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Molecular surveillance of aggressive large B-cell lymphoma using circulating tumor DNA duplexes

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Running head: ctDNA surveillance in B-cell lymphomas

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Conflicts of interests

H.H*: Genmab: Safety Committee; Incyte: Consultancy; Novartis: Research Funding; Elicera Therapeutics: Safety Committee; Serb SA: Advisory Board. J.J*: AbbVie: consultancy; BMS/Celgene: Consultancy; Gilead: Consultancy; Incyte: Consultancy; Novartis: Consultancy; Caribou Bioscience: Consultancy; Roche: Consultancy; SOBI: consultancy. P.B*: BMS: Consultancy; Gilead: Consultancy; Novartis: Consultancy; Roche: Consultancy. Ø.F*: Roche: Consultancy; Bayer: Consultancy. S.L*: AbbVie: Honoraria, Consultancy; AstraZeneca: Consultancy; BMS: Consultancy, Research Funding**; GENMAB: Research Funding**; GILEAD: Honoraria; Incyte, Consultancy,

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*All outside of the submitted work.

**To the institute

Author contributions

Conceptualization: LM, SL

Sample and clinical data collection and processing: MAr, JJ, MLK, KB, MP, KD, ØF, SJ, PB, HH, SL

Investigation: LM, MAu, SL

Formal analysis: LM

Data interpretation: All authors

Funding acquisition: SL

Writing - Original Draft Preparation: LM

Writing - Review & Editing: SL

Final approval of manuscript: All authors

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Ethics approval and trial registration

The study was conducted in accordance with the Guidelines on Good Clinical Practice from the International Conference on Harmonization and the principles outlined in the Declaration of Helsinki. The protocol was approved by the medical agencies and ethics

committees in Finland, Denmark, Norway, and Sweden, and the trial was registered at ClinicalTrials.gov (NCT03293173). All patients signed informed consent forms before participating in the study.

Data sharing statement

In accordance with European Union legislation, the General Data Protection Regulation (GDPR) and the Finnish Act on the Secondary Use of Health and Social Data, the clinical annotations and the linked sequencing data from this study cannot be deposited or shared publicly due to the sensitive patient data protection. Collaboration agreements that comply with the above and are accepted by the local ethics committees and authorities can be requested from the last author.

Abstract

Treatment failure affects one-third of patients with aggressive large B-cell lymphoma (LBCL). However, currently available clinical tools are inadequate for determining remission and fail to detect recurrence early. We investigated the added value of longitudinal monitoring of circulating tumor DNA (ctDNA) in plasma by targeted duplex sequencing during therapy and follow-up in high-risk LBCL patients treated in a Nordic phase II trial. Early clearance of ctDNA after the first two immunochemotherapy cycles provided a rapid pharmacodynamic measure of treatment efficacy with excellent negative predictive value (NPV, 91%) for progression. Later clearance after four cycles improved prognostication, and elevated interim ctDNA levels among those with a measurable residual disease (MRD) predicted refractory disease. At the end of therapy, MRD was a strong independent predictor of outcome, with two-year progression-free survival rates varying greatly (Hazard ratio = 18.7; 94.3% vs 18.2%, MRD negative and positive, respectively). Moreover, end-of-therapy ctDNA analysis resolved most false radiological response assessments and revealed poor targeting of post-therapy interventions to MRD-positive cases. On surveillance, ctDNA was detectable at least 3 months before systemic clinical relapse (median 6 months), while the patients with isolated central nervous system relapses had the shortest plasma ctDNA lead times. Overall, we demonstrate that ctDNA monitoring using an ultrasensitive technique provides a dynamic molecular tool to guide clinical decision-making during therapy and follow-up in patients with LBCL.

Introduction

Disease recurrence affects 30% of patients with aggressive large B-cell lymphoma (LBCL) and predicts poor outcomes(1). Treating LBCL relapse is challenging(2), and high tumor volumes predict poor survival(3). Early detection of recurrence could improve outcomes; therefore, treatment responses are monitored, and patients undergo imaging with computed tomography (CT) or positron emission tomography (PET), although the utility of these imaging modalities for surveillance is limited(4, 5). Repeated imaging exposes patients to radiation, the sensitivity of these methods is modest, and the poor specificity leads to invasive and unnecessary biopsies, which can potentially harm patients(6, 7). Consequently, more accurate methods are needed to determine remission and monitor recurrence in LBCL.

Liquid biopsies using plasma circulating tumor DNA (ctDNA) complement diagnostics, predict treatment response, and inform survival in LBCL(8). These tools are expected to transform clinical decision-making(9-12). The presence of ctDNA in plasma at diagnosis and recurrence is well established; however, detecting it before clinical relapse requires sensitive methods(8). Early studies used immunoglobulin high-throughput sequencing (Ig-HTS) to detect ctDNA, showing promising results for response evaluation and surveillance(9, 10). However, Ig-HTS lacks sensitivity, tracking only a single or a few genomic events, resulting in a disappointing 56% detection rate at relapse or before relapse in a prospective study(13). Hybrid-capture panel techniques can detect ctDNA more sensitively than Ig-HTS by tracing more mutations, but sequencing error rates limit their performance(11, 14, 15). Phased variants (PVs) are unlikely to be detected by error and can be harnessed to improve the sensitivity of disease monitoring(16), or they can

be used to suppress the error rate of sequencing(14). Still, lymphoma driver mutations are rarely phased events, which are mostly limited to hypermutable genomic regions affected by clonal heterogeneity, and disease detection mainly depends on the number of informative molecules(17).

Duplex sequencing, which relies on the recovery of both strands of the original DNA helix for complementary error correction(18), achieves an extremely low error rate and can detect mutations down to parts per million (ppm)(19). As an over-a-decade-old technique, duplex sequencing relies on barcoding input DNA duplexes with two unique molecular identifiers (UMIs). Newman and colleagues previously studied this innovation for detecting ctDNA in lung cancer by incorporating duplex UMIs into their CAPP-seq approach(20, 21). However, limited duplex recovery and the high sequencing capacity required to reach library exhaustion were beyond what was routinely feasible with previous generations of sequencers. Since then, exhaustive sequencing has become achievable through dramatically increased sequencer output and lower sequencing costs. Recently, duplex error correction has been adapted for ultrasensitive ctDNA detection in solid tumors and lymphoid cancers where phased variants are not abundant in stereotyped loci, yielding highly clinically significant results(22). We reasoned that longitudinal duplex sequencing of lymphoma ctDNA in LBCL could demonstrate the potential of detecting measurable residual disease (MRD) over time. Thus, we aimed to determine the added clinical value of ctDNA detection by targeted duplex sequencing in serial plasma samples from LBCL patients treated in a uniform protocol during therapy and follow-up(23).

Methods

Ethics approval and trial registration

The LBC-06 phase II trial was conducted in accordance with the Guidelines on Good Clinical Practice from the International Conference on Harmonization and the principles outlined in the Declaration of Helsinki. The protocol was approved by the medical agencies and ethics committees in Finland, Denmark, Norway, and Sweden, and the trial was registered at ClinicalTrials.gov (NCT03293173). All patients signed informed consent forms before participating in the study.

Patients and samples

We evaluated 447 longitudinal plasma cfDNA samples drawn at various time points from 119 of the 123 available patients with primary LBCL treated in a trial (Figure 1A)(23). The patients were young (aged 18-64 years), had high-risk LBCL, and were treated with a curative intent with early high-dose-methotrexate, dose-intensive immunochemotherapy (DA-EPOCH-R or R-CHOEP), and high-dose-cytarabine in a Nordic phase II trial(23). During an updated median follow-up of 60.4 months, 22 of the study patients experienced progression and 13 deaths.

Plasma samples were collected according to study protocol at pretreatment, on-therapy after the second and fourth cycles (interim computer tomography [CT] stagings), and at the end-of-therapy (EOT) at the time of PET-CT end-staging (Figure 1A). Sample selection of pretreatment, after 2 cycles (n = 112), and EOT (n = 99) plasma was based on sample availability. Samples drawn after cycle 4 (n=55) were enriched for patients with

events and included all available samples from patients with measurable residual disease (MRD) after 2 cycles of therapy (MRD^{CYC2+}, n=27/55). We analyzed all available post-therapy surveillance samples from patients with progression on follow-up with a control series of patients with no follow-up events selected based on baseline ctDNA positivity and sample availability (n=54). Additionally, we included 4 plasma samples drawn upon recurrence. MRD results and survival after cycle 2 and EOT were reported in the original manuscript, with a median follow-up of 3 years(23). The MRD decisions remained unchanged and are analyzed in full detail here with complete clinical data and an updated final 5-year follow-up. Surveillance samples were collected on follow-up at 3, 6, 9, 12, 18, and 24 months and at relapse if present. Lead times to recurrence were calculated as the difference between the date of first ctDNA-positive plasma sample draw and the date of recurrence.

Circulating tumor DNA detection

A previously established platform using a targeted panel sized ~750 kilobases with exhaustive duplex sequencing was used for ctDNA analysis(24). Only duplex consensus reads supported by sequencing of both DNA strands were considered for the MRD analyses, with no further requirements of duplex family size or support criteria per individual strand in a duplex family. A median of 131 million sequencing reads were produced per the plasma cfDNA sample, resulting in a median family depth of unique molecular identifiers of 4.39x, representing sufficient sequencing capacity allocation as previously established. The relevant sequencing metrics per sample are available in the Supplementary Table S4A. The MRD test was performed as previously described and

interpreted according to a cut-off for 95% specificity among withheld healthy donor cfDNA samples(23). The lowest ctDNA fractions detected were in the ppm range, and the median limit of detection was 1.69×10^{-5} , with 26% of samples having a limit of detection in the ppm range. We included four patients with non-informative exploratory genotyping at diagnosis (<20 MRD reporters) in the analyses and considered them in the tested follow-up samples. One of these four cases experienced progression on follow-up, and their separate survival is shown in Supplementary Figure S1. Additional sequencing and ctDNA analysis details and metrics are described in the Supplementary methods and Tables S1-S4.

