**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

Theodoros Moysiadis1, Panagiotis Baliakas2, Davide Rossi3, Mark Catherwood4, Jonathan C. Strefford5, Julio Delgado6, Achilles Anagnostopoulos7, Chrysoula Belessi8, Niki Stavroyianni7,Sarka Pospisilova9, David Oscier10, Gianluca Gaidano11, Elias Campo12, Richard Rosenquist2,13, Paolo Ghia14, Kostas Stamatopoulos1,2

1. Institute of Applied Biosciences, Center for Research and Technology Hellas, Thessaloniki, Greece
2. Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Sweden
3. Division of Hematology, Oncology Institute of Southern Switzerland, Bellinzona, Switzerland
4. Department of Hemato-Oncology, Belfast City Hospital, Belfast, United Kingdom
5. Cancer Genomics, Academic Unit of Cancer Sciences, Cancer Research UK Centre and Experimental Cancer Medicine Centre, Faculty of Medicine, University of Southampton, Southampton, United Kingdom
6. Hematology Department, Hospital Clinic, Barcelona, Spain
7. Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece
8. Hematology Department, Nikea General Hospital, Pireaus, Greece
9. Central European Institute of Technology, Masaryk University and University Hospital Brno, Czech Republic
10. Department of Haematology, Royal Bournemouth Hospital, Bournemouth, United Kingdom
11. Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy
12. Hematopathology Section, Laboratory of Pathology, Hospital Clinic of Barcelona, University of Barcelona, IDIBAPS, Spain
13. Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden
14. Division of experimental Oncology, IRCCS Istituto Scientfico San Raffaele and Università Vita-Salute San Raffaele, Milan, Italy

**Corresponding author**

Kostas Stamatopoulos

Institute of Applied Biosciences

Center for Research and Technology Hellas

57001 Thermi, Thessaloniki, Greece

Phone: +302310498271

Fax: +302310498270

e-mail: kostas.stamatopoulos@gmail.com

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**Abstract**

Molecular biomarkers are essential for accurate risk stratification in chronic lymphocytic leukemia (CLL). However, when utilizing such biomarkers, the prognosis is assessed assuming stable predictability over the disease course, which is often unrealistic, especially for genomic aberrations. Here we followed an alternative approach and explored whether the prognostic power of particular biomarkers may depend on time distance from diagnosis. To this end, we employed hazard plots instead of Kaplan-Meier curves and assessed the over-time risk for CLL progression in 1900 early-stage patients. We focused on the impact inflicted by the immunoglobulin-heavy-variable (IGHV) gene somatic hypermutation (SHM) status, namely the identification of mutated (M-CLL) or unmutated CLL (U-CLL). M-CLL displayed a rather stable, gradually decreasing over-time risk. In U-CLL the risk was markedly influenced by the elapsed time and the genomic background: hazard evolution was decreased for U-CLL carrying del(13q) or del(11q) and *NOTCH1* mutations, whereas it remained stable for patients carrying *SF3B1* mutations. U-CLL with *TP53* aberrations or trisomy 12 were found to display intensified risk after the fifth year from diagnosis. In conclusion, the risk for disease evolution in each genomic subgroup in CLL is not stable over-time but rather highly influenced by the SHM status.

**INTRODUCTION**

Chronic lymphocytic leukemia (CLL) is characterized by remarkable clinical heterogeneity(1, 2). Not paradoxically, therefore, this has spurred intense activity towards identifying biomarkers that might assist in accurate risk stratification, including both overall disease evolution (prognostic biomarkers) and response to treatment (predictive biomarkers)(3-11). Precise prognostication represents an even more urgent need within early stage patients, who reach up to 85% of all CLL at the time of diagnosis(3). For these patients, determination of who will require treatment and when is of vital importance for both treatment and lifestyle decisions, thus relevant to both the patient and his/her carers but also the attending physician and healthcare at large.

Ample evidence indicates that the pronounced clinical heterogeneity of CLL is likely attributed to and explained by the underlying biological heterogeneity(2, 12). Support to this argument is provided by the fact that certain patient profiles defined by immunogenetic features or genomic aberrations identify subgroups of CLL patients with distinct prognosis and outcome(10, 13-20). Indeed, there is now consensus that 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 genomic aberrations involving the *TP53* gene are essential for decision making in both clinical trials but also, most importantly, general practice(21). A cautionary note appears warranted when utilizing such 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(22-24). Therefore, it is not unreasonable to claim that the prognostic power of a given biomarker may, instead, heavily depend on the time distance from diagnosis.

