Lymphocyte doubling time in chronic lymphocytic leukemia modern era: a real-life study in 848 unselected patients

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Running title: Lymphocyte Doubling Time in Chronic Lymphocytic Leukemia

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Abstract

The prognostic significance of lymphocyte doubling time (LDT) in chronic lymphocytic leukemia (CLL) was identified when the biology of the disease was poorly understood and therapy was not effective. We assessed the clinical and biological significance of LDT in 848 CLL patients in a real-life setting and the context of new biomarkers and effective therapy. A short LDT (≤ 12 months) was enriched for adverse biomarkers. Patients with a rapid LDT did need therapy shortly after diagnosis (median 23 months vs. not reached ; p<0.001) and had a poorer overall survival (median 95 months vs. not reached p <0.001). LDT, IGHV mutational status, Beta-2 microglobulin, and Rai clinical stage were independent predictors for time to first treatment in the whole series and in Binet stage A patients. No correlation was observed between LDT and response to chemoimmununotherapy. However, a short LDT along with age ≥65 years, high-risk FISH (del(17p), del(11q)), unmutated IGHV, increased Beta-2 microglobulin, and TP53 mutations predicted short survival. Moreover, the prognostic significance of LDT was independent of the CLL-IPI and the Barcelona/Brno prognostic model. LDT remains an important outcome marker in the modern CLL era and should be incorporated into the clinical assessment and stratification of CLL patients.

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Introduction

Tumor kinetics are an important determinant of the outcome of patients with cancer. In chronic lymphocytic leukemia (CLL), the blood lymphocyte doubling time (LDT) reflects the birth rate and pace at which neoplastic lymphocytes accumulate in the organism. LDT is a validated independent biomarker that correlates with overall survival[1-3]. However, the seminal studies on the significance of LDT in CLL were conducted when the understanding of the biology of CLL was limited and treatment for this disease was not effective[1,4-6].

The objectives of this study were twofold. First, to correlate LDT with new genetic and molecular features of CLL; second, to determine the prognostic and predictive value of LDT in the general population of CLL patients treated with effective regimens in daily practice.

Material and Methods

This is a retrospective observational study in 848 unselected CLL patients from two European academic centers, the Hospital Clinic, University of Barcelona, Spain and the Amedeo Avogadro University of Eastern Piedmont, Novara, Italy. Patients in all clinical stages (Binet A 780, Binet B 61; Binet C 6; Rai 0 614; Rai I+II 208; Rai III +IV 25) diagnosed between 2000 and 2016 were included in the study. The study was approved by the local Institutional Review Boards and was performed in accordance with the Declaration of Helsinki. LDT was measured at the time of diagnosis if prior WBC counts were available or calculated after diagnosis by linear regression analysis, over a minimal observation, treatment-free period of 3 months[1]. Diagnosis and criteria for starting therapy were as recommended by the International Workshop on CLL[2]. Data at diagnosis retrieved from databases included age, sex, clinical stage, absolute lymphocyte count, Hb level, platelet count, β_2-microglobulin (B2M) and lactate dehydrogenase (LDH) levels. Fluorescent *in situ* hybridization (FISH) studies for del(11q), del(13q) and del(17p) deletions and trisomy 12 were performed using the Vysis CLL probe kit (Abbott, Del Plains, IL,

USA). IGHV rearrangements and mutational status were analyzed according to the European Research Initiative on CLL (ERIC) recommendations[7]. Mutations of *NOTCH1, SF3B1, ATM and TP53* were determined using previously described methods[8.] Data were retrieved within the 3 months from diagnosis; in some cases, samples stored at diagnosis were retrospectively analyzed. Due to the nature of the study, not all variables were available for all patients. Although well balanced for most variables, patients from the Novara series were older (71 vs. 67 years; p<0.01) and presented mutated IGHV more frequently than those from Barcelona (70% vs. 62%; p<0.02) (supplementary material Table 1). The median OS of the two series was similar (144 months vs. 155 months *p*>.05). The main endpoint of the study was OS.

