 4 1 0 0


Interim LiqBio-MRD.
MRD neg (n=17)
MRD pos (n=6)
PFS
P = 0.001
Months 0 10 20 30 40 50 60
MRD neg 17 16 13 7 3 2 1
MRD pos 6 5 2 1 0 0 0

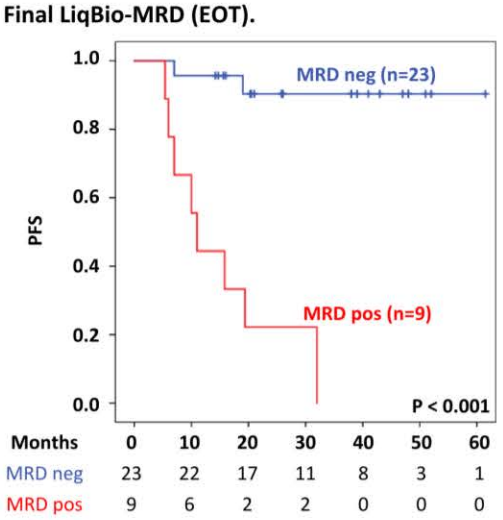

Final LiqBio-MRD (EOT).
MRD neg (n=23)
MRD pos (n=9)
PFS
P < 0.001
Months 0 10 20 30 40 50 60
MRD neg 23 22 17 11 8 3 1
MRD pos 9 6 2 2 0 0 0

