INTEGRATED MUTATIONAL AND CYTOGENETIC ANALYSIS IDENTIFIES NEW PROGNOSTIC SUBGROUPS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Running title: Integrated genetic profiling in CLL

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ABSTRACT

The identification of new genetic lesions in chronic lymphocytic leukemia (CLL) prompts a comprehensive and dynamic prognostic algorithm including gene mutations, chromosomal abnormalities, and their changes during clonal evolution. By integrating mutational and cytogenetic analysis in 1274 CLL samples, and by using both a training-validation and a time-dependent design, four CLL subgroups were hierarchically classified: i) high-risk, harboring TP53 and/or BIRC3 abnormalities (10-year survival: 29%); ii) intermediate-risk, harboring NOTCH1 and/or SF3B1 mutations and/or del11q22-q23 (10-year survival: 37%); iii) low-risk, harboring +12 or a normal genetics (10-year survival: 57%); iv) very low-risk, harboring del13q14 only, whose 10-year survival (69.3%) did not significantly differ from a matched general population. This integrated mutational and cytogenetic model independently predicted survival, improved CLL prognostication accuracy compared to FISH karyotype (p<0.0001), and was externally validated in an independent CLL cohort. Clonal evolution from lower to higher risk implicated the emergence of NOTCH1, SF3B1 and BIRC3 abnormalities in addition to TP53 and 11q22-q23 lesions. By taking into account clonal evolution through time-dependent analysis, the genetic model maintained its prognostic relevance at any time from diagnosis. These findings may have relevant implications for the design of clinical trials aimed at assessing the use of mutational profiling to inform therapeutic decisions.

Keywords: chronic lymphocytic leukemia, mutations, cytogenetics, prognosis
INTRODUCTION

The course of chronic lymphocytic leukemia (CLL) ranges from very indolent with a nearly normal life expectancy, to rapidly progressive leading to early death.\textsuperscript{1,2} To better understand the genetic basis of CLL heterogeneity and improve patients' prognostication, all recurrent and clinically relevant molecular lesions should be combined into a comprehensive prognostic model.

Chromosomal aberrations and \textit{TP53} mutations are of key importance for predicting CLL outcome.\textsuperscript{3,4} Recently, next generation sequencing has disclosed a further degree in the molecular complexity of CLL by revealing novel genetic lesions affecting the \textit{NOTCH1}, \textit{SF3B1}, \textit{MYD88} and \textit{BIRC3} genes.\textsuperscript{5–9} Alterations of these genes occur in ~5-10\% CLL at diagnosis and, in the case of \textit{NOTCH1}, \textit{SF3B1} and \textit{BIRC3}, have individually shown significant correlations with survival in consecutive series from independent institutions.\textsuperscript{5–11} These findings prompt the integration of the newly discovered genetic lesions into a model based on both chromosomal abnormalities and gene mutations.

Available genetic prognostic models in CLL are based on the evaluation of risk factors detected at a specific time point.\textsuperscript{3–4} The appearance of additional genetic lesions during CLL course may dynamically modify patients' survival, as suggested by the association between clonal evolution and poor prognosis, treatment resistance and transformation.\textsuperscript{12–22} On these bases, a dynamic prognostic model based on a time-dependent analysis of CLL genetic lesions may prove useful for a better understanding of disease outcome.

Here we report an integrated mutational and cytogenetic model for CLL survival prediction that maintains its prognostic relevance in a time-dependent fashion.
METHODS

