Increased rituximab exposure does not improve response and outcome of patients with chronic lymphocytic leukemia after fludarabine, cyclophosphamide, rituximab. A French Innovative Leukemia Organization (FILO) study

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Supplemental information: material and methods.

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Flow cytometry minimal residual disease investigators

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Patients

140 treatment-naive patients (aged 18-65 years) diagnosed with confirmed Binet stage C or active Binet stages A or B CLL according to IWCLL 2008 criteria has included.[1] An additional inclusion criterion was the absence of 17p deletion, assessed by FISH (<10% positive nuclei). Patients with abnormal renal function with creatinine clearance < 60 ml/min calculated according to the Cockcroft and Gault formula were excluded. Each patient provided a written informed consent before

enrolment and the study was registered on ClinicalTrials.gov with the number NCT 01370772. The study was approved by the institutional ethics committee of each participating center according to the principles of the Declaration of Helsinki. Patients were stratified according to *IGHV* mutational status, FISH analysis (11q deletion or not) and were randomly assigned in one-to-one ratio to receive either 6 cycles of standard chemoimmunotherapy FCR (intravenous RTX 375 mg/m2 for the first course, D1 and 500 mg/m^2 for the others, oral fludarabine 40 mg/m^2/d D2-4, oral cyclophosphamide 250 mg/m^2/d D2-4) every 28 days or Dense-FCR with an intensified RTX prephase (500 mg on D0, and 2000 mg on D1, D8 and D15) before the standard FCR treatment starting at D22 (Supplemental Figure 1).

Premedication recommendation before each rituximab infusion: dexchlorpheniramine, Polaramine® (one immediate release 2mg-tablet or one intravenous infusion of 5 mg) and paracetamol (1000 mg) 30 minutes before infusion. Any patient considered at risk of an Infusion Related Reaction (IRR), and/or tumour lysis syndrome (TLS) (patients with lymphocytosis > 25 G/L, and/or splenomegaly >5 cm), should receive: (i) appropriate hydration and allopurinol (300 mg per os once daily) 48 to 72 hours prior to initiating treatment and thereafter until the risk of the IRR/TLS is ruled out; (ii) prednisone/prednisolone (100 mg intravenous) used 30 min prior to start infusion. No specific premedication before dose dense RTX was recommended (Dense-FCR arm).

Patients were assessed 9 months after the first course of FCR in the two arms (M9) and at least 3 months after the end of treatment whatever the number of cycles. All CT-scans were centrally reviewed for response assessment. According to IWCLL 2008 guidelines, the complete remission patients group includes patients in complete remission with incomplete bone marrow recovery.[1]

CD20 expression cell detection

CD20 expression was quantified using the commercial kit QuantiBRITETM CD20PE (Ratio 1:1) according to manufacturer's recommendations (BD Biosciences, Le Pont-de-Claix, France). Calibration and quantification were performed using a FACSCANTO II cytometer (BD, Biosciences, San Jose, CA, USA).

Initial cytometer setup was performed to allow the study of CD20 expression on T-lymphocytes as negative control, normal B-cells as positive control and CLL cells. Then fluorescence target values were determined for PE channel using 8-peak Rainbow bead calibration particles (Spherotech, Lake Forest IL, USA) and a calibration curve for CD20 QuantiBRITETM assay was established based upon these settings. Before making any new calibration curve, the cytometer setup was adjusted to reproduce the initial settings using the same lot of 8-peak Rainbow bead calibration particles. Supplementary Figure 1 shows representative cases of CD20 expression on CLL cells. By using a

calibration curve, the measure of CD20 fluorescence intensity on CLL cells allows calculating the number of equivalent CD20 molecules present at the cell surface.