Statistical methods

Comparisons between groups were performed using the χ^2 for continuous variables and Mann-Whitney test for categorical variables. Overall survival (OS) was defined as the time between diagnosis and the date of death or last follow-up using the Kaplan-Meier method. Time to first treatment (TTFT) was calculated from the time of diagnosis to the time therapy was initiated or patient's death. Survival curves were compared by the log-rank test. Analyses of the independent prognostic value for OS and TTFT was performed with Cox regression multivariate models. The minimal observation period before any event (initiation of treatment or death) was 3 months. Actuarial plots were obtained after a landmark time of 12 months from diagnosis and taking time of diagnosis as time 0. Unadjusted *p*-values <0.05 were considered statistically significant.

Results

Correlation of LDT with clinical features, biomarkers, and outcomes

Among the 848 CLL patients, all clinical stages included, the proportion of patients with an LDT≤12 months was 94 (11%) and the proportion of patients with a LDT >12 months was 754 (89%). The median follow-up up was 85 months (range 44-201) and 103.5 months (range 4-224) for patients with a short and a long LDT, respectively. At the time of the analysis, 61/94 (65%) patients with short LDT and 283/754 (38%) of those with a long LDT had died.

The correlation of LDT (≤12 months vs. > 12 months) with clinical features, biomarkers, and outcomes is shown in Table 1. Patients with short LDT were predominantly male (p=0.02), had more advanced clinical stage (p<0.001), higher absolute lymphocyte counts (p<0.001) and increased serum B2M (p=0.004), and a tendency to increased serum LDH levels (p=0.065). Patients with short LDT had a trend towards lower levels of Hb and lower platelet counts, the difference not being statistically significant. A short LDT was also associated with unmutated *IGHV* status (p<0.001) and poor FISH cytogenetics (del17p; p=0.002 and del11q; p<0.001). In addition, patients with short LDT presented more frequently mutations in *NOTCH1* (p=0.002), and *TP53* (p=0.012) as compared to those with a long LDT; similarly, a tendency to a higher proportion of patients with *SF3B1* (p=0.064) and *ATM* mutations (p=0.102) was observed in patients with short LDT.

In contrast, patients with a long LDT were enriched for initial clinical stages (Binet A, Rai 0), del(13q), lower blood lymphocyte counts and normal FISH analysis (Table1). LDT did not predict response to initial therapy, which in 50% of cases consisted of chemoimmunotherapy (FCR, BR, Chlorambucil + anti-CD20 mononoclonal antibodies) (ORR 73% (CR 44%) vs. ORR 66% (CR 36%); p=0.326) (Table 1 and supplementary material Table 2).

LDT is an independent prognostic biomarker

LDT and time-to-first therapy

Altogether, 310 of 848 patients (37%) required therapy. All patients considered, the median TTFT was 174 months. Patients with a short LDT needed therapy more frequently (77/94 or 82% vs.233 /754 or 31%) ($p<0.001$) and more rapidly (median TTFT 23 months (range 16-30) vs. not reached) ($p<0.001$) than those with a long LDT, independently of clinical stage (Figure 1A and supplementary material Table 3). Among 780 patients with Binet stage A disease, the median TTFT for those with a short LDT (n=76) was 25 months (17-32) while in patients with a long LDT (n=704) the median TTFT had not been reached ($p<0.001$), independently of Rai stage. In 61 patients with Binet stage B disease, the median TTFT in cases with a short LDT (n=17) was 12 months (2-22) vs. 34 months (20-48) in those with a long LDT (n=44) ($p=0.074$), independently of Binet clinical stage. In 61 patients with Binet stage B disease, the median TTFT in cases with a short LDT (n=17) was 12 months (range 2-22) vs. 34 months (range 20-48) in those with a long LDT (n=44) ($p=0.074$). There were only 6 patients in Binet stage C disease, precluding a meaningful analysis of this group of patients (supplemental material Table 3). Results did not significantly differ when considering Rai instead of Binet clinical stages (data not shown). In addition, no differences were observed when the actuarial plots were obtained from the time of diagnosis or after a landmark of 12 months after diagnosis (supplementary material Figure 2A).

