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The negative influence of baseline cell-free DNA on long-term survival in DLBCL depends on frontline treatment intensity

Running title: cfDNA and DLBCL overall survival

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53 **TRANSLATIONAL RELEVANCE**

54 *Tumor-derived plasma cell free DNA (cfDNA), as a surrogate marker of disease*
55 *burden and early predictor of response to therapy, offers opportunities for therapeutic*
56 *intervention, particularly in DLBCL. The gold standard treatment for de novo DLBCL*
57 *is R-CHOP, but some studies have shown benefit for patients with dose escalation in*
58 *first-line therapy. In our study, we took advantage of a single randomized clinical trial*
59 *comparing R-CHOP with conventional high-dose chemotherapy plus autologous*
60 *stem cell transplantation (ASCT) for first-line treatment of advanced DLBCL to*
61 *explore the effect of cfDNA on patients' clinical outcome. We confirmed that cfDNA*
62 *concentration at diagnosis was an adverse prognostic factor for patients in the R-*
63 *CHOP arm but not for those in the high-dose arm. Interestingly, patients with positive*
64 *PETscan at the interim evaluation who underwent salvage chemotherapy*
65 *supplemented with ASCT had significantly improved survival compared with good*
66 *responders at the interim evaluation but treated with R-CHOP and with high cfDNA*
67 *levels at diagnosis. Our results demonstrate that high-dose first-line therapy mitigates*
68 *the negative influence of high cfDNA levels at diagnosis. Moreover, once CR was*
69 *achieved, for 11 out of 24 R-CHOP patients, the cfDNA concentration did not fall*
70 *back to normal levels. Overall, these results raise the question of whether R-CHOP*
71 *should be maintained as first-line therapy for DLBCL patients with elevated cfDNA*
72 *levels at diagnosis; alternative treatments could be considered, including high-dose*
73 *chemotherapy combined with ASCT.*

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ABSTRACT

Purpose: In this study, we address the question of the effect of first-line treatment intensity in de novo DLBCL on the impact of baseline cfDNA on long-term survival.

Experimental Design: The GOELAMS 075 randomized clinical trial compared R-CHOP with high-dose R-chemotherapy plus autologous stem cell transplantation (R-HDT) for patients aged ≤ 60 . An interim PET assessment was used to refer patients for salvage therapy. With a median follow-up of more than 5.8 years, we analyzed the effects of the treatment arm, salvage therapy, and cfDNA level at diagnosis on overall survival (OS).

Results: In a representative group of 123 patients, a high cfDNA concentration (>55 ng/ml) at diagnosis was associated with poor clinical prognostic factors and constituted a prognostic marker, independently of the age-adjusted International Prognostic Index. A cfDNA level above a threshold value of 55 ng/ml at diagnosis was associated with significantly worse OS. In an intention-to-treat analysis, high-cfDNA R-CHOP patients (but not high-cfDNA R-HDT patients) had worse OS (hazard ratio [95% confidence interval]: 3.99 [1.98-10.74], $P=0.006$). In patients with high cfDNA levels, salvage therapy and transplantation were associated with a significantly higher OS rate. Among 50 patients with complete response 6 months after the end of treatment, for 11 out of 24 R-CHOP patients, the cfDNA did not fall back to normal values.

Conclusions: In this randomized clinical trial, intensive regimens mitigated the negative influence of high cfDNA levels in de novo DLBCL, relative to R-CHOP.

INTRODUCTION

The first-line treatment for diffuse large B-cell lymphoma (DLBCL) combines rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone. This R-CHOP regimen is the standard of care, regardless of the cancer's immunohistochemical profile or molecular subtype ¹. However, 30 to 40% of patients are refractory to this treatment or relapse within 2 years of an initial response. After relapse, the overall cure rate ranges from 25 to 35% ¹. Refractory patients have a poor prognosis, with a median overall survival (OS) time of around 6 months ². It is therefore very important to provide the most appropriate treatment as soon as *de novo* DLBCL has been diagnosed. In the early 2000s (i.e., before the rituximab era), we showed that first-line high-dose chemotherapy with autologous stem cell transplantation (ASCT) was associated with longer disease-free survival in transplant-eligible patients with advanced DLBCL ^{3,4} and an OS benefit in a subset of patients ³. Since then, it has been shown that a combination of rituximab with high-dose conventional chemotherapy prolongs survival in young adults with a low-intermediate level of risk (according to the International Prognostic Index (IPI)) ⁵. At present, primary staging is based on the IPI and positron emission tomography-computed tomography (PET-CT) findings, which also allow assessment of the treatment response ⁶. In 2005, we initiated a French, open-label, randomized, Phase 3 trial (GOELAMS 075, NCT00561379) of R-CHOP vs. a combination of rituximab, high-dose conventional chemotherapy and ASCT (hereafter referred to as R-HDT) for the first-line treatment of advanced DLBCL adult patients aged ≤60. The median follow-up period after randomization was >5.8 years (70.4 months; 95% confidence interval (CI), 55.0 – 88.9).

The bloodstream of cancer patients usually contains circulating, cell-free DNA (cfDNA) released from tumor cells after apoptotic, caspase-dependent cleavage. The

predominant DNA peak is 166 base-pairs (bp) and is accompanied by a series of smaller peaks with a periodicity of 10 bp ⁷. Although tumor-specific alterations can have diagnostic and prognostic value, not all patients have detectable changes ⁸. The circulating cfDNA concentration varies significantly from one healthy individual to another and is higher in patients with lymphoma ⁹. The proportion of circulating tumor (ct) DNA is especially high in patients with aggressive lymphomas ¹⁰. The concept of ctDNA as a surrogate marker of disease burden and an early predictor of the treatment response offers opportunities for therapeutic intervention. Although the optimal ctDNA measurement time points and the thresholds for quantitative changes have not been validated extensively, a post-treatment fall in detectable ctDNA level is a good indicator of clinical benefit for various tumors and treatment contexts ¹¹. In the present study of patients with newly diagnosed, aggressive DLBCL, we examined the potential prognostic value of the cfDNA concentration in a 90-to-320 bp window. We also investigated the putative impact of the type of first-line therapy on the cfDNA concentration. To this end, we took advantage of the GOELAMS 075 study, in which plasma samples were collected (i) at diagnosis for 123 patients and (ii) at the time of a complete response (CR) 6 months after the end of treatment (EOT) for 50 of these 123 patients. The median follow-up time was 6.9 years. Our results revealed that the cfDNA concentration at diagnosis was an unfavorable prognostic factor for patients in the R-CHOP arm but not for those in the R-HDT arm. The achievement of CR parallels a clearance of cfDNA, except in a number of R-CHOP patients who maintained elevated levels of cfDNA.

