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

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

3

4 **Running title:** cfDNA and DLBCL overall survival

5

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13

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51

52

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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76  
77

78 **ABSTRACT**

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

81

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

88

89 Results: In a representative group of 123 patients, a high cfDNA concentration  
90 ( $>55\text{ng/ml}$ ) at diagnosis was associated with poor clinical prognostic factors and  
91 constituted a prognostic marker, independently of the age-adjusted International  
92 Prognostic Index. A cfDNA level above a threshold value of  $55\text{ ng/ml}$  at diagnosis  
93 was associated with significantly worse OS. In an intention-to-treat analysis, high-  
94 cfDNA R-CHOP patients (but not high-cfDNA R-HDT patients) had worse OS (hazard  
95 ratio [95% confidence interval]:  $3.99 [1.98-10.74]$ ,  $P=0.006$ ). In patients with high  
96 cfDNA levels, salvage therapy and transplantation were associated with a  
97 significantly higher OS rate. Among 50 patients with complete response 6 months  
98 after the end of treatment, for 11 out of 24 R-CHOP patients, the cfDNA did not fall  
99 back to normal values.

100

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

103 **INTRODUCTION**

104 The first-line treatment for diffuse large B-cell lymphoma (DLBCL) combines  
105 rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone. This R-  
106 CHOP regimen is the standard of care, regardless of the cancer's  
107 immunohistochemical profile or molecular subtype <sup>1</sup>. However, 30 to 40% of patients  
108 are refractory to this treatment or relapse within 2 years of an initial response. After  
109 relapse, the overall cure rate ranges from 25 to 35% <sup>1</sup>. Refractory patients have a  
110 poor prognosis, with a median overall survival (OS) time of around 6 months <sup>2</sup>. It is  
111 therefore very important to provide the most appropriate treatment as soon as *de*  
112 *novo* DLBCL has been diagnosed. In the early 2000s (i.e., before the rituximab era),  
113 we showed that first-line high-dose chemotherapy with autologous stem cell  
114 transplantation (ASCT) was associated with longer disease-free survival in  
115 transplant-eligible patients with advanced DLBCL <sup>3,4</sup> and an OS benefit in a subset of  
116 patients <sup>3</sup>. Since then, it has been shown that a combination of rituximab with high-  
117 dose conventional chemotherapy prolongs survival in young adults with a low-  
118 intermediate level of risk (according to the International Prognostic Index (IPI)) <sup>5</sup>. At  
119 present, primary staging is based on the IPI and positron emission tomography-  
120 computed tomography (PET-CT) findings, which also allow assessment of the  
121 treatment response <sup>6</sup>. In 2005, we initiated a French, open-label, randomized, Phase  
122 3 trial (GOELAMS 075, NCT00561379) of R-CHOP vs. a combination of rituximab,  
123 high-dose conventional chemotherapy and ASCT (hereafter referred to as R-HDT)  
124 for the first-line treatment of advanced DLBCL adult patients aged ≤60. The median  
125 follow-up period after randomization was >5.8 years (70.4 months; 95% confidence  
126 interval (CI), 55.0 – 88.9).

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

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

151

## 152 **METHODS**

153

154 **Patients**

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

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

177

178 **Data and tissue samples collected in the G075 cohort**



179 Plasma samples collected at the time of diagnosis (i.e. before any treatment) were  
180 available for 123 of the 340 patients enrolled in the trial. Furthermore, post-treatment  
181 plasma samples were available for 50 of the 123 patients who had maintained a CR  
182 six months after the EOT (detailed information for CR patients in **Supplemental table**  
183 **1**). For 178 of 670 patients of the GAINED cohort, plasma samples were collected at  
184 randomization and control plasma was obtained from healthy volunteer (HV) blood  
185 donors at the French National Blood Agency (age, 18-60). Plasma was collected in  
186 BD™ P100® tubes (BD Diagnostics, Le Pont-De-Claix, France), shipped at room  
187 temperature within 24 hours of collection to the core lab in Rennes (France),  
188 centrifuged, and stored at -80°C.