Statistical analysis

Statistical analyses were performed in the R environment. Statistical details accompany the results in the text and/or related figure legends. The used tests were non-parametric and two-sided, and *P* values < 0.05 were considered statistically significant. Survival analyses were performed for progression-free survival (PFS), defined as months from recruitment to lymphoma progression or death from any cause, and overall survival (OS) defined as months from recruitment to death from any cause.

Results

Early ctDNA dynamics provide a rapid pharmacodynamic measurement of treatment efficacy

Pretreatment ctDNA levels declined in all evaluable patients after two cycles of immunochemotherapy, and 75 of 112 patients (67%) achieved early molecular remission (MRD^{CYC2-}, Figure 1B). MRD^{CYC2+} was associated with elevated pretreatment ctDNA concentration ($P = 0.0038$, Mann-Whitney U test), and all patients with less than 25 haploid cancer genomes per milliliter of plasma (hGE/ml) at baseline reached MRD^{CYC2-}. Biological risk group assigned in the trial or *TP53* mutation status were not significantly associated with MRD^{CYC2+} status ($P = 0.2319$ and $P = 0.117$, respectively, Fisher's test). Notably, partial response rates by concurrent CT evaluation were similar between patients with MRD^{CYC2+} (33/36; 92%) and MRD^{CYC2-} (61/74; 82%, Fisher's $P = 0.257$).

MRD^{CYC2} was prognostic for PFS and OS (Figure 1C, Table S2-3). Yet, the positive predictive value (PPV) for progression was modest, with only 13 (35%) of 37 patients with MRD^{CYC2+} progressing during follow-up. In contrast, patients with early ctDNA clearance had excellent outcomes, and the negative predictive value (NPV) of MRD^{CYC2} evaluation was high (91%, Table S2). Notably, among the 37 MRD^{CYC2+} cases, exploratory analysis of the ctDNA levels revealed a strong prognostic impact of elevated ctDNA concentration after cycle 2 for refractory disease and worse survival (Figure 1D). Previously described major molecular response(25) (MMR, ≥ 2.5 log reduction in ctDNA after the first two cycles) was achieved by 94 (85%) of 111 patients, and failure to reach MMR identified patients with poor survival; however, its sensitivity for progression during follow-up was

lower than that of MRD^{CYC2} alone (Figure 1E, Table S2). Taken together, clearance of ctDNA after 2 cycles by duplex sequencing predicts excellent survival, and in cases where ctDNA is present, high levels indicate a worse outcome.

Molecular remission after four cycles updates prognostication and high ctDNA levels anticipate end-of-therapy refractoriness

Next, we analyzed the plasma samples of 55 available patients collected after 4 cycles (MRD^{CYC4}). Among these patients, MRD^{CYC4+} was prognostic with higher PPV than MRD^{CYC2} (Figure 2A, S2-3). Of the 27 available patients with MRD^{CYC2+}, 14 (52%) had become MRD-negative, whereas 13 (48%) remained MRD-positive, which was associated with poor OS (Figure 2B). Among patients with MRD^{CYC4+}, high ctDNA levels predicted poor survival, similar to MRD^{CYC2+} in the exploratory analysis, and primary refractory cases at EOT were identified by ctDNA levels above 10 hGE/ml (Figure 2C-D). After the last cycles of therapy (MRD^{EOT}), 7 (54%) of 13 patients with MRD^{CYC4+} became MRD-negative, while 6 (46%) remained MRD-positive. These findings reveal different molecular response kinetics that update prognostic information about therapy(26), with interim ctDNA quantification identifying patients unlikely to respond.

End-of-therapy ctDNA clearance determines response, complements radiological evaluation and reveals inadequate targeting of clinical interventions

At the end of therapy, 89% (88/99) of the evaluated patients were in molecular remission, while 11/99 (11%) were MRD positive (MRD^{EOT}). Positive MRD^{EOT} was associated with

TP53-mutation status, with 6 of the 11 MRD^{EOT+} patients (55%) having a mutation at baseline ($P = 0.034$, Fisher's test). Nine of the 11 patients (82%) were also positive for MRD after two cycles of therapy ($P = 0.00056$). By contrast, the biological risk-profile ($P = 0.357$), age-adjusted IPI (aaIPI 2-3, $P = 1.000$) or stage (Ann Arbor III/IV, $P = 1.000$) were not associated with the MRD^{EOT} status.

Among all evaluable patients, MRD^{EOT} identified a dramatic difference in outcomes, with all patients who died within two years of follow-up being MRD^{EOT+} (Figure 3A, Tables S2-3). Among patients who progressed, median PFS was 8.4 months for MRD^{EOT+} patients compared to 31.2 months for MRD^{EOT-} patients ($P=0.0008$, Mann-Whitney-U). MRD^{EOT} was positive in all 4 patients with primary refractory disease and in 6 (75%) of the 8 patients who experienced progression within 12 months of follow-up (early progression). Notably, the two early MRD-negative progressions concerned isolated CNS recurrences (explored below). Patients with late progression (PFS > 12 months, $n = 12$) were less likely to be MRD-positive at EOT, with only 3 (25%) having a positive test followed by an event within the second year of follow-up. Of the 2 MRD^{EOT+} patients with initial ctDNA clearance after two therapy cycles, one progressed after 13 months of follow-up, whereas the other completed follow-up after 60 months in remission. Two of the evaluable patients progressed late, presenting with an indolent lymphoma on follow-up (55 and 79 months), both being MRD-negative at EOT. Consistent with these observations, NPVs of MRD^{EOT} for 1-, 2-, and 4-year PFS progressively declined to 98%, 94%, and 91%, respectively, with an overall NPV of 88% and PPV of 82% over the entire follow-up. These findings indicate that MRD^{EOT} accurately predicts systemic disease progression during the first years of follow-up, whereas it is less sensitive for detecting later recurrence.

Survival impact of MRD^{EOT} was independent of concurrent metabolic responses measured by PET-CT (Figure 3B-C, Table S3B). MRD^{EOT} was associated with metabolic responses, with an MRD-positive rate of 4/71 (5.6%) in patients with complete metabolic response (CMR, Deauville score [DS] 1-3) versus 6/13 (46%) in non-CMR patients (DS 4-5, Fisher's $P=0.0051$, Figure 3D). MRD^{EOT} was positive in 5 (71%) of the 7 non-CMR and 3 (33%) of the 9 CMR patients with progression on follow-up, with 1 false positive in each group (Figure 3D).

Seven patients underwent tissue biopsies from PET-positive lesions after therapy, and two were confirmed to have lymphoma progression (29%). MRD^{EOT} was positive in 3 of the 7 patients, including both the biopsy-confirmed progressions. Twenty of 99 evaluable patients (20%) for MRD^{EOT} received radiotherapy (RT) after chemoimmunotherapy, which was not associated with MRD^{EOT} status (Fisher's exact, $P=0.69$, Figure 3E). Six patients received RT due to tumor bulk at diagnosis, and all were MRD-negative at EOT. Three of the 11 MRD^{EOT+} patients received RT for PET-avid lesions, and in two of those with available surveillance samples taken after RT, the MRD remained positive, and the patients progressed during follow-up. These results highlight the limitations of current clinical tools in providing appropriate interventions for patients at high risk of progression after immunochemotherapy, a gap that could be addressed with ultrasensitive ctDNA detection.

Longitudinal ctDNA surveillance on follow-up unmask LBCL recurrence with significant lead-time to clinical relapse

To investigate whether molecular surveillance could detect late recurrences before clinical presentation in asymptomatic patients, we examined the clinical relevance of MRD in longitudinal plasma samples collected per study protocol (Figure 1A). We analyzed a case series of cured and relapsing patients, including all available samples from those with any follow-up events.

During post-treatment surveillance, all evaluable patients with MRD^{EOT+} remained MRD-positive in subsequent plasma samples, and 6 (86%) of 7 relapsing patients with MRD^{EOT-} became MRD-positive before clinical relapse (Figure 4A-B). Only one patient presenting with a late relapse remained MRD-negative for more than a year of follow-up before turning MRD-positive. Once ctDNA was detected, 14/15 (93%) of subsequent tests remained positive, while specificity remained 100% in patients without a relapse, including 1 patient with a non-lymphoma-related death (22 tests, Figure 4B).

Surveillance provided lead time to relapse in 11 (92%) of 12 patients. Median lead time to clinical recurrence was 6 months, and a 3-month lead time was achieved in 10 (83%) of 12 cases (Figure 4C). Three patients had a lead time of over 12 months, including one with ctDNA detected throughout measurements (Figure 5A). The patient developed a symptomatic parailiacal tumor 3 months after CMR and responded to corticosteroids; however, tissue biopsies showed an unspecific histiocytic reaction twice before a conclusive diagnosis was made using genomic techniques, as tumor cells had lost B-cell phenotypic markers (Figure 5A).