METHODS

Patients

The GOELAMS 075 study (referred to hereafter as the G075 cohort study) is a multicenter trial in which adults aged 18 to 60 with untreated, histologically-proven, CD20-positive DLBCL were randomly assigned to receive several courses of R-CHOP or R-HDT (supplemental Figure 1A). Patients were required to have an Ann Arbor stage of I or II with bulk >7 cm or a stage of III or IV with zero to three adverse prognostic factors, as defined by the age-adjusted IPI (aa-IPI). Patients with transformed low-grade, lymphoblastic, mantle cell or Burkitt's lymphoma were excluded. Diagnostic slides were reviewed centrally by two hematopathologists (D.D. and M-C.P.). The trial was approved by the investigational review board at Nantes University Medical Center (Nantes, France), and all patients gave their written, informed consent. The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Patients with DLBCL (N=178; median follow-up time: 44 months) randomized into the GAINED trial (NCT01659099) were used as an independent validation cohort for the OS analysis ⁶. The GAINED study is a multicenter randomized phase 3 trial comparing Obinutuzumab to Rituximab in association with a chemotherapy administered every 14 days (doxorubicin, cyclophosphamide, prednisone plus vindesine, bleomycin [ACVBP] or vincristine [CHOP]) using an interim PET-scan driven strategy (supplemental Figure 1B). Transplant-eligible patients (18-60 years, n=670), enrolled between 2012 and 2017, were diagnosed with a de novo DLBCL with an age-adjusted International Prognostic Index (aaIPI) score ≥ 1 . The median follow-up time of the GAINED cohort was 35.4 months.

Data and tissue samples collected in the G075 cohort

Plasma samples collected at the time of diagnosis (i.e. before any treatment) were available for 123 of the 340 patients enrolled in the trial. Furthermore, post-treatment plasma samples were available for 50 of the 123 patients who had maintained a CR six months after the EOT (detailed information for CR patients in **Supplemental table 1**). For 178 of 670 patients of the GAINED cohort, plasma samples were collected at randomization and control plasma was obtained from healthy volunteer (HV) blood donors at the French National Blood Agency (age, 18-60). Plasma was collected in BD™ P100® tubes (BD Diagnostics, Le Pont-De-Claix, France), shipped at room temperature within 24 hours of collection to the core lab in Rennes (France), centrifuged, and stored at -80°C.

For all G075 patients, baseline clinical data and treatment responses were obtained during inclusion, interim and final assessments. For the 50 patients with a CR 6 months after the EOT, clinical data were obtained 7 to 10 years after inclusion. Interim and final assessment responses were based on the PET-CT and standard CT findings. The interim PET results were based on visual criteria and not the Deauville criteria, which were established after the G075-cohort study had started. As per protocol, patients with a positive interim imaging result received salvage therapy. The collection of data and tissue samples for all patients in the present study had been approved by the French national investigational review board.

cfDNA extraction, quantification, and size distribution analysis

Samples from both trials were processed in exactly the same way and a maximum of 2 ml of plasma was reserved for this cfDNA study (**supplemental Figure 2A**). In our study, the concentration of cfDNA corresponds to the amount of DNA detected in the 90- to 320-bp window (**supplementary Figure 2B**), and the median DNA fragment

size was significantly smaller for patients than for HVs (supplementary Figure 2C). We established the best threshold above which patient samples at diagnosis were considered cfDNA-enriched using two different approaches. First, we applied the maximum selected rank statistics (maxstat R package¹²) to determine the threshold that predicts the most significant differences in OS of patients independently in the GOELAMS 075 and GAINED cohort (supplementary Figure 2D). Next, the threshold was defined as 10 times the upper limit of cfDNA concentration in HV, this value is clinically relevant and easily applicable to a patient population (supplementary Figure 2E). Because the maxstat and HV cohort results were close in value (49.2 and 52.1 vs. 54.9 ng/ml, respectively), the cutoff was set at 55 ng/ml for the entire study, defining high and low cfDNA levels as above and below 55 ng/ml, respectively (supplementary Methods).

Statistical analysis

Analysis was performed on an intention-to-treat (ITT) basis, for evaluable patients. Descriptive statistics were quoted as the median [interquartile range (IQR)] or the number (percentage). Groups were compared using a chi-squared test, the Mann-Whitney U-test, Wilcoxon's signed-rank test, or the Kruskal-Wallis test, depending on the type of data and the number of groups. OS (defined as the time from randomization to the date of last follow-up or death from any cause) was estimated using the Kaplan–Meier method. Differences in survival curves were assessed in a log-rank test. A Cox proportional hazards model was used to determine hazard ratios (HRs) and their 95%CI. All statistical tests were two-sided, and the threshold for statistical significance was set to $P < 0.05$. Statistical

analyses were performed using GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, CA) and R software (version 4.1.1, R Core Team 2021).

Raw data for this study were generated at Hematology Laboratory of Rennes University Hospital. Derived data supporting the findings of this study are available from the corresponding author upon request.

RESULTS

R-HDT is not superior to standard R-CHOP with regard to OS

Between May 2005 and August 2010, 340 patients were enrolled in the G075 cohort. Seventeen patients were deemed to be ineligible, and the remaining 323 were randomized to receive either R-CHOP (n=161) or R-HDT (n=162). The two groups were similar, except for performance status (supplemental Table 2). There were no differences in demographic and clinical characteristics between the parent cohort and the 123 patients with cfDNA assay data (supplemental Table 2). Of the 123 patients, 68 were assigned to the R-CHOP and 55 were assigned to the R-HDT arm (Figure 1A); the median follow-up time after randomization was 69 months (95%CI, 55.3-88.0). With the exception of the sex ratio, the R-CHOP and R-HDT arms were well balanced with regard to their demographic and clinical characteristics (Table 1). Fifty-nine patients (87%) from the R-CHOP arm achieved a confirmed or unconfirmed complete response (CR) or partial response at the EOT assessment, compared with 51 (93%) in the R-HDT arm. There was no difference in 5-year OS between the randomization arms for the 123 patients (80.0% [70.1-91.3] for R-HDT vs. 77.7% [68.3-88.3] for R-CHOP; $P=0.57$) or for the 323 patients in the parent cohort (85.5% [80.2-91.2] for R-HDT vs. 81.1% [75.3-87.5] for R-CHOP; $P=0.39$) (Figures 1B-C).

Bone marrow involvement, the presence of more than one extranodal site, the aa-IPI as well as age >50 were found to be significantly associated with poor OS (supplemental Table 3).

The cfDNA concentration is elevated in patients with adverse prognostic factors

The cfDNA fragment distribution in the 90- to 320-bp range of patients differed from that of HVs, with a higher proportion of fragments shorter than 150 bp⁷. The median cfDNA concentration was significantly higher in patients (17.8 ng/ml, IQR 8.4-56.8) than in HVs (1.04 ng/ml, IQR 0.2-3.9) (Figure 2A), but did not differ significantly when comparing the two treatment arms (Table 1). The cfDNA concentration at diagnosis did not differ significantly when comparing patients with or without salvage therapy, or with negative or positive iPET. Patients with high-risk clinical factors at diagnosis (e.g., more than one extranodal site, a poor Revised-IPI (R-IPI), and a high-risk aa-IPI) had significantly higher cfDNA levels (Figures 2B, C and D).