In the univariate analysis, biomarkers correlated with a shorter time to first treatment (TTFT) were advanced Rai clinical stage, increased B2M, short LDT, unmutated IGHV, high risk FISH

cytogentics as defined by presence of del(11q) or del(l17)p, mutations in *NOTCH1, SF3B1*, and *ATM* (all p <0.001), as well as mutations in *TP53* (*p*=0.003) (Table 2 and supplementary Table 5).

In multivariate analysis including age, Rai stage, B2M, LDT, IGHV, high-risk FISH cytogenetics, and mutations in *TP53, NOTCH1, SF3B1*, and *ATM*, a short LDT maintained its independent prognostic value for TTFT (HR 4.3 (95% CI: 3.0-6.1), *p*<0.001) along with Rai stage (HR 2.6 (95% CI: 2.0-3.5), *p*<0.001), B2M (HR 1.5 (95% CI: 1.1-2.1), *p*=0.005), *IGHV* mutations (HR 3.0 (95% CI: 2.3-4.1), *p*<0.001), *NOTCH1* mutations (HR 1.6 (95% CI: 1.1-2.4), *p*=0.012), and *SF3B1* mutations (HR 1.99 (95% CI: 1.2-2.9), *p*=0.008) (Table 2 and supplementary Table 5).

LDT and overall survival

At the time of the analysis, 359 patients had died. The median follow-up of surviving patients was 100 months (range 4-224). The median OS of the whole series was 150 months (range 5-224). In those subjects with a LDT ≤ 12 months (n=94), the median OS was 95 months (range: 15-201) in comparison to 161 months (range: 5-224) in patients with a LDT > 12 months (n=754) (p<001) (Figure 1), independently of clinical stages (supplementary material Table 4). In an exploratory analysis LDT showed a tendency to behave as a continuous variable (supplementary material Figure 1).

To investigate the prognostic significance of LDT uni- and multivariate adapted analyses were performed (Table 2 and supplementary material Table 6). In univariate analysis there was a correlation between OS and advanced clinical stage (Rai 0 vs. I-IV), age ≥65 years, increased B2M, higher LDH, short LDT, unmutated *IGHV*, and high-risk FISH genetics (del(17p) and/or del(11q)) (all *p*<0.001). Likewise, mutations in *NOTCH1* (*p*<0.001) and *TP53* (*p*<0.001) were associated with OS while no association was found for *SF3B1* (*p*=0.25) and ATM (*p*=0.35).

In multivariate analysis including age, Rai stage, B2M, LDT, IGHV, FISH cytogenetics (alterations of del(17p) or del(11q)), *TP53* mutations, and *NOTCH1* mutations, a short LDT maintained its prognostic value for OS (HR 1.5 (95% CI: 1.1-2.1), $p<0.017$) along with age $\geq$ 65 years (HR 2.9 (95% CI: 2.1-3.9), $p<0.001$), high-risk FISH (del(17p), del(11q))(HR 1.9 (95% CI: 1.3-2.8), $p=0.002$), unmutated *IGHV* (HR 1.8 (95% CI: 1.3-2.3), $p<0.001$), increased B2M (HR 2.1 (95% CI: 1.5-2.8), $p<0.001$), and *TP53* mutations (HR 1.6 (95% CI: 1.1-2.6), $p=0.045$) (Table 2).