Finally, examination of the patients with the shortest ctDNA lead times to recurrence (0 and 1.6 months) revealed that these were the only cases with isolated CNS recurrence in the cohort (n=2, Figure 4C). Since mutations in primary CNS lymphomas can be more easily detected in cerebrospinal fluid (CSF)(27, 28), we analyzed cfDNA from CSF samples of one patient who had CSF flow cytometry positivity at inclusion. Exploratory analysis revealed lymphoma reporter mutations in the pretreatment CSF, all of which became undetectable after two cycles of therapy, reflecting ctDNA response in plasma (Figure 5B). Despite flow cytometry clearance after therapy, however, lymphoma reporters were again detectable in the CSF at EOT, indicating CNS-restricted resistance with an additional three months' lead time compared to plasma-based detection (Figure 5B). Altogether, these findings demonstrate that repetitive ctDNA surveillance after therapy provides significant lead time to systemic LBCL relapse by complementing EOT analysis, while CSF analysis could augment the early detection of CNS recurrence.

Discussion

Here, we showed that repetitive, longitudinal plasma ctDNA surveillance with targeted duplex sequencing accurately predicts survival, determines therapy responses, and informs decision-making in various clinical scenarios involving LBCL recurrence. Our results from a prospective trial suggest that ctDNA measurements obtained during treatment, at the end of therapy, and post-therapy could better inform treatment decisions. Clinical interventions could be directed more precisely and earlier to patients with emerging resistance that evades detection with concurrent clinical tools. These findings

add to a compelling body of evidence anticipating a precision-medicine leap toward ctDNA-guided clinical decisions in LBCL.

Thus far, studies of LBCL ctDNA with ultrasensitive tools that can detect MRD down to ppm levels have relied on the detection of phased variants and primarily focused on the relevance of end-of-therapy evaluation(17, 29, 30). Here, we adopted another technique based on targeted duplex sequencing and show that it allows similar MRD detection in LBCL. The analysis translated into highly significant clinical results comparable to those recently reported with phased variants at the end of therapy(17, 29, 30). We note, however, that, as with the caveats concerning phased variant-based approaches, the achieved limit of detection varied across patients and tests, reflecting differences in sample and sequencing characteristics and variable number of available reporters(17). Additionally, in four patients ctDNA was undetectable in follow-up samples due to failure to recover sufficient reporters. Moreover, neither technique routinely meets the 1-ppm limit of detection required by the National Comprehensive Cancer Network guidelines, which recommend ctDNA analysis for patients with EOT PET-CT positivity when tissue biopsy is not feasible. In light of our findings and other recent results, international efforts are warranted to standardize measurements, technical assay reporting, sample and reporter inclusion quality criteria, and clinical utility of ctDNA-based MRD, and to determine clinically relevant practical limits of detection(31).

Deciphering molecular remission at the end of therapy represents a highly appealing first clinical application of ultrasensitive ctDNA analysis, and MRD^{EOT} has been proposed as a future endpoint for clinical trials. Here, we observed a striking difference in outcomes according to MRD^{EOT}, with a long median follow-up of five years. The independent

prognostic significance of MRD positivity observed in patients with either radiological remission or residual PET-CT positivity supports incorporating molecular remission into LBCL response criteria to complement imaging(29). The clinical NPV of MRD^{EOT} for PFS in our cohort was high (88%), and comparable to recent reports with phased variants at 89% by Wang et al.(30) and 89% for 24-month PFS by Roschewski et al(29). Notably, we observed that the NPV was highest during the first two years of follow-up, and the only two MRD-negative patients with progression within the first year of follow-up concerned cases with isolated CNS recurrence, a similar limitation observed by Roschewski et al(29). Furthermore, the patients in our cohort had excellent outcomes, yet the high clinical specificity for progression (97%) was maintained, which is relevant in a cohort with low rate of events even on extended follow-up. These results encourage further investigations on the feasibility of end-of-therapy MRD as a clinical surrogate for early PFS and OS in LBCL. Moreover, the mismatch between MRD^{EOT} and clinical interventions suggests that some invasive confirmatory tissue biopsies could be spared, and consolidative treatments, such as radiotherapy, could be more accurately administered based on ctDNA detection, which remains to be established prospectively.

Our findings show, with high resolution, that on-therapy ctDNA kinetics were strongly linked to outcome, PET-CT response, and EOT ctDNA status(15, 25). Clearance of ctDNA after two cycles was observed in most cases and characterized patients with excellent survival, with high NPV. We note that pivotal clinical trials with biological stratification, such as the negative PHOENIX(32) and REMoDL-B(33) studies, allowed two cycles of induction prior to investigational therapy, and the presence of such a strong prognostic factor at this time point should be considered a potential confounder in future studies.

Moreover, early clearance could also be used to stratify patients, and those with MRD^{CYC2-} could benefit from de-escalation of therapy, for instance. By contrast, the positive predictive value of MRD^{CYC2+} was limited, and most of these patients achieved molecular response later, after four cycles or at the end of therapy, suggesting that MRD^{CYC2+} is not necessarily an ideal marker for intensifying therapy. Patients with MRD-positive plasma after four cycles had a higher rate of progression than those with MRD^{CYC2+}, suggesting that this time point could guide early treatment intensification. Besides detection, interim quantification of ctDNA concentration revealed clinically relevant heterogeneity and could accompany MRD detection to identify patients earlier who are unlikely to reach CMR, and could be used to stratify patients for consolidative treatments. Altogether, the on-therapy ctDNA dynamics provide an accurate molecular window into distinct patient responses that can inform and update prognostication(26), and could be used to stratify patients for different therapies in future clinical trials.

Despite excellent stratification of patients at EOT, the NPV for PFS declined with longer PFS, and most (75%) patients with late progression were MRD-negative at EOT evaluation. Our finding aligns with observations from phased variant-based approaches; for instance, Wang et al. detected ctDNA at EOT only in 3 of 11 patients (27%) with progression after 12 months(30). Although some patients in both studies experienced non-lymphoma-related deaths, the findings suggest that the identification of late progressions would require technological improvements or repetitive surveillance. A seminal study by Roschewski et al. recognized the potential of ctDNA surveillance in LBCL patients using IG-HTS with a median 3.5-month lead-time to recurrence(9). However, longitudinal, post-treatment ctDNA surveillance has not been previously

reported in LBCL using an ultrasensitive methodology. Here, we observed a marked improvement in clinical relevance, with ctDNA re-emerging in plasma in all evaluable cases with systemic recurrence at least 3 months prior to clinical relapse. With a long median lead time of 6 months and most cases having multiple positive MRD tests prior to relapse, the opened time window would be clinically significant and enough to apply appropriate second-line therapeutic interventions earlier to preclude the development of higher tumor burden and symptomatic disease. Intensity of surveillance could be tailored according to pretherapeutic and dynamic risk profiling, such as pretreatment ctDNA levels or early ctDNA clearance. Furthermore, our exploratory CSF analysis suggests that CSF surveillance could supplement plasma surveillance in patients with risk factors or suspected CNS recurrence(27, 28) for earlier detection. However, these hypotheses remain to be investigated in future studies with appropriate designs and sample collection. Our study provides a limited, yet highly compelling first insight into the enormous potential of ultrasensitive ctDNA post-treatment surveillance in LBCL.

Taken together, we show that dynamic molecular monitoring of ctDNA using targeted duplex sequencing, a scalable and increasingly available routine sequencing technique, outperforms current clinical tools and accurately distinguishes patients with different treatment responses and patterns of refractoriness. The prospective, uniform cohort with comprehensive clinical data, sample collection, and long median follow-up, together with a technologically advanced ctDNA detection down to the parts-per-million range, represents the key strengths of our study. Further enhancements in sequencing design, such as personalized panels, analysis of larger plasma volumes, and inclusion of phased variants, could further improve the practical sensitivity of ctDNA detection. The patients

in our study were treated under an experimental protocol with early CNS prophylaxis and a biologically stratified immunochemotherapy regimen, which may limit the direct extrapolation of our findings to other clinical settings. Prospective studies are warranted to confirm the feasibility and establish any potential benefits of ctDNA-guided therapy.

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Figure Legends

Figure 1. Study design, on-therapy circulating tumor DNA (ctDNA) kinetics and prognostic relevance of early measurements after two cycles of chemoimmunotherapy.

- A) Collection of plasma samples according to the study protocol.
- B) Dendrogram of ctDNA detection and levels per patient in therapy sampling time points. Colors according to end-of-therapy (EOT) response by PET-CT. Red, progressive disease (PD); Orange, partial response (PR); Blue, complete response (CR).
- C–E) Kaplan-Meier (KM) survival estimates for progression-free survival (above, PFS) overall survival (below, OS) after two therapy cycles according to (C) presence of measurable residual disease (MRD) in all evaluable patients, (D) ctDNA concentration after two cycles among MRD-positive cases (categorized according to median concentration of 2.19 haploid genome equivalents per milliliter of plasma [hGE/ml]) and (E) major molecular response (MMR).

Figure 2. Circulating tumor DNA (ctDNA) measurements after four cycles of chemoimmunotherapy.

- A–C) Kaplan-Meier (KM) survival estimates for progression-free survival (above, PFS) overall survival (below, OS) after four therapy cycles according to (A) presence of measurable residual disease (MRD) in evaluated patient set, (B) all available patients who were MRD-positive in cycle two and (C) ctDNA concentration (categorized according to median concentration of 6.31 haploid genome equivalents per milliliter of plasma [GE/ml]).
- D) Box and dot plot of ctDNA concentration after four cycles (y-axis, GE/ml) according to end-of-therapy PET-CT responses.

Figure 3. Circulating tumor DNA (ctDNA) measurements and detection at the end-of-therapy(EOT) evaluation after chemoimmunotherapy.