Minimal Residual Disease determination

An 8-color combination comprising CD19, CD20, CD5, CD43, CD79b, CD81, CD22 and CD3 was performed by flow cytometry (FACSCANTO II cytometer, BD, Biosciences, Le Pont-de-Claix, France) to evaluate the immunophenotypic response of CLL patients in both peripheral blood and bone marrow (BM) at M9, as previously described.[2] Presence of MRD was defined as the detection of one or more CLL cells per 10 000 leukocytes.[1]

Tumor burden

Whole body CT-scan (neck, thorax, abdomen and pelvis) was performed before the beginning of treatment. Three-dimensional tumor volume measurements were performed on the six largest lesions according to a previously described semi-automated accurate measurement technique with thin slicing.[3]

The baseline total quantity of the CD20 antigenic target was estimated on B-cells both in the circulation ($CD20_{cir}$) and in the lymph nodes ($CD20_{LN}$), as follows:

$CD20_{cir}$ = CD20 expression per cell x Number of circulating target cells

$CD20_{LN}$ = CD20 expression per cell x Number of cells for 6 largest scanned lesions

where CD20 expression per cell is expressed in MESF (Molecules of Equivalent Soluble Fluorophores), number of circulating target cells was determined as follows:

Number of circulating target cells = $CD19^+$ cell concentration x blood volume

$CD19^+$ cell concentration = total lymphocyte concentration x % $CD19^+$ cells

Blood volume (L) is calculated according to Pearson et al: [4]

Blood volume = 0.3669 x $(Height)^3$ + 0.03219 x Weight + 0.6041 (male)

Blood volume = 0.3561 x $(Height)^3$ + 0.03308 x Weight + 0.1833 (female)

Number of cells for 6 scanned lesions was assessed as follows:

Number of cells for 6 scanned lesions = volume of 6 scanned lesions (cm^3) / CLL lymphocyte volume, where CLL lymphocyte volume is assumed to be 54 fL.[5]

Exposure to rituximab

RTX concentration data were available in a total of 118 patients (PK population) from both treatment arms. In these patients, RTX population pharmacokinetic (PK) was assessed using a semi-mechanistic pharmacokinetic model, as previously described.[6] Exposure to RTX was assessed by the area under the concentration-time curve (AUC) from the beginning of treatment to the last concentration

measurement time at M12 (6 months after the end of the FCR treatment, $AUC_{0\text{-}12M}$) or D22 (before C1 of FCR treatment in the Dense-FCR arm, $AUC_{0\text{-}22D}$). RTX $AUC_{0\text{-}12M}$ and $AUC_{0\text{-}22D}$ values were obtained by integrating the concentrations predicted in the PK model.

Statistical analysis

The primary endpoint of this randomized phase II study was the rate of CR with nMRD as assessed 3 months after the end of treatment. To calculate the number of patients, we used the Simon method in one step with the following hypothesis: in our previous study we observed a complete response (CR) with negative minimal residual disease (nMRD) of 35% in patients having received the combination of fludarabine, cyclophosphamide and rituximab (FCR) at standard dose. We assume an increase of 15% of CR rate with nMRD by using high dose of rituximab. Taking into account a rate of 10% of patients not assessable for response, 140 patients was included in this study, 70 in each arm.

Distributions of data were tested with the Shapiro-Wilk test. Chi-squared or Fisher's exact tests were used to compare categorical data. For numerical data, medians were compared using Student's T test or Mann-Whitney's test. Spearman's correlation test was used to assess the association between two numerical variables. The association between covariates and patients' response was assessed using multivariate logistic regression analysis. The receiver operating characteristics (ROC) curve was used to determine the threshold able to predict complete response with BM nMRD associated with the best sensitivity and specificity according to the Youden index.[7] Progression-free survival (PFS) was measured from the date of the initiation of treatment to the date of relapse and/or progression. PFS was estimated using the Kaplan-Meier method and comparisons were made using the log-rank test. Hazard ratios and their 95% confidence intervals in univariate and multivariate analyses were calculated using Cox regression analyses. All statistical analyses were performed at the conventional two-tailed α level of 0.05 using R software version 3.0.2.10.

