ORIGINAL ARTICLE

Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia

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Telomere length (TL) has been associated with outcome in chronic lymphocytic leukemia (CLL). The aim of this extensive analysis carried out on 401 CLL patients was to assess TL conclusively as a prognostic biomarker. Our study included two cohorts used as learning (191 patients) and blinded validation series (210 patients). A TL cutoff of 5000 bp was chosen by receiver operating characteristic (ROC) analysis and Youden's index in the learning series. In this series, TL ⩽ 5000 bp was independently associated to a worse outcome for both overall survival (OS; 105.5 vs 281 months, $P < 0.001$) and treatment-free survival (TFS; 24.6 vs 73 months, $P < 0.001$). In the blinded validation series, TL ⩽ 5000 bp was confirmed as an independent outcome predictor for OS (79.8 vs not reached, $P < 0.001$) and TFS (15.2 vs 130.8 months, $P < 0.001$). Moreover, TL ⩽ 5000 bp independently predicted the risk of Richter's syndrome (5-year risk: 18.9 vs 6.4%, $P = 0.016$). Within CLL subsets defined by biological predictors, TL consistently identified patient subgroups harboring unfavorable prognosis. These results demonstrate that TL is a powerful independent predictor of multiple outcomes in CLL, and contributes to refine the prognostic assessment of this disease when utilized in combination with other prognostic markers. We thus believe that this prognostic biomarker has the potential for a more widespread use in CLL.
Leukemia (2009) **23**, 1062–1072; doi:10.1038/leu.2008.399; published online 2 April 2009
Keywords: chronic lymphocytic leukemia; telomere; prognosis; Richter's syndrome; kinetics

Introduction

Chronic lymphocytic leukemia (CLL) has a highly hetero-geneous clinical course: some patients have a normal or nearly normal life expectancy, whereas others shortly succumb to their disease.[1–4] In recent years, several biomarkers have been used for outcome prediction in this neoplasm. Currently, the most widely used biomarkers are cytogenetics, immunoglobulin heavy-chain variable gene mutation status (IGHV-MS) and expression of ZAP70 and CD38.[4–14] Despite the established prognostic value of these biomarkers, a stratification according to these parameters does not fully explain the heterogeneity of CLL. In addition, scant information is available on independent predictors of some critical events in CLL, such as clinicopatho-logical transformation to aggressive lymphoma, also known as

Richter's syndrome (RS).[15] Thus, further improvement of our current predictive tools would be advisable.

Telomeres are complex structures composed of repeated nucleotide sequences and by a number of proteins having both structural and regulatory functions. Telomeres have a major function ensuring genetic stability and regulating critical cellular functions, including proliferation and replicative senescence.[16–18] Several lines of evidence suggest that cancer cells have dysfunctional telomeres, often characterized by marked short-ening of the DNA component and upregulation of telomerase, possibly aimed at stabilizing critically shortened telomeric sequences.[19–22] Telomeric dysfunction has been recently docu-mented to be a prominent feature of CLL cells, which are characterized by major defects of telomere-related proteins.[23]

On the basis of these considerations, it is not surprising that telomere-related parameters are linked to outcome in CLL. A number of reports have shown that short telomeres and expression of h-tert, the catalytic subunit of telomerase, are poor prognostic factors in this neoplasm.[24–27] Although early studies postulated that telomere length (TL) was mostly acting as a surrogate marker for IGHV-MS, a number of subsequent reports indicated that in discordant cases TL performed better than IGHV-MS, suggesting that TL deserved further consideration as a prognostic biomarker in CLL.[28,29]

Despite these encouraging results, additional experience needs to be accumulated on TL as a prognostic marker in CLL. First, a larger patient sample is required to establish the prognostic role of TL fully, define the optimal cutoff value and confirm results in a blinded validation series. Second, the independent prognostic value of TL needs to be proven by multivariate Cox regression analysis not only for treatment-free survival (TFS), but also for overall survival (OS). Finally, the prognostic value of TL should be tested in the context of well-established CLL prognostic subgroups, to understand the outcome of cases with discordance between TL and other CLL predictors. These issues have been addressed on a CLL learning cohort and validated on an independent blinded cohort, overall accounting for 401 CLL patients. Also, we have tested TL as a risk factor of RS, an extremely severe event that most of the currently available biomarkers fail to predict.[15,30]

Patients and methods

Patient population and development of a blinded validation series
The patient sample of this study consisted of two large consecutive CLL cohorts (Table 1). The first cohort belonged

Table 1 Biological and clinical characteristics of the learning CLL series from the UT ($n = 191$) and the validation CLL series from the AAUEP ($n = 210$)

Characteristics at CLL diagnosis	University of Turin		Amedeo Avogadro University		P
Age (years)[a]	61	53–69	69	62–76	**<0.001**
Male	118/191	61.8%	118/210	56.2%	0.256
Binet stage					
A	129/191	67.5%	154/210	73.3%	0.437
B	31/191	16.2%	29/210	13.8%	
C	31/191	16.2%	27/210	12.9%	
Nodal areas ⩾3	NA	NA	37/210	17.6%	NA
Largest lymph node ⩾3 cm	NA	NA	29/210	13.8%	NA
Splenomegaly	NA	NA	41/210	19.5%	NA
Peripheral blood lymphocytes (× 10⁹ per liter)	NA	NA	11.7	6.3–20.0	NA
Hb (g/100 ml)	NA	NA	13.5	12.3–14.6	NA
Platelets (× 10⁹ per liter)	NA	NA	190	140–226	NA
Bone marrow lymphocytes (%)	NA	NA	40	25–70	NA
Diffuse bone marrow pattern	NA	NA	48/207	23.2%	NA
β-2-Microglobulin (mg/l)	NA	NA	2.5	1.8–3.5	NA
LDH (U/l)	NA	NA	350	296–430	NA
Alkaline phosphatase (U/l)	NA	NA	155	126–180	NA
Albumin (g/l)	NA	NA	42	39–45	NA
IGHV homology ⩾98%	78/191	40.8%	75/208	36.1%	0.327
FISH stratification					
del(13q14)/normal/+12	107/131	81.7%	164/201	82.0%	0.941
del(1q22–q23)/del(17p13)	24/131	18.3%	37/201	18.0%	
Number of FISH lesions >1	16/131	12.2%	37/201	18.4%	0.157
CD38 ⩾30%	59/150	39.3%	65/209	31.1%	0.106
ZAP70 ⩾20%	43/106	40.6%	54/177	30.5%	0.084
Telomere length (bp)[a]	6024	4566–7688	5959	4667–7002	0.343
Telomere length ⩽5000 bp	61/191	31.9%	62/210	29.5%	0.601

