Circulating tumor DNA at baseline as a prognostic marker in untreated follicular lymphoma

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Abstract

Follicular lymphoma (FL) is an indolent disease with favorable outcome in patients with symptomatic disease treated with anti-CD20 based immunochemotherapy. However, a subset of patients experiencing progression within 24 months (POD24) have poor prognosis and have shown shorter overall survival at 5 years. Identification of patients at high risk that would benefit for alternative treatment is an unmet medical need, but available clinicogenetic scores show suboptimal performance for predicting early progression. We report a circulating tumor (ct) DNA analysis in baseline plasma from a large cohort of the global phase III GALLIUM trial, which evaluated the efficacy of obinutuzumab plus chemotherapy *versus* rituximab plus chemotherapy in patients with untreated FL. We showed that ctDNA measured at baseline is prognostic for the identification of high-risk *versus* low-risk patients. In this large dataset, mutant molecules per milliliter (MMPM) levels add prognostic value beyond known factors to POD24 (area under the receiver operating characteristic curve [AUROC] 0.69) compared to FLIPI (AUROC 0.57), FLIPI-2 (AUROC 0.59), and SPD (AUROC 0.58) in a univariate analysis, and consistently increased the AUROC of these scores in multivariate models. Using a cross-validated cutoff of 168.57 MMPM, high-baseline ctDNA was significantly associated with shorter progression-free survival (hazard ratio =2.2; 95% confidence interval: 1.8-2.6). This prognostic value was maintained across CHOP or CVP and bendamustine regimens. These results provided a robust assessment of baseline ctDNA as stratification tool in clinical trials for patients treated with standard of care chemoimmunotherapy, and corroborates the growing scientific evidence proposing ctDNA as a novel prognostic biomarker for untreated FL.

Introduction

Follicular lymphoma (FL) is a prototypically indolent disease, and most patients experience favorable outcomes with anti-CD20 antibody-based immunochemotherapy. However, FL remains an incurable disease characterized by subsequent relapse.[1-3] A subset of patients, around 10-20% of untreated FL with symptomatic disease, suffer from a more aggressive disease and have poor prognosis. This group includes patients who experience disease progression within 24 months (POD24) and patients showing histological transformation to a higher-grade lymphoma (transformed FL [trFL]). In particular, POD24 is associated with significantly shorter overall survival (OS) 5 years after rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) treatment.[4-6]

Identification of high-risk patients at diagnosis is an urgent medical need that could enable tailored treatment approaches and identify patients that may be candidates for alternative regimens.[7] From a clinical development perspective, accurate identification of patients at high risk or at low risk of early progression on standard-of-care treatment has the potential to streamline clinical trials and allow for smaller and faster studies to investigate the efficacy of new drugs based on patient need, including treatment intensification or treatment de-escalation approaches.[8]

Several clinical risk scores like FLIPI, FLIPI-2, PRIMA PI and, more recently, FLEX, have been developed with the aim

of identifying high-risk patients with previously untreated FL receiving chemoimmunotherapy. However, all of these scores have shown suboptimal performance in predicting early progression or disease transformation.[9-11] Alternative approaches, such as m7-FLIPI, 23-gene signature or FL20, combined gene mutation and/or gene expression information with clinical features to predict outcome.[9,12-14,15] These scores have a strong biological component and were not generalizable across all treatment regimens as they showed a different prognostic effect on anti-CD20 plus CHOP *versus* anti-CD20 plus bendamustine or anti-CD20 plus lenalidomide treatment. This reflects the high heterogeneity of FL disease and the importance of understanding the underlying disease biology to improve tumor classification and select appropriate treatment for patients based on disease characteristics. Additionally, genetic scores rely on biological features of individual, localized biopsies and this might not recapitulate the diversity of the disease, which often have a clinical presentation characterized by multiple tumor lesions. The practical challenge of obtaining tumor biopsies is a further limitation for the clinical applicability of some of these scoring approaches.

Circulating tumor (ct) DNA measurement offers a novel technology for the identification of high-risk patients. This approach could lead to a more comprehensive understanding of disease heterogeneity by capturing tumor DNA shed into the bloodstream from various lesions and circumvents the need for tumor biopsies.[16]

Recent literature has highlighted the potential of ctDNA as a non-invasive biomarker for prognostication and high-risk patient identification in lymphoid malignancies. While initial proof-of-concept studies largely focused on aggressive entities like diffuse large B-cell lymphoma (DLBCL),[17-19] growing evidence reported the value of ctDNA analysis in FL.[20-24] Baseline ctDNA levels in FL correlate significantly with established clinical parameters of tumor burden including high-risk FLIPI and elevated serum lactate dehydrogenase (LDH) levels. High ctDNA levels are independently associated with inferior progression-free survival (PFS), and ctDNA dynamic in combination with total metabolic tumor volume (TMTV) has shown promising results for POD24 identification.

Given the generally favorable prognosis of FL, patients experiencing POD24 represent a small, albeit critical, subset of the overall patient population and large datasets are key to understanding prognostic/predictive biomarkers for this high-risk subgroup. It is important to acknowledge that the definition and prognostic assessment of POD24 presents some complexity. The POD24 high-risk group includes patients who relapse following front line systemic immunotherapy, and does not carry the same prognostic weight in patients with low-risk diseases at presentation.[25] Additionally, disease transformation to a more aggressive histology (i.e., trFL) is part of early progression events if it occurs within 24 months, adding a level of heterogeneity within this high-risk group. Assessment of POD24 prognostic value is further complicated by how progression is defined and identified (imaging-detected *vs.* clinically meaningful progression) and by statistical issues such as immortal-time bias.[25,26] Despite these complexities, POD24 remains a clinically meaningful prognostic factor, and its utility was confirmed in major clinical trials, including GALLIUM.[27]

Here, we report results from baseline ctDNA analysis in a large patient cohort from the global phase III GALLIUM trial, which evaluated the efficacy of obinutuzumab or rituximab in combination with different chemotherapy backbones including CHOP; cyclophosphamide, vincristine, and prednisone (CVP) and bendamustine in patients with untreated FL.[28,29] This is a unique cohort thanks to the substantial sample size and the possibility to investigate the prognostic value of baseline ctDNA in patients treated with standard of care anti-CD20 immunochemotherapy.