- A–C) Kaplan-Meier (KM) survival estimates for progression-free survival (above, PFS) overall survival (below, OS) at EOT according to presence of measurable residual disease (MRD) in (A) all available patients, (B) patients with complete metabolic response (CMR, Deauville score [DS] 1-3), and (C) patients with non-CMR (DS 4-5).
- D) Sankey plot of study patients at EOT according to PET-CT DS, MRD^{EOT} and status on follow-up (FU). Numbers in bold indicate the number of patients per group.
- E) Sankey plot of the study patients at EOT according to radiotherapy (RT) administration status, RT indications, MRD^{EOT} and relapse status on FU. Numbers in bold indicate the number of patients per group.

Figure 4. Circulating tumor DNA (ctDNA) detection on surveillance provides lead-time to clinical recurrence.

- A) Dendrogram of ctDNA detection and levels per patient during treatment and surveillance. Patients with ctDNA detected are shown. MRD, measurable residual disease.
- B) Swimmer's plot of the patients (rows) and their follow-up (x-axis). MRD test results from surveillance samples drawn at protocol-specified time points are indicated in red (positive) and blue (negative) rectangles. Lead time to recurrence visualized with green bar.
- C) Bar plot of lead times in evaluable patients with late relapse during surveillance. Grey bars represent cases with isolated recurrence in the central nervous system.

Figure 5. Analysis of circulating tumor DNA (ctDNA) in plasma and cerebrospinal fluid informs in clinical challenges.

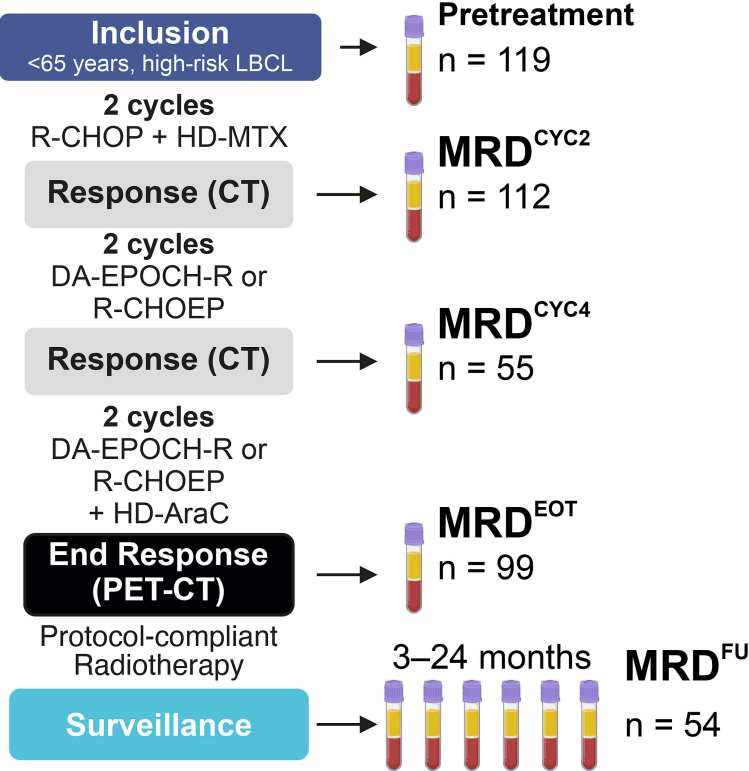
- A) Longitudinal analysis of plasma ctDNA, radiological imaging and tumor biopsies in a patient presenting with recurrence. Array comparative genomic hybridization was performed in clinical routine on primary and relapse tissues, revealing a shared

copy number profile and regions of loss of heterozygosity between the biopsies, which confirmed a clonal relationship between the primary and relapse tumors.

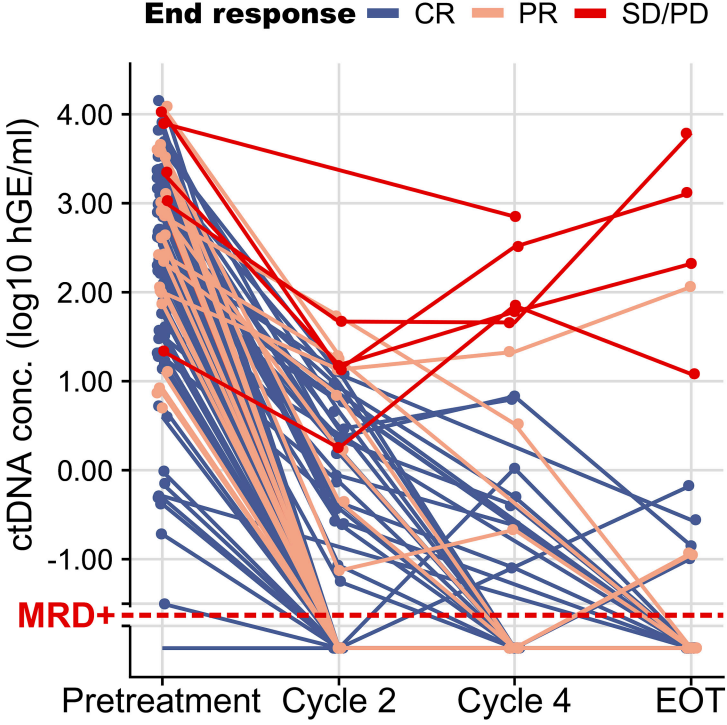
B) Dot and line graph of reporter variants (dots) variant allele fractions (y-axis) in serially collected (x-axis) plasma (top) and cerebrospinal fluid (bottom) samples in a case with isolated central nervous system recurrence on follow-up. Illustrative graphics created using biorender.com.

Figure 1.