A high cfDNA level at diagnosis is associated with worse outcomes

Based on the 55 ng/ml threshold for cfDNA, 21 of 55 (38%) R-HDT patients had high cfDNA levels compared to 14 of 68 (21%) R-CHOP patients ($P=0.05$) (Table 1). The OS rate at 60 months was significantly worse for the 35 high-cfDNA patients than for the 88 low-cfDNA patients (65.7%, 95%CI, 51.7-83.5 vs. 83.9%, 95%CI, 76.5-92.0, respectively; HR=2.4, 95%CI, 1.1-5.2) (Figure 3A). Progression-Free Survival (PFS), taking progression, relapse or death from lymphoma as the first event, was performed on 118 of 123 patients. Indeed, of the 27 patients who died, five died in CR from a cause unrelated to the lymphoma (supplemental Table 4). Of the 118

patients, the PFS showed a better result for patients with a cfDNA below the threshold value of 55ng/ml, with a *P* value at the limit of significance (*P*=0.076), which was not the case in the GAINED cohort (supplemental Figure 4A-B).

Similar results were observed in patients from the GAINED cohort. The median DNA fragment size was 174 bp (IQR 167-231), and median [IQR] cfDNA levels were significant higher in patients than in HVs (9.24 ng/ml [4.1-31] vs. 1.04 [0.2-3.9], respectively; supplemental Figure 2E). Twenty-six of the 178 GAINED patients were in the high-cfDNA group and had a significantly worse OS at 48 months (73.1%, 95%CI, 57.9-92.3 vs. for low-cfDNA 88.5%, 95%CI, 83.3-94.1; HR=2.67, 95%CI, 1.11-6.53) (Figure 3B). Interestingly, the 55ng/ml cutoff is close to the optimal threshold at 52ng/ml obtained for the GAINED cohort using the maxstat method (supplemental Figure 2F).

As for known risk clinical factors, when combined with the randomization arm, abnormal serum LDH, age \geq 50 years, and aalPI=2 or 3 had significantly worse OS compared with their respective low-risk counterpart (LDH, *P*=0.038; age, *P*=0.019; aalPI=2, *P*=0.036; aalPI=3, *P*=0.011). These significant differences were observed only in the R-CHOP arm, as for cfDNA. Only high-risk (HR) aalPI (=3) distinguished patients with a low OS at 60 months by 50%, as did cfDNA, but did not differentiate R-HDT patients from R-CHOP patients in the HR aalPI category (supplemental Figure 4C).

In a multivariate Cox analysis, the cfDNA levels (in ng/ml) and the aa-IPI risk still had statistically significant relationships with OS in the 075 cohort: HR [95%CI] =5.48 [1.53-19.56] for high- vs. low-risk aa-IPI (*P*=0.0088) and 7.33 [2.33-23.09] for cfDNA (*P*=0.00066), whereas the intermediate vs. low risk aa-IPI (HR=2.91, 95%CI, 0.96-8.75, *P*=0.057) was not significant.

High-cfDNA patients in the R-CHOP arm had significantly worse survival

With regard to the randomization arm (Figure 4A), patients in the R-CHOP arm had significantly worse OS when they were in the high-cfDNA category (HR=3.99, 95%CI, 1.48-10.74, $P=0.006$); this was not the case for patients in the R-HDT arm (HR=1.40, 95%CI, 0.43-4.59, $P=0.58$). This finding is consistent with the fact that high-cfDNA patients in the R-HDT arm tended to do better than high-cfDNA patients in the R-CHOP arm (HR=2.74, 95%CI, 0.87-8.69, $P=0.084$). Overall, long-term OS was worse among the 14 high-cfDNA R-CHOP patients than the other subgroups, with a plateau of 5-year survival at 50% [29.6-84.4], vs. 76.2% [60-96.8] for high-cfDNA R-HDT, 82.4% [70.5-96.2] for low-cfDNA R-HDT, and 82.9% [73.3-93.8] for low-cfDNA R-CHOP.

Intensive treatment mitigates the negative outcome expected in patients with high cfDNA levels

In the parent cohort ($n=323$), the OS rate at 60 months was 76.9% [69.5-85.1] for the 114 interim PET-positive patients and 89.0% [84.7-93.6] for the 196 PET-negative patients ($P=0.009$).

In the studied G075-cohort, only for iPET-negative patients at the interim assessment, those with high cfDNA had a statistically significantly shorter OS than low-cfDNA patients (HR=3.48, 95%CI, 0.68-5.63, $P=0.02$) (supplementary Figure 5A).

When considering the 82 patients (regardless of the randomization arm) who did not receive salvage therapy after the interim assessment, OS was significantly worse for high-cfDNA patients than for low-cfDNA patients (HR=3.82, 95%CI, 1.55-9.43,

$P=0.0036$) (Figure 4B). In contrast, for individuals receiving salvage therapy, high-cfDNA patients had much the same OS rate as low-cfDNA patients. Thus, for high-cfDNA patients and regardless of randomization arm, those who did not receive salvage therapy at the interim assessment tended to survive for a shorter time than those who received salvage therapy (HR=3.08, 95%CI, 0.83-11.41, $P=0.092$). The outcome was particularly unfavorable for the nine high-cfDNA patients who received 8 R-CHOP cycles, (supplemental Figure 5B). Six of the nine died, resulting in a 60-month OS rate of 33.3% (HR=5.70, 95%CI, 2.87-14.22, $P=0.0002$) (supplemental Figure 5C). As with OS, analysis of PFS showed that high-cfDNA patients who did not receive salvage treatment had a statistically significantly worse outcome than low-cfDNA patients with no salvage ($P=0.003$) and high-cfDNA patients who did receive salvage treatment ($P=0.026$) (supplemental Figure 5D).

Lastly, we looked at the effect of ASCT in our study. ASCT was performed in 69 of the 123 patients, either as an integral part of the R-HDT protocol or as elective salvage treatment. We found that ASCT abolished the unfavorable outcome associated with the high-cfDNA category because the 60-month OS rate among the 24 high-cfDNA patients with ASCT was 83.3% (95%CI: 69.7-99.7), which is similar to that observed in the low-cfDNA subgroup. In contrast, only three of the 11 high-cfDNA patients who did not undergo ASCT were still alive after 36 months of follow-up with an OS of 27.3% (95%CI: 10.4 – 41.6) (Figure 4C).

High plasma cfDNA levels may persist at CR in R-CHOP patients

The 50 patients with a CR 6 months after the EOT and for whom we had a plasma sample were evenly distributed between the two randomization arms (24 in R-CHOP

and 26 in R-HDT) and did not differ from the study population as a whole with regard to their clinical characteristics (Table 1 and Supplemental Table 1). New follow-up time points were assessed, giving a median documented follow-up time of 83 months (6.9 years). Three of the 50 patients experienced a late relapse of lymphoma 5.5, 8.8, and 10.6 years after the initial diagnosis, all were treated with eight courses of R-CHOP. One died as a result of the relapse.