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

198

### 199 ***cfDNA extraction, quantification, and size distribution analysis***

200 Samples from both trials were processed in exactly the same way and a maximum of  
201 2 ml of plasma was reserved for this cfDNA study (**supplemental Figure 2A**). In our  
202 study, the concentration of cfDNA corresponds to the amount of DNA detected in the  
203 90- to 320-bp window (**supplementary Figure 2B**), and the median DNA fragment

204 size was significantly smaller for patients than for HVs (supplementary Figure 2C).  
205 We established the best threshold above which patient samples at diagnosis were  
206 considered cfDNA-enriched using two different approaches. First, we applied the  
207 maximum selected rank statistics (maxstat R package<sup>12</sup>) to determine the threshold  
208 that predicts the most significant differences in OS of patients independently in the  
209 GOELAMS 075 and GAINED cohort (supplementary Figure 2D). Next, the threshold  
210 was defined as 10 times the upper limit of cfDNA concentration in HV, this value is  
211 clinically relevant and easily applicable to a patient population (supplementary Figure  
212 2E). Because the maxstat and HV cohort results were close in value (49.2 and 52.1  
213 vs. 54.9 ng/ml, respectively), the cutoff was set at 55 ng/ml for the entire study,  
214 defining high and low cfDNA levels as above and below 55 ng/ml, respectively  
215 (supplementary Methods).

216

### 217 **Statistical analysis**

218 Analysis was performed on an intention-to-treat (ITT) basis, for evaluable patients.  
219 Descriptive statistics were quoted as the median [interquartile range (IQR)] or the  
220 number (percentage). Groups were compared using a chi-squared test, the Mann-  
221 Whitney U-test, Wilcoxon's signed-rank test, or the Kruskal-Wallis test, depending on  
222 the type of data and the number of groups.

223 OS (defined as the time from randomization to the date of last follow-up or death  
224 from any cause) was estimated using the Kaplan–Meier method. Differences in  
225 survival curves were assessed in a log-rank test. A Cox proportional hazards model  
226 was used to determine hazard ratios (HRs) and their 95%CI. All statistical tests were  
227 two-sided, and the threshold for statistical significance was set to  $P < 0.05$ . Statistical

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

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

233

## 234 RESULTS

235

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

237 Between May 2005 and August 2010, 340 patients were enrolled in the G075 cohort.  
238 Seventeen patients were deemed to be ineligible, and the remaining 323 were  
239 randomized to receive either R-CHOP (n=161) or R-HDT (n=162). The two groups  
240 were similar, except for performance status (supplemental Table 2). There were no  
241 differences in demographic and clinical characteristics between the parent cohort and  
242 the 123 patients with cfDNA assay data (supplemental Table 2). Of the 123 patients,  
243 68 were assigned to the R-CHOP and 55 were assigned to the R-HDT arm (Figure  
244 1A); the median follow-up time after randomization was 69 months (95%CI, 55.3-  
245 88.0). With the exception of the sex ratio, the R-CHOP and R-HDT arms were well  
246 balanced with regard to their demographic and clinical characteristics (Table 1). Fifty-  
247 nine patients (87%) from the R-CHOP arm achieved a confirmed or unconfirmed  
248 complete response (CR) or partial response at the EOT assessment, compared with  
249 51 (93%) in the R-HDT arm. There was no difference in 5-year OS between the  
250 randomization arms for the 123 patients (80.0% [70.1-91.3] for R-HDT vs. 77.7%  
251 [68.3-88.3] for R-CHOP;  $P=0.57$ ) or for the 323 patients in the parent cohort (85.5%  
252 [80.2-91.2] for R-HDT vs. 81.1% [75.3-87.5] for R-CHOP;  $P=0.39$ ) (Figures 1B-C).

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

256

257 ***The cfDNA concentration is elevated in patients with adverse prognostic***  
258 ***factors***

259 The cfDNA fragment distribution in the 90- to 320-bp range of patients differed from  
260 that of HVs, with a higher proportion of fragments shorter than 150 bp<sup>7</sup>. The median  
261 cfDNA concentration was significantly higher in patients (17.8 ng/ml, IQR 8.4-56.8)  
262 than in HVs (1.04 ng/ml, IQR 0.2-3.9) (Figure 2A), but did not differ significantly when  
263 comparing the two treatment arms (Table 1). The cfDNA concentration at diagnosis  
264 did not differ significantly when comparing patients with or without salvage therapy,  
265 or with negative or positive iPET. Patients with high-risk clinical factors at diagnosis  
266 (e.g., more than one extranodal site, a poor Revised-IPI (R-IPI), and a high-risk aa-  
267 IPI) had significantly higher cfDNA levels (Figures 2B, C and D).