Patients

Time-fixed analysis at diagnosis was based on a training series of 637 newly diagnosed and previously untreated CLL patients of whom 583 (91.5%) were provided with regular follow-up. Among CLL from the training series that required treatment, 122/266 (45.8%) cases received rituximab-based regimens (i.e. fludarabine-cyclophosphamide-rituximab, fludarabine-rituximab or pentostatin-cyclophosphamide-rituximab), 64/266 (24.0%) fludarabine-based regimens (i.e. fludarabine or fludarabine-cyclophosphamide), and 80/266 (30.1%) alkylator-based regimens (i.e. chlorambucil). To externally validate the results of the time-fixed analysis, an independent cohort of 370 newly diagnosed and previously untreated CLL was also investigated. Time-dependent analysis and analysis of clonal evolution were based on a mono-institutional cohort of 257 CLL out of the training series that was provided with sequential tumor samples (n=469) and clinical information prospectively collected at clinically relevant time points (i.e. diagnosis, progression, last follow-up). Further details of the study populations are available in table S1 and in the Supplementary Methods. CLL diagnosis was according to 2008 IWCLL-NCI criteria and confirmed by a flow cytometry score >3 in all cases. Monoclonal B-cell lymphocytosis (MBL) were excluded. The study was approved by the institutional ethical committee (Protocol Code 59/CE; Study Number CE 8/11). Patients provided informed consent in accordance with local IRB requirements and Declaration of Helsinki.

Samples

Overall, 1274 CLL samples were subjected to mutational and FISH analysis. CLL samples were extracted from fresh or frozen peripheral blood mononuclear cells (PBMC) isolated by Ficoll-Paque gradient centrifugation. In all cases, the fraction of tumor cells corresponded to >70% as assessed by flow cytometry. Matched normal DNA from the same patient was obtained from saliva.
or from purified granulocytes and confirmed to be tumor-free by PCR of tumor-specific IGHV-D-J rearrangements. High-molecular-weight (HMW) genomic DNA was extracted from tumor and normal samples according to standard procedures. DNA was quantified by the NanoDrop 2000C spectrophotometer (Thermo Scientific).

**Molecular studies**

The mutation hotspots of TP53 (exons 4-9, including splicing sites; RefSeq NM_000546.5), NOTCH1 (exon 34; RefSeq NM_017617.2), SF3B1 (exons 14, 15, 16, 18, including splice sites; RefSeq NM_012433.2), MYD88 (exons 3, 5, including splicing sites; RefSeq NM_002468.4), and BIRC3 (exons 6-9, including splicing sites; RefSeq NM_001165.4) genes were analyzed by PCR amplification and DNA direct sequencing of high molecular weight genomic DNA.8,10,11 The mutant allele frequency was estimated by next generation sequencing.5 Amplicons known to harbor TP53, NOTCH1, SF3B1, MYD88, or BIRC3 mutations by Sanger sequencing were reamplified from genomic DNA by oligonucleotides containing the gene-specific sequences, along with 10-bp MID tag for multiplexing and amplicon library A and B sequencing adapters. The obtained amplicon library was subjected to deep sequencing on the Genome Sequencer Junior instrument (454 Life Sciences).5 In order to obtain ~700-fold coverage per amplicon, no more than 100 amplicons/run were analyzed. The obtained sequencing reads were mapped to reference sequences and analyzed by the Amplicon Variant Analyzer software (Roche) to establish the mutant allele frequency. Probes used for FISH analysis were: i) LSID13S319, CEP12, LSIp53, LSIATM (Abbott); ii) RP11-177O8 (BIRC3) BAC clone.11 Further details of the molecular studies are available in the Supplementary Appendix.