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Number of SAE per patient (grade >3), n (%)	Prephase	FCR		
	Dense-FCR arm n=68	Cohort n=137	FCR arm n=69	Dense-FCR arm n=68
IRR	2 (3)	-	0	-
Hematological				
Anemia	3 (4)	19 (14)	8 (12)	11 (16)
Neutropenia	6 (9)	88 (64)	40 (58)	48 (71)
Thrombocytopenia	3 (4)	29 (21)	12 (17)	17 (25)
Infection	0	9 (7)	5 (7)	4 (6)
General	2 (3)	25 (18)	12 (17)	13 (19)

Supplemental Table 1. List of grade 3/4 adverse events during the treatment period in ITT population (n=137)

Abbreviations: SAE, severe adverse event; n, number; IRR, infusion-related reaction.

	OR (95% CI)	p
Age (years)	1.03 (0.97-1.10)	0.409
Women	1.06 (0.43-2.81)	0.906
Binet stage C*	0.19 (0.04-0.60)	0.010
IGHV unmutated	0.56 (0.25-1.28)	0.171
Cytogenetic abnormalities		
Del(13q)	1.69 (0.69-4.33)	0.257
Del(11q)	0.97 (0.32-2.64)	0.958
Trisomy 12	4.74 (1.06-24.93)	0.045
Lymphocytes (G/L)	0.99 (0.99-1.00)	0.062
β2 microglobulin (mg/L)	0.74 -0.51-1.00)	0.086
MESF CD20 (per cell) ($x10^3$)	1.00 (0.98-1.03)	0.715
CT-Scan characteristics**		
Cheson (mm^2) ($x10^3$)	0.97 (0.84-1.08)	0.627
Volume (cm^3) ($x10^2$)	0.92 (0.56-1.34)	0.689
Number of lesions	1.25 (0.84-1.48)	0.369
Tumor burden•		
$CD20_{LN}$ ($x10^{15}$)	1.01 (0.98-1.03)	0.666
$CD20_{cir}$ ($x10^{15}$)	0.90 (0.76-1.01)	0.135
$CD20_{patient}$ ($x10^{15}$)	1.00 (0.97-1.02)	0.834
FCGR3A-158VV	2.85 (0.74-11.01)	0.118
Treatment arm	1.04 (0.46-2.33)	0.927

Supplemental Table 2. Univariate logistic regression response analysis in ITT population (n=137)

Patients not belonging to complete response with bone marrow undetectable minimal residual disease group were used as the odds ratio reference group. *None of patients in CR with BM nMRD were in Binet stage A. **CT-scan characteristics included classical Cheson volume determination according to Cheson standardized guidelines of radiology for non-Hodgkin's lymphomas[8] and a three-dimensional tumor volume described in methods section. •Tumor burden for a patient ($CD20_{patient}$) is composed of the lymph node ($CD20_{LN}$) and the circulating B cells ($CD20_{cir}$) part, evaluated as explained in methods section. Abbreviations: OR, odds ratio; CI, confidence interval; *IGHV*, immunoglobulin heavy chain variable; MESF, molecules of equivalent soluble fluorochrome; nd, not determined; $CD20_{LN}$, lymph nodes CD20 antigen; $CD20_{cir}$, CD20 antigen count on circulating B cells; $CD20_{patient}$, patient's CD20 antigen count.

A	HR (95% CI)	p
Age ≥58.34 (years), n=67	1.03 (0.54-1.99)	0.923
Male Gender, n=100	1.06 (0.50-2.26)	0.877
Binet stage AB, n=101	0.80 (0.39-1.63)	0.543
*IGHV u*nmutated, n=82	2.96 (1.29-6.81)	0.008
Cytogenetic abnormalities		
Del(13q), n=59	0.63 (0.30-1.32)	0.215
Del(11q), n=25	1.93 (0.90-4.13)	0.085
Trisomy 12, n=9*	nc	0.058
Lymphocyte count >74.65 (G/L), n=68	2.63 (1.29-5.35)	0.006
β2 microglobulin >2 (mg/L), n=111	2.20 (0.53-9.19)	0.266
Treatment arm FCR, n=69	1.09 (0.57-2.10)	0.792
Number of cycles received = 6 cycles, n=111	0.94 (0.41-2.15)	0.889
MESF CD20 >9169 (per cell), n=61	0.29 (0.14-0.61)	<0.001
CT-Scan characteristics**		
Cheson >3535 (mm^2), n=51	2.54 (1.22-5.27)	0.010
Volume >51.4 (cm^3), n=61	3.39 (1.51-7.63)	0.002
Number of lesions =6, n=117	3.07 (0.42-22.59)	0.245
Tumor burden•		
$CD20_{LN}$ >6.40 $.10^{15}$, n=65	1.83 (0.74-4.54)	0.184
$CD20_{cir}$ >3.58 $.10^{15}$, n=41	1.57 (0.78-3.18)	0.205
$CD20_{patient}$ >4.42 $.10^{15}$, n=79	4.92 (0.67-36.34)	0.083
FCGR3A-158VV, n=14	0.25 (0.03-1.85)	0.144