The Kaplan–Meier survival curves are the gold standard approach for visually assessing the impact of a specific biomarker on survival(25). An alternative suggestion can be the use of hazard curves(26). The main advantage of the hazard curve compared to the Kaplan-Meier survival curve is that it represents the “instant” risk for the event at each time point, instead of the cumulative risk. This alternative approach has been applied in myelodysplastic syndromes (MDS)(27), where the evaluation of different prognostic risk scoring systems based on the hazard curves resulted in the conclusion that hazards in the distinct risk groups became essentially equivalent after five years, indicating a significant loss of prognostic power over time.

Motivated by these results, we used the hazard curve representation to investigate over time the impact of SHM within the IGHV genes, i.e. the segregation into mutated (M-CLL) and unmutated CLL (U-CLL), that was inflicted on the evolution of risk for CLL progression and need of treatment within 1900 early stage patients with CLL. Our analysis focused on how the risk for CLL progression evolved over time, between the two SHM categories in both the entire cohort as well as within subgroups of patients defined by a specific genomic background. We used SHM status as a reference for our analysis, since it is a stable feature throughout disease evolution(28), contrasting other biomarkers such as cell-intrinsic aberrations (e.g. cytogenetic defects or gene mutations) which may change over time.

Based on these time-specific risk values, we suggest a new method to statistically evaluate the differences between the M-CLL and U-CLL patients regarding risk evolution for CLL progression. In addition, we propose a novel tool to visualize the risk evolution differences between M-CLL and U-CLL patients, which includes both their hazard differences and their hazard ratios at distinct time points, thus providing complete information of the over time risk evolution. Both approaches can also be applied to compare and visualize, respectively, when any biomarker or risk system with more than two categories is considered.

**SUBJECTS AND METHODS**

**Patient cohort**

Overall, 1900 early stage, Binet A CLL patients from 10 European institutions were included in the present multicenter retrospective study. The diagnosis was established according to the 2008 iwCLL criteria(29). A concise summary of patient characteristics is given in Supplemental Table 1. Ethical approval was granted by the local review committees and informed consent was collected according to the Helsinki Declaration.

**Methods**

The methodologies used to analyze the prognostic biomarkers were as follows: (i) fluorescence in situ hybridization (FISH) was performed in 1476/1900 (77.7%) cases using probes for the 13q14, 13q34, 11q22, 17p13 regions and trisomy 12 and results were interpreted following Döhner’s hierarchical model(30); (ii) 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%); and (iii) sequence analysis of IGHV/IGHD/IGHJ rearrangementswas performed in all cases as described previously(31). All FISH and gene mutation screens were performed before the administration of any treatment; in 1702/1900 (90%) cases, these tests were performed within the first year from diagnosis.

**Statistical analysis**

In order to evaluate the risk for disease progression, we evaluated the time-to-first-treatment (TTFT) within different subgroups of Binet A patients. These subgroups were defined based on particular genomic aberrations. In the entire cohort as well as in each genomic subgroup, the risk regarding the need for treatment was evaluated separately for M-CLL and U-CLL, with risk evolution over time being presented by a hazard curve. Smoothed estimates of the hazard curve were computed, based on a non-parametric methodology resulting in smoothed hazard plots (“bshazard” package)(32). A hazard curve 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. On the other hand, a Kaplan-Meier survival curve estimates the proportion of patients who are treatment-free at a specific time point. The hazard plots are displayed along with the usual Kaplan-Meier survival plots to strengthen the understanding of risk evolution but also for comparison reasons.

To evaluate and compare the evolution pattern of the M-CLL and U-CLL hazard curves for each subgroup, we investigated over time both (a) their hazard differences, and (b) their hazard ratios. An interpolation method was initially used to estimate the values of the hazard curve at each distinct year from the time of diagnosis. The hazard differences between M-CLL and U-CLL were computed at each distinct year from the time of diagnosis. Years 5, 10 and 15 after diagnosis were considered as landmark time points for over time comparison. Then, the distributions of hazard differences were statistically compared between consecutive 5-year intervals with a non-parametric test (Mann-Whitney) 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. When more than 10 years of follow-up were assessed, an overall p-value was also calculated (Kruskal-Wallis test), which signified the overall comparison of hazard differences’ distributions in all 5-year intervals. In all other cases the overall p-value was identical to the p-value.