Abbreviations: CLL, chronic lymphocytic leukemia; FISH, fluorescence *in situ* hybridization; LDH, lactate dehydrogenase.
[a]Median and 25th–75th percentiles are reported for continuous variables.

to the Division of Hematology of the University of Turin (UT; $n = 191$) and was used as learning series. The second cohort belonged to the Division of Hematology of the Amedeo Avogadro University of Eastern Piedmont (AAUEP; $n = 210$) and was used as blinded validation series. Both series were updated for the analysis in February 2008. All patients provided informed consent in accordance with local institutional review board requirements and the Declaration of Helsinki Principles. In both series, diagnosis of CLL was based on National Cancer Institute (NCI) Working Group criteria and confirmed by a flow cytometry score >3 in all cases.[31,32] Also, in both series, patients were managed according to NCI Working Group guidelines.[32]

Median follow-up of patients from the UT learning series was 32 months. The following demographic and clinical variables were recorded for patients included in the UT learning series: date of diagnosis, age, sex, Binet stage, date of first-line treatment and date of death. The following biological variables were available for the UT learning series: TL, *IGHV* gene usage, homology to germ-line IGHV sequences (IGHV-MS) and presence of a complementarity-determining region (CDR) 3 belonging to published subsets of stereotyped B-cell receptors (BCR),[33] fluorescence *in situ* hybridization (FISH) karyotype for del(13q14), +12, del(17p13), del11(q22–q23), CD38 and ZAP70 expression.

Median follow-up of patients from the AAUEP blinded validation series was 54.2 months. The following demographic and clinical variables were recorded at presentation for patients included in the AAUEP validation series: date of diagnosis, age, sex, ECOG performance status, Rai stage, Binet stage, spleen size (cm below left costal margin), size of largest node (cm of the

largest transversal diameter), lymphocyte count, hemoglobin, platelet count, serum β-2-microglobulin (normal range 1.8–2.3 mg/100 ml), lactate dehydrogenase (LDH, normal range 200–450 U/l), alkaline phosphatase (normal range 90–360 U/l), albumin (normal range 34–48 g/l), immunoglobulin levels (normal ranges IgG 7–16 g/l, IgA 0.7–4 g/l, IgM 0.4–2.3 g/l), CD4+ lymphocyte count, presence of monoclonal component, direct antiglobulin test, percentage of bone marrow lympho-cytes, pattern of infiltration (diffuse vs non-diffuse) on bone marrow biopsy, date of first-line treatment according to NCI Working Group guidelines,[32] date of lymphocyte doubling, date of progression to a more advanced stage, date of transformation to RS (based on histology of lymph node or extranodal tissue biopsy in accordance to the World Health Organization *Classification of Tumors of the Haematopoietic and Lymphoid Tissues*)[34] and date of death. The following biological variables were available for the AAUEP validation series: TL, *IGHV* gene usage, homology to germ-line IGHV sequences (IGHV-MS) and presence of CDR3 belonging to published subsets of stereotyped BCR,[33] FISH karyotype for del(13q14), +12, del(17p13), del11(q22–q23), CD38 and ZAP70 expression.

All biological variables collected for both series were analyzed on fresh or cryopreserved peripheral blood mono-nuclear cells at the time of CLL diagnosis.

Normal B cells from a panel of 30 age-matched controls were used to compare TL of CLL versus normal cells.

Determination of TL
Peak TL was determined for the whole series by Southern blot analysis as reported elsewhere.[29,35] Peak TL was preferred to

mean TL because this approach allows accurate measurement of TL in the presence of up to 30% contaminating non-neoplastic cells, as documented by extensive cell dilution studies.[35] Briefly, 2 μg of genomic DNA was digested by mixing HinfI and RsaI (Roche Diagnostics, Mannheim, Germany). Telomere restriction fragments were separated by 0.8% agarose gel electrophoresis. Gels were transferred to a positively charged nylon membrane (Roche Diagnostics) and were UV cross-linked. Hybridization and detection were performed using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics), as previously described.[35] Membranes were scanned and analyzed with Kodak Digital Science 1D software (Scientific Imaging Systems, New Haven, CT, USA).

Data on TL were not available to clinicians and did not affect their therapeutic decisions. Because TL analysis was performed at UT, the AAUEP series was used as a blinded validation sample. All DNAs from the AAUEP series were anonymously sent to UT, without any information on clinical and biological features. The results were then sent back to AAUEP, where the final outcome analysis was performed.

IGVH analysis
Tumor-specific IGHV rearrangements were amplified starting from genomic DNA as described elsewhere.[29] IGHV sequences were aligned to ImMunoGeneTics directories (www.imgt.cines.fr), and considered mutated if homology to the corresponding germ-line gene was <98%. HCDR3-driven clustering was performed by converting all in-frame IGHV rearrangements into amino-acid sequences, and by aligning HCDR3 sequences by means of the multiple sequence alignment software ClustalW (www.ebi.ac.uk/clustalw/; EMBL-EBI, European Bioinformatics Institute).[36]

CD38 and ZAP70 expression by flow cytometry
A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used for flow cytometric analysis. Expression of CD38 and ZAP70 was analyzed as reported.[6,8,9] Cutoff points of 30 and 20% were used to define positivity for CD38 and ZAP70, respectively.

FISH analysis of chromosomal imbalances
Dual-color interphase FISH for del(13q14), +12, del(11q22–q23) and del(17p13) was performed using standard protocols. Briefly, probes (Vysis, London, UK) used for FISH analysis were: LSI13 and LSID13S319 for detection of del(13q14); CEP12 for detection of aneuploidy of chromosome 12; LSIp53 for detection of del(17p13) and LSIATM for detection of del(11q22–q23). Patients were classified as having del(13q14), +12, del(11q22–q23), del(17p13) and normal karyotype, according to the hierarchical stratification model proposed by Döhner et al.[5] Clinicians were aware of the FISH status of their patients and, for patients with NCI indications to treatment, this knowledge might have affected the choice of the therapeutic regimen adopted.

Statistical analysis
The series from UT was used as learning cohort in whom investigations aimed at (1) defining the best cutoff for TL and (2) testing TL at the selected cutoff as an independent prognosticator for OS and TFS of CLL. The series from AAUEP was used as validation cohort in whom investigations aimed at (1) confirming TL at the selected cutoff as an independent prognosticator for OS and TFS, (2) testing TL at the selected cutoff as an independent predictor of RS transformation.

The cutoff point for TL was selected according to receiver operating characteristic (ROC) analysis using death as state variable, and Youden's index calculated utilizing the sensitivity and specificity derived from ROC analysis.