In contrast to the R-CHOP patients, the R-HDT patients showed a significant decrease in cfDNA levels (Figure 5A and Supplemental Figure 3). We used the log 2 ratio of cfDNA concentration measured at CR to that measured at baseline. We thus identified three subgroups of patients: “A” with a log2 ratio > 1, “B” with a log2 ratio between -1 and 1 and “C” with a log2 ratio < 1 (Figure 5B). These three subgroups had similar clinical characteristics, with the exception of subgroup B, which had proportionally smaller tumors than the other two (supplemental Table 5). The distribution between the two randomization arms of subgroup A and subgroups B+C was significantly different (Fisher test, $P=0.045$). Thus, of the 7 patients in subgroup A, 6 were randomized to the R-CHOP arm and, with the exception of one, none of them were referred for catch-up treatment at the midterm assessment. More importantly, the three patients who relapsed are in subgroup A and thus had higher cfDNA concentrations at CR than at baseline. Finally, two of 26 patients in the R-HDT arm and 11 of 24 patients in the R-CHOP arm showed an increase in cfDNA concentration at 6Mo_CR compared to diagnosis (Supplemental Figure 3).

DISCUSSION

This study shows that the level of cfDNA measured between 90 and 320 bp at diagnosis is a reliable and robust marker (independent of the aa-IPI) of aggressive *de*

378 *novo* DLBCL in adults under 60. Firstly, a cfDNA concentration >55 ng/ml was
379 associated with a significantly worse clinical outcome. Secondly, in an intention-to-
380 treat analysis, R-HDT was not superior to standard R-CHOP with regard to OS and
381 so should not be recommended for young adults with DLBCL, in whom conventional
382 R-CHOP is equally effective, less toxic, and resource-saving – as shown previously
383 in other studies ^{13–15}. Nevertheless, our present results showed that the R-HDT
384 regimen mitigates the harmful influence of a cfDNA level ≥ 55 ng/ml at diagnosis.
385 Thirdly, we found that cfDNA levels fell significantly after a CR had been achieved
386 but only in patients in the R-HDT arm.

387
388 Today's techniques for measuring tumor-derived cfDNA are based on genotyping by
389 deep DNA sequencing ¹⁶; this technique requires specific preprocessing steps and a
390 sensitive analytical platform ^{10,17}. The GOELAMS 075 multicenter clinical trial began
391 in 2005, and we obtained 1 to 2 ml of plasma from a BDTM P100 blood collection tube
392 that contains potassium EDTA anticoagulant and proprietary protein stabilizers. This
393 unique sample collection provided reliable information on cfDNA levels and disease
394 status, with the expected distribution pattern (in terms of the median DNA fragment
395 size and cfDNA concentration) relative to HVs ¹⁸. Higher cfDNA concentrations were
396 significantly associated with adverse clinical factors in DLBCL. Overall, our results
397 suggest that cfDNA is a relevant, simple, cost-effective biomarker of aggressive
398 DLBCL in newly diagnosed patients. We established 55 ng/ml cfDNA to be the
399 optimal cutoff for the G075 cohort and independently confirmed it on the GAINED
400 cohort. We show that patients with cfDNA >55 ng/ml had a significantly worse
401 outcome only when in the standard R-CHOP arm.

The clinical trial's interim assessment included a visual interpretation of the PET data. Depending on the randomization arm, PET-negative patients received either four additional courses of R-CHOP or a BEAM regimen with ASCT. PET-positive patients were offered salvage therapy (a combination of rituximab and intensive chemotherapy) and then (if there was a disease response) the same BEAM regimen with ASCT. In line with previous reports^{19,20}, our analysis of 323 patients confirmed that BEAM with ASCT was effective and compared favorably with the two- and three-year OS rates of 61% and 62% reported respectively for the continuation of CHOP and R-CHOP in patients with a PET-positive interim assessment^{21,22}.

Our present results show that patients with cfDNA<55 ng/ml had a good OS rate (over 70%), whatever their treatment. Similarly good outcomes were achieved by patients with cfDNA>55 ng/ml at diagnosis receiving an intensive treatment regimen (R-HDT, salvage therapy and/or ASCT). However, patients with cfDNA>55 ng/ml who did not undergo intensive treatment (i.e., those having received eight cycles of R-CHOP) had a significantly worse outcome. Our results demonstrate that high-dose first-line treatment mitigates the negative influence of high cfDNA levels at diagnosis. Furthermore, in contrast to aalPI, cfDNA at baseline only identified R-CHOP>55ng/ml patients with statistically worse OS.

Overall, cfDNA assays have shown significant benefits in clinical settings, and the large range of applications continues to expand²³. The present study was based on a clinical trial with a long follow-up time, although the number of patients was low and the interim PET assessment did not include calculation of the Standardized Uptake Value²⁴⁻²⁶. The results showed that for patients who do not undergo salvage

therapy, R-CHOP is much less effective than R-HDT in reducing the risk of relapse when the cfDNA concentration at diagnosis exceeds 55 ng/ml. Furthermore, the results for patients with a CR 6 months after EOT showed that R-CHOP was associated with persistence in some patients with high plasma cfDNA levels. However, a lack of material prevented us from assessing the tumor's molecular/biological characteristics at diagnosis – characteristics that are known to be of value when evaluating DLBCL^{24–26}. Overall, the results showed that the cfDNA concentration was a prognostic factor (like the IPI) for newly diagnosed DLBCL patients in a clinical trial and that this biomarker could identify patients requiring additional drugs to the standard R-CHOP regimen²⁷, intensive therapies such as ASCT (in young, fit, chemosensitive patients)²⁸ or T-cell immunotherapy (in elderly, chemoresistant patients)²⁷. However, these results need to be confirmed in independent PET-driven clinical cohorts and using technical aspects that are now more widely used at the preanalytical and molecular level. For example, the availability of large amounts of material allows personalized cancer profiling by deep sequencing in B-cell lymphoma^{16,29}. New studies should include extensive comparisons between cfDNA measured in the 90-320 bp window and ctDNA to carefully determine whether cfDNA concentration may reflect tumor burden. In addition, characterization of plasma DNA genotyping at the time of CR after cessation of all treatments would also be critical to determine whether maintenance of elevated cfDNA is associated with mutations detected in the tumor and/or plasma at diagnosis. These new investigations would also provide insight into minimal residual disease and the prediction of late relapse or possibly a second cancer.

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AUTHOR CONTRIBUTIONS

F.D. designed the study, developed methodologies, performed analysis and wrote the manuscript; D.R. developed methodologies for database analysis and statistical approaches and wrote the manuscript; M.R. helped with the database analysis; C.P. collected samples; L.L. provided technical assistance with sequencing; M de S. J. analyzed plasma DNA; S. Le G. was the principal investigator of the GAINED study; V.D., R.G., J.C. G.D., H.M., T.L. recruited patients; D.D. and M-C. P. supervised the hematopathology analysis; F. K-B. performed PET analysis; N. M. was the principal clinical investigator of the GOELAMS 075 trial; T.F. (the principal investigator of the biological part of the GOELAMS 075 trial) designed and supervised the presented study and wrote the manuscript.