Next, we investigated whether LDT added prognostic value to CLL-IPI or to the Barcelona/Brno prognostic model[9,10]. For this, we first performed multivariate analysis including LDT and CLL-IPI variables (age, B2M, IGHV, del(17p) or TP53 alteration, Rai stage). LDT maintained its prognostic value for OS (HR 1.6 (95% CI 1.2-2.3), p=0.003) along with age ≥65 years (HR 3.0 (95% CI: 2.2-4.0), $p<0.001$), increased B2M (HR 2.4 (95% CI: 1.9-3.2), $p<0.001$), unmutated *IGHV* (HR 1.9 (95% CI: 1.5-2.5), $p<0.001$), and presence of alterations in 17p and/or TP53 (HR 1.9 (95% CI: 1.3-2.8), $p=0.001$) while Rai stage failed to enter the OS prognostic model. ($p>0.05$) (supplementary material Table 6). Additionally, in a multivariate analysis including LDT together with Barcelona-Brno variables (*IGHV* mutational status and FISH analysis). LDT showed independent prognostic value for OS (HR 1.6 (95% CI: 1.2-2.2), $p=0.002$) along with unmutated IGHV (HR 1.9 (95% CI: 1.5-2.4), $p<0.001$) and high-risk FISH cytogenetics (del(17p)/del(11q)) (HR 1.8 (95% CI: 1.3-2.5), $p<0.001$) (supplementary material Table 6). As for OS, no differences were observed when the actuarial plots were obtained from the time of diagnosis or after a landmark of 12 months (supplementary material Figure 2B).

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Discussion

CLL is characterized by a heterogeneous clinical course and variable response to therapy. Due to this, the management of patients with CLL highly relies on prognostic factors (which estimate overall survival) and predictive factors (which anticipate response to a given treatment). Age, clinical stage (i.e., Rai or Binet), serum B2M, *IGHV* mutational status, cytogenetic features (e.g., del(17p)/TP53 mutations, del(11q)) are considered the most relevant outcome biomarkers[11,12]

In the last decade, progress in the understanding of the biology and therapy of CLL has led to the identification of a huge number of potential biomarkers, although most of them have not been incorporated into daily clinical practice due their complexity, limited availability, lack of validation, or arguable clinical usefulness. In this regard, it is important to underline that biomarkers should be easily obtained, reproducible, and biologically and clinically meaningful[12]

In 1966, in a seminal study, David Galton scrutinized a large series of CLL patients with a long follow-up and observed several "blood lymphocyte trends" (from stable to rapidly increasing) which correlated with the clinical course of CLL, from a benign to an aggressive disorder[13]. This observation was at the origin of the identification of LDT as a biomarker in CLL[1--6]. LDT is easily calculated and applied, is reproducible, and can be used in any setting. Moreover, LDT is a biologically meaningful biomarker as it is mechanistically related to the replication and expansion of neoplastic lymphocytes[14,15], CLL cells birth-rate[16,17], driver mutations[18], and genomic aberrations[18].

There is a renewed interest on LDT in the context of progress in CLL molecular biology and therapy[19,20]. The German CLL Study Group (GCLLSG) has published a study based on 539 stage A patients from the CLL1 trial[21]. In this study, LDT emerged as a significant, independent prognostic biomarker for TTFT and OS. Other independent prognostic parameters were del(17p), unmutated *IGHV*, B2M >3.5 mg/ dL, and age >60 years, which are the building block

for the CLL-IPI [9]; del(11q) was also found to be prognostically significant; these results are validated in our study. Based on their results, the GCLLSG elaborated a prognostic index that efficiently discriminates four prognostic groups.

Our study is the first comprehensive analysis of LDT in the modern CLL era. We found that LDT captures a wide array of adverse (short LDT) or favorable (long LDT) biomarkers, which explains its robustness as biomarker. Also, LDT is an independent biomarker for TTFT and OS. The prognostic significance of LDT is relevant because of its simplicity and independence from other biomarkers and CLL outcome models[9,10], and thus can be used in conjunction with them. Whether LDT could be employed as a surrogate for IGHV mutational status has been raised[22]. This is not supported by our data since 35% of our patients with a short LDT had mutated *IGHV*. Likewise, the proportion of patients with no TP53 aberrations and a short LDT was 35%. Thus, LDT may complement but not replace *IGHV* mutational status nor TP53 aberrations in CLL prognostication.