Methods

Clinical, circulating tumor DNA, and disease progression within 24 months analysis cohorts

The reference cohort for outcome includes 1,202 patients with FL (intention-to-treat [ITT] population) enrolled in the randomized phase III GALLIUM trial (*clinicaltrials.gov. Identifier: NCT01332968*).[28] Briefly, patients were aged ≥18 years with previously untreated CD20–positive FL (histologic grades 1 3A), stage III/IV disease (or stage II with bulky disease) and an Eastern Oncology Group performance status (ECOG PS) of ≤2. In GALLIUM, patients were randomized 1:1 to receive 6-8 cycles of obinutuzumab based or rituximab-based induction immunochemotherapy, followed by maintenance with the same antibody for 2 years (or until disease progression or withdrawal) in responders. The chemotherapy backbone (CHOP, CVP or bendamustine) was selected upfront by each individual study center and was a stratification factor for the trial. The primary endpoint was investigator-assessed PFS. The study was conducted in accordance with the principles set out in the updated Declaration of Helsinki, the International Conference on Harmonization Guideline for Good Clinical Practice and all applicable local laws and regulations.

The ctDNA cohort includes 416 patients that were selected as follows (CONSORT in *Online Supplementary Figure S1*): (i) a first pilot analysis for signal seeking was conducted to assess the potential of ctDNA to identify high-risk patients. This initial cohort of 216 patients was assembled to include roughly 50% of high-risk patients, including all available patients with early progression (PFS <24 months) or disease histological transformation to aggressive disease, and roughly 50% of patients at low risk, defined as long-term responders with PFS >5 years. Additionally, patients

were selected to be evenly distributed among treatment arms.[30] (ii) As the signal-seeking cohort was not reflective of the GALLIUM population, we increased the ctDNA analysis cohort with an additional 200 patients to be analyzed in combination with the 216 of the pilot cohort. The new samples were selected by random stratified sampling to ensure the combined ctDNA cohort (N=416 patients) has adequate coverage of the GALLIUM trial population with respect to three characteristics consisting of: PFS (4 groups: censored <5 years [y], event =< 2y, 2y<event<5y, event or censored >=5y), FLIPI (3 groups: high, intermediate, low), and treatment arm (4 groups: obinutuzumab-bendamustine, obinutuzumab-CHOP/CVP, rituximab- bendamustine, rituximab-CHOP/CVP). Subsequent analyses used inverse probability weighting (IPW) among the combined strata to ensure that the weighted sample matched the characteristics of the GALLIUM trial population with respect to the strata and the PFS curves of the weighted cohort *versus* the ITT population is provided in *Online Supplementary Figure S2*.

The baseline characteristics of the ctDNA cohort compared to the GALLIUM study ITT are summarized in *Online Supplementary Table S1*.

POD24 were defined as patients with progressive disease (PD) or death due to PD within 24 months of randomization as in the original analysis for POD24 in the GALLIUM.[31] For the POD24 analysis cohort, patients with death unrelated to disease within 24 months (N=9), as well as those censored before 24 months, were excluded. Furthermore, patients with incomplete information on sum of the product of tumor lesion diameters (SPD) (N=3), FLIPI score 0 to 5 (N=1), FLIPI-2 score 0 to 5 (N=7), m7-FLIPI score (N=3) were excluded, resulting in a POD24 analysis cohort of N=369 patients.

Overall, 98 patients of 416 ctDNA cohort (23.6%) had progressive disease or death within 24 months compared to 12.9% in the GALLIUM trial.[27]

Circulating tumor DNA analysis

Blood was collected in 6 mL EDTA tubes to prevent clotting, yielding a maximum of 4 mL of plasma. To minimize genomic DNA contamination, plasma was isolated on-site after the blood draw via centrifugation to be performed at 1,500g for 10 minutes within 30 minutes from collection. Plasma samples were shipped frozen for long-term storage at <-20 °C. Circulating free (cf) DNA was isolated from plasma using the AVENIO cfDNA isolation kit (Roche Molecular Systems, Inc., Branchburg, USA, for research use only; not for use in diagnostic procedures).

Library preparation and next-generation sequencing (NGS) were completed using a modified version of the AVENIO ctDNA analysis workflow, based on previously described CAPP-Seq technology.[18,19,32] Up to 50 ng of cfDNA (average, 25 ng) was used as input for sequencing.

The targeted NGS panel was designed to cover ~314 kb of regions in or near 466 genes relevant for minimal residual disease and cell-of-origin detection in DLBCL. As matched normal samples were unavailable for these samples, a combination of filtering schemes was used to exclude likely non-tumor-specific variants. Germline variants were filtered out using databases as previously described[33] and we excluded variants with allelic frequency >20%. ctDNA was quantified as mutant molecules per milliliter (MMPM) based on single nucleotide variants (SNV). Molecular characterization was performed by identification of driver mutations, which include only mutation in protein coding region (i.e., missense, truncating, start loss) and splice variants, with an allele frequency (AF) of >0.5% and <=20%.

Mutation profile from tumor biopsy was performed by targeted exon sequencing as previously reported.[14]

Statistical analysis methods

The performance of MMPM to predict POD24 was evaluated by an area under the receiver operating characteristic curve (AUROC) analysis using 5-fold cross validation. The receiver operating characteristic curve was vertically averaged across the folds. The MMPM cutoff was derived by the Youden index, averaged across folds.

The strength of PFS association with the MMPM cutoff was evaluated by Kaplan-Meier methods and Cox proportional hazards models.

