**DIFFERENT TIME-DEPENDENT CHANGES OF RISK FOR EVOLUTION IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH MUTATED OR UNMUTATED ANTIGEN B-RECEPTORS**

**Running title:** Over-time risk for chronic lymphocytic leukemiaevolution

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Chronic lymphocytic leukemia (CLL) displays remarkable clinical heterogeneity, likely attributed to the underlying biological diversity(1). This claim is supported by the fact that certain immunogenetic and/or genomic features identify subgroups of CLL patients with distinct prognosis and outcome(2-4). Indeed, determination of the somatic hypermutation (SHM) status of the immunoglobulin heavy variable (IGHV) genes expressed by the clonotypic B cell receptor (BcR) and screening for aberrations οf the *TP53* gene are nowadays considered essential for clinical decision making(5). A cautionary note appears warranted when utilizing biomarkers, where the prognosis is usually assessed assuming stable predictability over the disease course; this hypothesis, however, is often unrealistic as it concerns genomic aberrations(6, 7). Therefore, arguably, the prognostic power of a given biomarker may, instead, heavily depend on the time distance from diagnosis.

To address this issue we investigated in early-stage CLL patients the impact over-time of SHM within the IGHV genes, i.e. the segregation into mutated (M-CLL) and unmutated CLL (U-CLL), on the evolution of risk for CLL progression and need of treatment. Our analysis was based on hazard curves instead of Kaplan–Meier survival curves, which represent, respectively, the “instant” risk for the event at each time-point instead of the cumulative risk(8, 9).

Overall, 1900 early-stage, Binet A CLL patients from 10 European institutions diagnosed according to the 2008 iwCLL criteria(10) were included in this retrospective study (summary of patient characteristics: Supplemental Table 1). Ethical approval was granted by the local review committees and informed consent was collected according to the Helsinki Declaration.

Fluorescence *in situ* hybridization (FISH) was performed in 1476/1900 (77.7%) cases using probes for the 13q14, 11q22, 17p13 regions and trisomy 12; results were interpreted following Döhner’s hierarchical model(11). Genes analyzed for mutations included *TP53* (exons 4-10, n=1186/1900, 62.4%), *SF3B1* (exons 14-16, n=1166/1900, 61.4%) and *NOTCH1* (entire exon 34 or targeted analysis for del7544-45/p.P2514Rfs\*4, n=1691/1900, 89%). Sequence analysis of IGHV/IGHD/IGHJ rearrangements was performed in all cases as described(12). All FISH, gene mutation screens and IG gene sequencing studies were performed once before the administration of any treatment; in 1702/1900 (90%) cases, these tests were performed within the first year from diagnosis.

In order to assess the risk for disease progression, we evaluated the time-to-first-treatment (TTFT) from diagnosis within different genomic subgroups, with risk evolution over-time represented by a hazard curve. Smoothed estimates of the hazard curve were computed separately for M-CLL and U-CLL, based on a non-parametric methodology (“bshazard” package)(13).

To compare the evolution pattern of hazard curves for each subgroup, we investigated over-time both their differences and ratios. Years 5, 10 and 15 after diagnosis were considered as landmark time-points for over-time comparison. The distributions of hazard differences were statistically compared between consecutive 5-year intervals to assess the evolution over-time (trend) of the distance between the hazard curves of the M-CLL and U-CLL patients. P-values less than 0.05 might indicate convergence or divergence of the curves within consecutive 5-year intervals. Regarding the hazard ratios for M-CLL and U-CLL, the proportional hazards assumption was checked. Moreover, a method able to identify the break points in the hazard was applied (“RPEXE.RPEXT” package)(14). The analysis was performed with R. Details about the statistical methodology are provided in Supplemental Material.

Based on the SHM status, 1224 (64.4%) and 676 (35.6%) patients were classified as M-CLL and U-CLL, respectively. The over-time risk for evolution was evaluated with SHM status as a reference using hazard plots in: (i) the entire cohort, (ii) cases with *TP53* aberrations (*TP53*abn: del(17p) and/or *TP53* mutations), (iii) cases carrying del(11q) with no *TP53*abn (del(11q), non *TP53*abn)), (iv) cases carrying +12 with no *TP53*abn (+12, non*TP53*abn), (v) cases carrying isolated del(13q) or normal FISH according to the Döhner model(11) (del(13q)/normal FISH), (vi) *NOTCH1* mutations, and (vii) *SF3B1* mutations.

In both the entire cohort and *TP53*abn patients, M-CLL exhibited gradual risk decrease over-time (Figure 1B,1D). In contrast, in U-CLL, a constant decrease was observed in the entire cohort, while in *TP53*abn cases the hazard curve initially decreased until the fifth year and then started to increase, indicating intensification of the risk for progression after the fifth year (Figure 1B,1D). Notably, the survival plots (Figure 1A,1C) failed to highlight any difference regarding the over-time risk between M-CLL and U-CLL and exhibited a similar behavior with slowly increasing distance between the M-CLL and U-CLL survival curves over-time.

In the remaining cases (Supplemental Figures 1-5), the M-CLL hazard curve slowly decreased except for del(11q) patients. In U-CLL, there was a wide range for hazard evolution: from decrease, such as for patients with del(13q)/normal FISH), del(11q), and *NOTCH1* mutations; to almost stable hazard over-time (*SF3B1* mutant patients). Interestingly, +12 patients showed a risk evolution similar to cases with *TP53*abn.