268

269 ***A high cfDNA level at diagnosis is associated with worse outcomes***

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

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

281 Similar results were observed in patients from the GAINED cohort. The median DNA  
282 fragment size was 174 bp (IQR 167-231), and median [IQR] cfDNA levels were  
283 significant higher in patients than in HVs (9.24 ng/ml [4.1-31] vs. 1.04 [0.2-3.9],  
284 respectively; supplementary Figure 2E). Twenty-six of the 178 GAINED patients were  
285 in the high-cfDNA group and had a significantly worse OS at 48 months (73.1%,  
286 95%CI, 57.9-92.3 vs. for low-cfDNA 88.5%, 95%CI, 83.3-94.1; HR=2.67, 95%CI,  
287 1.11-6.53) (Figure 3B). Interestingly, the 55ng/ml cutoff is close to the optimal  
288 threshold at 52ng/ml obtained for the GAINED cohort using the maxstat method  
289 (supplementary Figure 2F).

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

298 In a multivariate Cox analysis, the cfDNA levels (in ng/ml) and the aa-IPI risk still had  
299 statistically significant relationships with OS in the 075 cohort: HR [95%CI] =5.48  
300 [1.53-19.56] for high- vs. low-risk aa-IPI (*P*=0.0088) and 7.33 [2.33-23.09] for cfDNA  
301 (*P*=0.00066), whereas the intermediate vs. low risk aa-IPI (HR=2.91, 95%CI, 0.96-  
302 8.75, *P*=0.057) was not significant.

303

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

305 With regard to the randomization arm (Figure 4A), patients in the R-CHOP arm had  
306 significantly worse OS when they were in the high-cfDNA category (HR=3.99,  
307 95%CI, 1.48-10.74,  $P=0.006$ ); this was not the case for patients in the R-HDT arm  
308 (HR=1.40, 95%CI, 0.43-4.59,  $P=0.58$ ). This finding is consistent with the fact that  
309 high-cfDNA patients in the R-HDT arm tended to do better than high-cfDNA patients  
310 in the R-CHOP arm (HR=2.74, 95%CI, 0.87-8.69,  $P=0.084$ ). Overall, long-term OS  
311 was worse among the 14 high-cfDNA R-CHOP patients than the other subgroups,  
312 with a plateau of 5-year survival at 50% [29.6-84.4], vs. 76.2% [60-96.8] for high-  
313 cfDNA R-HDT, 82.4% [70.5-96.2] for low-cfDNA R-HDT, and 82.9% [73.3-93.8] for  
314 low-cfDNA R-CHOP.

315

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

318 In the parent cohort (n=323), the OS rate at 60 months was 76.9% [69.5-85.1] for the  
319 114 interim PET-positive patients and 89.0% [84.7-93.6] for the 196 PET-negative  
320 patients ( $P=0.009$ ).

321 In the studied G075-cohort, only for iPET-negative patients at the interim  
322 assessment, those with high cfDNA had a statistically significantly shorter OS than  
323 low-cfDNA patients (HR=3.48, 95%CI, 0.68-5.63,  $P=0.02$ ) (supplementary Figure  
324 5A).

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

328  $P=0.0036$ ) (Figure 4B). In contrast, for individuals receiving salvage therapy, high-  
329 cfDNA patients had much the same OS rate as low-cfDNA patients. Thus, for high-  
330 cfDNA patients and regardless of randomization arm, those who did not receive  
331 salvage therapy at the interim assessment tended to survive for a shorter time than  
332 those who received salvage therapy (HR=3.08, 95%CI, 0.83-11.41,  $P=0.092$ ). The  
333 outcome was particularly unfavorable for the nine high-cfDNA patients who received  
334 8 R-CHOP cycles, (supplemental Figure 5B). Six of the nine died, resulting in a 60-  
335 month OS rate of 33.3% (HR=5.70, 95%CI, 2.87-14.22,  $P=0.0002$ ) (supplemental  
336 Figure 5C). As with OS, analysis of PFS showed that high-cfDNA patients who did  
337 not receive salvage treatment had a statistically significantly worse outcome than  
338 low-cfDNA patients with no salvage ( $P=0.003$ ) and high-cfDNA patients who did  
339 receive salvage treatment ( $P=0.026$ ) (supplemental Figure 5D).