Statistical analysis to compare frequency of mutation status among two groups was performed by a χ^2 test with Bonferroni adjustment.

Results

Baseline circulating tumor DNA is prognostic in untreated follicular lymphoma

We analyzed a cohort of 416 baseline plasma samples from patients with untreated FL enrolled in the GALLIUM study to evaluate the prognostic value of baseline ctDNA. The cohort was designed to be representative of the overall GALLIUM trial patient population with respect to PFS, FLIPI and treatment arms (obinutuzumab or rituximab in combination with CHOP/CVP or bendamustine; see material and methods *Online Supplementary Table S1* and *Online Supplementary Figure S1*). In this ctDNA cohort, analysis of baseline ctDNA showed a median of 102 SNV and a median MMPM of 110.1.

We observed a significant association between baseline ctDNA, evaluated as MMPM levels, and known clinical prognostic factors; the median MMPM levels is higher in cases with higher SPD (defined as higher quartile), higher FLIPI score (high *vs*. low/intermediate), and higher FLIPI-2 (high *vs*. low/intermediate) (all $P<0.0001$) (Figure 1). These results indicate a good correlation between standard prognostic factors in chemo-immunotherapy and increased baseline ctDNA in untreated FL patients.

Baseline circulating tumor DNA has a superior prognostic value compared to established prognostic risk scores

To assess the utility of baseline ctDNA for clinical prognostication, we compared the MMPM levels in baseline plasma to known clinical prognostic factors including SPD, FLIPI and FLIP-2. As POD24 is one of the most clinically relevant high-risk endpoints in FL we tested the specificity and sensitivity of those prognostic tools in predicting it.[5,6] In a univariate analysis, baseline MMPM levels were the best biomarker to predict POD24 when compared to other clinical scores, with an AUROC for MMPM of 0.69, compared to FLIPI AUROC of 0.57, FLIPI-2 AUROC of 0.59 and SPD AUROC of 0.58 (Figure 2A).

In a multivariate model evaluating the potential additive value of baseline MMPM to FLIPI, FLIPI-2 or SPD to predict

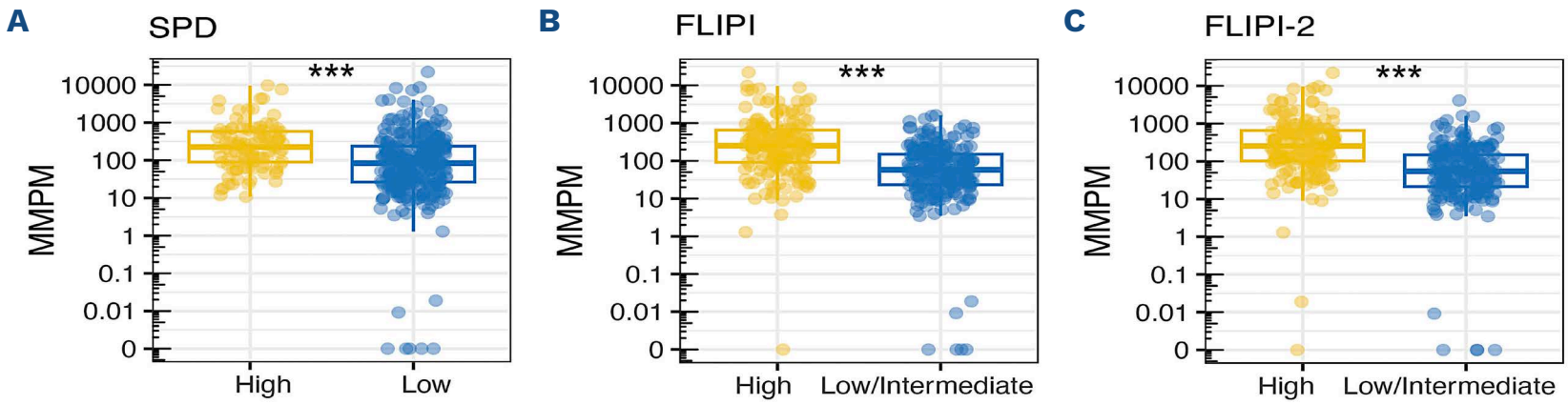

Figure 1. Association between baseline circulating tumor DNA, and prognostic factors. Box plot showing the association between (A) mutant molecules/mL (MMPM) levels at baseline and sum of the product of tumor lesion diameters (SPD) high (N=104, median MMPM=226) *versus* low (N=309, median MMPM=84.3), (B) Follicular Lymphoma International Prognostic Index (FLIPI) high (N=178, median MMPM=254) *versus* low/intermediate (N=238, median MMPM=57.8), (C) FLIPI-2 high (N=178 median MMPM=254) *versus* low/intermediate (N=229, median MMPM=54.5). Circulating tumor (ct) DNA levels were calculated as MMPM and the median and whiskers are shown. The statistical significance was assessed using the Mann-Whitney U test; ***P<0.001.