COMPETING INTERESTS

F.D., D.R., M.R., C.P., L.L., M. de S.J., D.D., J.C., M-C.P., R.G., H.M., D.D., V.D., F. K-B., T.L., M-C.P. and T.F. have no conflicts of interest to declare. N. M. reports

advisory board from Gilead and travel funds from Gilead and Roche; K.B. advisory board from Kite/Gilead and Takeda, travel funds from Kite/Gilead and Takeda, Honoraria from Kite/Gilead, Takeda, Sandoz, Incyte; G.D. reports advisory board from blueprint medicines, Takeda, Roche & Thermo fischer, travel funds from Gilead, BMS, Takeda, Pfizer; S.L.G. received grants, personal fees, or nonfinancial support from Roche Genentech, during the conduct of the study; personal fees from Celgene; and grants and personal fees from Janssen-Cilag, Gilead/Kite, and Servier outside of the submitted work.

FIGURE LEGENDS

Figure 1: Clinical results of the GOELAMS 075 trial. **A)** Study flow chart. R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CEEP/MC: rituximab, cyclophosphamide, epirubicin, vindesine, prednisone/methotrexate, cytarabine; ASCT: autologous stem cell transplantation (also referred to as rituximab high dose therapy (R-HDT)); CR: complete remission. Plasma was collected at baseline and 6 months after the EOT (6Mo_CR). **B)** Overall survival curves (in an intent-to-treat analysis) for the 123 patients. **C)** Overall survival curves (in an intent-to-treat analysis) for the parent cohort of 323 patients.

Figure 2: The cfDNA biomarker. **A)** Measurement of cfDNA in plasma from 22 HVs and in plasma collected at the time of diagnosis from 123 patients in the 075 cohort. The threshold of 10 times the maximum value found in HVs was used to classify the cfDNA levels as high or low. **B-D)** The cfDNA concentration as a function of the number of extranodal sites, the R-IPI, and the aa-IPI.

Figure 3: Clinical outcomes of patients, as a function of the 55 ng/ml threshold value for cfDNA. **A)** Overall survival curves as a function of the cfDNA concentration for all 123 patients. **B)** Overall survival curves as a function of the cfDNA concentration, for the 178 patients from the GAINED cohort.

Figure 4: Survival curves for the 123 patients in the 075 cohort, as a function of the 55 ng/ml cutoff value for cfDNA. **A)** Overall survival, analyzed on an intention-to-treat basis. **B)** Overall survival, taking into account the administration or not of salvage treatment. **C)** Overall survival of patients, depending on whether or not they had undergone ASCT.

Figure 5: cfDNA concentration in plasma of 50 patients at diagnosis and at CR 6 months after the end of treatment (6Mo_CR). **A)** Plasma cfDNA concentrations for 24 patients in the R-CHOP arm and 26 patients in the R-HDT arm. **B)** Ratio of Log 2 cfDNA concentrations measured at 6Mo_CR to that measured at baseline; patients were classified into three subgroups: A: $\log_2 \text{ ratio} > 1$; B: $-1 \leq \log_2 \text{ ratio} \leq 1$; C: $\log_2 \text{ ratio} < -1$.

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- 607

Table 1

	Cohort 123 patients (n = 123)	R-HDT (n = 55)	R-CHOP (n = 68)	P-value between both arms	Cohort 50 patients (n = 50)	P-value betw. Cohort 123 vs Cohort 50
Characteristics	No. of patients (%)	No. of patients (%)	No. of patients (%)		No. of patients (%)	
At diagnosis						
Intention To Treat						
R-CHOP	68 (55)				24 (48)	0.48
R-HDT	55 (45)				26 (52)	
Age, years						
median (IQR)	49.9 (41.5-55.5)	51.4 (42.9-55.5)	47.6 (40.8-55.5)	0.32	47.4 (40.3-53.8)	0.47
< 50 years old	62 (50)	24 (44)	38 (56)	0.24	32 (64)	0.14
≥ 50 years old	61 (50)	31 (56)	30 (44)		18 (36)	
Gender						
Male	75 (61)	40 (73)	35 (51)	0.027	29 (58)	0.85
Female	48 (39)	15 (27)	33 (49)		21 (42)	
ECOG PS						
< 2	108 (88)	46 (84)	62 (91)	0.32	43 (86)	0.94
≥ 2	15 (12)	9 (16.4)	6 (8.8)		7 (14)	
Symptoms						
A	74 (60)	34 (62)	40 (59)	0.88	33 (66)	0.59
B	49 (40)	21 (38)	28 (41)		17 (34)	
Bulky disease						
< 7 cm	54 (45)	20 (38)	34 (51)	0.22	19 (38)	0.5
≥ 7 cm	66 (55)	33 (62)	33 (49)		31 (62)	
Bone marrow involvement						
Not involved	100 (83)	43 (80)	57 (86)	0.46	44 (92)	0.25
Involved	20 (17)	11 (20)	9 (14)		4 (8.3)	
Extranodal sites						
< 2	77 (63)	33 (60)	44 (65)	0.73	36 (72)	0.32
≥ 2	46 (37)	22 (40)	24 (35)		14 (28)	
Ann Arbor Stage						
I-II	24 (20)	12 (22)	12 (18)	0.73	11 (22)	0.87
III-IV	99 (80)	43 (78)	56 (82)		39 (78)	
Serum LDH						
Normal	32 (26)	10 (19)	22 (32)	0.13	14 (28)	0.96
Elevated	90 (74)	44 (81)	46 (68)		36 (72)	
aalPI score						
0-1	45 (37)	18 (33)	27 (40)	0.64	20 (40)	0.92
2	65 (53)	30 (55)	35 (51)		25 (50)	
3	13 (11)	7 (12.7)	6 (8.8)		5 (10)	
R-IPI score						
0	10 (8.1)	3 (5.5)	7 (10.3)	0.43	3 (6)	0.54
1-2	70 (57)	30 (55)	40 (59)		33 (66)	
3-5	43 (35)	22 (40)	21 (31)		14 (28)	
Histology						
de novo DLBCL	87 (71)	42 (76)	45 (66)	0.47	32 (64)	0.48
PMBL	22 (18)	8 (15)	14 (21)		13 (26)	
others	14 (11) [†]	5 (9.1) ⁺⁺	9 (13.2) ⁺⁺⁺		5 (10) ⁺⁺⁺	
Cell-of-origin subtype (IHC)						
GCB	31 (39)	17 (42)	14 (36)	0.71	11 (35)	0.88
NGC	48 (61)	23 (58)	25 (64)		20 (65)	
cfDNA						
median (IQR)	17.8 (8.4-56.8)	34.2 (9.7-94.4)	13.0 (6.8-35.7)	0.51	19.6 (8.9-36.6)	0.16
< cutoff	88 (72)	34 (62)	54 (79)	0.05	41 (82)	0.22
> cutoff	35 (28)	21 (38)	14 (21)		9 (18)	