**Statistical analysis**

Overall survival (OS) was the primary endpoint and was measured from date of initial presentation to date of death from any cause (event) or last follow-up (censoring). Treatment free
survival (TFS) was measured from date of initial presentation to date of progressive disease requiring treatment according to IWCLL-NCI guidelines (event), death or last follow-up (censoring). Survival analysis was performed by the Kaplan-Meier method. The crude and adjusted association between time-fixed exposure variables at diagnosis and OS was estimated by Cox regression. The stability of the Cox model was internally validated using bootstrapping procedures. Recursive partitioning was applied to divide patients in genetic subgroups with different outcome. The stability of the recursive decision tree was validated by the random survival forest method. An amalgamation algorithm was used to merge terminal nodes showing homogenous survival. Relative survival, defined as the ratio between the actuarial survival observed in the CLL cohort and the expected survival of the general Italian population matched to CLL patients by sex, age and calendar year of diagnosis, was calculated using the Ederer II method. Time to acquisition of a risk factor was estimated considering death as a competing risk and was compared across groups with Gray’s test. The crude and adjusted effect of time-varying prognostic factors was assessed by time-dependent Cox regression. Landmark analysis was utilized to illustrate the effect of time-varying prognostic factors. Associations and anti-associations between genetic lesions were assessed by Fisher’s exact test corrected for multiple comparisons by Bonferroni’s test. The number of cases (n=637) allowed to identify all possible co-occurring genetic lesions present in this study, as well as exclusive alterations in genetic lesions that occurred in at least 8.6% of cases. Categorical variables were compared by chi-square test and Fisher’s exact test when appropriate. Continuous variables were compared by Mann-Whitney (two unrelated samples) or Wilcoxon (two related samples) tests. 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 v.20.0 (Chicago, IL) and with R statistical package 2.15.1 (http://www.r-project.org). Further details of the statistical analysis are available in the Supplementary Appendix.
RESULTS

Mutational complementation groups and independent prognostic value of genetic lesions in newly diagnosed CLL

As a preliminary step toward the construction of an integrated mutational and cytogenetic model, we assessed the prevalence and independent prognostic value of the candidate genetic lesions in the training series of 637 CLL (Tables S1-S2-S3). Del13q14 and +12 distributed in a mutually exclusive fashion (p<0.0001) (Fig. S1, Fig. S2). With the sole exception of the expected association between NOTCH1 mutations and +12 CLL (p=0.0014),27,28 the prevalence of other genetic lesions did not differ among molecular subgroups (Fig. S1, Fig S2). Consistent with a dual hit mechanism of inactivation, mutations of both TP53 and BIRC3 frequently co-occurred with deletion of the corresponding locus (p<0.0001 and p=0.0065, respectively) (Fig. S1, Fig S2).

Analysis of FISH abnormalities reproduced the previously described prognostic groups in this study cohort (Table S3, Fig. S3).3 Patients harboring del17p13 and patients harboring TP53 mutations in the absence of del17p13 showed an identical outcome in the study cohort and therefore were combined for the analysis of outcome.13 Among new CLL lesions, survival analysis confirmed the independent prognostic value of NOTCH1, SF3B1 and BIRC3 abnormalities (Table S3). MYD88 mutations had no prognostic effect (p=0.1728), although the study was adequately powered (90%) for detecting the impact of these low frequency (~4%) mutations on survival.

This preliminary assessment provided the rationale for including alterations of NOTCH1, SF3B1, and BIRC3, in addition to standard FISH lesions and TP53 mutations, in the subsequent development of an integrated mutational and cytogenetic model.

Integrated mutational and cytogenetic model for CLL prognostication

The hierarchical order of relevance of the genetic lesions in predicting CLL survival was established by recursive partitioning analysis of the training series (Fig. 1).25,26 TP53 disruption was
the most predictive genetic variable in the survival tree, followed by BIRC3 disruption, mutations of SF3B1 and NOTCH1, and del11q22-q23 (Fig. 1). In CLL lacking all the aforementioned abnormalities, +12 and del13q14 further stratified patients’ outcome. Measure of the variable importance validated the hierarchical order of relevance of the genetic lesions established by the recursive partitioning analysis and confirmed the stability of the decision tree (Table S4). Based on the application of the amalgamation algorithm to the terminal nodes, cases harboring TP53 abnormalities and cases harboring BIRC3 abnormalities were grouped into a single category, as well as cases harboring NOTCH1 mutations, SF3B1 mutations or del11q22-q23. This approach allowed to establish an integrated mutational and cytogenetic model for classifying newly diagnosed CLL patients according to risk of death.