Regarding the hazard ratios for M-CLL and U-CLL, they were also computed at each distinct year from the time of diagnosis. In addition, the proportional hazards assumption was checked, based on the Schoefeld residuals, as it would be typically checked when applying a simple Cox model with SHM status being the sole predictor. The proportional hazards assumption meaning in this case is that the hazard ratio between a U-CLL and a M-CLL patient does not depend on time. Rejection of the assumption would indicate significant differences in the hazard ratio over time. The analysis was performed with R.

**RESULTS**

**Over time risk evolution for CLL progression**

Based on the SHM status, 1224 (64.4%) and 676 (35.6%) patients were classified as M-CLL and U-CLL, respectively. The distribution of M-CLL and U-CLL in the current cohort is concordant with the literature, particularly regarding “general practice” patients with CLL(33). The over time risk for evolution was evaluated with SHM status as a reference in: (i) the entire cohort, (ii) cases carrying aberrations within the *TP53* gene (*TP53*abn, deletion of chromosome 17p and/or *TP53* mutations), (iii) cases carrying deletion of chromosome 11q with no *TP53*abn (del(11q), non *TP53*abn)), (iv) cases carrying trisomy 12 with no *TP53*abn (+12, non*TP53*abn), (v) cases carrying isolated deletion of chromosome 13q or normal FISH according to the Döhner hierarchical model (del(13q)/normal FISH), (vi) *NOTCH1* mutations, and (vii) *SF3B1* mutations.

To better understand the importance of the hazard plot and the advantages compared to the typical survival plot, the entire cohort and the *TP53*abn patients are displayed in Figure 1 in both visualizations. For both subgroups, the standard Kaplan-Meier survival curves (Figure 1A, 1C) exhibited a similar behavior with slowly increasing distance between the M-CLL and U-CLL survival curves over time. When considering the hazard curves (Figure 1B, 1D), no difference was seen for M-CLL, where a gradual decrease was noted for both the entire cohort and the *TP53*abn cases. On the other hand, the corresponding U-CLL hazard curves exhibited significant differences over time. In particular, while in the entire cohort the hazard curve of the U-CLL patients constantly decreased over time (Figure 1B), the respective hazard curve for the *TP53*abn patients initially decreased until the fifth year and then started to increase (Figure 1D), indicating intensification of the risk for progression after the fifth year. Thus, the hazard plot showed a time effect in hazard amongst *TP53*abn patients not evident in the standard Kaplan-Meier survival plot, enabling to follow the “instant” risk of need for treatment over time and detect critical time points, such as the fifth year in the example above signifying a sudden increase in the risk for treatment for the U-CLL *TP53*abn patients.

The hazard plots and the corresponding survival plots for all the remaining subgroups considered are displayed in Supplemental Figures 1-5. In all cases, the M-CLL hazard curve was found to slowly decrease with the exception of del(11q) patients. However, the very small number of events for M-CLL in this case hinders definitive conclusions from being drawn. On the other hand, for the U-CLL patients 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); and, even more interestingly, to increase in +12 patients, similarly to cases with *TP53*abn.

**Time effect in hazard differences**

The distributions of hazard differences between M-CLL and U-CLL patient curves were statistically compared between consecutive 5-year intervals for each subgroup to assess the evolution over time of the distance between the curves. In addition to these consecutive comparisons, an overall comparison was performed for the whole time span considered in each case (overall p-values are given in Table 1). For both the entire cohort as well as del(13q)/normal FISH patients, the time span reached up to 20 years, while for the remaining subgroups, the corresponding time span was limited to the first 10 years due to their more aggressive clinical courses and the limited number of available events after the 10th year.

In the entire cohort, significant differences were found between M-CLL vs 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, p(10,15]Vs(15,20]=0.009, and poverall<0.001. These results reflected the constant decrease of the distance between the M-CLL and U-CLL hazard curves (Fig 2A) and indicated that this decrease was statistically significant in all pairwise comparisons and in the overall setting. The same evolution rule was followed by the del(13q)/normal FISH patients with statistically significant results between intervals with p[0,5]Vs(5,10]=0.006, p(5,10]Vs(10,15]=0.009, p(10,15]Vs(15,20]=0.009, and poverall<0.001. These results indicate little additional impact on risk evolution beyond that dictated by the SHM status (Fig 2E). Similar results were obtained for the del(11q) and the *NOTCH1* patients with p[0,5]Vs(5,10]=0.006 in both cases (Fig 2C, 2F).

In sharp contrast, amongst patients with *TP53*abn, the distance between the hazard curves for M-CLL and U-CLL statistically significantly increased after the 5th year with p[0,5]Vs(5,10]=0.006 (Fig 2B). Similarly, amongst +12 patients, the hazard curve of the U-CLL patients constantly increased from diagnosis, and this increase was statistically significant after the 5th year with p[0,5]Vs(5,10]=0.006 (Fig 2D).

