

# Progressive telomere shortening is part of the natural history of chronic lymphocytic leukaemia and impacts clinical outcome: evidences from long term follow-up

Many biological markers at diagnosis have improved the prediction of outcome in chronic lymphocytic leukaemia (CLL) (Nabhan *et al*, 2015). Previous studies on telomere length (TL) have demonstrated that shorter telomeres at diagnosis are associated with poor outcome, along with unmutated *IGHV* status, high levels of CD38, CD49d and ZAP70 (Grabowski *et al*, 2005; Roos *et al*, 2008; Lin *et al*, 2014; Dos Santos *et al*, 2015). Moreover, our previous study, on a large CLL population ( $n = 401$ ), showed that short TL (<5000 bp) at diagnosis is an independent predictor of shorter overall survival (OS), treatment-free survival (TFS) and progression to Richter syndrome (Rossi *et al*, 2009).

Here we report TL dynamics over time and its predictive value in 90 CLL patients with a longer follow-up.

Patients from the previous cohort that were willing to donate further samples were analysed. Diagnosis and treatment of CLL were managed according to institutional guidelines, based on the National Cancer Institute (NCI) Working Group (Hallek *et al*, 2008). Clinical and biological data were recorded (Table SI). All patients provided informed consent.

TL was assessed at two time-points; telomere loss over time was calculated in terms of absolute loss, defined as the loss of telomeric DNA (in base pairs, bp) between the first and second determination of TL, and then adjusted for time, as Yearly Loss [YL = absolute loss (bp)/time (months)], in order to limit the bias of different timing in the second sample acquisition among patients.

The second TL analysis was performed on peripheral blood mononuclear cells, recovered using density gradient stratification procedure (Ficoll-Hypaque, GE Healthcare,

Buckinghamshire, UK), as previously described (Rossi *et al*, 2009). Genomic DNA was extracted using DNAzol (Invitrogen, Carlsbad, CA, USA). DNA yields and quality were measured by Nanodrop2000 (ThermoScientific, Waltham, MA, USA). TL was determined by Southern blot analysis.

Primary end-point was TFS, defined as the time from the first TL evaluation to the time of first treatment. For univariate analyses, the TFS curve was estimated by the Kaplan–Meier method and compared using the log-rank test. TFS was then analysed by the Cox proportional hazards model comparing, by the Wald test, relevant risk factors (Table I). All reported *P*-values were two-sided, at the 5% significance level. Data were analysed as of June 2016 by R v.3.3.0 (R Foundation for Statistical Computing, Vienna, Austria).

Ninety CLL patients from the original series were clinically monitored for a median time of 128 months (range, 21–336 months). The first TL determination (baseline TL) was assessed at early stage of the disease, while the second, follow-up, determination (FU TL) was not uniform for all patients, with a median time between the two TL measures of 44 months (range: 11.5–231 months). At the time of the second determination, 26 patients had already relapsed after a first-line treatment, while 64 patients were still in Watch and Wait (WW). Among these, 33 progressed and required a treatment between FU TL and the last follow-up.

Telomeres were shorter at FU TL compared to baseline TL (median YL −137 bp; range +174 to −1906 bp,  $P < 0.001$ ). This was particularly evident in *IGHV*-mutated patients, as compared to unmutated (median YL −205 bp vs. +63 bp;  $P < 0.05$ ). Patients with longer telomeres at baseline showed

**Table I.** Treatment-free survival analysis by Cox univariate and multivariate hazard models for the main clinical and biological variables in the chronic lymphocytic leukaemia Watch and Wait' population (all patients were Binet Stage A and without *TP53* mutations at diagnosis).

Variable	Cox univariate analysis			Multivariate analysis		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Age >65 years	1.47	0.74–2.95	0.272			
Male/Female	1.01	0.5–2.03	0.98			
%YL > 6	2.13	1.02–4.44	0.043	2.20	1.01–4.82	0.049
TL at diagnosis <5000 bp	5.35	2.51–11.39	0.001	3.92	1.56–9.85	0.004
CD38	1.48	0.69–3.16	0.316			
ZAP70	2.25	1.05–4.84	0.037	1.17	0.49–2.76	0.729
<i>IGHV</i> unmutated status	4.38	2.09–9.19	0.01	2.48	1.05–5.89	0.039

95% CI, 95% confidence interval; HR, Hazard ratio; TL, telomere length; %YL, per cent yearly loss.