340

341 Lastly, we looked at the effect of ASCT in our study. ASCT was performed in 69 of  
342 the 123 patients, either as an integral part of the R-HDT protocol or as elective  
343 salvage treatment. We found that ASCT abolished the unfavorable outcome  
344 associated with the high-cfDNA category because the 60-month OS rate among the  
345 24 high-cfDNA patients with ASCT was 83.3% (95%CI: 69.7-99.7), which is similar to  
346 that observed in the low-cfDNA subgroup. In contrast, only three of the 11 high-  
347 cfDNA patients who did not undergo ASCT were still alive after 36 months of follow-  
348 up with an OS of 27.3% (95%CI: 10.4 – 41.6) (Figure 4C).

349

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

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

353 and 26 in R-HDT) and did not differ from the study population as a whole with regard  
354 to their clinical characteristics (Table 1 and Supplemental Table 1). New follow-up  
355 time points were assessed, giving a median documented follow-up time of 83 months  
356 (6.9 years). Three of the 50 patients experienced a late relapse of lymphoma 5.5,  
357 8.8, and 10.6 years after the initial diagnosis, all were treated with eight courses of R-  
358 CHOP. One died as a result of the relapse.

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

374

## 375 **DISCUSSION**

376 This study shows that the level of cfDNA measured between 90 and 320 bp at  
377 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 <sup>13–15</sup>. Nevertheless, our present results showed that the R-HDT  
384 regimen mitigates the harmful influence of a cfDNA level  $\geq 55$  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 <sup>16</sup>; this technique requires specific preprocessing steps and a  
390 sensitive analytical platform <sup>10,17</sup>. The GOELAMS 075 multicenter clinical trial began  
391 in 2005, and we obtained 1 to 2 ml of plasma from a BD<sup>TM</sup> 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 <sup>18</sup>. 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.

402

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

412

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

422

423 Overall, cfDNA assays have shown significant benefits in clinical settings, and the  
424 large range of applications continues to expand <sup>23</sup>. The present study was based on  
425 a clinical trial with a long follow-up time, although the number of patients was low and  
426 the interim PET assessment did not include calculation of the Standardized Uptake  
427 Value <sup>24-26</sup>. The results showed that for patients who do not undergo salvage

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

451

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462

#### 463 **AUTHOR CONTRIBUTIONS**

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

474

#### 475 **COMPETING INTERESTS**

476 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.  
477 K-B., T.L., M-C.P. and T.F. have no conflicts of interest to declare. N. M. reports

478 advisory board from Gilead and travel funds from Gilead and Roche; K.B. advisory  
479 board from Kite/Gilead and Takeda, travel funds from Kite/Gilead and Takeda,  
480 Honoraria from Kite/Gilead, Takeda, Sandoz, Incyte; G.D. reports advisory board  
481 from blueprint medicines, Takeda, Roche & Thermo fischer, travel funds from Gilead,  
482 BMS, Takeda, Pfizer; S.L.G. received grants, personal fees, or nonfinancial support  
483 from Roche Genentech, during the conduct of the study; personal fees from Celgene;  
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485 the submitted work.

486

#### 487 **FIGURE LEGENDS**

488

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

497

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

503

504 **Figure 3:** Clinical outcomes of patients, as a function of the 55 ng/ml threshold value  
505 for cfDNA. **A)** Overall survival curves as a function of the cfDNA concentration for all  
506 123 patients. **B)** Overall survival curves as a function of the cfDNA concentration, for  
507 the 178 patients from the GAINED cohort.