For the *SF3B1* patients, we observed that the distance between the two curves remained almost stable over time with no significant differences, p[0,5]Vs(5,10]=0.465 (Fig 2G).

**Hazard ratio over time**

Next, we tested the proportional hazards assumption based on the Schoefeld residuals (see Table 1 and Supplemental Fig 6) to test whether the hazard ratio between a U-CLL and a M-CLL patient depended on time. Significant differences and/or variation in the hazard ratio over time could cause the rejection of the assumption, and solidify even further the need for an over time analysis of the hazard evolution.

The assumption of proportional hazards between M-CLL and U-CLL was rejected only for the *TP53*abn patients with p-value=0.045. This signified statistically significant differences in the hazard ratio between U-CLL and M-CLL *TP53*abn patients over time, reflecting the great variation observed for the hazard ratios in the case of *TP53*abn patients, ranging from 1.75 (at diagnosis) to 8.09 (10th year). In all other cases, the deviation from over time hazard proportionality was not statistically different (see Table 1).

**Visualization of risk evolution in CLL subgroups with different somatic hypermutation status**

Finally, we introduced a novel tool to visualize the comparison of risk evolution between the M-CLL and U-CLL patients, per subgroup, in terms of both the hazard differences and the respective hazard ratios (Figure 3). Both the hazard difference (U-CLL – M-CLL) and the hazard ratio (U-CLL/M-CLL) evolution at distinct years from diagnosis are simultaneously displayed as dotted lines, for all subgroups considered. The variation of each line represents the trend of the hazard differences and of the respective hazard ratios. A parallel line in either case would indicate stable difference or stable ratio over time, respectively. Thus, this visualization enables to easily follow the evolution pattern of hazard comparison between M-CLL and U-CLL patients for each subgroup considered. Subsequently, such a visualization could aid in detecting critical time points when assessing the comparison of hazard evolution between two groups of patients in the same way that the hazard plot could aid in detecting critical time points when assessing the hazard evolution for a specific group of patients.

A characteristic example concerns *TP53*abn patients (Figure 3B), where before the 5th year, the hazard ratio of U-CLL to M-CLL increased linearly and after the 5th year it increased exponentially. This indicated that the 5th year is important for the *TP53*abn patients not only within the U-CLL category who exhibited at this point a sudden increase in the risk for treatment, but also when comparing U-CLL versus M-CLL, where we noted significant difference in both the hazard differences and the hazard ratios evolution after this specific time point. Moreover, amongst *TP53*abn cases, both the differences and the ratios exhibited the most pronounced change of all the subgroups considered with ranges of 0.22 and 6.33, respectively. The subgroups of *TP53*abn and +12 patients were the only ones where both the differences and the ratios increased monotonically over time. Both in the entire cohort and for the del(13q)/normal FISH subgroup, we observed a monotonic decrease in the differences and a concave evolution in the ratios, which was depicted as an initial increase followed by a subsequent decrease. The subgroup of del(11q) patients is the only one which exhibited a monotonic ratio decrease. Finally, both the *NOTCH1* and *SF3B1* subgroups exhibited monotonic increase in hazard ratios over time, while the differences remained almost stable over time.

**DISCUSSION**

Risk assessment in CLL has so far been attempted assuming stable predictability of each considered prognosticator over the disease course (5), whereas the possibility that the impact of each marker may change over time has been overlooked. This may hinder revealing the true impact of any given biomarker which is especially relevant considering that CLL represents a dynamic setting where changes in both the tumor and the host occur over time. Thus, conceivably, the impact of any individual biomarker at a specific time point during the disease course may differ from that defined at the time of the initial evaluation(34, 35).

A systematic PubMed search using as query terms “hazard curves”, “prognosis” and “chronic lymphocytic leukemia” (CLL) revealed that the concept of hazard curves 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. Since this hypothesis is often unrealistic concerning genomic aberrations, it is crucial to evaluate the temporal effect on the factors’ prognostic power regarding CLL progression.

On these grounds, here we assessed for the first time in CLL the over time risk for disease evolution based on an alternative statistical approach by using hazard plots, evaluating both hazard differences and hazard ratios. Our methodology allows assessing the actual risk at specific time points rather than the cumulative risk from the time of diagnosis, which is the displayed output when employing the traditional survival curves.