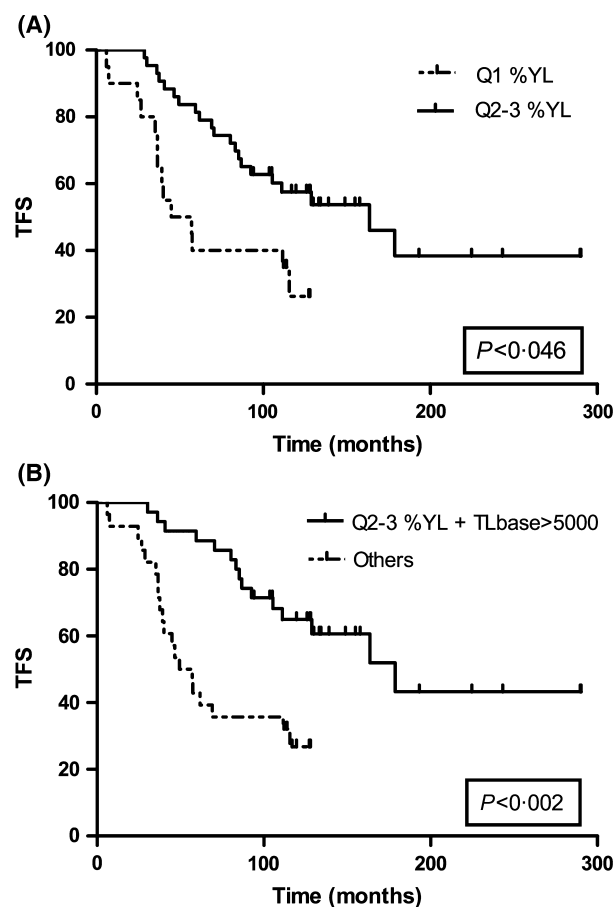


Fig 1. (A) TFS of WW patients according to Q %YL (Q1, %YL > 6% vs. Q2–3, %YL < 6%). (B) TFS of WW patients with good telomere-related prognostic factors (telomere length at baseline > 5000 bp and Q2–3%YL < 6%) vs. WW patients with at least one telomere-related risk factor. %YL, per cent yearly loss; Q, quartile; TFS, treatment-free survival; TLbase, telomere length at baseline; WW, watch and wait.

deeper erosion than those with shorter telomeres (median YL –49 bp vs. –129 bp for patients in the 25° and 100° TL percentile, respectively,  $P < 0.0001$ ). For this reason, YL was adjusted based on the baseline TL with this formula: %YL = (YL/baseline TL)  $\times$  100 and this parameter was used as an outcome predictor.

Univariate analysis for TFS was conducted on 64 WW patients, untreated between the two TL determinations, avoiding the bias of treatment impact on telomere erosion. In this population, only four biomarkers were associated with a shorter TFS: unmutated *IGHV*, ZA P70 positivity, TL < 5000 bp and %YL > 6% (representing the Q1 of %YL). The %YL was predictive for an inferior median TFS [72 vs. 163 months;  $P < 0.046$ ; Hazard ratio (HR) 2.13; 95% confidence interval (CI) 1.02–4.44 in univariate analysis; Fig 1A]. In multivariate analysis, unmutated *IGHV*, TL < 5000 bp and %YL > 6% was independently associated with poor TFS in this population, while ZAP70 lost statistical significance (Table I).

The outcome analysis identified a highly favourable group of patients, characterized by both baseline TL > 5000 bp and %YL < 6% and showing a median TFS of 179 vs. 63 months compared to the other patients, defined by the presence of at least one telomere-related risk factor ( $P = 0.002$ ; HR 0.3; 95% CI 0.14–0.65; Fig 1B).

This is the first study on CLL focusing on TL dynamics on a large cohort of patients. This study shows that: (i) progressive telomere erosion occurs during the natural history of CLL and is higher than the YL described in healthy subjects (20–59 bp) (Wang *et al*, 2014); (ii) telomere loss is more pronounced when baseline TL is higher; (iii) accelerated telomere loss is associated with an inferior TFS, identifying a subgroup of patients that, despite a TL still far from critical levels, are likely to progress.

Telomere erosion appears related to the aggressiveness of the disease; %YL was able to describe this, even in patients with shorter TL. In WW patients, characterized at diagnosis by Binet Stage A and no *TP53* mutations, the %YL has an independent TFS predictive value. Moreover, a %YL > 6 was predictive of inferior TFS, despite being more common in patients with longer baseline telomeres and mutated *IGHV*.

Combining the two TL parameters describing telomere biology (baseline TL > 5000 bp; %YL < 6), a highly favourable patient group was defined. These data raise the possibility that TL dynamics over time could identify “highly stable” patients in the WW population, eventually requiring less frequent observation.

Lin *et al* (2014) reported their study on TL using high resolution Single Telomere Length Analysis, a technique that excludes sub-telomeric regions, and a telomere fusion assay. It would be interesting to apply this approach for future TL dynamic studies.

In conclusion, this study confirmed that baseline TL < 5000 bp, unmutated *IGHV*, and the dynamic parameter %YL > 6 were independently associated with shorter TFS in a CLL population monitored for a long-term follow-up.

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## Author contributions

PG, EG performed the research and wrote the paper, ML, SF, GG, DR designed the research study, DD, BM, DB, EB, LM, MC, MR, PO, CD, LDP contributed in the laboratory experiments and data collection, RP, PG, EG analyzed the data, MC, FC, MM, MB provided critical organizational support.

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## Conflict of interest

The authors have no conflict of interest to declare.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table SI.** Clinical and biological characteristics of the total CLL population (n=90) at diagnosis

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