508

509 **Figure 4:** Survival curves for the 123 patients in the 075 cohort, as a function of the  
510 55 ng/ml cutoff value for cfDNA. **A)** Overall survival, analyzed on an intention-to-treat  
511 basis. **B)** Overall survival, taking into account the administration or not of salvage  
512 treatment. **C)** Overall survival of patients, depending on whether or not they had  
513 undergone ASCT.

514

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

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- 607

Table 1

Characteristics	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
	No. of patients (%)	No. of patients (%)	No. of patients (%)		No. of patients (%)	
<b>At diagnosis</b>						
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)	
aaIPI 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)	R-HDT (n = 55)	R-CHOP (n = 68)	P-value	Cohort 50 patients (n = 50)
	No. of patients (%)	No. of patients (%)	No. of patients (%)		No. of patients (%)
<b>At intermediate evaluation</b>					
[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)
<b>At end of treatment (EOT)</b>					
[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
<b>Survival data</b>					
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 2

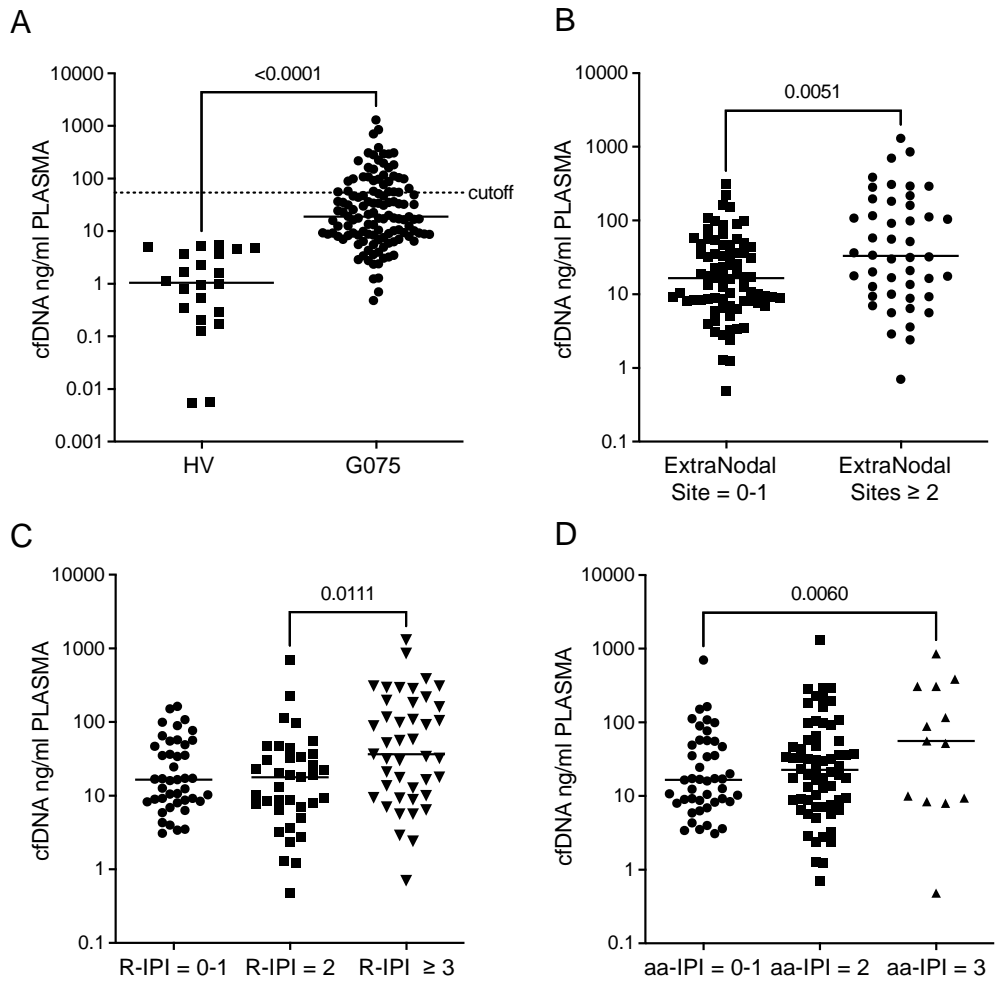
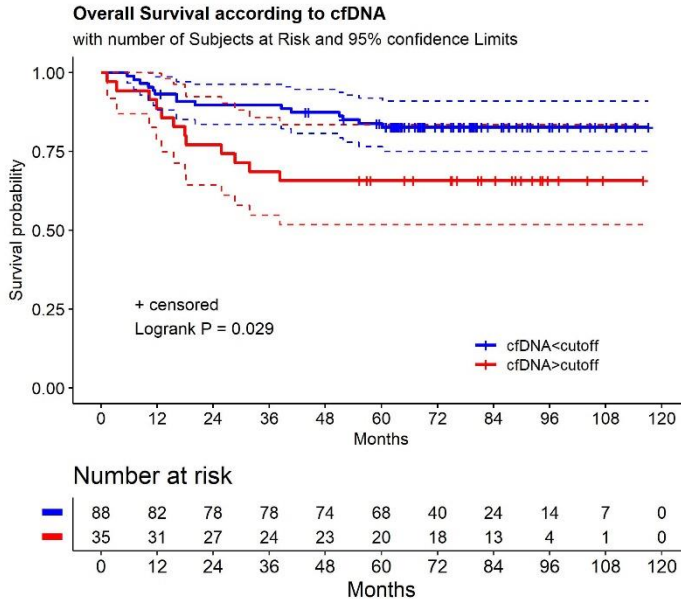