B	AUC (95% CI)	Threshold	Sp (%)	Se (%)
MESF CD20 (per cell)	**0.6880 (0.5817-0.7942)**	**9169**	**68**	**67**
CT-Scan characteristics				
Cheson (mm^2)	**0.6293 (0.5168-0.7417)**	**3535**	**66**	**60**
Volume (cm^3)	**0.6476 (0.5352-0.7601)**	**51.4**	**60**	**73**
Tumor burden				
$CD20_{LN}$	**0.5489 (0.4225-0.6753)**	**$6.40 .10^{15}$**	**39**	**78**
$CD20_{cir}$	**0.4986 (0.3724-0.6247)**	**$3.58 .10^{15}$**	**63**	**52**
$CD20_{patient}$	**0.5534 (0.4259-0.6809)**	**$4.42 .10^{15}$**	**19**	**96**

Supplemental Table 3. Cox analysis for PFS in ITT population (n=137)

(A) Univariate associations between baseline characteristics and PFS. (B) Parameters of ROC curve constructions. *Trisomy 12 determination on 92/137 samples. **CT-scan characteristics included classical Cheson volume determination according to Cheson standardized guidelines of radiology for non-Hodgkin's lymphomas[8] and a three-dimensional tumor volume described in methods section. •Tumor burden for a patient ($CD20_{patient}$) is composed of the lymph node ($CD20_{LN}$) and the circulating B cells ($CD20_{cir}$) part, evaluated as explained in methods section. Abbreviations: HR, hazard ratio; CI, confidence interval; *IGHV*, immunoglobulin heavy chain variable; MESF, molecules of equivalent soluble fluorochrome; nc, not converged; $CD20_{LN}$, lymph nodes CD20 antigen count; $CD20_{cir}$, CD20 antigen count on circulating B cells; $CD20_{patient}$, patient's CD20 antigen count; AUC, area under the ROC curve; Sp, specificity; Se, sensitivity; ROC, receiver operating characteristic.