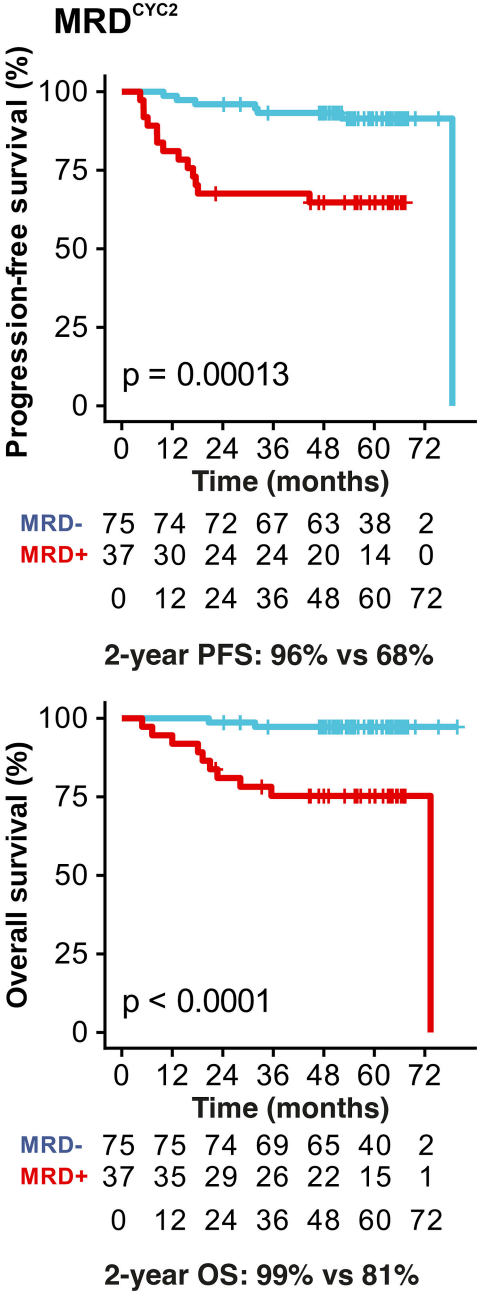
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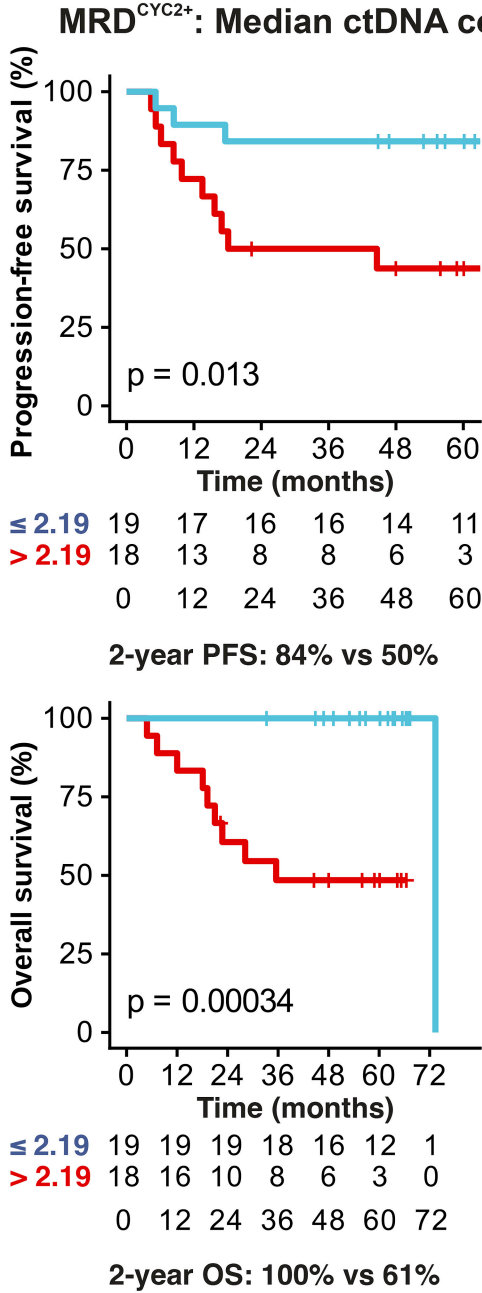
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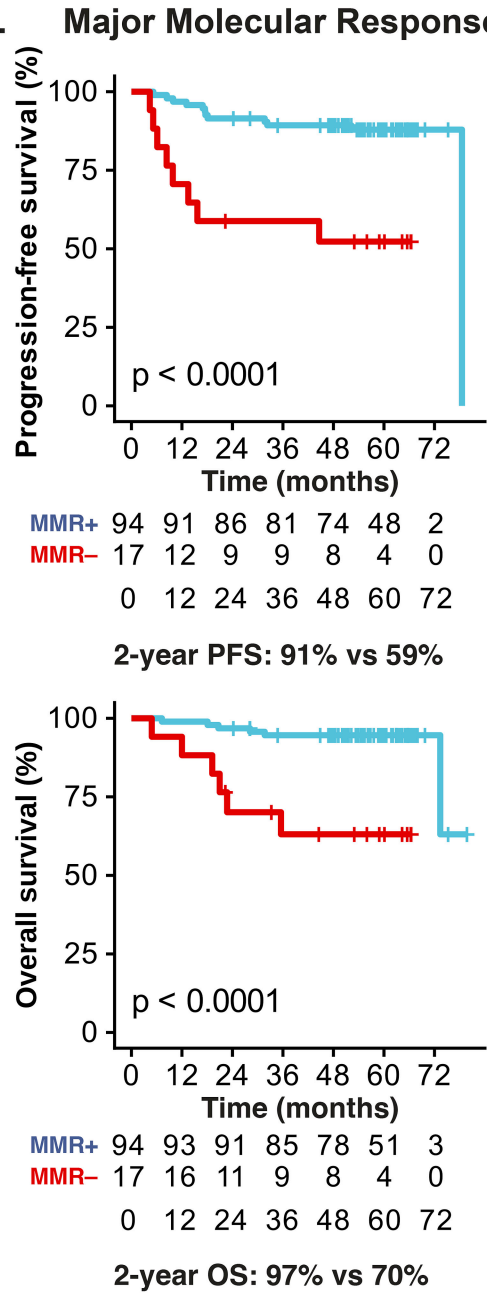
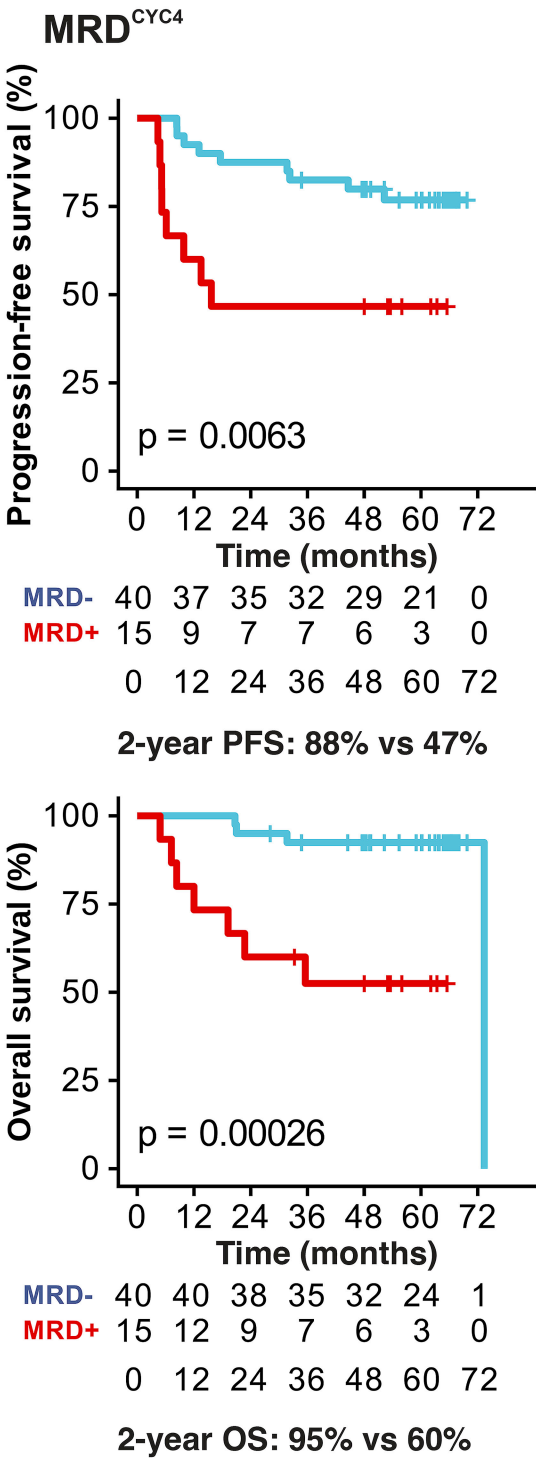
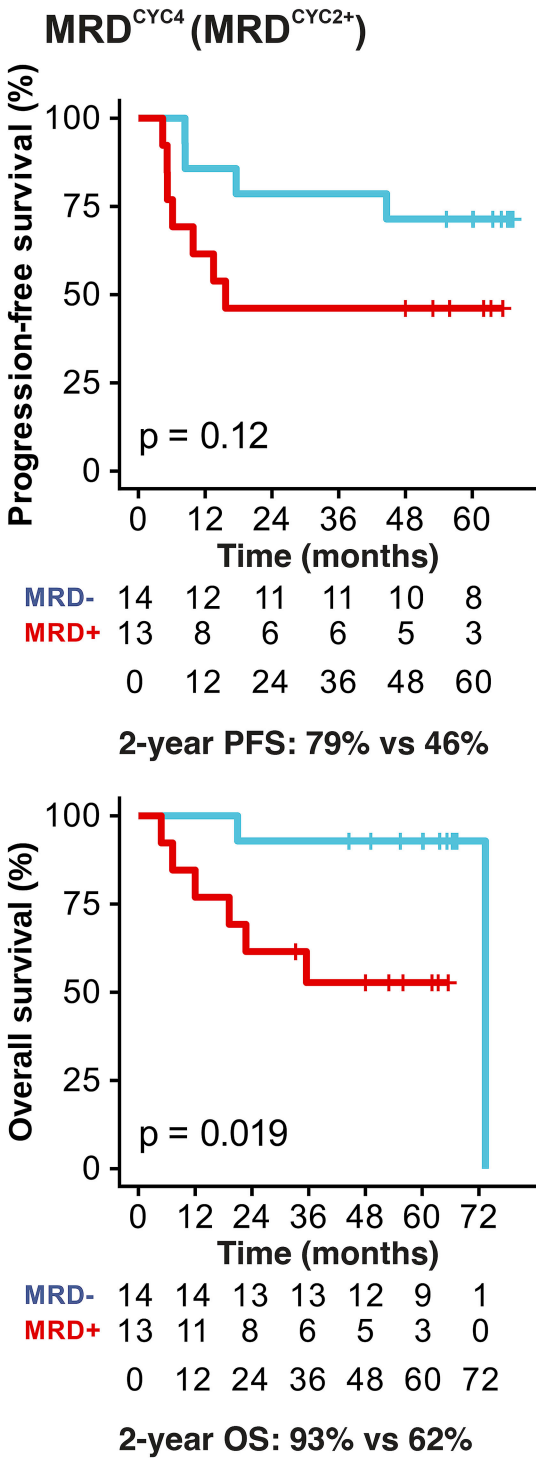


Figure 2.

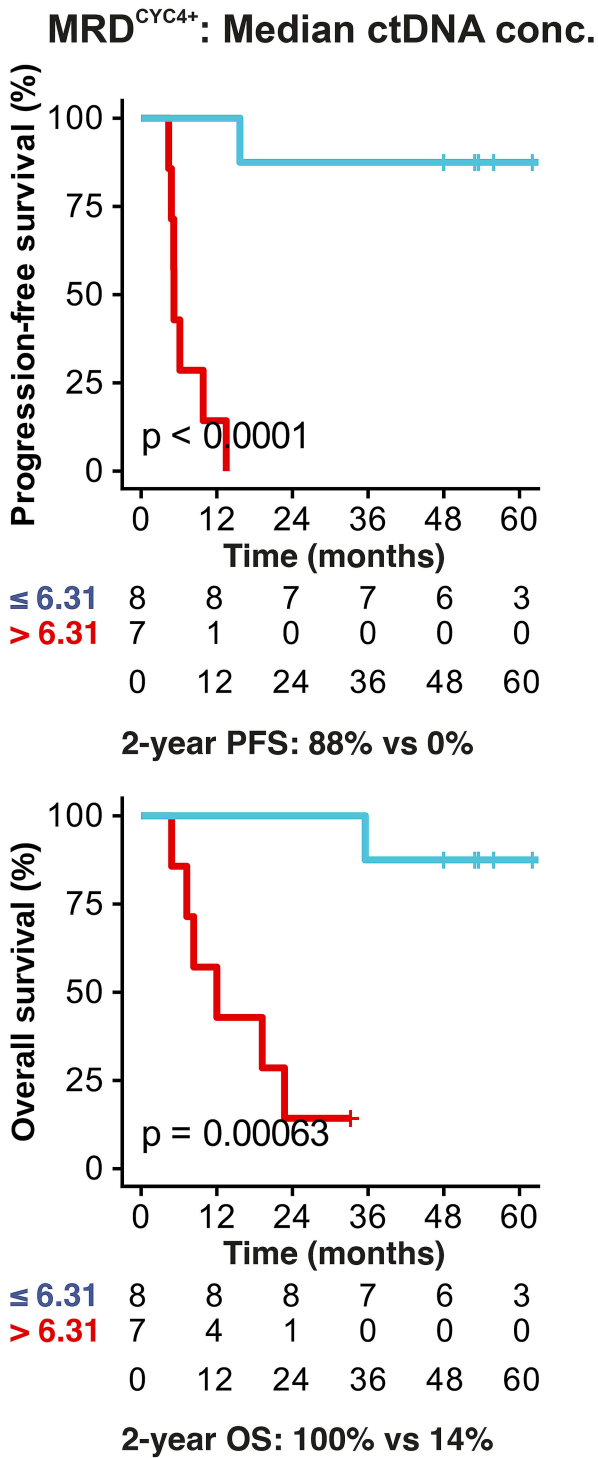
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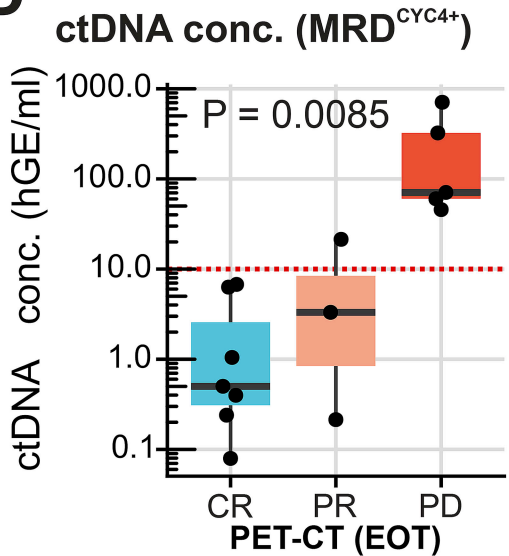