Regarding the distribution of hazard differences, significant differences were found in the entire cohort between M-CLL and U-CLL in all consecutive pairs of five-year intervals with p[0,5]Vs(5,10]=0.006, p(5,10]Vs(10,15]=0.009, and p(10,15]Vs(15,20]=0.009 (Supplemental Figure 7A) reflecting a statistically significant decrease of the distance between the two hazard curves in all pairwise comparisons. The same evolution rule was followed by the del(13q)/normal-FISH patients as well as patients carrying del(11q) or *NOTCH1* mutations (Supplemental Figures 7D,7B,7E). For patients with *SF3B1* mutations, the distance between the two curves remained almost stable, p[0,5]Vs(5,10]=0.465 (Supplemental Figure 7F). In sharp contrast, within *TP53*abn patients, the distance between the hazard curves for M-CLL and U-CLL increased significantly after the 5th year with p[0,5]Vs(5,10]=0.006 (Figure 2A). Similarly, in +12 patients, the U-CLL hazard curve constantly increased from diagnosis, with p[0,5]Vs(5,10]=0.006 (Supplemental Figure 7C).

Next, we tested the proportional hazards assumption (see Supplemental Table 2, Supplemental Figure 9) to test whether the hazard ratio between an U-CLL and an M-CLL patient depended on time. The assumption was rejected only for the *TP53*abn patients (p-value=0.045), reflecting the great variation observed with hazard ratios ranging from 1.75 to 8.09.

We then introduced a novel tool to visualize the comparison of risk evolution between M-CLL and U-CLL patients, per subgroup, in terms of both the hazard differences and ratios. A characteristic example concerns *TP53*abn patients (Figure 2B), where the hazard ratio of U-CLL to M-CLL increased linearly before and exponentially after the 5th year. Moreover, amongst *TP53*abn cases, both the differences and the ratios exhibited the most pronounced change of all the subgroups considered with ranges 0.22 and 6.33, respectively. *TP53*abn and +12 patients (Supplemental Figure 8C) were the only subgroups where both the differences and the ratios increased monotonically over-time, reflecting the divergence of the hazard curves. By applying piecewise exponential distribution no breakpoints were observed.

The concept of hazard curves(15) has not been yet explored in CLL. The principal advantage of a hazard curve compared to the standard Kaplan-Meier survival curve is that it represents the “instant” risk for the event of interest at each time-point, instead of the cumulative risk until that point. This might prove important in CLL, where, typically, the prognostic power of any biomarker is assumed stable over the disease course, although this is often unrealistic concerning genomic aberrations. Hence, it is crucial to evaluate the temporal effect on the factors’ prognostic power regarding CLL progression.

Our approach was grounded on the fundamental segregation of CLL patients into M-CLL and U-CLL, since the SHM status remains stable over-time(1); furthermore, M-CLL and U-CLL have distinct biological background underlying distinct clonal behavior and eventual outcome(1, 12). Within each subgroup we assessed how time distance from diagnosis impacted the prognostic power of several biomarkers on CLL progression. Taking a step further, we proposed a new method to statistically evaluate the differences in risk evolution between these patient groups.

In M-CLL, the risk for disease evolution was rather homogeneous across different genomic subgroups, tending to gradually decrease over-time. In contrast, within U-CLL the pattern of over-time risk evolution was remarkably heterogeneous, greatly affected by the genomic background of the malignant clone. In particular, *TP53*abn cases exhibited a significant increase of disease evolution especially after the 5th year from diagnosis (further highlighted by the rejection of the proportional hazards assumption). A similar pattern was observed in +12 cases. A possible explanation for the hazard increase amongst U-CLL cases with *TP53*abn and +12 may relate to either the expansion of the clonal size over-time or the acquisition of extra genomic abrnormalities, reflecting potential genomic instability.

In conclusion, differential patterns of risk evolution for disease progression in M-CLL versus U-CLL support the notion that the SHM status represents more than a simple prognostic/predictive marker, and that segregation of CLL patients based on SHM might aid to detect important time effects on risk evolution within genomic subgroups of CLL patients. Moreover, they imply that specific genomic abnormalities may be linked to differential risk for disease progression over-time, while their prognostic impact may be modulated with the time elapsing from the initial diagnosis. This new methodology for evaluating and visualizing the over-time risk for disease evolution in CLL is easy to apply and can be generalized to cover the case of scoring systems where the number of categories compared is more than two, arguably also in other disease contexts.

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**CONFLICT OF INTEREST**

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**FIGURE LEGENDS**

**Figure 1: Standard Kaplan-Meier survival plot and hazard plot for the entire cohort and the *TP53*abn patients.** The hazard plot shows the estimated proportion of patients who received treatment for the first time in a defined time interval, given that they were still treatment-free at the start of this interval. The p-value corresponding to the log-rank test for the comparison of the survival distributions is displayed in the survival plot. The table including the number of patients at risk, and the cumulative number of events/censoring, applies in both plots. For both subgroups, the survival curves (Figure 1A, 1C) exhibited a similar behavior. When considering the hazard curves (Figure 1B, 1D), M-CLL showed a gradual decrease in both subgroups, while U-CLL exhibited significant differences over-time with a constant decrease over-time in the entire cohort (Figure 1B) and initial decrease until the fifth year and sudden increase for the *TP53*abn patients (Figure 1D).

**Figure 2:** **Hazard plot and evolution of hazard differences/ratios for the *TP53*abn patients.** **2A:** The hazard plot shows the estimated proportion of patients who received treatment for the first time in a defined time interval, given that they were still treatment-free at the start of this interval. The hazard differences between the M-CLL and U-CLL curves are represented by vertical dashed lines. The p-values of the comparison within consecutive 5-year intervals of the distributions of hazard differences between M-CLL and U-CLL are also displayed. **2B:** The evolution of the hazard difference, U-CLL – M-CLL, with its scale displayed in the left vertical axis in red, and the evolution of the hazard ratio, U-CLL/M-CLL, with its scale displayed in the right vertical axis in black, are simultaneously displayed for all subgroups considered.