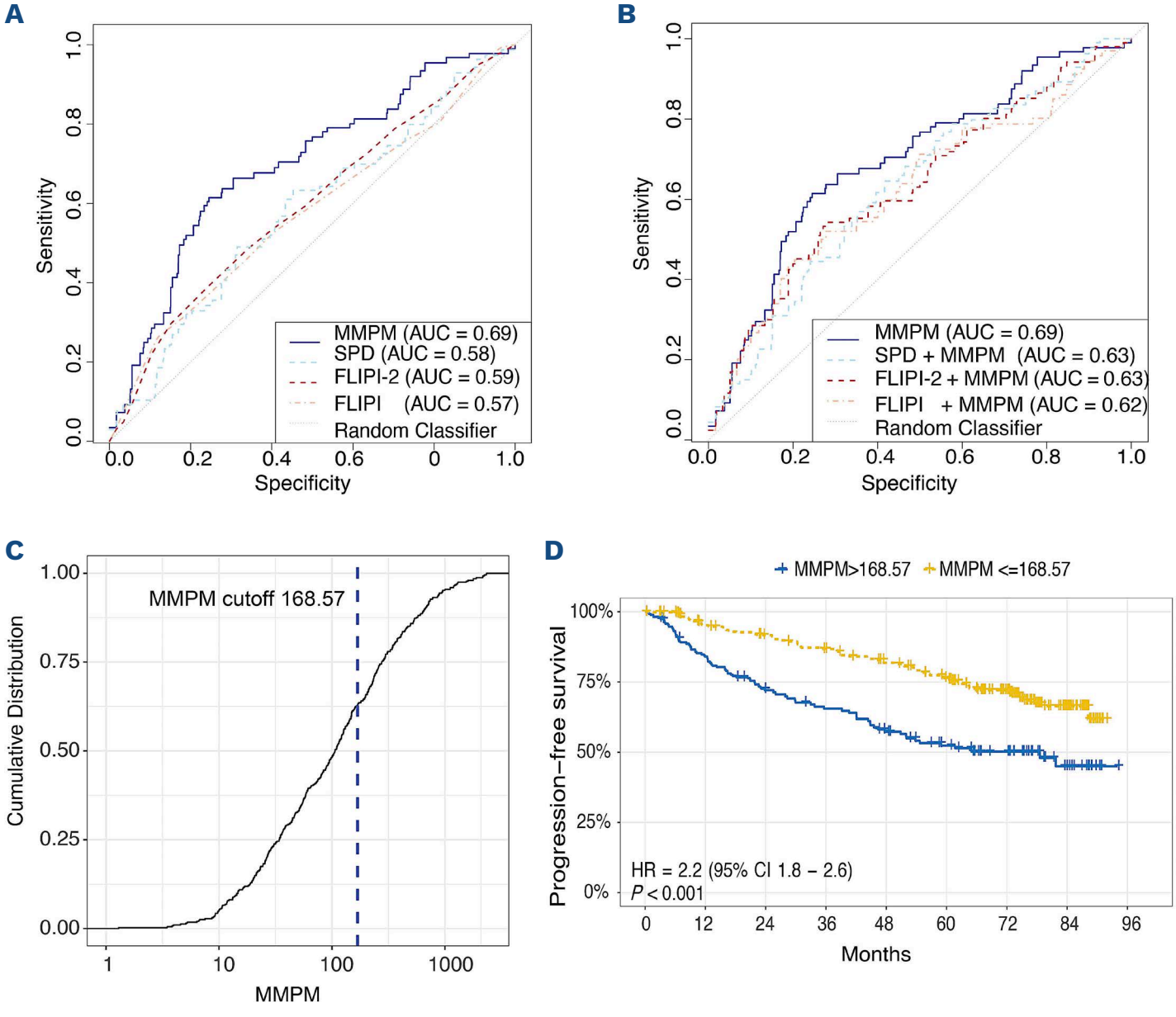

Figure 2. Baseline mutant molecules/mL as prognostic factor for progression of disease within 24 months. (A) Receiver operating characteristic (ROC) curves showing the performance of baseline mutant molecules/mL (MMPM) levels, sum of the product of tumor lesion diameters (SPD), Follicular Lymphoma International Prognostic Index (FLIPI) and FLIPI-2 in a univariate analysis. (B) ROC curves showing the performance of baseline MMPM levels, SPD, FLIPI and FLIPI-2 in a multivariate analysis. (C) Cumulative distribution of MMPM in the analysis cohort and MMPM cutoff indicated by dotted line. (D) Kaplan-Meier analysis of progression-free survival (PFS) based on MMPM cutoff=168.57. AUC: area under the curve. CI: confidence interval; HR: hazard ratio.

POD24, baseline MMPM level consistently increased the AUROC of FLIPI (from AUROC 0.57 to 0.62), FLIPI-2 (from AUROC 0.59 to AUROC 0.63) and SPD (from AUROC 0.58 to AUROC 0.63) (Figure 2B). Importantly, baseline MMPM value as a univariate marker showed the highest AUROC, underscoring its potential critical value for evaluating baseline risk in untreated FL patients.

To evaluate the use of baseline ctDNA as a patient stratification/selection tool, we selected a ctDNA cutoff to predict POD24. The MMPM cutoff of 168.57 was determined by an average Youden index in a 5-fold cross-validation model (Figure 2C). A Cox proportional hazard analysis of PFS demonstrated that patients with high baseline ctDNA (MMPM >168.57) had a significantly higher probability of experiencing shorter PFS (hazard ratio [HR]=2.2; 95% confidence interval [CI]: 1.8-2.6) (Figure 2D). The performance of this MMPM cutoff in classifying patients at risk of POD24 showed a sensitivity of 0.66, indicating the ability to correctly identify true POD24 cases, and a specificity of 0.69, indicating the ability to correctly identify non-POD24 cases. To assess the clinical utility of the MMPM cutoff in the context of the study population, we calculated the predictive values in the weighted population (*Online Supplementary Table S2A*). This analysis yielded a positive predictive value (PPV) of 0.29 and a negative predictive value (NPV) of 0.91, indicating a stronger performance in identifying low-risk patients rather than patient and high-risk of POD24.