Overall survival was measured from the date of CLL diagnosis to the date of last follow-up or death. TFS was measured from diagnosis to first-line treatment, death or last follow-up. All patients underwent first treatment at the time of documentation of progressive and symptomatic disease according to NCI Working Group guidelines.[32] Time to lymphocyte doubling was measured from diagnosis to lymphocyte doubling, death or last follow-up. As previously reported,[8] time to progression to a more advanced stage was defined as the time elapsed from diagnosis to progression to a more advance stage, first-line treatment according to NCI Working Group guidelines, death or last follow-up. Date of transformation was defined as the date of the biopsy showing that RS transformation occurred. Time to RS transformation was measured from date of CLL diagnosis to date of transformation, death or last follow-up.

Categorical variables were compared by χ^2-test and Fisher's exact test when appropriate. Continuous variables were compared by Mann–Whitney test. Survival analysis was performed by Kaplan–Meier method, using log-rank statistics to test for significant associations.[37] Cox proportional hazard regression was used to build a multivariate model.[38]

Stratification of continuous clinical variables was based on the best predictive cutoff value or usual limit of normal. All statistical tests were two sided. Statistical significance was defined as P-value <0.05. The analysis was performed with the Statistical Package for the Social Sciences (SPSS) software version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Identification of the best TL cutoff for prediction of TFS and OS in CLL
Median TL of the CLL patients in the learning series was 6311 bp and was similar to that reported in other CLL series.[25,39] The median TL observed in the CLL learning series was significantly lower compared to the median TL observed in normal B cells, which showed a median TL of 7396 bp (P<0.001; data not shown) in CLL cells was not associated with age or sex (data not shown; P = 0.20 and 0.161, respectively). The best TL cutoff point was generated in the learning CLL series from UT (n = 191) by ROC analysis and Youden's index using death as state variable. Accordingly, the best cutoff point for TL was 5000 bp (Supplementary Figure 1S). On the basis of selected cutoff, 61/191 (31.9%) CLL patients of the learning series harbored TL ⩽5000 bp. Because a sizeable fraction of cases in the learning cohort lacked data on FISH karyotype (60/191, 31.4%), and expression of CD38 (41/191, 21.4%) and ZAP70 (85/191, 44.5%), these variables were not included in the univariate and multivariate models applied to the learning series.

After a median follow-up of 32 months from diagnosis, 90/191 patients of the learning series had progressed to symptomatic disease requiring treatment according to NCI Working Group guidelines. Median TFS was 49.8 months (95% CI 31.9–67.6). Univariate analysis identified TL ⩽5000 bp as a predictor of reduced TFS (TL ⩽5000 bp: 24.6 months vs TL >5000 bp: 73.0 months; P = 0.001) (Figure 1a; Supplementary Table 1S). Other predictors of reduced TFS by univariate analysis were
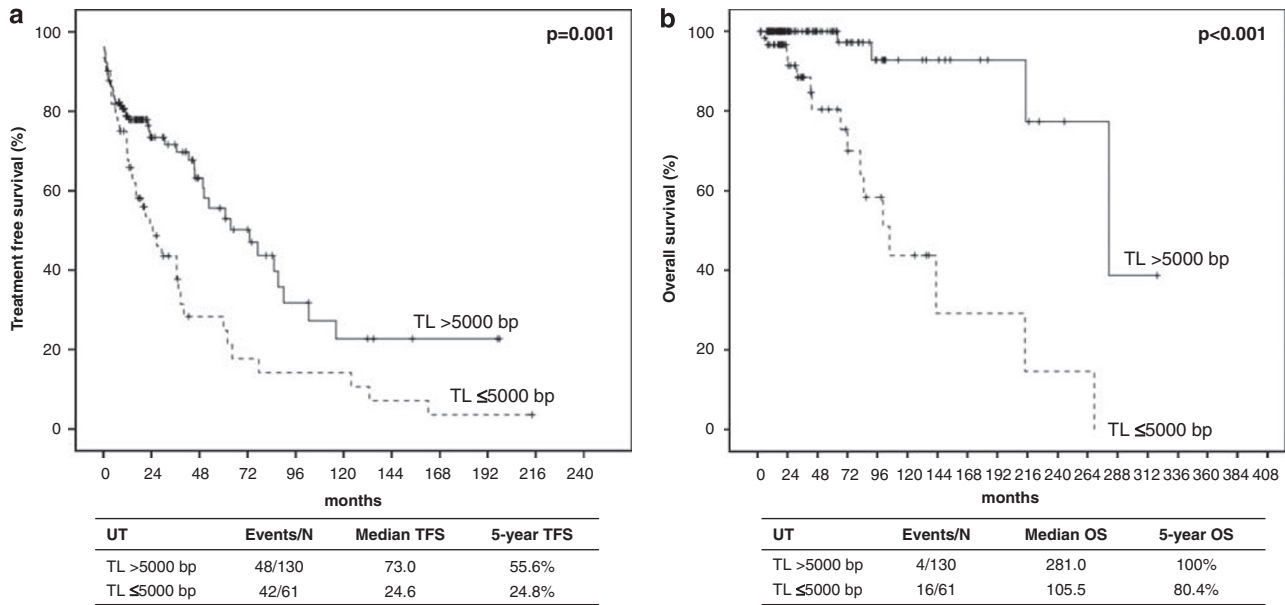
Figure 1 Performance of telomere length (TL) in the UT learning series. TL is predictive of treatment-free survival (TFS; **a**) and overall survival (OS; **b**) in the UT learning series of 191 patients.

UT	Events/N	Median TFS	5-year TFS
TL >5000 bp	48/130	73.0	55.6%
TL ≤5000 bp	42/61	24.6	24.8%

UT	Events/N	Median OS	5-year OS
TL >5000 bp	4/130	281.0	100%
TL ≤5000 bp	16/61	105.5	80.4%

advanced Binet stage and IGHV homology ≥98% (Supplementary Table 1S) ($P < 0.05$ in all instances). Multivariate analysis selected TL ≤5000 bp (hazard ratio (HR) 1.77; 95% CI 1.16–2.69; $P = 0.008$) as an independent predictor of reduced TFS along with advanced Binet stage (HR 3.97; 95% CI 2.57–6.13; $P < 0.001$). Advanced Binet stage at diagnosis may be *per se* an indication to first-line treatment. Therefore, multivariate analysis for TFS was also assessed after removing Binet B-C stage from covariates. TL ≤5000 bp was selected in this model as the sole independent predictor of reduced TFS (HR 2.02; $P = 0.001$; Table 2).