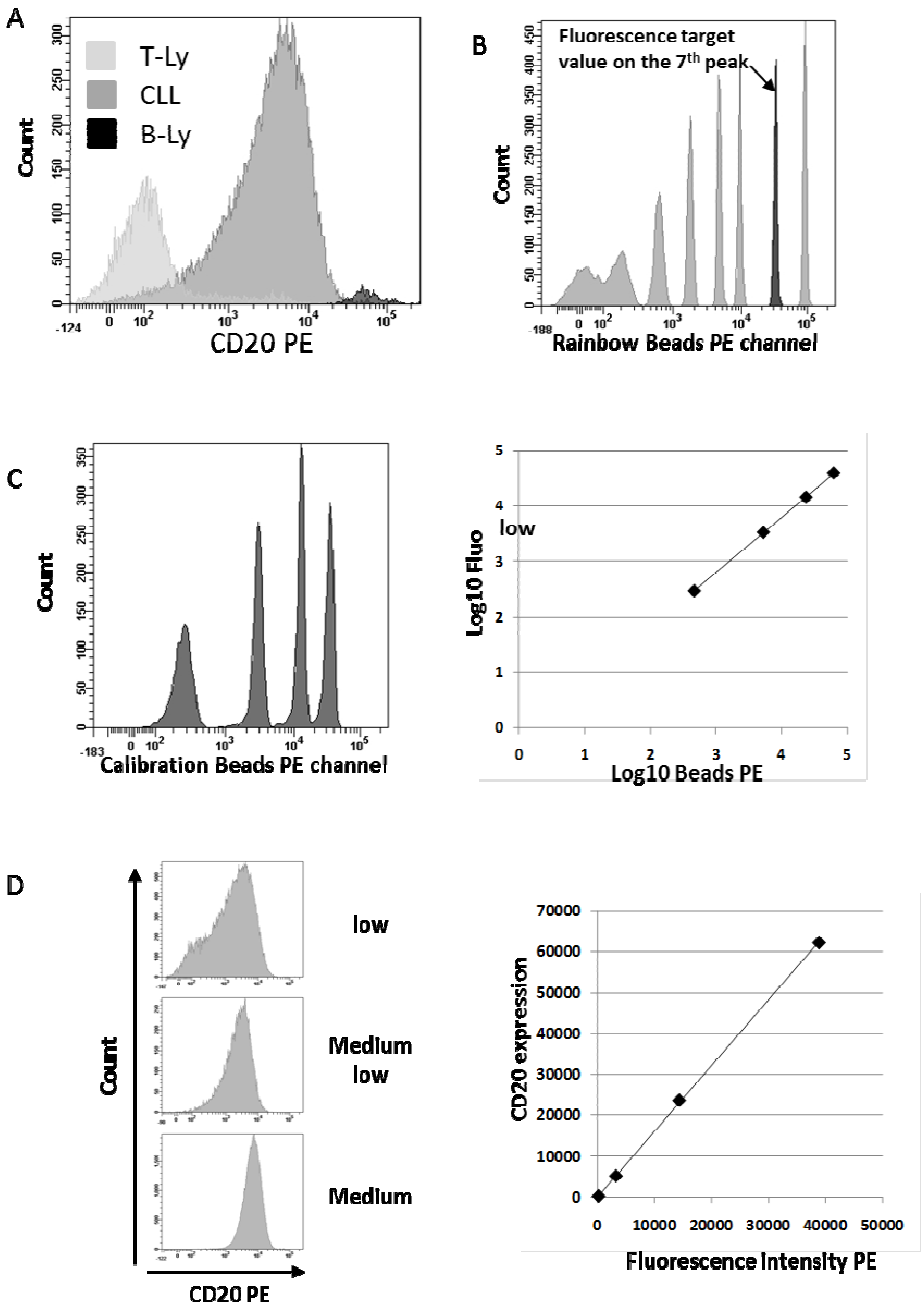
	PK Cohort (n=118)		ITT Cohort (n=137)		p
	n (%)	Median (IQR)	n (%)	Median (IQR)	
Age (years)	†	58 (52-62)	†	58 (53-62)	0.968
women	30 (25)	-	37 (27)	-	0.887
Binet stage					0.888
A	2 (2)	-	3 (2)	-	
B	84 (71)	-	98 (72)	-	
C	32 (27)	-	36 (26)	-	
IGHV unmutated	67/114 (59)	-	82/133 (62)	-	0.696
Cytogenetic abnormalities					
Del(13q)	53/95 (56)	-	59/106 (56)	-	1
Del(11q)	20/115 (17)	-	25/134 (19)	-	0.869
Trisomy 12	8/82 (10)	-	9/92 (10)	-	1
Lymphocyte count (G/L)	†	63.00 (26.63-113.00)	†	74.65 (29.60-114.30)	0.808
β2 microglobulin (mg/L)	†	2.90 (2.40-3.70)	124 (91)	3.07 (2.39-4.10)	0.494
MESF CD20 (per cell)	†	10580 (7095-13980)	107 (78)	10552 (6577-15240)	0.862
CT-Scan characteristics**					
Cheson (mm^2)	†	2939 (2146-4885)	128 (93)	2978 (2196-5044)	0.931
Volume (cm^3)	†	47.86 (27.71-88.99)	128 (93)	47.85 (26.45-95.80)	0.952
Tumor burden•					
$CD20_{LN}$ ($.10^{14}$)	†	102.10 (52.79-167.50)	99 (72)	91.70 (41.50-159.90)	0.495
$CD20_{cir}$ ($.10^{14}$)	†	29.27 (11.57-55.71)	99 (72)	30.70 (12.60-50.70)	0.849
$CD20_{patient}$ ($.10^{14}$)	†	138.80 (76.30-277.10)	93 (68)	134.50 (75.70-260.40)	0.588
FCGR3A-158VV	13 (11)	-	14/127 (11)	-	1
FCR arm	55 (47)	-	69 (50)	-	0.615