Notably, the prognostic value of ctDNA at baseline, using the MMPM cutoff of 168.57, was observed across treatment arms. High MMPM correlated with shorter PFS in patients treated with obinutuzumab or rituximab CHOP/CVP (HR=2.5; 95% CI: 1.9-3.53), and in patients treated with obinutuzumab or rituximab bendamustine (HR=32; 95% CI: 1.5-2.6) chemotherapy backbone (Figure 3A, B). High MMPM correlated with shorter PFS in patients treated with obinutuzumab or rituximab bendamustine (HR=2; 95% CI: 1.5-2.6), and in patients treated with obinutuzumab or rituximab CHOP/CVP (HR=2.5; 95% CI: 1.9-3.53) chemotherapy backbone (Figure 3A, B). An interaction test for the MMPM by treatment group (CHOP/CVP *vs*. bendamustine) was performed using a Cox regression model, yielding a non-significant interaction term (P=0.63). Altogether these data point to-

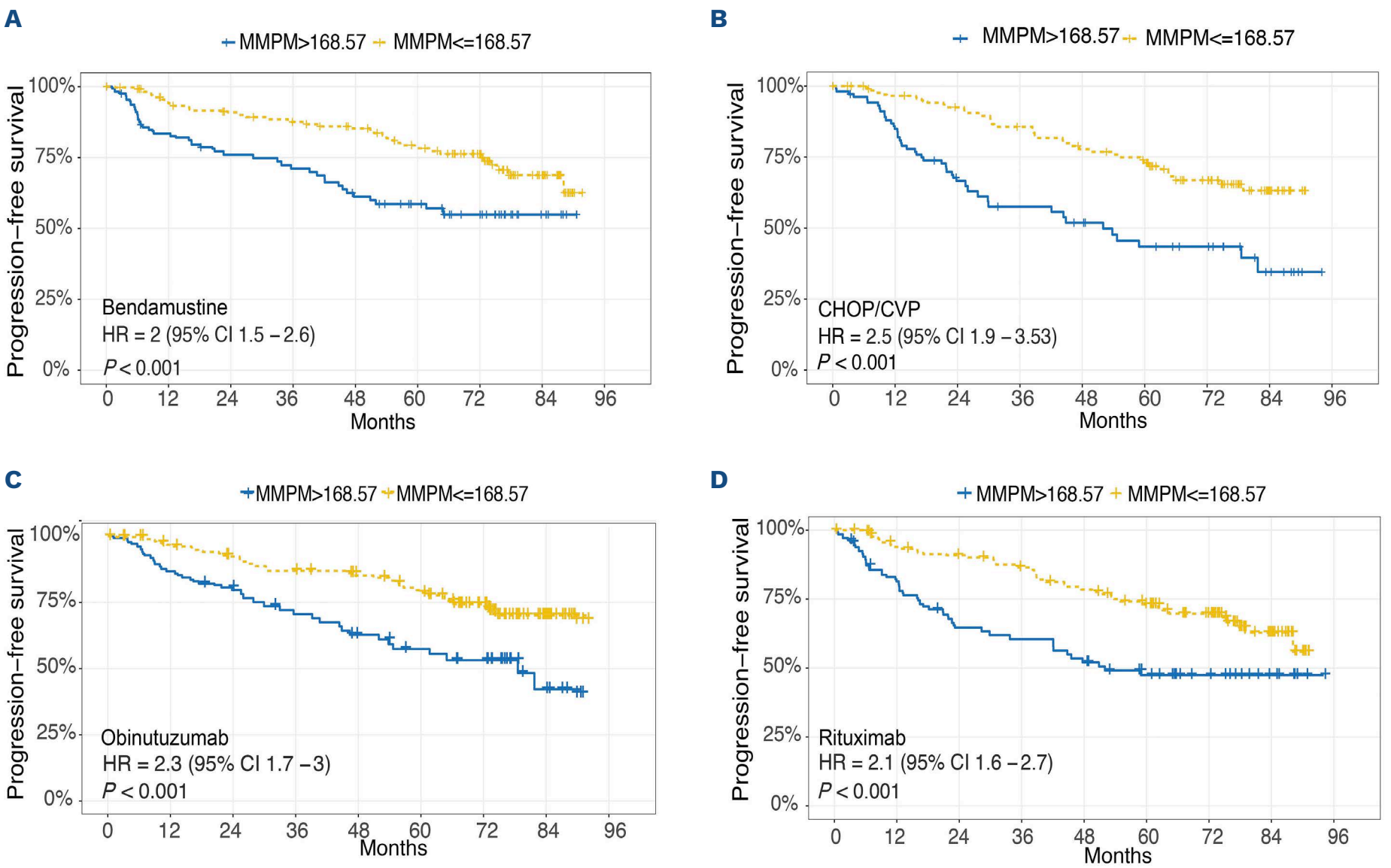

Figure 3. Prognostic value of circulating tumor DNA across chemotherapy backbones. (A) Kaplan-Meier analysis of progression-free survival (PFS) based on mutant molecules/mL (MMPM) cutoff=168.57 in patients treated with bendamustine. (B) Kaplan-Meier analysis of PFS based on MMPM cutoff=168.57 in patients treated with cyclophosphamide, doxorubicin, vincristine, and prednisone/cyclophosphamide, vincristine, and prednisone CHOP/CVP. (C) Kaplan-Meier analysis of PFS based on MMPM classification in patients treated with obinutuzumab. (D) Kaplan-Meier analysis of PFS based on MMPM classification in patients treated with rituximab. CI: confidence interval; HR: hazard ratio.

ward a prognostic, rather than predictive, role for baseline ctDNA, and suggest that it could be developed as a tool for patient stratification or patient selection in a clinical trial setting thanks to the ability to capture high-risk features across treatment regiments.

Baseline circulating tumor DNA levels correlate with histological transformation to aggressive lymphoma

Transformation of indolent follicular lymphoma to a more aggressive histology of trFL is another poor prognostic event that requires changes in clinical management.[31] As the ability to predict occurrence of transformation at diagnosis could inform different patient treatment strategies, we investigated the potential for baseline ctDNA to predict transformation in the GALLIUM study.

In the ctDNA cohort from the GALLIUM study 26 patients (26/416, 6.2%) experienced histologically confirmed disease transformation. The median PFS of this cohort was 9.1 months. Patients who experienced transformation during the study had higher ctDNA level at baseline (median MMPM 324.9 in trFL *vs*. MMPM 103.6 in non-trFL) (Figure 4A). MMPM level at baseline showed an AUROC value for predicting transformation of 0.66 (Figure 4B). As mutations in *TP53* have been previously associated with disease transformation,[19] we investigated if adding *TP53* mutation status to the MMPM level could improve the ability to predict trFL. In a multivariate model, *TP53* mutation status did not improve the prognostic value of MMPM (Figure 4B). These results should be considered anecdotal due to the small cohort of 26 patients with trFL.