After a median follow-up of 32 months from diagnosis, 20/191 patients of the learning series died. Median OS was 269.3 months (95% CI 212.2–337.4). According to univariate analysis, TL ≤5000 bp was associated with poor OS (TL ≤5000 bp: 105.5 months vs TL >5000 bp: 281.0 months; $P < 0.001$) (Figure 1b; Supplementary Table 2S). Other variables predicting reduced OS by univariate analysis were advanced Binet stage and IGHV homology ≥98% ($P < 0.05$ in all instances; Supplementary Table 2S). Multivariate analysis identified TL ≤5000 bp (HR 13.31; $P < 0.001$) as an independent predictor of reduced OS along with advanced Binet stage (HR 5.34; $P = 0.002$; Table 2).

Validation of short TL at the cutoff of 5000 bp as an independent predictor of progressive CLL according to NCI criteria
The prognostic value of short TL at the cutoff of ≤5000 bp as a predictor of TFS was validated in the independent CLL series ($n = 210$) from AAUEP. When comparing clinical and biological features of the learning and validation series, the two series were similar in terms of TL, Binet stage distribution and prevalence of unfavorable biological prognosticators (Table 1). The only significant difference between the two series was patient median age ($P < 0.001$; Table 1). Again, also in the AAUEP validation series, TL in CLL cells is not associated with age or sex (data not shown; $P = 0.28$ and 0.24, respectively). The AAUEP validation series was provided with additional variables at

diagnosis that were not available for the learning series. Also, only a low number of cases in the validation series lacked data on FISH karyotype (9/210, 4.2%), CD38 (1/210, 0.4%) and ZAP70 (33/210, 15.7%) expression (Table 1). All these parameters were thus included in univariate and multivariate analyses for this series.

On the basis of selected cutoff, 62/210 (29.5%) CLL patients of the validation series harbored TL ≤5000 bp. After a median follow-up of 54.2 months from diagnosis, 95/210 patients progressed to symptomatic disease requiring treatment according to NCI guidelines. Median TFS was 67.4 months (95% CI 37.0–97.8). Univariate analysis identified TL ≤5000 bp as a predictor of reduced TFS (TL ≤5000 bp: 15.2 months vs TL >5000 bp: 130.8 months; $P < 0.001$; Figure 2a). Other predictors of reduced TFS by univariate analysis are listed in Supplementary Table 3S ($P < 0.05$ in all instances). Multivariate analysis selected TL ≤5000 bp (HR 1.85; 95% CI 1.13–3.03; $P = 0.013$) as an independent predictor of reduced TFS, along with advanced Binet stage (HR 6.19; 95% CI 3.55–10.79; $P < 0.001$), peripheral blood lymphocytes (HR 3.10; 95% CI 1.87–5.12; $P < 0.001$), CD38 expression (HR 2.76; 95% CI 1.65–4.60; $P < 0.001$) and β-2-microglobulin levels (HR 2.22; 95% CI 1.30–3.79; $P = 0.003$).

For the reasons already outlined for the learning series, multivariate analysis for TFS in the validation series was also assessed after removing Binet B-C stage from covariates. This analysis confirmed TL ≤5000 bp as an independent predictor of reduced TFS (HR 2.14; $P = 0.002$) along with peripheral blood lymphocytes (HR 3.27; $P < 0.001$), CD38 expression (HR 2.68; $P < 0.001$), β-2-microglobulin levels (HR 2.53; $P = 0.001$) and unfavorable FISH karyotype (HR 1.72; $P = 0.046$) (Table 2).

Short TL at the cutoff of 5000 bp associates with markers of proliferation and rapid kinetics of CLL
At CLL diagnosis, TL ≤5000 bp was associated with clinical markers of leukemic disease, such as Binet C stage ($P = 0.020$), lymphocyte count ($P < 0.001$), bone marrow pattern (< 0.001) and extent of infiltration ($P < 0.001$), anemia ($P < 0.001$) and

Table 2 Multivariate analysis for OS, TFS, RS transformation and recurrent infections

	HR	95% CI	P
TFS UT[a]			
Telomere length ⩽5000 bp	2.02	1.33–3.07	0.001
TFS AAUEP[b]			
Peripheral blood lymphocytes >20 × 10⁹ per liter	3.27	1.97–5.42	<0.001
CD38 ⩾30%	2.68	1.64–4.39	<0.001
β-2-Microglobulin >2.5 mg/l	2.53	1.49–4.29	0.001
del(11q22–q23)/del(17p13)	1.72	1.01–2.95	0.046
Telomere length ⩽5000 bp	2.14	1.32–3.47	0.002
OS UT[c]			
Binet B-C	5.34	1.85–15.41	0.002
Telomere length ⩽5000 bp	13.31	3.76–47.05	<0.001
OS AAUEP[d]			
Age >65 years	4.02	1.85–8.74	<0.001
Binet B-C	2.14	1.15–3.96	0.015
Telomere length ⩽5000 bp	1.91	1.04–3.52	0.035
RS transformation AAUEP[e]			
Largest lymph node ⩾3 cm	7.46	2.65–20.96	<0.001
No del13q14	4.18	1.15–15.14	0.029
Telomere length ⩽5000 bp	2.70	1.04–7.40	0.048

Abbreviations: AAUEP, Amedeo Avogadro University of Eastern Piedmont; HR, hazard ratio; 95% CI, 95% confidence interval; OS, overall survival; P, P-value calculated by Cox analysis; RS, Richter's syndrome; TFS, treatment-free survival; UT, University of Turin.
Only significant covariates for each outcome are listed above.
[a]Covariates that entered the analysis were telomere length, IGHV gene homology, age, sex and Binet stage.
[b]Covariates that entered the analysis were telomere length, IGHV gene homology, unfavorable FISH karyotype, CD38 expression, ZAP70 expression, Binet stage, peripheral blood lymphocytes and β-2-microglobulin levels.
[c]Covariates that entered the analysis were telomere length, IGHV gene homology and Binet stage.
[d]Covariates that entered the analysis were telomere length, IGHV gene homology, unfavorable FISH karyotype, age, Binet stage and β-2-microglobulin levels.
[e]Covariates that entered the analysis were telomere length, del(13q14) and large lymph node size.

splenomegaly ($P=0.003$) (Supplementary Table 4S; Figure 3). Moreover, at CLL diagnosis, TL⩽5000 bp was associated with clinical markers of proliferation, such as LDH ($P=0.010$) and β-2-microglobulin levels ($P=0.015$). On the contrary, TL⩽5000 bp was not associated with clinical markers of solid disease, such as Binet B stage, lymph node size ($P=0.285$) or extent of nodal involvement ($P=0.222$). Among biological variables at diagnosis, TL⩽5000 bp was associated with IGHV homology ⩾98% ($P<0.001$), unfavorable FISH karyotype ($P<0.001$), complex FISH karyotype as depicted by higher number of FISH lesions ($P=0.018$) and expression of CD38 ($P<0.001$) and ZAP70 ($P=0.038$).