Supplemental Table 4. Comparison of PK (n=118) and ITT (n=137) population

†For the pharmacokinetic population, n=118; intent-to-treat population, n=137. *MESF CD20 corresponds to the number of CD20 molecules expressed on a CLL cell surface. Quantification is detailed in supplemental methods. **CT-scan characteristics included classical Cheson volume

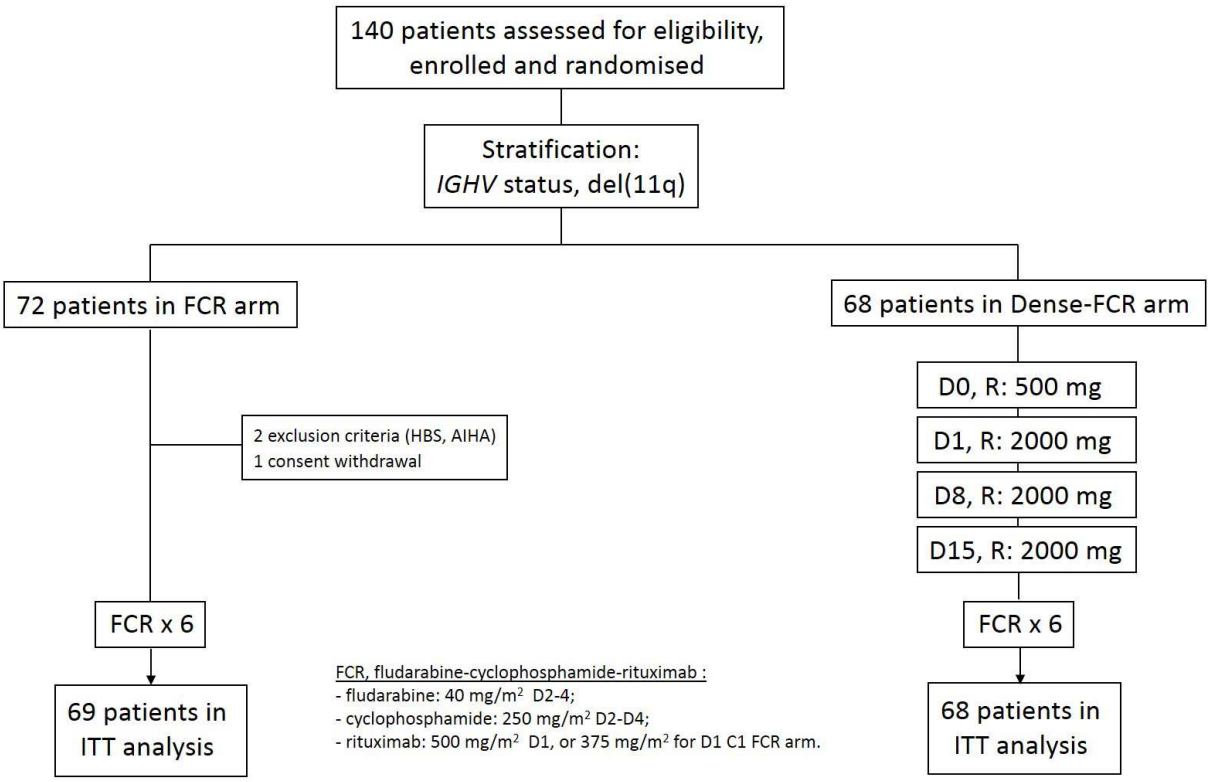
determination according to Cheson standardized guidelines of radiology for non-Hodgkin's lymphomas[8] and a three-dimensional tumor volume described in methods section. •Tumor burden for a patient ($CD20_{patient}$) is composed of the lymph node ($CD20_{LN}$) and the circulating B cells ($CD20_{cir}$) part, evaluated as explained in methods section.