Figure 3

A



B

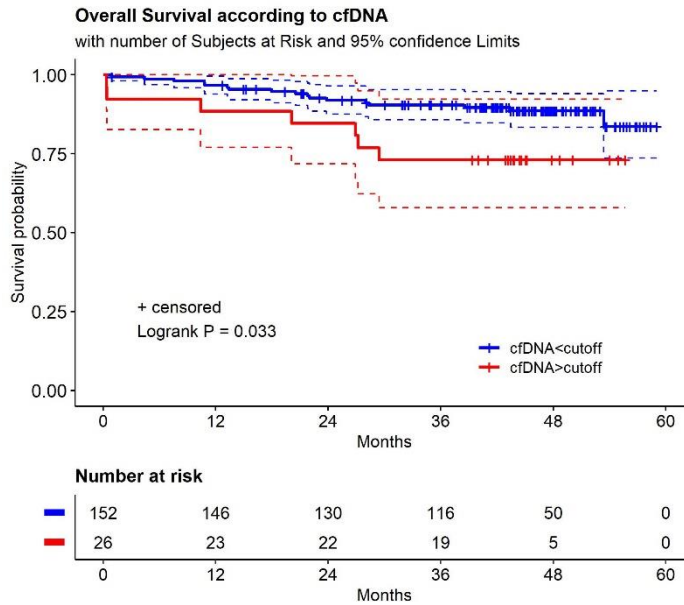
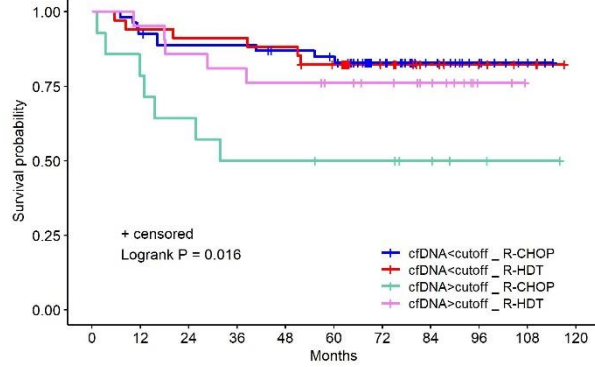


Figure 4

A

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

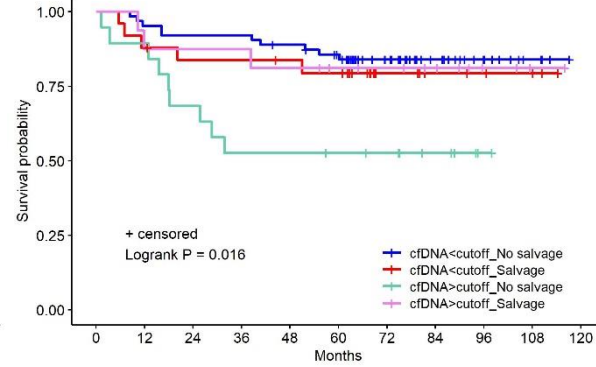


Number at risk

	0	12	24	36	48	60	72	84	96	108	120
cfDNA<cutoff _ R-CHOP	54	50	47	47	44	42	26	16	9	4	0
cfDNA<cutoff _ R-HDT	34	32	31	31	30	26	14	8	5	3	0
cfDNA>cutoff _ R-CHOP	14	11	9	7	6	6	4	2	1	0	0
cfDNA>cutoff _ R-HDT	21	20	18	17	16	14	12	9	2	0	0