Molecular characterization of patients with high baseline circulating tumor DNA

To characterize molecular features of patients with high ctDNA defined using the MMPM cutoff, we examined differences in the AF, number of SNV and mutated genes in patients with high MMPM (>168.57) and low MMPM (≤168.57). The MMPM high group showed a significantly higher number of somatic mutations (median number of SNV 127 *vs*. 87; *P*<0.001), a higher AF (median 5.37% vs. 1.61%; *P*<0.001), and a higher number of mutated genes (median 40 *vs*. 27; *P*<0.001) compared to the MMPM low group, suggesting that both an increase in the amount and number of detected SNVs contribute to higher MMPM levels (Figure 5A-C).

Taking advantage of the possibility to derive mutation profiles from the cancer personalized profiling by deep sequencing (CAPP-seq) technology, we investigated frequently mutated genes in plasma, focusing on SNV occurring in the coding region with an AF greater than 0.5% to improve the specificity of mutation calls. When compared to the mutation profile derived from targeted exon sequencing in tumor biopsies, 24 of 27 of the most frequent mutations observed in ctDNA by CAPP-seq were also detected by targeted exome sequencing in tumor biopsies (*Online Supplementary Figure S3*). We observed that those additional filters increased the concordance of negative mutation calls between CAPP-seq and targeted sequencing, maybe by reducing low-level noise (*Online Supplementary Figure S3B, C*). No major effects were observed for the concordance of positive mutation calls. Although comparison of mutation profile data from CAPP-seq in ctDNA with targeted sequencing in tumor biopsies requires caution due to differences in regions targeted for sequencing, we consider these results valuable to gain insight on the information that can be gathered from the two technologies/compartments.

Most frequent potential driver mutations in ctDNA by CAPP-seq were identified in *BCL2*, *TNFRSF14*, *CREBBP* and *EZH2* (Figure 5B). Comparing the frequency of mutations in patient with MMPM high *versus* MMPM low did not show major differences, however in patients with MMPM high *versus* MMPM low we observed more frequent mutations in *IGLL5* (24.8% *vs*. 19.2%; *P*=not significant), *TP53* (16.1% *vs*. 6.3%; *P*=0.0542) and *B2M* (10.6% *vs*. 2.4%; *P*=0.0221) and less frequent mutation status in *TNFRSF14* (21.7% *vs*. 39.6%; *P*=0.0064) and *CREBBP* (19.3% *vs*. 40.4%; *P*=0.0003) (*Online Supplementary Figure S4*).

This descriptive analysis suggested that high MMPM is likely driven not only by a higher allele frequency in the genes analyzed but also by an increased detection of SNV,

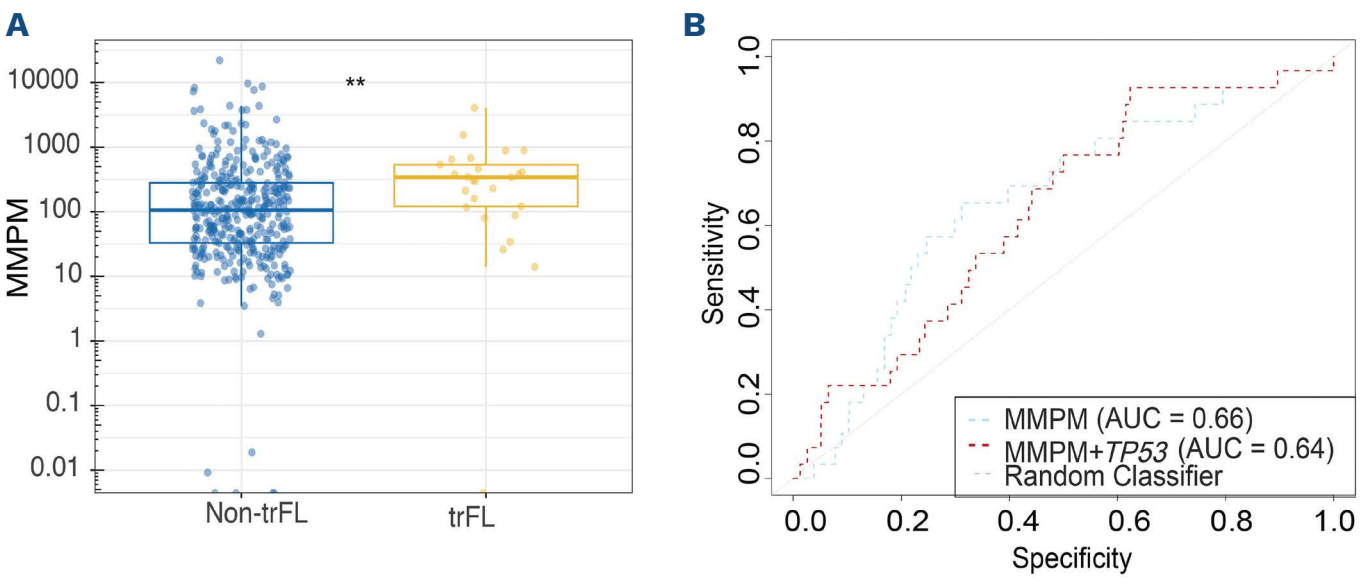

Figure 4. Correlation between baseline mutant molecules/mL levels and histological transformation status. (A) Box plot showing the correlation between mutant molecules/mL (MMPM) levels and transformed follicular lymphoma (trFL) *versus* non-trFL. Median and whiskers are shown; **P<0.01. (B) Receiver operating characteristic (ROC) curves showing the performance of baseline MMPM levels and MMPM level plus *TP53* status to predict trFL.

which collectively contribute to the high circulating tumor burden.