Abbreviations: PK, pharmacokinetic; ITT, intent-to-treat; n, number; IQR, interquartile range; *IGHV*, immunoglobulin heavy chain variable; MESF, molecules of equivalent soluble fluorochrome; $CD20_{LN}$, lymph nodes CD20 antigen count; $CD20_{cir}$, CD20 antigen count on circulating B cells; $CD20_{patient}$, patient's CD20 antigen count.



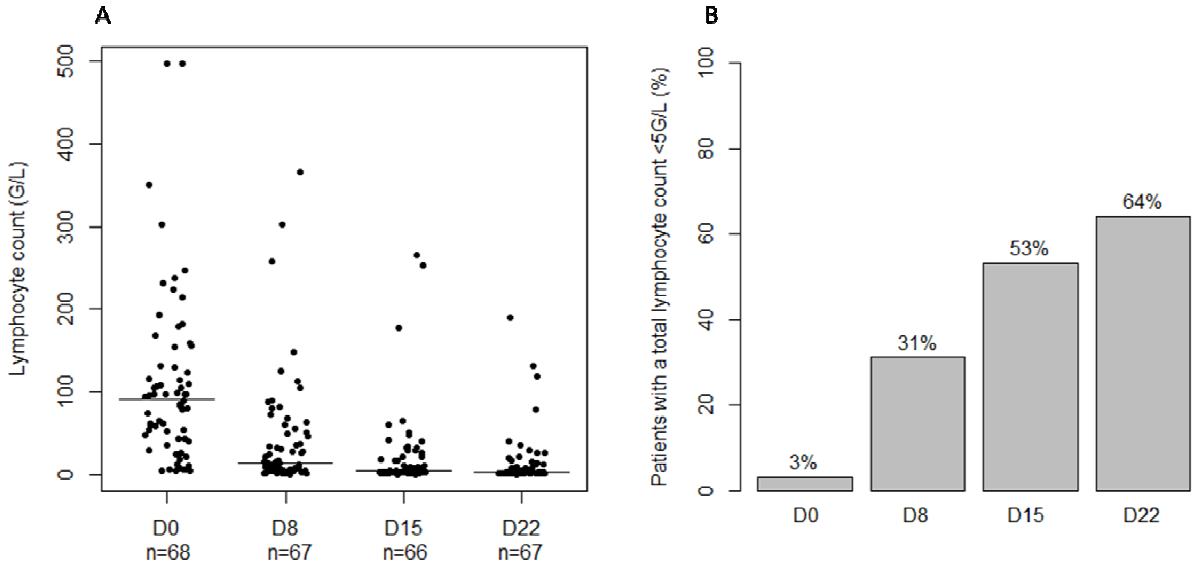
Supplemental Figure 1. Study of CD20 expression on CLL cells using QuantiBRITE™ CD20PE commercial kit.

Panel A shows the initial setting for the study of CD20 expression on T-lymphocytes ▒, normal B-cells ■ , and CLL cells ▒ . Panel B shows the determination of the fluorescence target value for PE channel using Rainbow beads. Panel C shows the use of the calibration beads to measure the fluorescence intensity of the beads and construct the calibration curve. Panel D shows representative cases of CD20 labeling on CLL cells. The determination of the fluorescence intensity and the use of an antiCD20 reagent certified with a PE to monoclonal antibody ratio of 1:1, allows calculating (with the calibration curve of panel C) the number of equivalent CD20 molecules present at the cell surface.