Figure 3

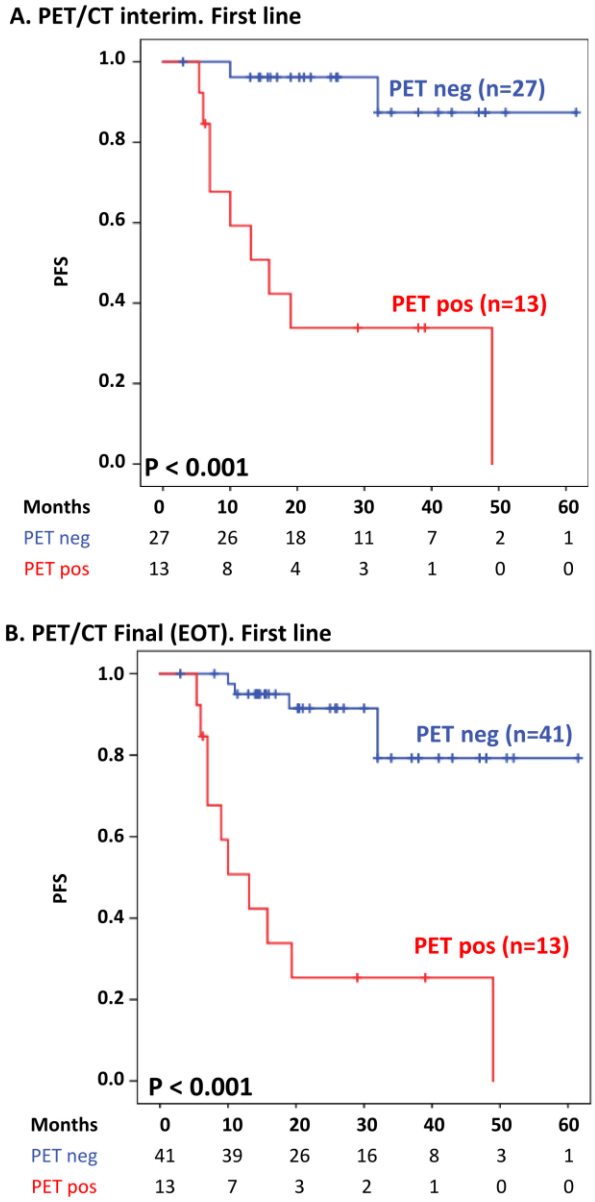


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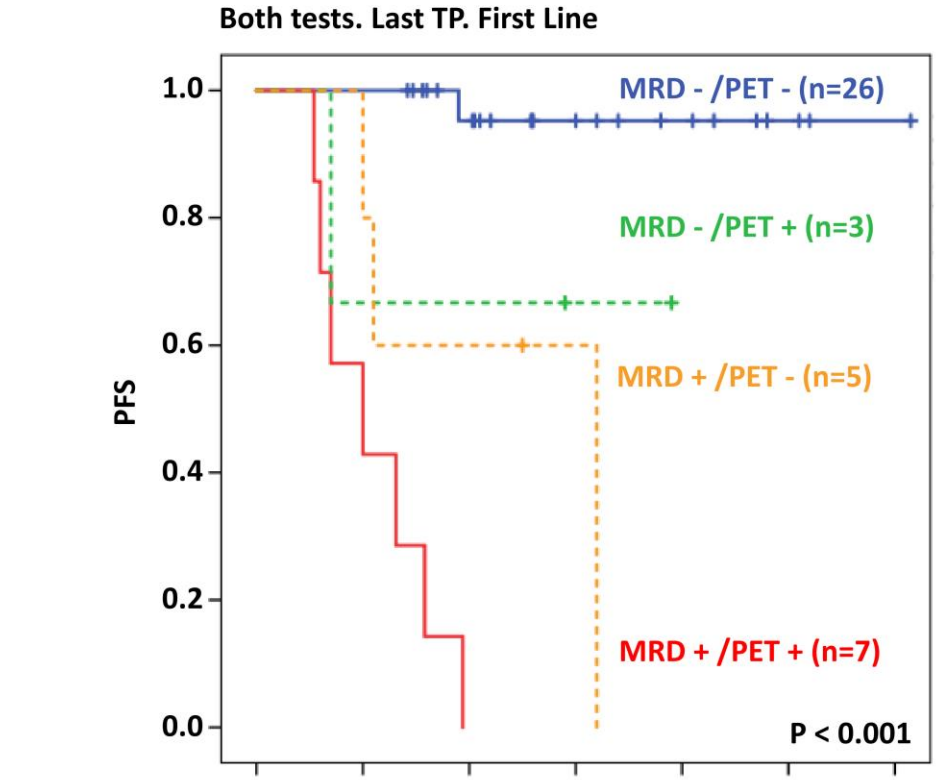
Figure 4



Months	0	10	20	30	40	50	60
MRD - /PET -	26	26	20	13	8	3	1
MRD - /PET +	3	2	2	2	1	0	0
MRD + /PET -	5	5	3	2	0	0	0
MRD + /PET +	7	4	0	0	0	0	0