Four CLL subgroups were hierarchically classified (Fig. 2). The high-risk category included patients harboring TP53 disruption and/or BIRC3 disruption independent of co-occurring lesions (5-year OS: 50.9%; 10-year OS: 29.1%) (Fig. 2A). When the demographic effects of age, sex and year of diagnosis were compensated, the 10-year life expectancy of high-risk patients was only 37.7% of that expected in the matched general population (p<0.0001) (Fig. 3B).

The intermediate-risk category included patients harboring NOTCH1 and/or SF3B1 mutations and/or del11q22-q23 in the absence of TP53 and BIRC3 abnormalities (5-year OS: 65.9%; 10-year OS 37.1%) (Fig. 2A). The 10-year life expectancy of intermediate-risk patients was only 48.5% of that expected in the matched general population (p<0.0001) (Fig. 3B).

The low-risk category (5-year OS: 77.6%; 10-year OS: 57.3%) comprised both patients harboring +12 and patients wild type for all genetic lesions (i.e. normal) (Fig. 2A). Though experiencing an indolent behavior, the 10-year life expectancy of low-risk patients was 70.7% of that expected in the matched general population (p<0.0001) (Fig. 3B).

The very low-risk category included patients harboring del13q14 as the sole genetic lesion (5-year OS: 86.9%; 10-year OS 69.3%) (Fig. 2A). Notably, the 10-year life expectancy of very low-risk patients was only slightly (84.2%) and not significantly (p=0.1455) lower than that expected in
the matched general population (Fig. 3B). Consistent with the small excess mortality experienced by very low-risk CLL compared to the matched general population, the cause of death in this subgroup was unrelated to CLL in many patients (16/27, 59.2%). In the remaining cases, the cause of death was second cancer (4/27, 14.8%), infection (4/27, 14.8%), and progressive CLL (3/27, 11.1%).

Differences in outcome among the four subgroups were consistent with: i) differences in prevalence of unfavorable clinical and biological features at presentation (Table S5); and ii) differences in disease progression, as indicated by TFS (Fig. 2B).

Because the higher risk genetic groups contain more patients with advanced stage disease, we also limited the survival analysis to Rai 0-I CLL. Consistent with the results obtained in the whole CLL population, the genetic model stratified four genetic subgroups also in early stage CLL (Fig. S4).

In order to provide a preliminary signal on the reproducibility of the prognostic model in patients treated with rituximab-containing regimens, we have analyzed the impact of the model on: i) OS from presentation in patients from the training series who received the diagnosis in 2005 or afterwards\textsuperscript{29} (in this time frame, 67.5\% of cases requiring treatment received rituximab-based regimens); and ii) OS from first treatment in a consecutive mono-institutional cohort of 62 patients who received fludarabine-cyclophosphamide-rituximab and were provided with tumor samples and clinical information prospectively collected at first progression. Consistent with the results obtained in the whole CLL population, the genetic model stratified four genetic subgroups also in these patients (Fig. S5).

Overall, these data establish the hierarchical order of relevance of recurrent mutations and cytogenetic lesions in CLL and identify four distinct prognostic subgroups.
The integrated mutational and cytogenetic model is an independent and reproducible predictor of OS in CLL

Multivariate analysis selected the genetic model as an independent risk factor of OS in the training series ($p_{\text{trend}}=0.0010$), along with age ($p<0.0001$), Rai stage ($p_{\text{trend}}<0.0001$) and unmutated $IGHV$ genes ($p=0.0036$) (Table 1). Internal validation on 1000 bootstrap samples confirmed the stability of the genetic model as a prognostic factor of OS in the training series (Table 1).

Survival analysis in an independent external validation series of 370 newly diagnosed CLL confirmed: i) the more general reproducibility of the genetic model in predicting OS (Fig. S6); ii) its capacity of discriminating patients' outcome (c-index in the validation series 0.662; vs c-index in the training series: 0.642), and iii) its independent prognostic value ($p_{\text{trend}}=0.0053$) when adjusted for other confounding covariates (Table S6).