The association between TL⩽5000 bp and markers of proliferation prompted investigations on the impact of TL⩽5000 bp on disease kinetics. TFS was used as the primary end point of progressive disease according to NCI criteria (see above). We also reasoned that TFS may not capture all events relevant to CLL kinetics, such as lymphocyte doubling and progression to a more advanced stage, because a fraction of these events does not meet NCI criteria for progressive disease requiring treatment. Therefore, lymphocyte doubling and time to progression to a more advanced stage were used as additional end points of disease kinetics. Univariate log-rank analysis identified TL⩽5000 bp as a risk factor of reduced time to lymphocyte doubling and of reduced time to progression to a more advanced stage (Figures 2b and c). Median time to lymphocyte doubling for patients with TL⩽5000 bp was 10.5 months (95% CI 5.5–15.5) compared to 41.4 months (95% CI 30.8–51.9) for patients with TL>5000 bp ($P<0.001$). Also, median time to progression to a more advanced stage for

patients with TL⩽5000 bp was 10.5 months (95% CI 5.3–15.8), compared to 49.8 months (95% CI 32.9–66.6) for patients with TL>5000 bp ($P<0.001$).

Validation of short TL at the cutoff of 5000 bp as an independent predictor of overall survival in CLL
The prognostic value of short TL at the cutoff of ⩽5000 bp as a predictor of OS was validated in the independent series ($n=210$) from AAUEP. At the time of the analysis, 51/210 patients of the validation series had died and median OS was 144 months (95% CI 107.6–180.4). According to univariate analysis, TL⩽5000 bp was associated with poor OS (TL⩽5000 bp: 79.8 months vs TL>5000 bp: not reached; $P<0.001$; Figure 2d). Other variables predicting reduced OS by univariate analysis were age, Binet stage, β-2-microglobulin levels, unfavorable FISH karyotype and ZAP70 expression ($P<0.05$ in all instances; Supplementary Table 5S). Multivariate analysis identified TL⩽5000 bp (HR 1.91; $P=0.035$) as an independent predictor of reduced OS along with age >65 years (HR 4.02; $P<0.001$) and advanced Binet stage (HR 2.14; $P=0.015$) (Table 2).

TL is an independent predictor of Richter's syndrome transformation
The AAUEP CLL series ($n=210$) was managed according to a uniform biopsy policy for detection of RS transformation.[15] Accordingly, 17/210 CLL patients had transformed to RS, accounting for a 5-year cumulative risk of transformation of
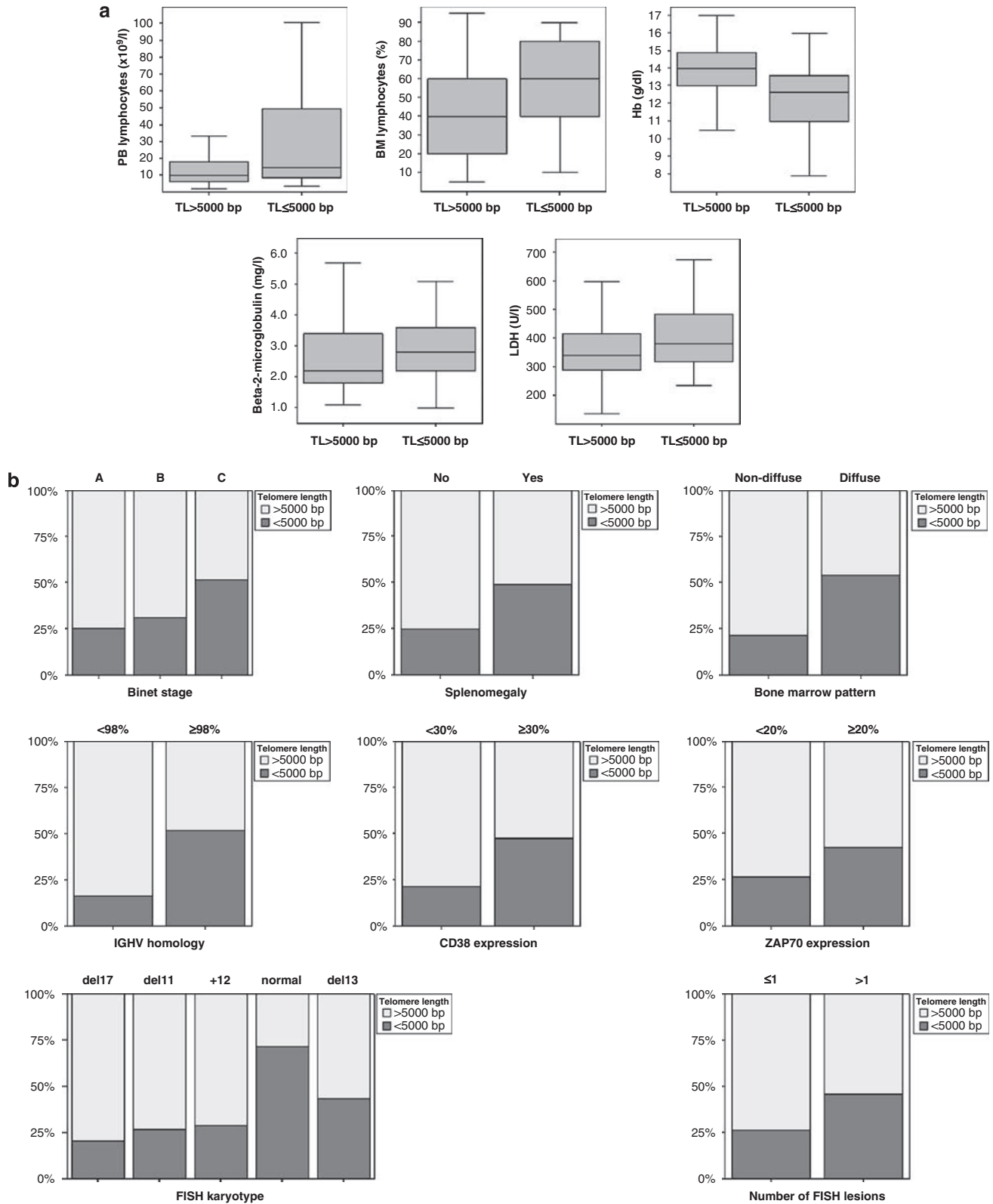
Figure 2 Performance of telomere length (TL) in the AAUEP blinded validation series. TL is predictive of treatment-free survival (TFS; **a**), time to lymphocyte doubling (**b**).