In our study, LDT did not correlate with response to chemoimmunotherapy, which agrees with a recent report in patients treated with FCR[23]. This strongly suggests that differences in OS according to LDT are due to the heterogeneous biology of the disease, which is revealed by LDT, rather than by differences in treatment response rates.

Historically, the usefulness of LDT as biomarker has been questioned on the basis that in many cases it is not available at the time of diagnosis and that a short LDT is a criterion to initiate therapy. However, CLL rarely constitutes a treatment emergency. Indeed, it is recommended that after diagnosis patients are observed for 4-8 weeks to complete the diagnostic workup and to assess the pace of the disease, this including LDT if not available. On the other hand, a short LDT is infrequently the only reason to start therapy (<1% of cases in the Barcelona series [24]). In this regard, it is also worth emphasizing that in our analysis either time of diagnosis or a

12 months landmark after diagnosis were used as time 0 in statistical analysis, with no significant differences.

This paper has the limitations inherent to all retrospective analysis, including that not all biomarkers were available in the entire cohort of patients. Also, the proportion of patients treated upfront with BTK and BCL2 inhibitors was quite small. In a limited independent cohort of patients initially treated with ibrutinib, a raw analysis showed a likely correlation between LDT and OS (supplementary material Figure 3). However, the prognostic and predictive value of LDT in patients treated with ibrutinib must be prospectively determined in large series of patients. The most important strength of this paper is the demonstration that LDT remains an important marker for OS in the modern CLL era.

The fact that the conclusions from this paper are based on OS rather than progression-free-survival (PFS) is important and deserves comment. Thus, in most cases CLL runs a protracted clinical course characterized by consecutive episodes of disease progression and need for therapy. Consequently, the OS depends on the response to different treatments given during the disease. Therefore, progression-free-survival (PFS), which largely depends on treatment modality, cannot replace OS as ultimate endpoint[25,26]. Nevertheless the relationship of LDT and PFS in cohorts of homogeneously treated patients within trials warrants study.

In conclusion, this study shows that LDT (1) significantly correlates with CLL biomarkers, a short and a long LDT being significantly enriched for adverse and favorable biomarkers, respectively; (2) remains an independent biomarker for TTFT and OS in the modern CLL era; and (3) pending of further study, it does not appear to be a treatment-dependent biomarker. For these reasons and because of its applicability in all settings, it is advisable to include LDT in the assessment and stratification of CLL patients and in prognostic studies pursuing the identification of new biomarkers for this form of leukemia.

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Figure legends

Figure 1A. Time-to-first therapy (TTFT) of patients with CLL according to LDT (median 23 months vs. not reached; $p<0.001$).

Figure 1B. Overall survival (OS) of patients with CLL according to LDT (median 95 months vs. 161 months; $p<0.001$).1919
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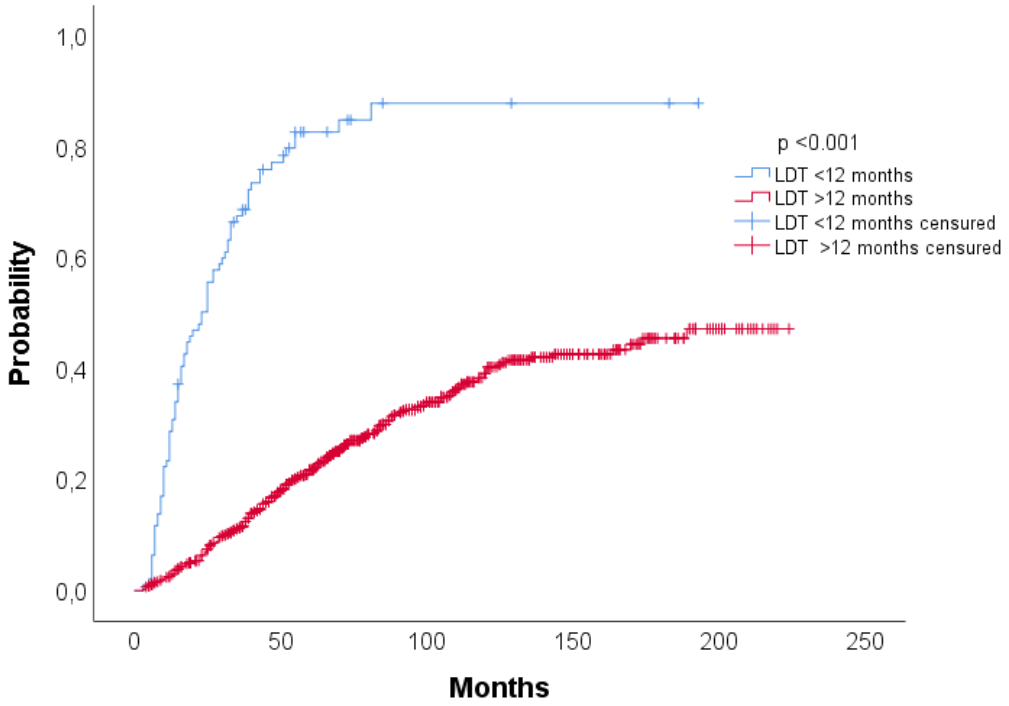
Table 1. Demographics, clinico-biological features, and outcomes in 848 patients according to LDT