Characteristics	Cohort 123 patients (n = 123) No. of patients (%)	R-HDT (n = 55) No. of patients (%)	R-CHOP (n = 68) No. of patients (%)	P-value	Cohort 50 patients (n = 50) No. of patients (%)
At intermediate evaluation					
[18]FDG-PET scan response				0.64	
Negative PET	75 (62)	32 (59)	43 (65)		34 (68)
Positive PET	45 (38)	22 (41)	23 (35)		16 (32)
Salvage therapy				0.65	
No	82 (67)	35 (64)	47 (69)		35 (70)
Yes	41 (33)	20 (36)	21 (31)		15 (30)
Autologous stem cell				<0.0001	
No	54 (44)	4 (7.3)	50 (73.5)		17 (34)
Yes	69 (56)	51 (93)	18 (26)		33 (66)
At end of treatment (EOT)					
[18]FDG-PET scan response				0.57	
Negative PET	82 (80)	37 (84)	45 (78)		38 (90)
Positive PET	20 (20)	7 (16)	13 (22)		4 (10)
Final response				0.38	
CR, unCR, PR	110 (89)	51 (93)	59 (87)		50 (100)
SD, PD, Death	13 (11)	4 (7)	9 (13)		0
Survival data					
Status				0.80	
Alive	96 (78)	44 (80)	52 (76)		50 (100%)
Dead	27 (22)	11 (20)	16 (24)		0
Overall Survival				0.60	
36-months estimates (95% CI)	83.7 (77.4-90.5)	87.3 (78.9-96.5)	80.7 (71.8-90.7)		100.0
60-months estimates (95% CI)	78.7 (71.7-86.3)	80.0 (70.1-91.3)	77.7 (68.3-88.3)		100.0

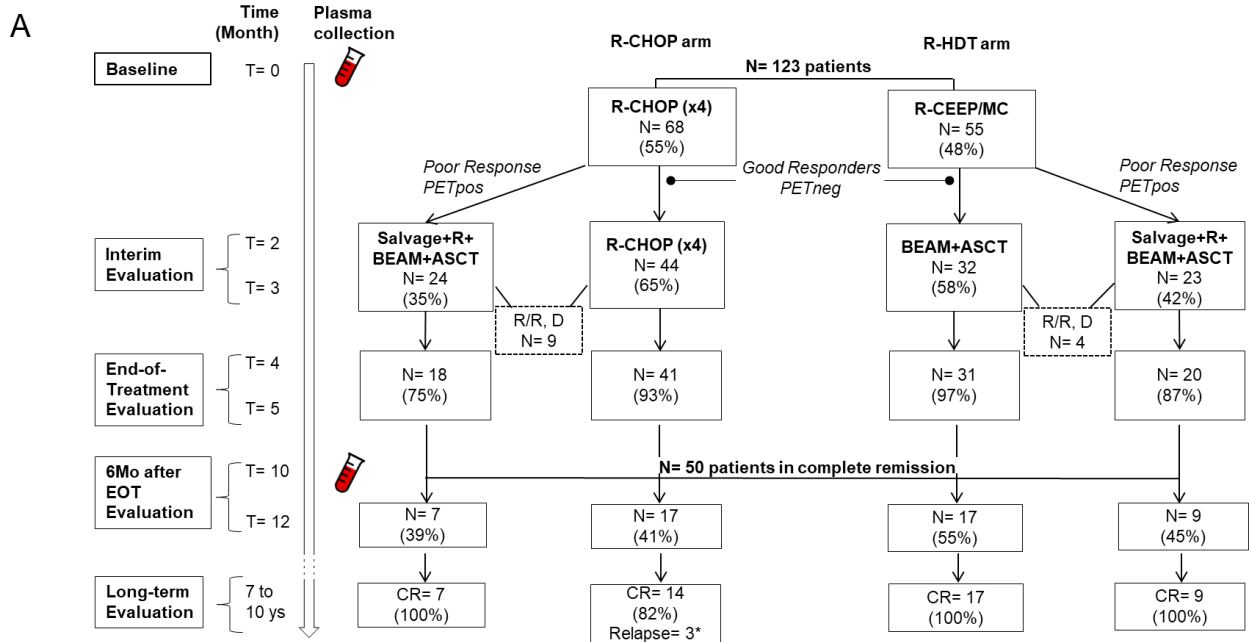
Table 1 (cont'n)

Table 1. Baseline demographic, clinical and survival characteristics of the patients.

Abbreviations: R-HDT, rituximab plus high dose therapy; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone; ECOG PS, Eastern Cooperative Oncology Group performance status, ECOG PS ranges from 0-4, where higher score indicates greater degree of impairment; LDH, lactate dehydrogenase; aaIPI, age-adjusted International Prognostic Index; R-IPI, revised IPI; Cell-of-origin, IHC algorithm according to Hans classification (Hans *et al.* Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* **103**, 275–282 (2004)); GCB, germinal center B-cell like; NGC, nongerminal center B-cell like; gated-cfDNA, circulating tumor DNA, cutoff defined as equal to 10 times the maximal value of cfDNA in the healthy cohort; IQR, interquartile range. Histology: † secondary DLBCL, n=3; DLBCL rich in T cells, n=7; CD25+ DLBCL, n=1, non determined (ND), n=1; †† secondary

DLBCL, n=1; DLBCL rich in T, n=3; ND, n=1; +++ secondary DLBCL, n=2; DLBCL rich in T, n=4; CD25+ DLBCL, n=1; ND, n=2; ++++ secondary DLBCL, n=1; DLBCL rich in T, n=3; ND, n=1.

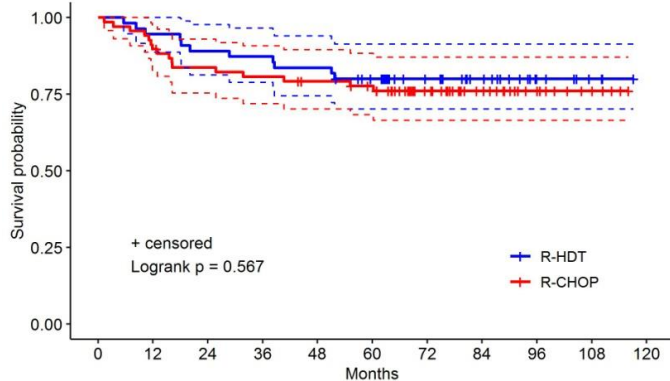
Figure 1



B

Overall Survival according to Intention To Treat

with number of Subjects at Risk and 95% confidence Limits



Number at risk

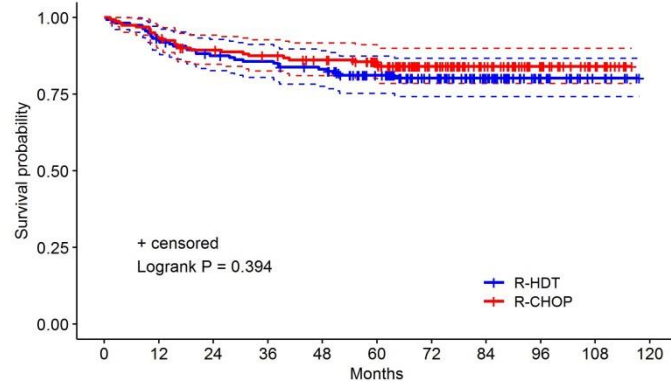
55	52	49	48	46	40	26	17	7	3	0
68	61	56	54	51	48	32	20	11	5	0
0	12	24	36	48	60	72	84	96	108	120