10.0% (95% CI 5.0–15.0%). This allowed the assessment of short TL at the cutoff of $\leqslant 5000$ bp as a predictor of RS transformation. By univariate analysis, TL $\leqslant 5000$ bp was associated with an increased risk of RS (5-year risk for TL $\leqslant 5000$ bp:

18.9% vs 5-year risk for TL > 5000 bp: 6.4%; $P = 0.016$) (Supplementary Table 6S; Figure 4). Large lymph node size and absence of del(13q14) at diagnosis have been previously identified as independent predictors of RS transformation in the
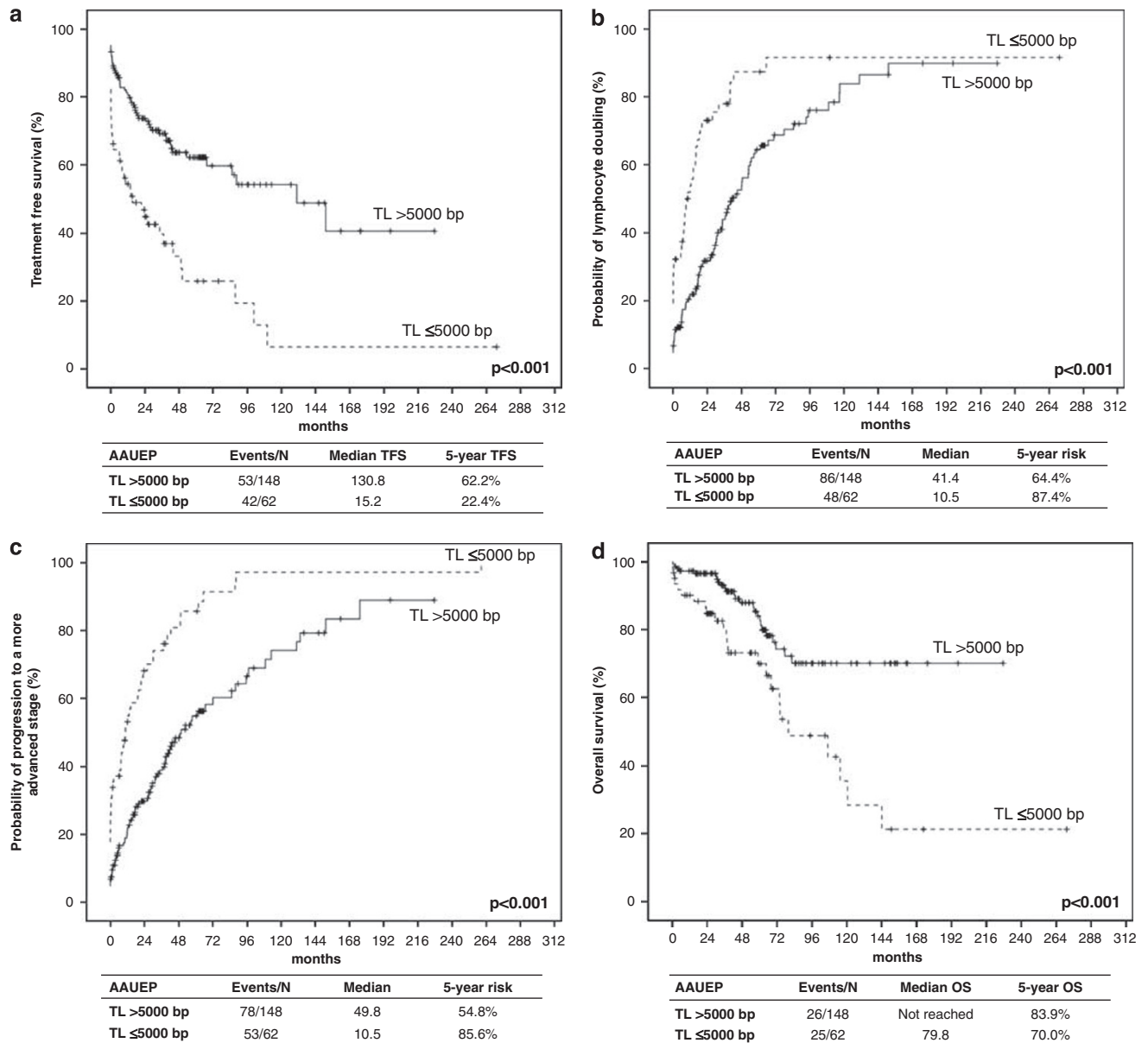
Figure 3 Significant associations between telomere length (TL) and clinical markers of chronic lymphocytic leukemia (CLL) in the AAEUP blinded validation series. (**a**, **b**) Continuous variables, (**c**, **d**) categorical variables.

AAUEP series.[15] On these bases, lymph node size and del(13q14) entered multivariate analysis along with TL.[15] Multivariate analysis selected TL $\leqslant$ 5000 bp as an independent predictor of RS transformation (HR 2.70; $P = 0.048$), along with lymph node size (HR 7.46; $P < 0.001$) and absence of del(13q14) (HR 4.18; $P = 0.029$) (Table 2).

TL refines the prognostication of CLL subgroups identified by conventional CLL risk factors

The learning and the validation series were combined into a single group of CLL patients ($n = 401$) for further detailed analysis of the interactions between TL and other CLL risk factors. Of note, a consistent proportion of patients showed a short TL in the presence of favorable prognosticators, and *vice versa*. These cases were defined as discordant and accounted for 147/401 (36.6%) patients for TL vs Binet stage, 105/399 (26.3%)

for TL versus IGHV-MS, 103/331 (31.1%) for TL vs cytogenetics, 108/283 (38.1%) for TL versus ZAP70 expression and 130/359 (36.2%) for TL versus CD38 expression. Short TL in our series identified a CLL subgroup that displayed rapid disease progression and reduced survival despite being characterized at diagnosis by favorable predictors. This observation is reproducible in all favorable risk categories.