Discussion

In this study, we demonstrated that baseline ctDNA is an independent prognostic factor in patients with untreated FL enrolled in the GALLIUM study, and it adds prognostic value to existing clinical factors like SPD, FLIPI and FLIPI-2. Being able to identify high-risk patients at diagnosis could enable personalized patient care, including therapeutic modification/intensification and enhanced monitoring for a better disease outcome. From a drug development perspective, the POD24 patient segment could offer the opportunity to investigate novel therapeutic approaches and help design shorter and smaller clinical trials focused on patients with critical needs.

Our findings show that ctDNA at baseline can predict patients at risk of early relapse, with a focus on POD24 as a clinical meaningful endpoint for untreated FL. This molecular tool was built using ctDNA mutations detected from plasma samples at baseline with need of neither tumor biopsy nor additional samples for identification of germline mutations. This could provide a biomarker for POD24 from a single blood draw to be collected before treatment initiation to assess risk of early relapse. As many patients experience transformation within 24 months, it is important to highlight the potential histological heterogeneity of this high-risk group.

This work adds on a number of evidence pointing to ctDNA as a prognostic tool in lymphoma.[20-23] This is, to

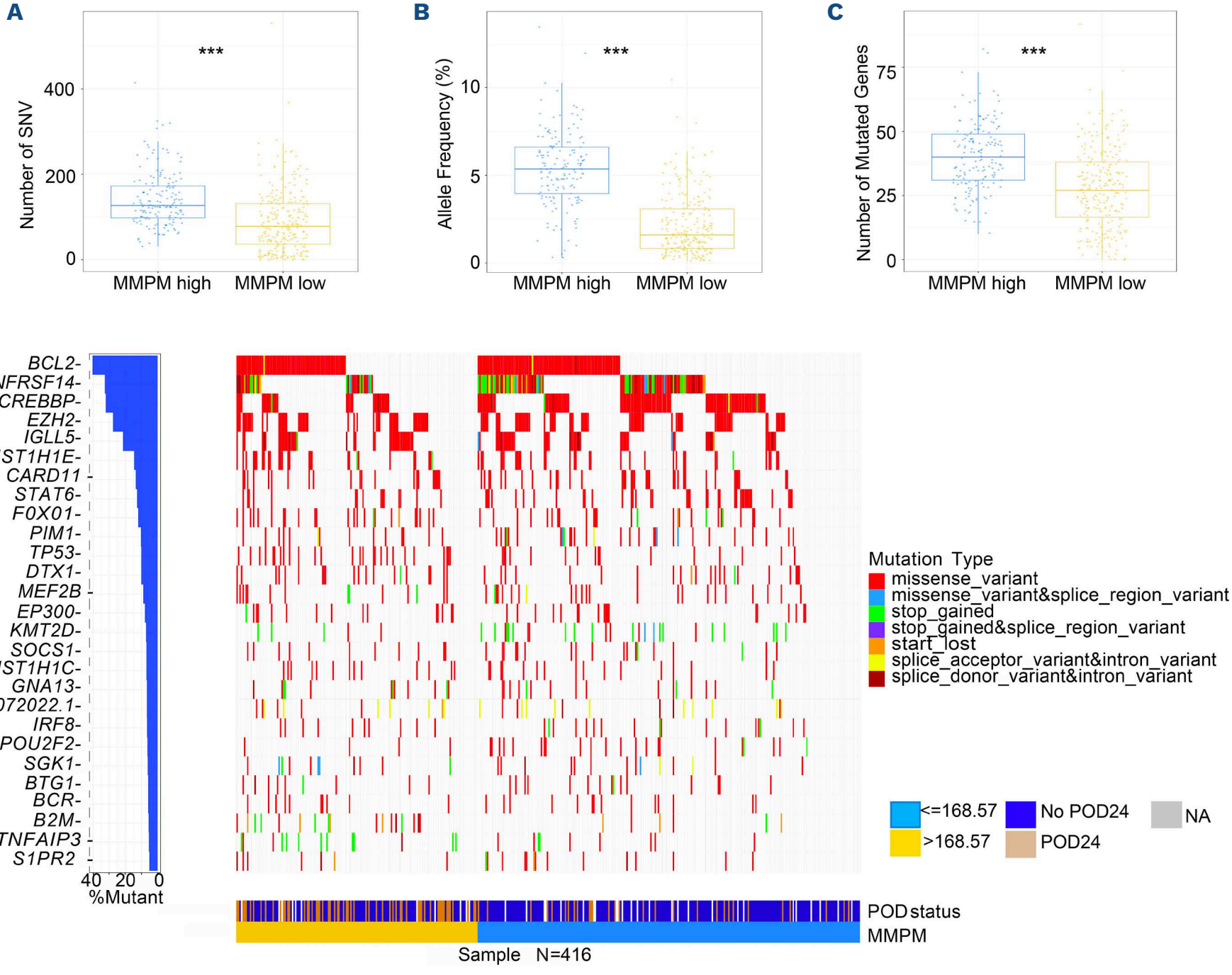

Figure 5. Molecular characterization of mutant molecules/mL (MMPM) high *versus* MMPM low. (A) Box plot showing the correlation between number of single nucleotide variants (SNV) and mutant molecules/mL (MMPM)-high *versus* MMPM-low patients. (B) Box plot showing the correlation between allele frequency (AF) and MMPM-high *versus* MMPM low patients. (C) Box plot showing the correlation between number of mutated genes and MMPM-high *versus* MMPM-low patients. For (A-C) the median and whiskers are shown. The statistical significance was assessed using the Mann-Whitney U test; ***P<0.001. (D) Oncoplot showing the most frequent driver mutations in the cohort. NA: not applicable; POD24: disease progression within 24 months.