Months

C

Overall Survival according to Intention To Treat

with number of Subjects at Risk and 95% confidence Limits



Number at risk

162	149	139	135	129	107	78	47	22	9	0
161	150	141	136	130	117	78	54	33	14	0
0	12	24	36	48	60	72	84	96	108	120

Months

Figure 2

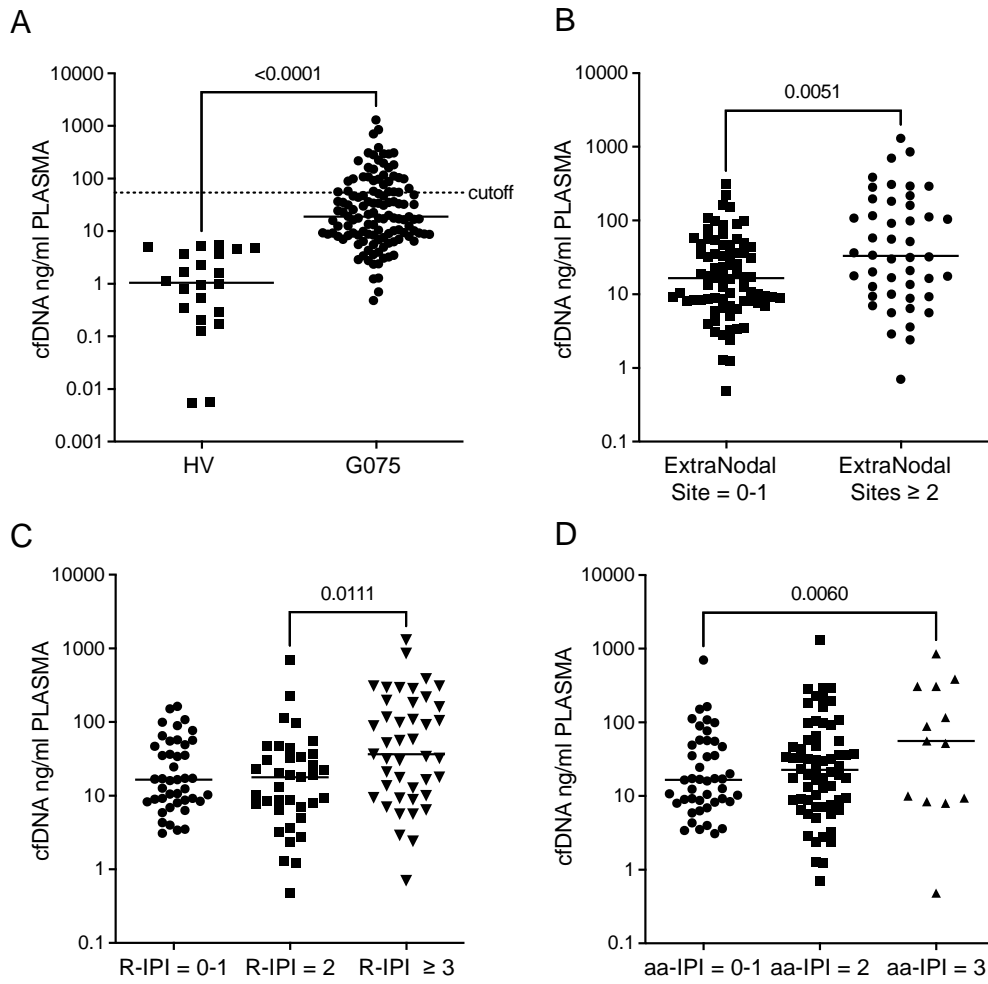
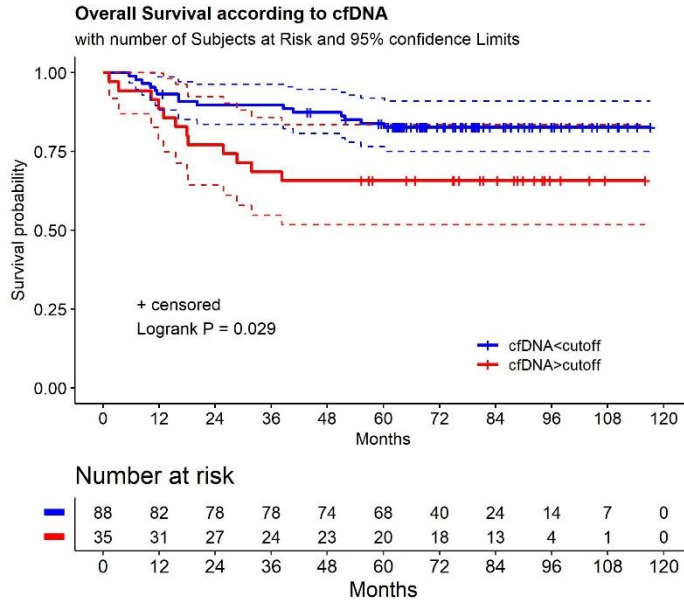