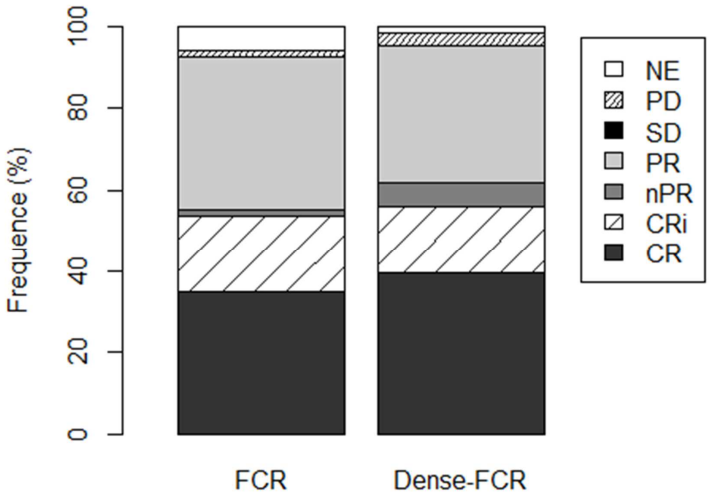
Supplemental Figure 2. Study design of the CLL2010FMP protocol

Abbreviations: AIHA, Autoimmune hemolytic anemia; C, Cyclophosphamide; D, Day; F, Fludarabine; HBs, HBs Antigen; ITT, Intent to treat; R, Rituximab.



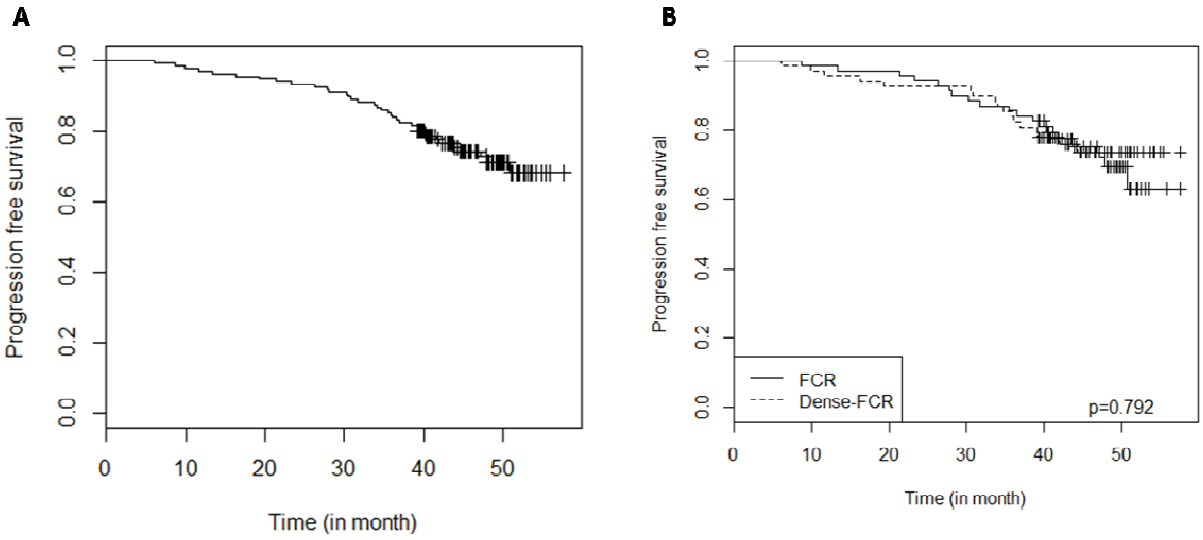
Supplemental Figure 3. Changes in lymphocyte counts during rituximab pre-phase in the Dense-FCR arm (n=68).

Total lymphocyte counts at D0 (n=68), D8 (n=67), D15 (n=66) and D22 (n=67) (A). Percentage of patients with a total lymphocyte count ≤ 5.0 G/L (B).



Supplemental Figure 4. Response rate according to treatment (ITT population, n=137)

Abbreviations: NE, not evaluable ; PD, progressive disease ; SD, stable disease ; PR, partial response ; nPR, nodular partial response ; CRi, complete response with incomplete bone marrow recovery ; CR, complete response.



Supplemental Figure 5. Estimates of PFS curves (ITT population, n=137)

PFS of the whole cohort (A) and according to treatment arm (B).