In the whole CLL series, reduced TFS was predicted by TL $\leqslant$ 5000 bp ($P < 0.001$) along with advanced Binet stage ($P < 0.001$), IGHV homology $\geqslant$ 98% ($P < 0.001$), unfavorable FISH ($P < 0.001$), CD38 ($P < 0.001$) and ZAP70 ($P = 0.001$) expression. Interaction analysis showed that TL $\leqslant$ 5000 bp segregated a group of CLL displaying reduced TFS despite being characterized by Binet stage A ($P < 0.001$), IGHV homology < 98% ($P < 0.001$), favorable FISH karyotype ($P < 0.001$), CD38 < 30% ($P < 0.001$) or ZAP70 < 20% ($P < 0.001$) (Supplementary Table 7S; Figures 5a–c).
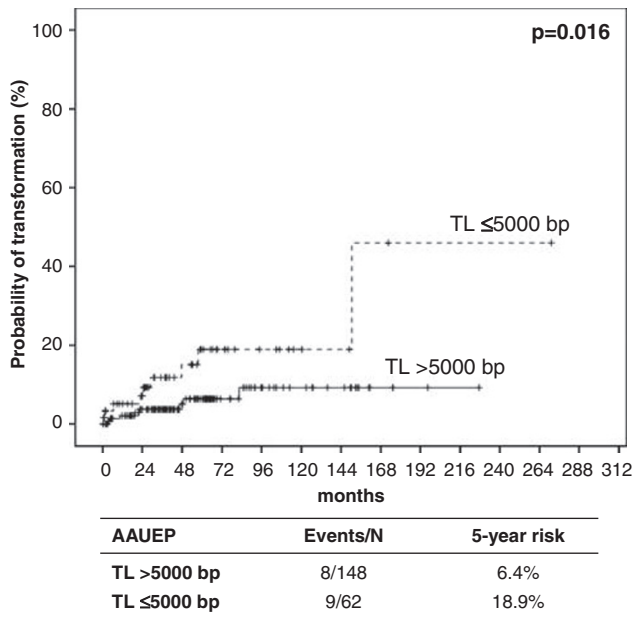
Figure 4 Telomere length (TL) as a predictor for transformation to Richter's syndrome (RS). The 5-year cumulative risk of transformation was 18.9% in patients with TL ⩽ 5000 bp, as opposed to 6.4% for those with long telomeres.

AAUEP	Events/N	5-year risk
TL >5000 bp	8/148	6.4%
TL ⩽5000 bp	9/62	18.9%

In the whole CLL series, reduced OS was predicted by TL ⩽ 5000 bp ($P<0.001$) along with age >65 years ($P<0.001$), advanced Binet stage ($P<0.001$), IGHV homology $\geqslant 98\%$ ($P=0.001$) and unfavorable FISH karyotype ($P=0.001$). Regarding OS, TL ⩽ 5000 bp segregated a group of CLL displaying reduced OS despite being characterized by age $\leqslant 65$ years ($P<0.001$), Binet stage A ($P=0.001$) or IGHV homology $<98\%$ ($P<0.001$) (Supplementary Table 8S; Figures 5d–f). Also, among CLL patients carrying unfavorable prognosticators, TL >5000 bp identified a CLL subgroup displaying longer OS despite being characterized by age >65 years ($P=0.004$), Binet stage B-C ($P<0.001$) or unfavorable cytogenetics ($P=0.001$) (Supplementary Table 8S; Figures 5d–f).

TL in CLL with and without stereotyped B-cell receptors
We tested whether TL differed in CLL with and without a stereotyped BCR. Our series included 89/336 (26.5%) evaluable patients who showed a stereotyped BCR.[36] Patients with a stereotyped BCR carried a shorter TL (TL ⩽ 5000 bp: 42/89, 47.7%) compared to patients without stereotyped BCR (TL ⩽ 5000 bp: 56/247, 27.1%) ($P=0.001$). No differences were found by comparing stereotyped and nonstereotyped CLL in the context of IGHV-mutated or -unmutated CLL ($P>0.05$ for all comparisons), suggesting that TL differences between stereotyped and nonstereotyped cases should be ascribed to IGHV-MS. The patient sample was not large enough to allow a correlation of TL with specific stereotyped BCR clusters considered independently.

Discussion

We report the largest available analysis of the prognostic role of TL in CLL. The prognostic impact of TL and optimal choice of cutoff value have been investigated on a learning series, and validated on an independent blinded series. Our results show

that (1) 5000 bp is the best cutoff point for the definition of short TL; (2) short TL associates with markers of proliferation and rapid disease kinetics; (3) short TL is an independent predictor of all CLL outcomes including TFS, OS and RS transformation and (4) TL contributes to refine the prognostic assessment of CLL when used in combination with other prognostic markers.

Short TL is a prominent feature of several neoplasms and has been linked to outcome in several cancer types.[40–44] Short telomeres are not only a by-product of tumor development. In fact, telomeric loss could be instrumental in maximizing the malignant potential of tumor cells by boosting genetic instability and by interfering epigenetically with genes located in sub-telomeric regions.[22,45,46] Also, the effort of preserving telomeres from critical shortening can lead to upregulation of telomerase and deregulation of other telomere-related proteins that might contribute to neoplastic growth by multiple mechanisms.[22]

On the basis of these premises, it is not surprising that TL bears independent prognostic value in CLL.[28,29] Previous studies have shown that TL associates with IGHV homology, high-risk cytogenetics and poor TFS.[25,26] Subsequent reports outlined the existence of patients in which discordance exists between TL and IGHV-MS and suggested a superior predictive value of TL.[28,29] Finally, Roos *et al.*[47] were able to link TL with poor cytogenetics in a series of 152 patients, including samples collected both at diagnosis and after treatment. Notably, the independent effect of TL on OS was not demonstrated in any of the previously published reports. Our report adds further evidence for consideration, including the identification and validation of a cutoff point for the definition of short TL, the demonstration that TL predicts survival and RS transformation in a manner that is independent of conventional clinical and biological parameters, and the contribution of TL to refining CLL prognostic categories.

Our strategy included the use of two institutional databases (UT and AAUEP) used as learning and blinded validation series. This two-step approach is considered optimal for successful identification of powerful and reproducible prognostic markers suitable for clinical use. This same validation procedure used for TL is commonly used to corroborate clinically and/or biologically based prognostic indicators and has been used also in the CLL field in a recent analysis comparing the relative value of CD38, ZAP70 and VHMS.[47–49]

The identification and validation of a robust cutoff point is a prerequisite for widespread application of any biomarker. Previous studies in CLL used the median or the mean value of TL, ranging from 4300 to 6000 bp among series, to perform survival comparisons.[24,25,28,47] With the aim of defining a highly reproducible cutoff, we used a stringent statistical approach, that is, ROC analysis and Youden's index for cutoff identification, followed by validation in an independent series. The 5000 bp cutoff identified by this strategy was confirmed as an outcome predictor in both series analyzed.