	LDT ≤12 months n= 94	**LDT >12 months n= 754**	***P***
Age			
Median (range)	69 (40-90)	69 (34-100)	.93
≤65 years (%)	42/94 (45)	301/754 (40)	.375
Gender, male (%)	64/94 (68)	418/754 (55)	**.02**
Rai, 0 vs I-IV (%)	42 vs 156 (45 vs 55)	572 vs 181 (76 vs 24)	**<.001**
Lymphocyte count, $x10^9$/L (mean±SD)	20.7 ± 23.2	11.7 ± 11.6	**<.001**
Hb, $x10^9$/L (mean±SD)	13.7 ± 1.3	13.9 ± 1.5	.662
Platelets, $x10^9$/L (mean±SD)	205 ± 69	217 ± 72	.109
LDH increased (%)	15/94 (16)	69/746 (9)	.065
B2M increased (%)	57/91 (63)	337/727 (46)	**.004**
Unmutated *IGHV* (%)	53/82 (65)	192/637 (30)	**<.001**
FISH			
del13q (%)	48/92 (52)	366/696 (53)	1.0
Normal (%)	15/93 (16)	228/698 (33)	**.001**
Tris12 (%)	23/93 (25)	117/687 (17)	.083
del11q (%)	17/92 (19)	40/694 (6)	**<.001**
del17p (%)	11/92 (12)	26/694 (4)	**.002**
Complex karyotype (%)	3/42 (7)	23/337 (7)	1.0
NOTCH1 mutated (%)	16/73 (22)	47/540 (9)	**.002**
SF3B1 mutated (%)	8/70 (11)	29/526 (6)	.064
ATM mutated (%)	7/65 (11)	29/524 (6)	.102
TP53 mutated (%)	11/75 (15)	34/576 (6)	**.012**
Treatment (%)	77/94 (82)	233/754 (31)	**<.001**

Chemoimmunotherapy (%)	38/77 (49)	117/233 (50)	1.0
Response			
CR	34 (44)	84 (36)	
PR	22 (29)	70 (30)	.58
Failure (F)	7 (9)	23 (10)	
Not assessable (NA)	14 (18)	56 (24)	
Response			
CR/PR	56 (73)	154 (66)	.326
F/NA	21 (27)	79 (34)	