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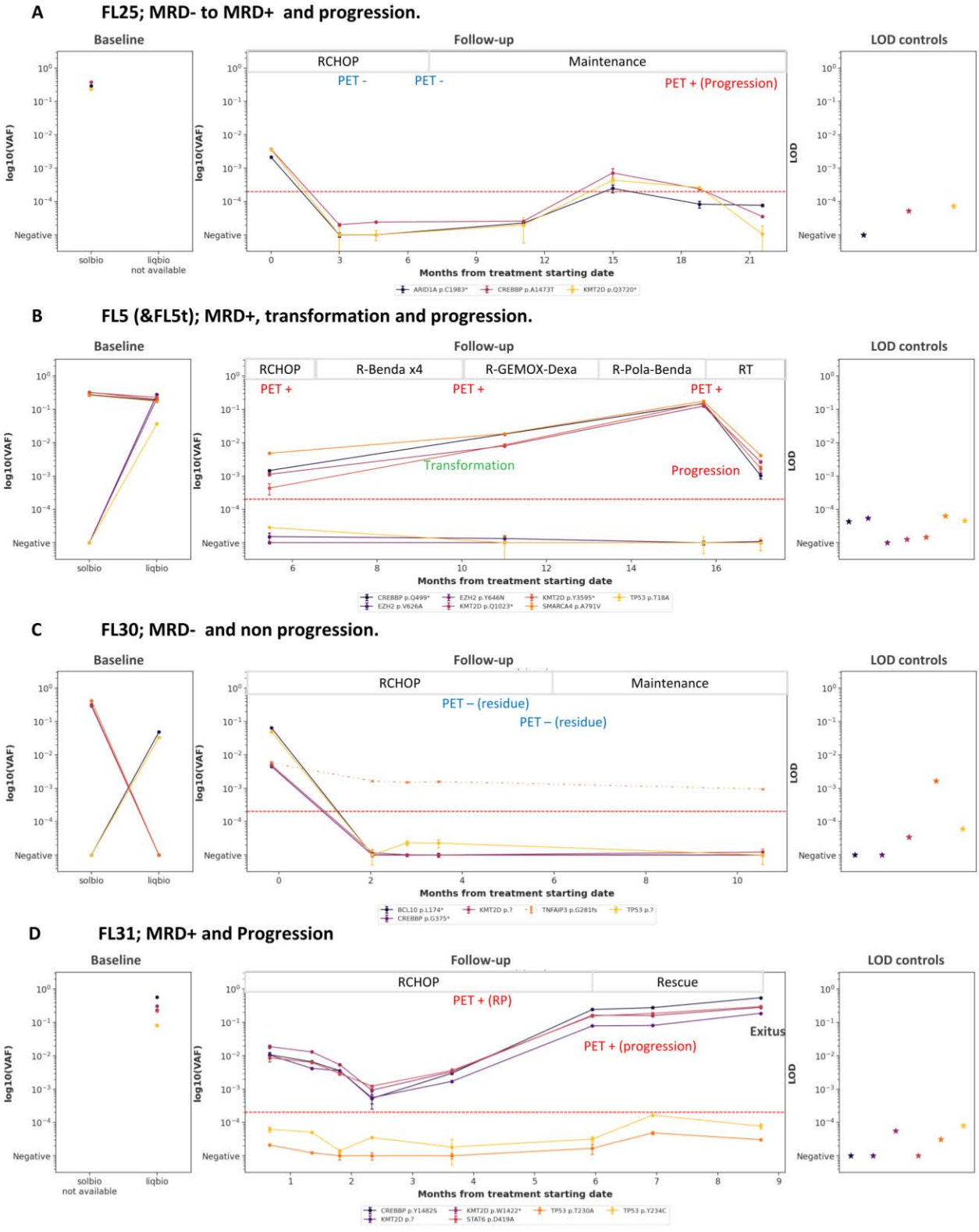
Figure 5


A
FL25; MRD- to MRD+ and progression.
Baseline
Follow-up
LOD controls
RCHOP
Maintenance
PET -
PET -
PET + (Progression)
log10(VAF)
LOD
Negative
solbio
liqbio not available
Months from treatment starting date
ARID1A p.C1983*
CREBBP p.A1473T
KMT2D p.Q3720*
B
FL5 (&FL5t); MRD+, transformation and progression.
RCHOP
R-Benda x4
R-GEMOX-Dexa
R-Pola-Benda
RT
PET +
PET +
PET +
Transformation
Progression
CREBBP p.Q499*
EZH2 p.V626A
EZH2 p.Y646N
KMT2D p.Q1023*
KMT2D p.Y3595*
SMARCA4 p.A791V
TP53 p.T18A
C
FL30; MRD- and non progression.
PET – (residue)
PET – (residue)
RCL10 p.L174*
CREBBP p.G375*
KMT2D p.?
TNFAIP3 p.G281fs
TP53 p.?
D
FL31; MRD+ and Progression
Rescue
PET + (RP)
PET + (progression)
Exitus
CREBBP p.Y1482S
KMT2D p.?
KMT2D p.W1422*
STAT6 p.D419A
TP53 p.T230A
TP53 p.Y234C