Figure 3

A



B

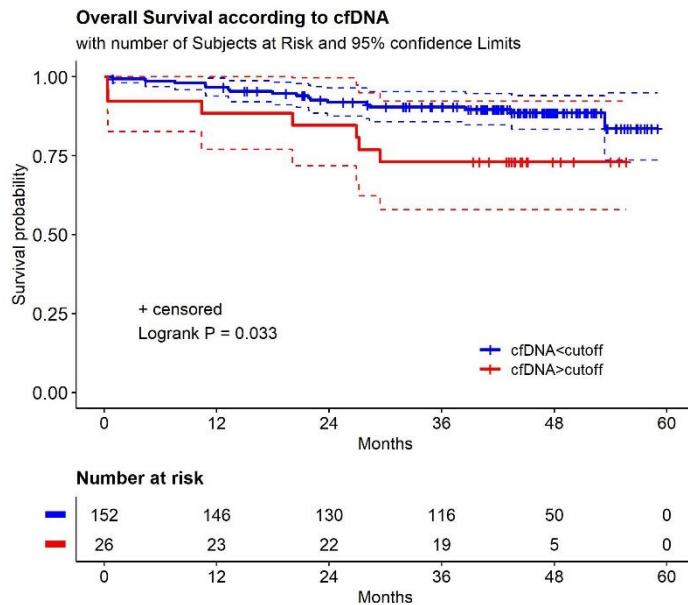
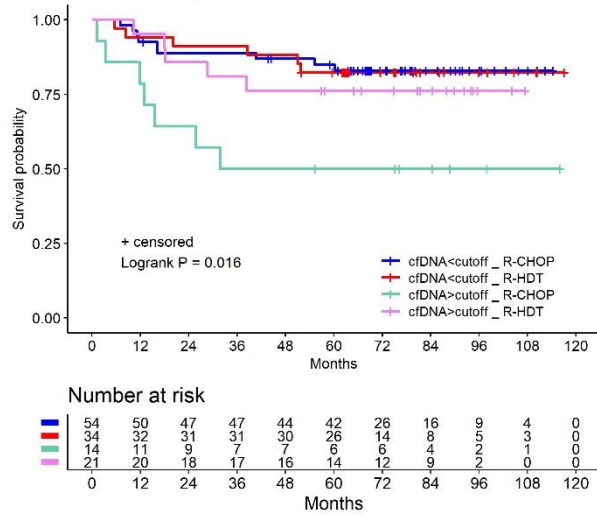


Figure 4

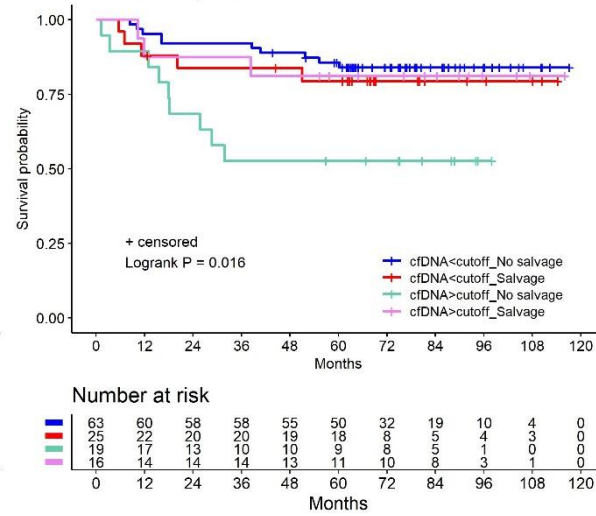
A

OS according to cfDNA + Intention To Treat
with number of Subjects at Risk



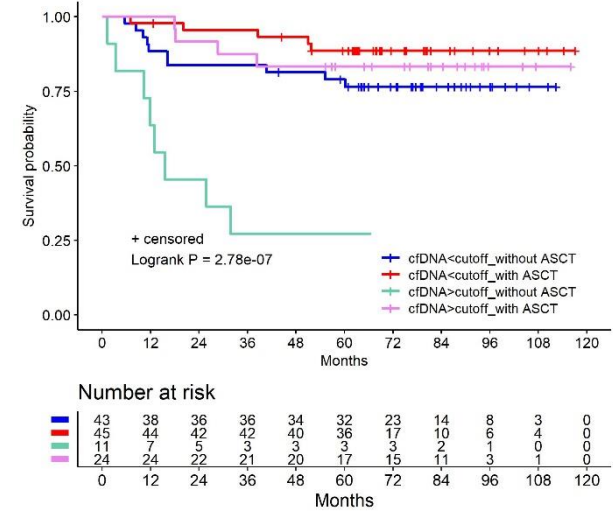
B

OS according to cfDNA + Salvage therapy
with number of Subjects at Risk



C

Overall Survival according to cfDNA + ASCT
with number of Subjects at Risk



		Cox analysis	
		HR (95% CI)	P-value
cfDNA>cutoff _ R-CHOP versus	cfDNA<cutoff _ R-CHOP	3.99 (1.98-10.74)	0.006
cfDNA>cutoff _ R-HDT versus	cfDNA<cutoff _ R-HDT	1.40 (0.43-4.59)	0.58
cfDNA>cutoff _ R-CHOP versus	cfDNA>cutoff _ R-HDT	2.74 (0.87-8.69)	0.084
cfDNA<cutoff _ R-CHOP versus	cfDNA<cutoff _ R-HDT	0.98 (0.34-2.73)	0.95
cfDNA>cutoff _ R-CHOP versus	cfDNA>cutoff _ R-HDT	3.86 (0.23-11.51)	0.0154
cfDNA<cutoff _ R-CHOP versus	cfDNA>cutoff _ R-HDT	1.45 (0.49-4.33)	0.51

		Cox analysis	
		HR (95% CI)	P-value
cfDNA>cutoff _ No salvage versus	cfDNA<cutoff _ No salvage	3.82 (1.55-9.43)	0.0036
cfDNA>cutoff _ Salvage versus	cfDNA<cutoff _ Salvage	0.89 (0.21-3.73)	0.87
cfDNA>cutoff _ No salvage versus	cfDNA>cutoff _ Salvage	3.08 (0.83-11.41)	0.092
cfDNA<cutoff _ No salvage versus	cfDNA<cutoff _ Salvage	0.72 (0.25-2.10)	0.55
cfDNA>cutoff _ No salvage versus	cfDNA>cutoff _ Salvage	2.74 (0.92-8.21)	0.071
cfDNA<cutoff _ No salvage versus	cfDNA>cutoff _ Salvage	0.81 (0.22-2.93)	0.74

		Cox analysis	
		HR (95% CI)	P-value
cfDNA>cutoff _ without ASCT versus	cfDNA<cutoff _ without ASCT	5.36 (2.09-13.73)	0.00046
cfDNA>cutoff _ with ASCT versus	cfDNA<cutoff _ with ASCT	1.52 (0.41-5.66)	0.53
cfDNA>cutoff _ without ASCT versus	cfDNA>cutoff _ with ASCT	7.99 (2.39-26.75)	0.00075
cfDNA<cutoff _ without ASCT versus	cfDNA<cutoff _ with ASCT	2.26 (0.77-6.62)	0.14
cfDNA>cutoff _ without ASCT versus	cfDNA>cutoff _ with ASCT	12.13 (3.93-37.47)	0.000014
cfDNA<cutoff _ without ASCT versus	cfDNA>cutoff _ with ASCT	1.49 (0.47-4.75)	0.50

	Negative iPET	Positive iPET	NA
cfDNA<cutoff _ No salvage	60	2	1
cfDNA<cutoff _ Salvage	0	24	1
cfDNA>cutoff _ No salvage	15	4	1
cfDNA>cutoff _ Salvage	0	15	0

	60-month OS (95% CI)
cfDNA>cutoff _ without ASCT	27.3 (10.4-41.6)
cfDNA>cutoff _ with ASCT	83.3 (69.7-99.7)
cfDNA<cutoff _ without ASCT	79.0 (67.7-92.2)
cfDNA<cutoff _ with ASCT	88.6 (79.6-98.5)

Figure 5