A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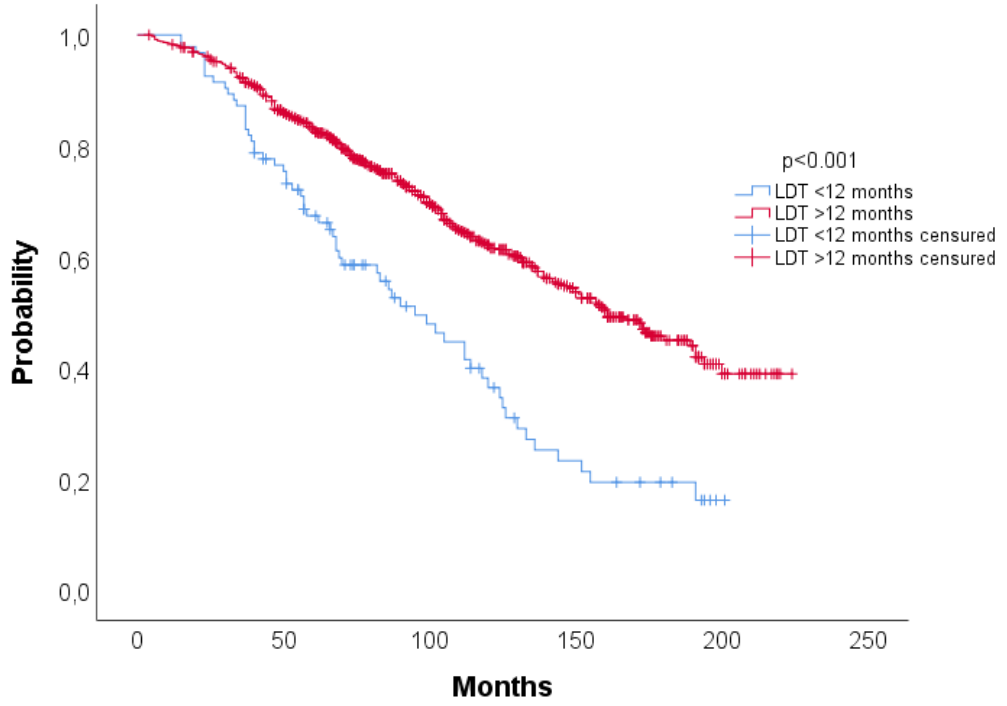
Probability
0
50
100
150
200
250
Months
p <0.001
LDT <12 months
LDT >12 months
LDT <12 months censured
LDT >12 months censured

B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Probability
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100
150
200
250
Months
p<0.001
LDT <12 months
LDT >12 months
LDT <12 months censured
LDT >12 months censured

Table 2.

Univariate análisis and multivariate regression for time-to-first-therapy (TTFT) and overall survival (OS) according to clinico-biological features in 848 patients with CLL.

		Time-to-first-therapy (TTFT)			**Overall survival (OS)**		
		Univariate Log-rank	**Multivariate Cox regression**		**Univariate Log-rank**	**Multivariate Cox regression**	
Parameter	**Risk category**	*P* value	HR (95% CI)	*P* value	*P* value	**HR (95% CI)**	***P* value**
Age	>65 years	NS (0.67)	-	NS (0.09)	<.001	2.9 (2.1-3.9)	<0.001
Rai stage	I-IV	<.001	2.6 (2.0-3.5)	<.001	<.001	-	NS (0.34)
B2M	>UNL	<.001	1.5 (1.1-2.1)	.005	<.001	2.1 (1.5-2.8)	<.001
LDT	≤ 12 months	<.001	4.3 (3.0-6.1)	<.001	<.001	1.5 (1.1-2.1)	.017
IGHV	Unmutated	<.001	3.0 (2.3-4.1)	<.001	<.001	1.8 (1.3-2.3)	<.001
FISH	del11q or del17p	<.001	-	NS (0.7)	<.001	1.9 (1.3-2.8)	.002
TP53	Mutated	.003	-	NS (.14)	<.001	1.6 (1.1-2.6)	.045
NOTCH1	Mutated	<.001	1.6 (1.1-2.4)	.012	<.001	-	NS (0.27)
SF3B1	Mutated	<.001	1.99 (1.2-2.9)	.008	0.25	.	NS
ATM	Mutated	<.001	-	NS (.051)	0.35	-	NS

HR-Hazard ratio; CI- confidence interval; NS- not significant; UNL- upper normal limit