Short TL is an independent predictor of all major CLL outcomes, including TFS, OS and transformation to RS, both in the learning and in the validation series. A potential explanation of the poor outcome characterizing CLL with short TL may be derived from the rapid kinetics of progression observed in this subgroup of patients, as documented by short time to lymphocyte doubling and time to progression to a more advanced stage. The association between TL and OS is of particular relevance, as it is independent of all conventional CLL predictors, either clinical or biological. The ability of predicting RS transformation is another important feature of TL. RS is a severe event occurring in 10–15% of CLL patients.[15,50] Very few studies have investigated the features that at CLL diagnosis may
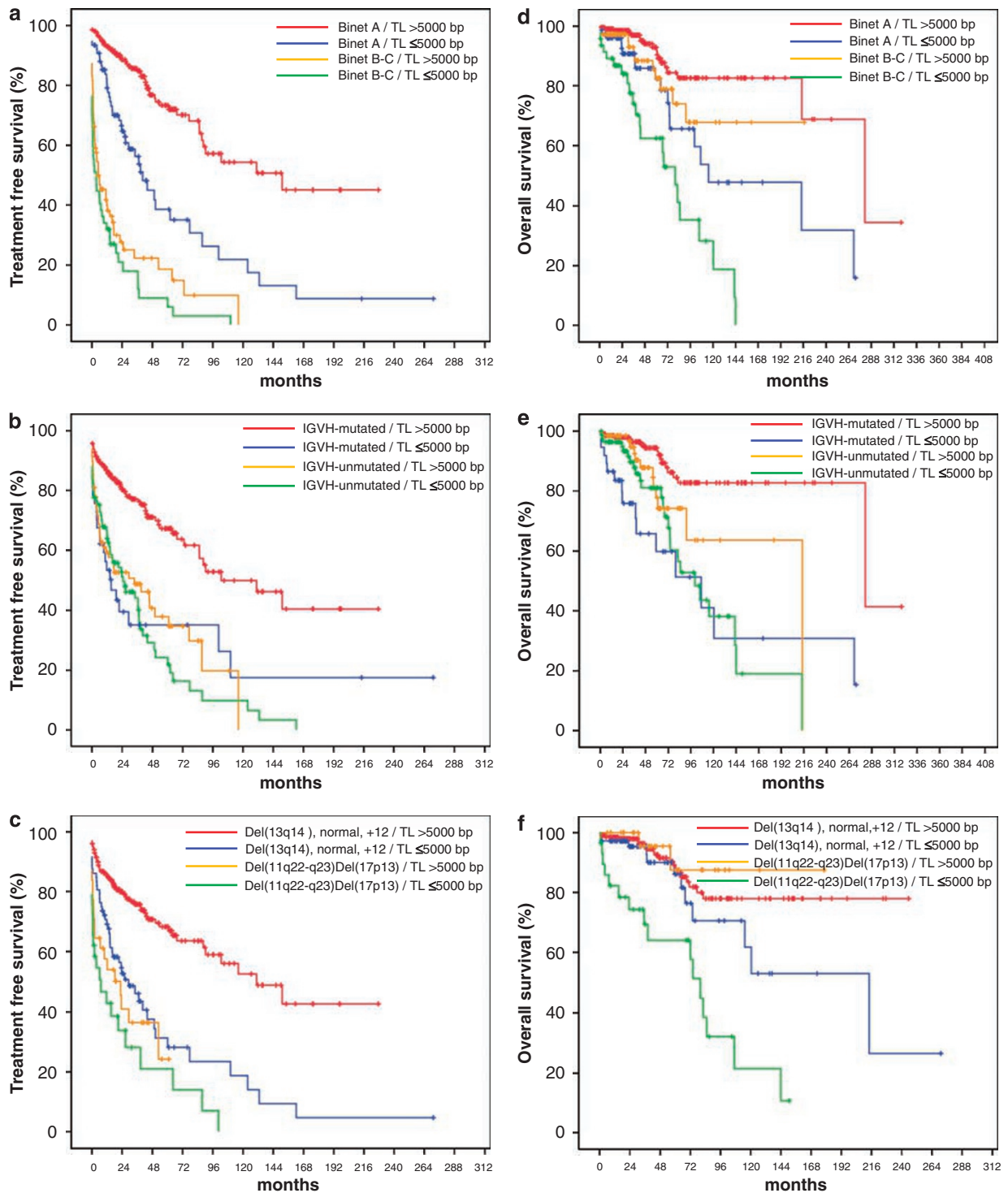
Figure 5 Paired analysis of telomere length (TL) with Binet stage, immunoglobulin heavy-chain variable gene mutation status (IGHV-MS) and fluorescence *in situ* hybridization (FISH) karyotype. Patients with TL<5000 bp display reduced treatment-free survival (TFS) despite being characterized by Binet stage A (**a**), IGHV homology <98% (**b**) and favorable FISH karyotype (**c**). TL refines the overall survival (OS) of patients classified according to Binet stage (**d**), IGHV homology (**e**) and FISH (**f**).

herald the subsequent development of RS. Analysis of the risk of RS transformation was allowed by the strict and uniform biopsy policy adopted at AAUEP. This analysis shows that long telomeres, along with del(13p14), are a major biological protector against RS, further stressing the indolent nature of CLL bearing TL>5000 bp.

Given the increasing availability of biological CLL prognosticators, novel molecular risk factors should provide information that is useful for refining the prognostication of risk categories identified by already existing markers. In the case of TL, the relevance of this issue is documented by the frequency of discordant cases (approximating 30%), exemplified by patients

harboring short TL in the presence of a favorable asset of conventional markers or *vice versa*. We have addressed the issue of discordant cases by investigating interactions of TL with conventional clinical and biological risk factors. Short TL in our series identified a CLL subgroup that displayed rapid disease progression and reduced survival despite being characterized at diagnosis by favorable predictors, including IGHV homology, FISH and Binet stage. Conversely, TL > 5000 bp softens the unfavorable prognosis of patients with high-risk cytogenetics, that is, del(11q22–q23) and del(17p13). Consistently, CLL patients with del(11q22–q23) or del(17p13) but harboring a TL > 5000 bp show an OS that is comparable to that of cases without these cytogenetic abnormalities.

Although a large bulk of data on the prognostic role of TL at diagnosis have been generated, a large longitudinal analysis of TL at different time points is still lacking. Some preliminary findings indicate that modest variations of TL exist over prolonged periods of time, particularly in IGHV-unmutated cases.[25] However, given the long natural history of CLL, a large patient series with a very prolonged follow-up is required for a conclusive assessment of the potential interest for prognostic discrimination of longitudinal variation of TL.

One potential drawback of TL is the presence of different methods for the analysis. Previous studies have shown good concordance between the approaches most commonly used, for example, Southern blot and real-time PCR.[28] Concordance of results observed with different methods further adds to the reliability and potential for wide applicability of TL analysis.[28] Nevertheless, a multicenter standardization effort involving multiple laboratories from different countries would be advisable in the future for harmonization of TL studies. In particular, a direct comparison between Southern blot, real-time PCR and flow cytometry–FISH analyses would be advisable as well as a careful analysis of factors that might generate intercenter variability. Such efforts may be justified in view of the fact that TL independently recapitulates all major CLL outcomes and, in addition, adds novel prognostic information when used in combination with existing biological markers.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)