Our approach was grounded on the fundamental segregation of CLL patients into mutated M-CLL and U-CLL. Within each of these two groups we assessed how time distance from diagnosis impacted on 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 groups of patients (based on the distance between the hazard curves). According to this method, the 5th, 10th and 15th years are considered as reference time points in the sense that we are interested to compare what happens between the first 5-year interval and the second, the second and the third and so on. The 5-year interval choice is not arbitrary but arises from the usual practice of reporting the 5-year and 10-year treatment probability(36). The proposed visualization enables to detect landmark time points in CLL evolution.

In M-CLL, the risk for disease evolution was rather homogeneous across different subgroups defined by recurrent genomic aberrations, tending to gradually decrease over time. On the contrary, 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, cases with *TP53*abn exhibited a significant increase of disease evolution especially after the 5th year from diagnosis. A similar pattern was observed for U-CLL carrying trisomy 12. On the other hand, amongst cases with isolated del(13q)/normal FISH, *NOTCH1* mutations or del(11q), a tendency for decreased risk over time was observed, while amongst cases with *SF3B1* mutations the risk for disease evolutions appeared to remain stable over time. The distinct risk for disease evolution according to the SHM status for patients carrying *TP53*abn is also highlighted by the rejection of the assumption of proportional hazards. 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 within U-CLL carrying the particular genetic aberrations.

When comparing the over time hazard differences between M-CLL and U-CLL, we found statistically significant differences in all genomic subgroups except of *SF3B1* mutant patients. This indicated that the temporal effect on the risk evolution for CLL progression might be better projected, when SHM is taken into account, reflecting at the same time the fundamendal biological differences between these two groups. In addition, hazard curves aided in revealing valuable latent information regarding over time risk comparison for CLL progression, which was otherwise unavailable.

In this study we used the SHM status of the clonotypic BcR IG as a reference in order to assess risk evolution separately within M-CLL and U-CLL. To large extent, this decision was grounded on the fact that the SHM status remains stable over time while other biomarkers such as cytogenetic aberrations may change. Our decision was also supported by ample evidence that M-CLL and U-CLL have distinct biological background underlying distinct clonal behavior and eventual outcome(37, 38). Moreover, differential SHM status is also associated with different responses to the gold standard treatment for fit patients lacking *TP53*abn, namely the Fludarabine-Cyclophosphamide-Rituximab (FCR) regimen(39-41).

In conclusion, the different patterns of risk evolution for disease progression observed between M-CLL and U-CLL support the notion that the SHM status represents more than a simple prognostic/predictive classification marker, and that segregation of CLL patients based on SHM might aid to detect important time effects on risk evolution within specific genomic subgroups of CLL patients. Moreover, our results strongly imply that each genomic abnormality may be linked to a differential risk for disease progression over time while its prognostic impact may be modulated with the time elapsing from the initial diagnosis. This new methodology for the evaluation and visualization of 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, respectively.** 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 TP53abn patients (Figure 1D).

**Figure 2:** **Hazard plot displayed for all the subgroups considered.** 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. Based on the number of patients at risk and the number of events at different time points, for some subgroups specific time points were selected as landmarks and the hazard curves from those points and onwards (indicated by an asterisk) were dotted to indicate the small number of patients at risk and/or the small or inexistent number of events (see e.g. 2C).

**Figure 3: Evolution of hazard differences and hazard ratios.** 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. Based on the number of patients at risk and the number of events at different time points, for some subgroups specific time points were selected as landmarks and the bullets from those points and onwards (indicated by an asterisk) were replaced by triangles to indicate the small number of patients at risk and/or the small or inexistent number of events. All plots are given in the same scale for both the left and the right vertical axis to enable straightforward comparison between the subgroups.

**TABLES**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | Binet A | *TP53*abn | del(11q)  (no *TP53*abn ) | +12  (no *TP53*abn ) | del(13q)/ normal FISH | *NOTCH1* | *SF3B1* |
| Over time differences | overall  p-value | **<0.001** | **0.006** | **0.006** | **0.006** | **<0.001** | **0.006** | 0.465 |
| Proportional hazard assumption | p-value | 0.381 | **0.045** | 0.550 | 0.365 | 0.787 | 0.603 | 0.243 |

**Table 1:** The p-values of the overall comparison within consecutive 5-year intervals of the distributions of hazard differences between M-CLL and U-CLL are displayed in the first row for all the subgroups considered. The p-values referring to the evaluation of the proportional hazards assumption, based on the Schoefeld residuals are displayed in the second row for all the subgroups considered.