Figure 3.

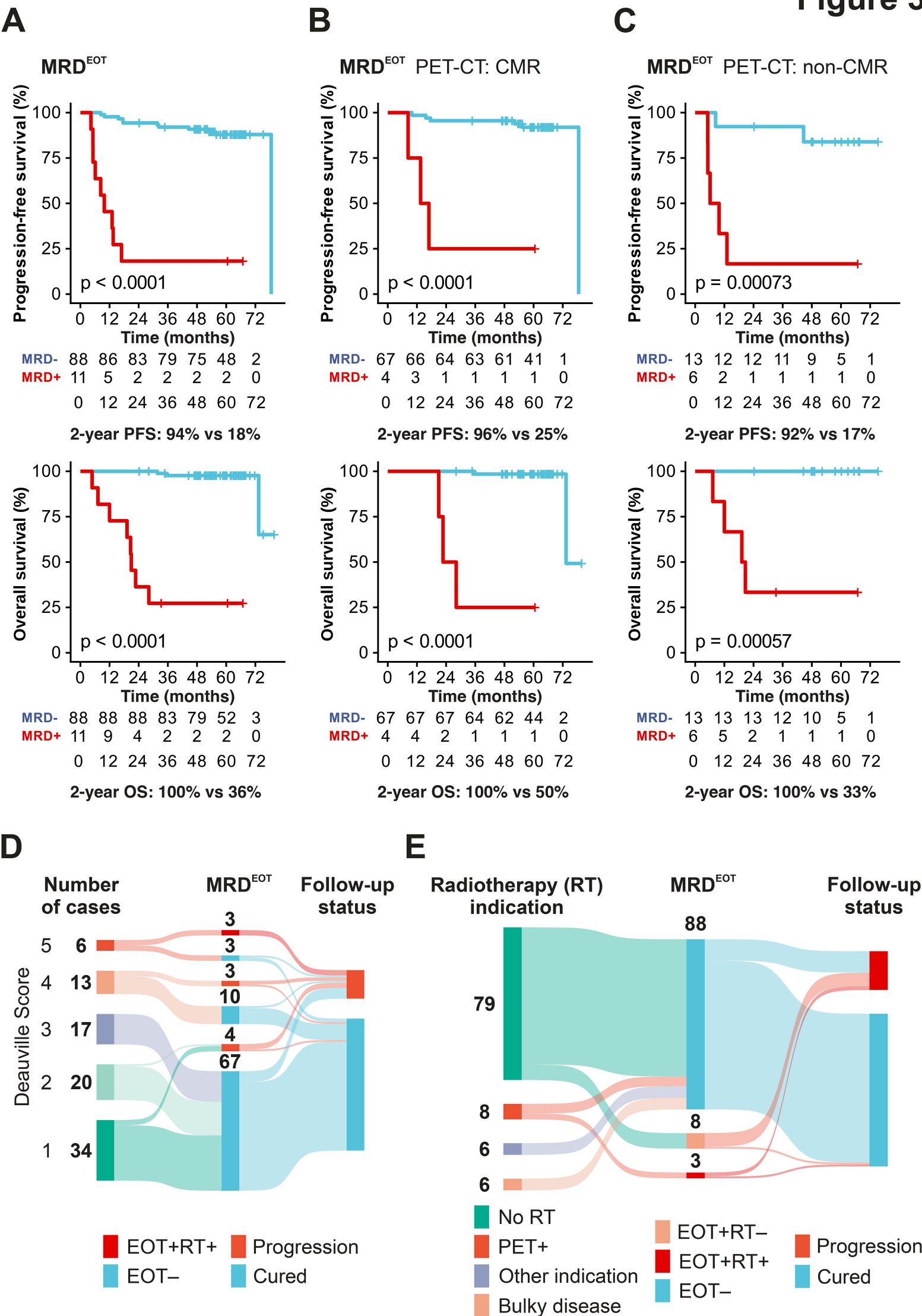
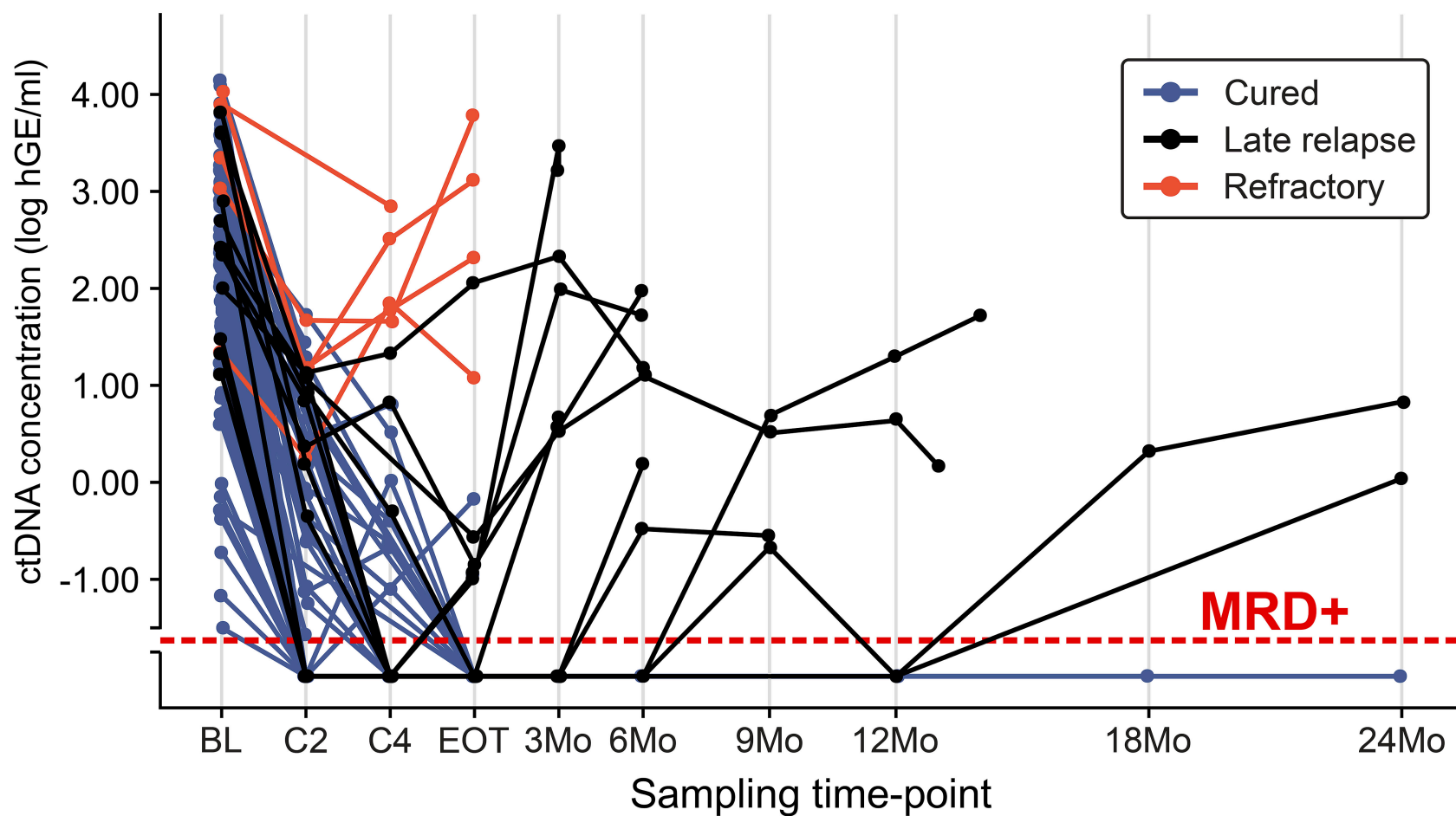
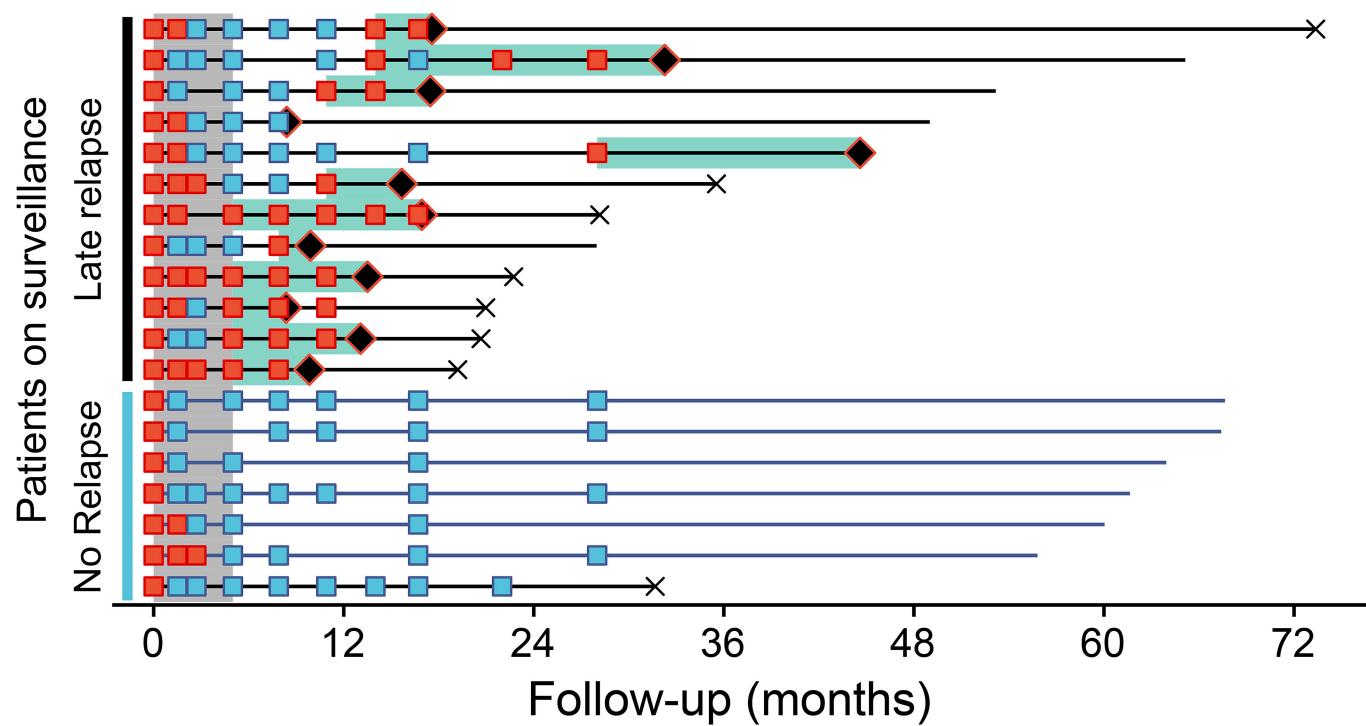