B

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

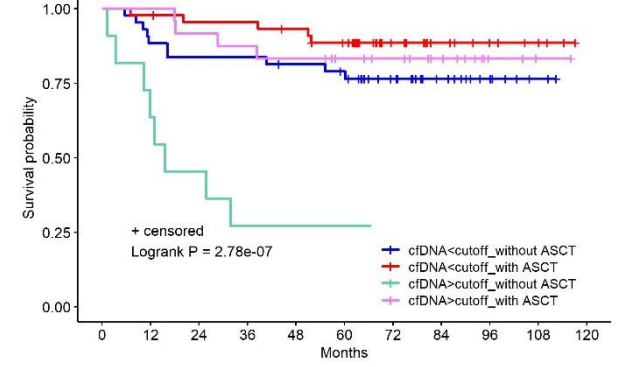


Number at risk

	0	12	24	36	48	60	72	84	96	108	120
cfDNA<cutoff _ No salvage	63	60	58	58	55	50	32	19	10	4	0
cfDNA<cutoff _ Salvage	25	22	20	20	19	18	8	5	4	3	0
cfDNA>cutoff _ No salvage	19	17	13	10	10	9	8	5	1	0	0
cfDNA>cutoff _ Salvage	16	14	14	14	13	11	10	6	3	1	0

C

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



Number at risk

	0	12	24	36	48	60	72	84	96	108	120
cfDNA<cutoff _ without ASCT	43	38	36	36	34	32	23	14	8	3	0
cfDNA<cutoff _ with ASCT	45	44	42	42	40	36	17	10	6	4	0
cfDNA>cutoff _ without ASCT	11	7	5	3	3	3	3	2	1	0	0
cfDNA>cutoff _ with ASCT	24	24	22	21	20	17	15	11	3	1	0

		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

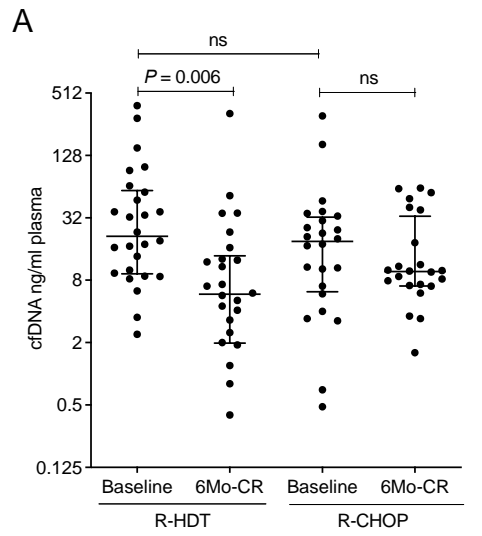
		Cox analysis	
		HR (95% CI)	P-value
cfDNA>cutoff _ without ASCT versus	cfDNA<cutoff _ without ASCT	5.36 (2.09-13.73)	0.00046
cfDNA>cutoff _ with ASCT versus	cfDNA<cutoff _ with ASCT	1.52 (0.41-5.66)	0.53
cfDNA>cutoff _ without ASCT versus	cfDNA>cutoff _ with ASCT	7.99 (2.39-26.75)	0.00075
cfDNA<cutoff _ 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



Baseline (50)		6Mo_CR (50)	
R-HDT (N=26)	R-CHOP (N=24)	R-HDT (N=26)	R-CHOP (N=24)
<b>cfDNA (ng/ml plasma) Median [IQR]</b>			
21.2	18.9	5.8	9.7
[9.2-58.6]	[6.1-32.4]	[1.9-13.7]	[7.0-33.1]