our best knowledge, the largest ctDNA cohort in untreated FL, compounded with a robust statistical approach to ensure comparability with the baseline clinical features of the GALLIUM population. The GALLIUM study reflects the current standard of care, and, thanks to the multiple treatment regimens, this cohort provides the unique opportunity to evaluate the prognostic value of ctDNA in patients treated with two different anti-CD20 antibodies (rituximab and obinutuzumab) in combination with different chemotherapy backbones (CHOP/CVP or bendamustine). Unlike other genomic scores like m7-FLIPI, 23 gene signature or FL20, ctDNA shows a significant prognostic value across chemotherapy backbones. Predictive biomarkers allowing personalized treatment based on specific tumor characteristics and vulnerabilities are the end goal of personalized medicine, and development of biomarkers based on a well distinct biology is critical for this scope. A critical finding of this study is that the prognostic value of baseline ctDNA is stable and consistent, regardless of whether patients received CHOP/CVP or bendamustine-based anti-CD20 immunochemotherapy. The role of ctDNA was prognostic, rather than predictive, with respect to treatment selection. Its clinical utility is particularly strong in identifying patients at low-risk of early relapse, demonstrated by its high negative predictive value. A tool with these characteristics might facilitate stratification for clinical trials, enrichment of patients with higher risk of progression or help identify patients at lower risk with potential for treatment reduction.

Baseline ctDNA levels appear to be a measure of tumor-burden, as suggested by strong correlation with the imaging-based SPD shown in this work, and with LDH and GELF as previously reported.[21] However, independent prognostic value compared to established tumor burden measurements suggests that ctDNA may capture lymphoma-biology aspects, such as high mutational burden and specific genomic alterations, providing a measure that integrates both tumor volume and intrinsic biological aggressiveness to improve risk stratification in FL.

Analysis of ctDNA using the CAPP-seq technology offers the possibility of deriving a comprehensive mutation profile.[17,34] Although the CAPP-seq panel was designed specifically for DLBCL, its use for prognostication in FL could be adequate thanks to the good overlap of prognostically relevant genes.[21] Indeed, epigenetic regulators (like *KMT2D*, *EZH2, TNFRSF14*) are frequently altered in FL and have shown a prognostic value.[14,35,36] Additionally, this panel may comprehensively capture features of high-tumor burden across both histology as shown in this and other works in the literature.[18,19] A generally good concordance was observed between mutated genes identified in plasma and in tumor biopsies. However, some genes like *KMT2D, IRF8, CREBBP* were underrepresented in plasma compared to the expected mutation profile in tumor biopsies[14]. This might be due to differences in the sequencing panel as previously reported also in the RELEVANCE dataset,[24] and future work will be important to further dissect potential discordance between plasma and tumor biopsies. ctDNA could become an important biomarker for the diagnosis and prognostication of patients with untreated FL; in addition to mitigating challenges in tumor accessibility, this approach may provide a more comprehensive view of disease heterogeneity across multiple tumor lesions. Additionally, ctDNA can be used for evaluation of longitudinal disease evolution and for minimal residual disease monitoring application.[37] A recent study conducted on a sub-cohort of patients enrolled in the RELEVANCE study showed the enhanced value of combining ctDNA kinetics and TMTV for POD24 identification.[24] While combining multiple biomarkers has the advantage of improving high-risk definition, it comes with more operational burden due to multiple testing, which might pose more challenges for implementation.

Future studies are warranted to further corroborate our findings on the prognostic value of baseline ctDNA levels, which are currently limited to the GALLIUM study. It will be key to validate the identified ctDNA cutoff in independent studies and to investigate if ctDNA levels are prognostic also in patients treated with emerging novel therapies like bispecifics or immunomodulators. The integration of ctDNA with additional biomarkers and the evaluation of on-treatment ctDNA kinetics are a key area for further improvement to guide treatment decisions.

Altogether, these results show that baseline ctDNA has the potential to capture high-risk *versus* low-risk features independent from established clinical scores and could be implemented for patient stratification in clinical trials. As the availability of drugs and treatment modalities with different safety and efficacy profiles become available for patients with FL, the availability of biomarkers to predict outcome becomes essential to inform clinical decisions and advancing personalized healthcare.

Disclosures

DK, AB, EP, PEB, and TN disclose employment and stock options at Roche and Genentech. CL discloses employment at Signature Diagnostics and Roche. CRB discloses employment and stock options at Roche and Natera. MS, AK, and HM dislcose employment at Roche. AD discloses research funding from Celgen, BMS, Roche, Kite, Gilead, Janssen, MSD, Acerta Pharma, and AstraZeneca; travel grants from Celgene, BMS, and Roche; consultancy or advisory role at Celgene, BMS, Roche, Kite, Gilead, Abbvie, Genmab, Prelude, Incyte, Sobi, and Serb; honoraria from Celgene, BMS, Roche, Kite, Gilead, Abbvie, Janssen, Acerta Pharma, AstraZeneca, and JW Therapeutics. OW discloses research support from InCyte; consultancy or advisory role at Roche.

Contributions

AB, TN, EP, and PEB contributed to the conceptualization and ideation of the project. CL, DK, CB, VA, and HM performed analysis. MS performed experiment. All authors contributed

to the acquisition, analysis, and interpretation of study data and critically reviewed the manuscript. AB and CL wrote the manuscript.

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Data-sharing statement

Requests for the exploratory biomarker data underlying this publication requires a detailed, hypothesis-driven statistical analysis plan that is collaboratively developed by the requestor and company subject matter experts to enforce terms such as security, patient privacy, and consent of specified data use, consistent with evolving, applicable data protection laws. Direct such requests to alessia.bottos@roche.com for consideration. Up-to-date details on Roche's Global Policy on the Sharing of Clinical Information and how to request access to related clinical study documents are available at https://go.roche.com/data_sharing. Anonymized records for individual patients across more than one data source external to Roche cannot and should not be linked because of a potential increase in risk of patient re-identification.

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