Figure 4.

A



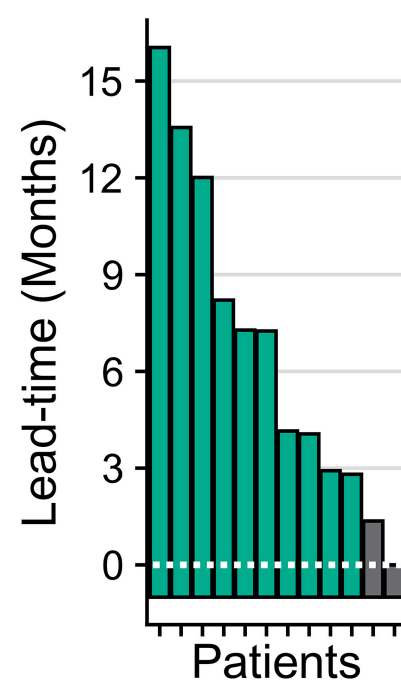
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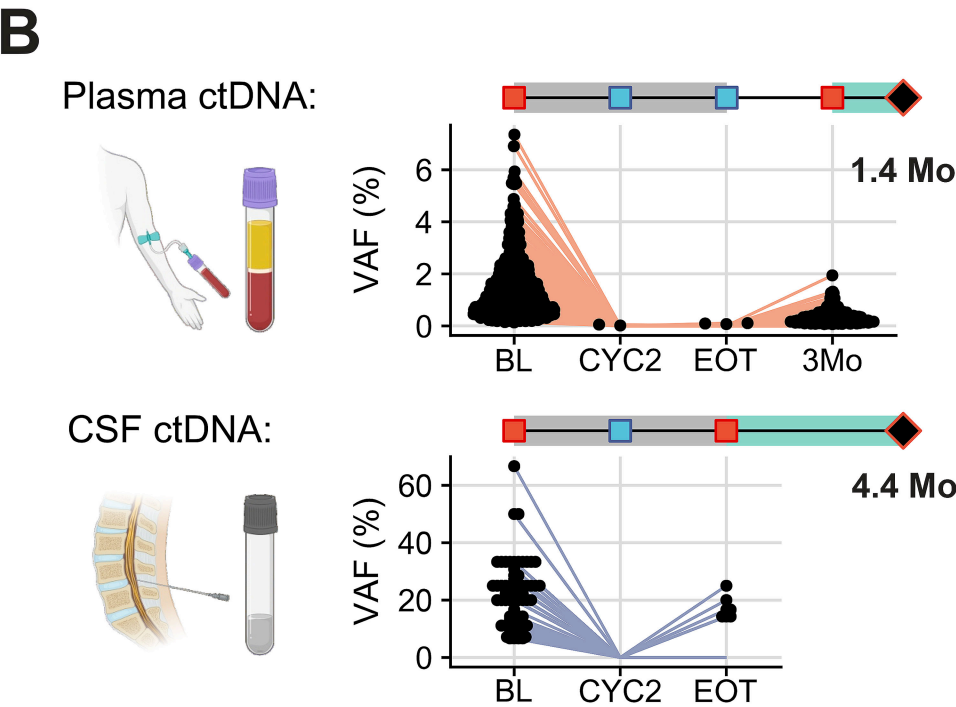
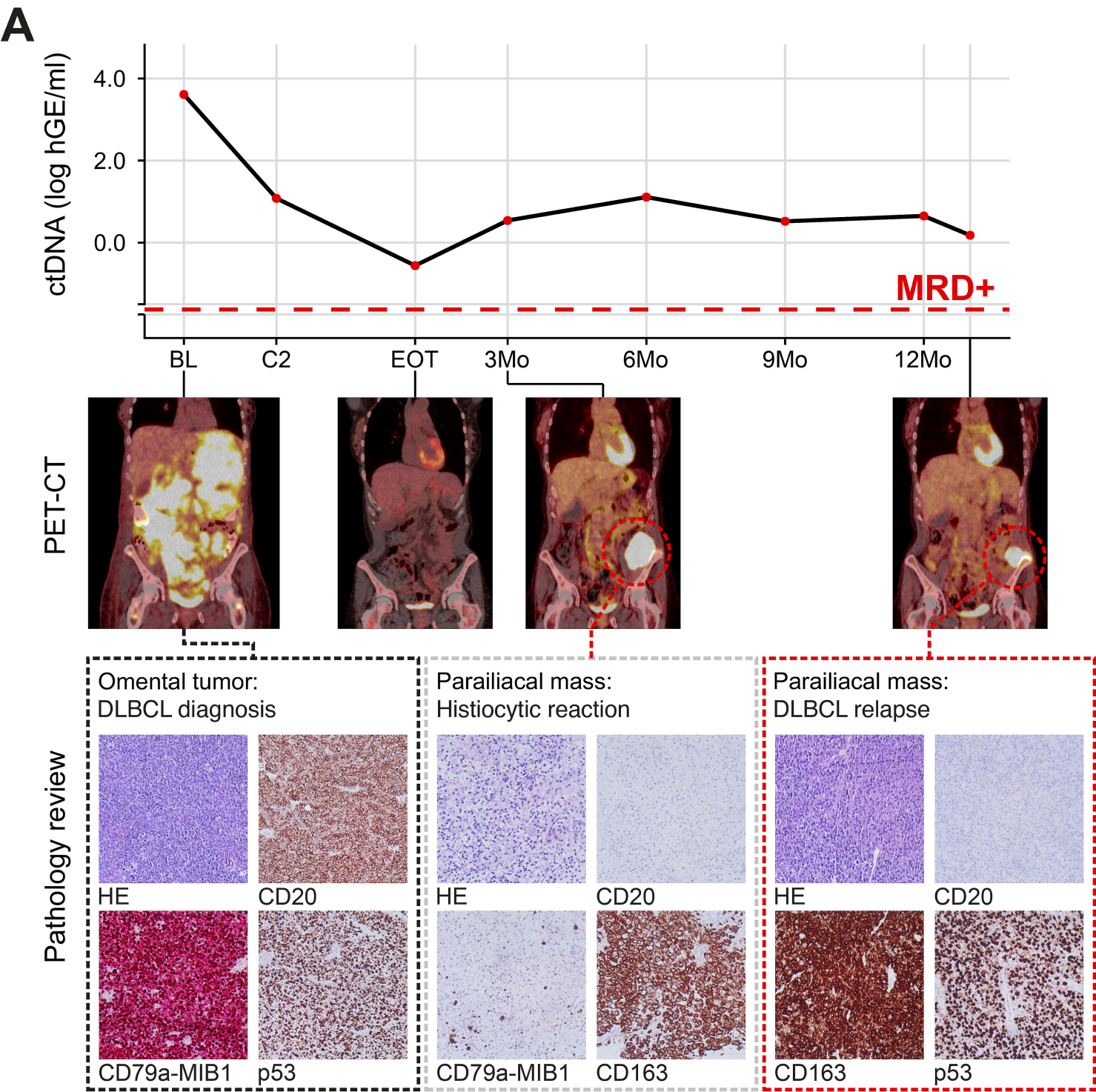
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Recurrence



Figure 5.



Supplementary Material

Molecular surveillance of aggressive large B-cell lymphoma using circulating tumor DNA duplexes

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Running head: ctDNA surveillance in B-cell lymphomas

Keywords: Circulating tumor DNA, duplex sequencing, measurable residual disease, B-cell lymphoma, surveillance, relapse, longitudinal

Supplementary Table Legends

Supplementary tables are available as Excel files.

Table S1. List of resources and reagents used in the study.

Table S2. Confusion matrices of MRD test results and clinical endpoints, with related statistics at measured time points during therapy.

Table S3A-B. Cox regression univariable (S3A) and multivariable (S3B) models according to on-therapy ctDNA risk factors and end-of-therapy radiological evaluation by PET-CT.

Table S4A-B. Table S4A-C. Sample and sequencing quality metrics, measurable residual disease assessments and reporter mutation data of the study samples.

Supplementary Methods

Clinical data

Of 123 patients enrolled in the Nordic phase II protocol (NCT03293173), 119 had samples evaluable for circulating tumor DNA (ctDNA). The clinical trial results, study treatment, and patient characteristics have been previously reported(1). Briefly, young patients (≥ 65 years of age) with de novo aggressive large B-cell lymphoma (LBCL) were recruited and treated with curative intent. The treatment consisted of early central nervous system (CNS) prophylaxis and dose-dense or dose-adjusted chemoimmunotherapy (R-CHOEP or DA-EPOCH-R) according to tumor biomarker profile. The follow-up data were updated for the current study.

Samples, sample processing, and sequencing

The reagents and resources used in the study are listed in Table S1. Peripheral blood drawn in K2-EDTA tubes at inclusion was used as a source of matched normal DNA and was processed as previously described(1). Residual tumor DNA derived from diagnostic fresh frozen biopsies or formalin-fixed paraffin-embedded tissue was analyzed when available.

Ten milliliters of peripheral blood was drawn to standard K2-EDTA or white blood cell preserving (cell-free DNA BCT, Streck) tubes, and the plasma was immediately separated, aliquoted and stored. In addition, cerebrospinal-fluid (CSF) samples were collected from spinal taps at pretreatment, and if tumor cells were detected by flow cytometry, CSF samples were collected after 2 cycles and at EOT (one patient in the study reported in Figure 5B, 7 CSF samples studied in total). 2-4 ml of cerebrospinal fluid was collected in a sterile test tube, which was centrifuged at 2,000g for 10 minutes at +4 C to remove cell debris, and the supernatant was transferred to vials and stored at -70 C. Extraction of cfDNA from CSF was performed similarly to plasma samples.

ctDNA analysis

Centralized cfDNA and DNA extraction, quantification and quality control steps, and library preparation, target enrichment, sequencing, and raw data processing were performed as previously described(2). Sequencing metrics of cfDNA samples are reported in Table S4A, analysis metrics are shown in Table S4B, and reporter variants and their counts are available in Table S4C.

We estimated the presence of MRD using adapted software(3), with concepts as previously described(4). All tests were run, and the samples analyzed blinded to the

clinical data using predetermined test parameters. Reporter mutations were single nucleotide variants not in predetermined clonal hematopoiesis target genes, identified in genotyping diagnostic tumor tissues with $\geq 10\%$ variant allele frequency (VAF) and/or in pretreatment plasma cfDNA with $\geq 1\%$ VAF. Patients with fewer than 20 reporters ($n=4/119$) were considered genotyping failures and were regarded as undetected MRD for all tested samples as intended. The median number of reporters per evaluable patient was 252 (range 24–1316). Only duplex corrected reads with a mapping quality of 60 and bases with a base quality of 60 were considered in the MRD analysis. A blacklist of genomic loci deemed prone to noise was determined previously(2) and is available in Supplementary Table S4D. The sample-specific background error rate was measured as any mismatch from the reference, and the median error rate was $2.74\text{e-}06$ (range $3.18\text{e-}07$ – $1.09\text{e-}05$). 128 (40%) plasma cfDNA samples were drawn in Streck tubes, and 196 (60%) in tubes with EDTA as anticoagulant, and no difference in background rate or number of informative duplex reads was detected between the starting materials (Mann-Whitney U tests, $P > 0.05$, data in Table S4B). Results from a Monte-Carlo-based MRD framework were interpreted with a predetermined cut-off for empirical p-value calibrated to 95% specificity among with-held controls determined previously(1). The ctDNA levels at interim time-points were measured as haploid genomic equivalents (hGE/ml), and the median levels were 2.19 hGE/ml after 2 cycles and 6.31 hGE/ml after 4 cycles of therapy. The analytical sensitivity of duplex sequencing allows mutation detection below parts per million(5). In the context of cfDNA with limited input material, the sample specific sensitivity depends on the number of informative molecules, and therefore, the sample-wise limit of detection (LOD) was estimated with a binomial-based detection model using

the informative duplex depth, assuming 95% sensitivity and a 5% false-positive rate (LOD95), similarly to previously described(6). The informative duplex depth per patient was defined as the sum of the altered and reference counts from reporter mutation positions that met the MRD test criteria above. 96% (301/313) of the samples from evaluable patients reached below 10^{-4} LOD95, the median LOD95 was 1.69×10^{-5} , and 26% (82/313) of the samples reached LOD95 in 10^{-6} range (Table S4B). In line with these models, the lowest ctDNA fractions in the study were in the parts-per-million range (Table S4B).

Immunohistochemical images

Slides of immunohistochemical stainings from routine diagnostics were performed at Helsinki University Hospital, Department of Pathology, and were available for the current study. Microscopy images were acquired with a Leica DM LB brightfield microscope (Leica Microsystems GmbH) and an Olympus DP50 camera.

Statistical analysis

Statistical analyses were performed in the R environment (R Foundation for Statistical Computing, Vienna, Austria, Table S1). The Kaplan-Meier method with the log-rank test was used to estimate survival rates between different patient groups, and Cox regression was used to estimate survival in univariate models, which are available in Table S3 (R package survival, 3.8-3). Survival was estimated and the results interpreted using the follow-up times without considering the time point of the ctDNA test. Test performance metrics were evaluated, and the cross-tabulation and confusion matrices were generated with the R package caret (version 7.0-1).

Supplementary References

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Figure S1.

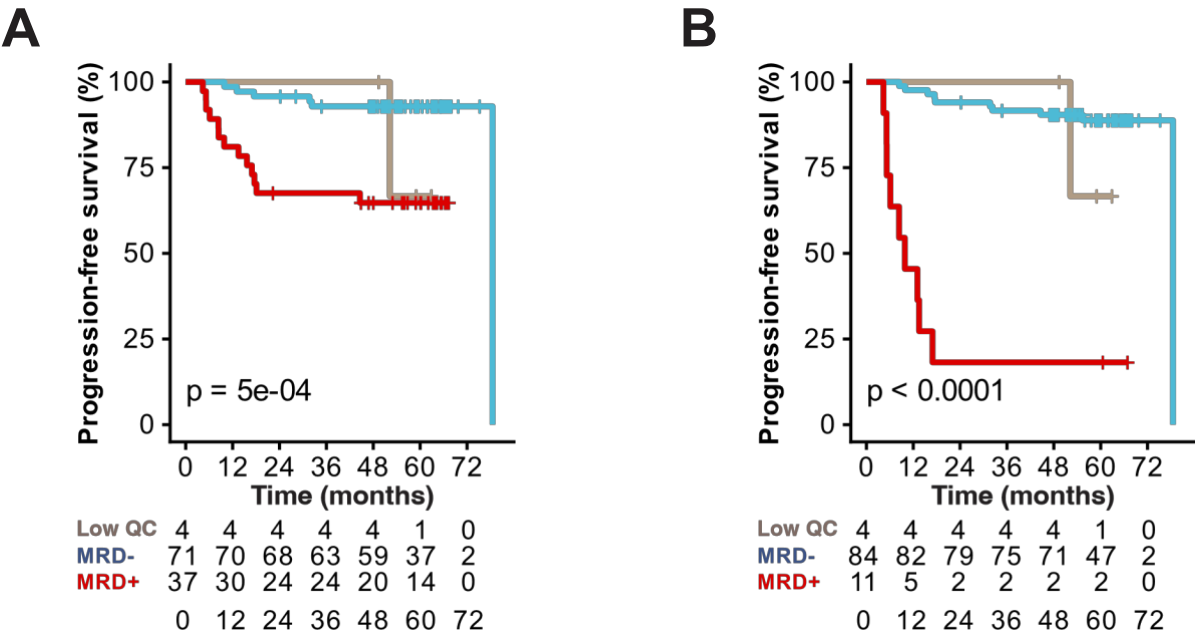


Figure S1. Kaplan-Meier survival estimates for progression-free survival according to measurable residual disease (MRD) detection a) after two cycles and b) at the end-of-therapy. The four patients with a low number of reporters (≤ 20 , 'Low QC') that were considered MRD negative in the main analyses are shown here separately with the brown curve.