Based on these data, the genetic model represents one of the most important independent prognosticators of CLL survival in both the training and the validation cohorts.

Inclusion of mutations in addition to FISH abnormalities significantly improves the accuracy of CLL prognostication

Overall, ~20% (105/488) of lower risk patients according to the FISH cytogenetic model, including 20.1% (39/194) del13q14 only CLL, were reclassified into higher risk subgroups by the integrated mutational and cytogenetic model because of the co-occurrence of $NOTCH1$, $SF3B1$, $TP53$ mutations or $BIRC3$ disruption (Table S7). Consistently, the inclusion of $TP53$, $NOTCH1$, and $SF3B1$ mutations, and of $BIRC3$ lesions in addition to FISH abnormalities significantly improved the accuracy of survival prediction (c-index: 0.617 vs c-index: 0.642 $p<0.0001$) (Fig. 3).

Based on the sole FISH assessment, the life expectancy of del13q14 only CLL was significantly lower (68.1%; $p<0.0001$) than that expected in the matched general population (Fig. 3A). Conversely, the genetic model segregated a subgroup of patients with del13q14 only who lacked other genetic alterations and showed an expected survival not significantly different from
that of the matched general population (Fig. 3B). This subgroup of del13q14 only CLL displayed a preferential usage of mutated \textit{IGHV} genes (80.9\% vs 42.1\% in del13q14 CLL with co-occurring mutations).

On these grounds, integrating mutations and cytogenetic lesions proves useful to refine the prognosis of CLL and helps identifying a subgroup of patients with an extremely indolent disease.

\textbf{Assessment of clonal evolution in CLL by integrated mutational and cytogenetic analysis}

In order to clarify the evolution of \textit{NOTCH1}, \textit{SF3B1}, and \textit{BIRC3} lesions, we repeatedly analyzed CLL patients provided with >2 sequential samples (n=202) followed for at least 2 years after presentation (median interval between baseline and last sequential sample: 62.8 months, range 24-150 months). FISH lesions and \textit{TP53} mutations were also investigated.

Overall, 36 (17.8\%) of the sequentially investigated patients developed 59 new genetic lesions during disease course, mainly represented by \textit{TP53}, \textit{NOTCH1}, \textit{SF3B1}, \textit{BIRC3} and del11q22-q23 abnormalities (54/59, 91.5\%) (Table S8 and S9). The median time to clonal evolution was 3 years (95\% CI: 1.9-4.0). The median follow-up of cases that developed clonal evolution was 6.1 years (95\% CI: 4.9-7.4 years). The median follow-up of cases that did not develop clonal evolution was 5.3 years (95\% CI: 4.8-5.7 years). No patient developed a new +12 or a new monoallelic del13q14, while development of biallelic del13q14 (i.e. new del13q14 on the second chromosome after initially monoallelic del13q14) was restricted to 4 (1.9\%) cases.

Among cases that were informative at presentation, \textit{TP53}, \textit{NOTCH1}, \textit{SF3B1}, \textit{MYD88} and \textit{BIRC3} mutant allele frequency did not significantly change during disease course (Fig. S7). Clonal fluctuation, defined as the disappearance of a mutated clone, occurred in only three cases. Notably, in all three cases the baseline mutated clone was substituted by a second clone harboring a new high risk mutation (Table S8, Fig. S8).

These data document that, similarly to \textit{TP53} abnormalities and del11q22-q23, also \textit{NOTCH1}, \textit{SF3B1} and \textit{BIRC3} lesions may emerge during clonal evolution.
Baseline factors associated with high risk clonal evolution

To investigate the dynamics of acquisition of high risk genetic lesions during follow-up, we evaluated the time to clonal evolution and its modifications according to the disease characteristics at diagnosis after adjusting for death as a competing risk.

At 10 years from diagnosis, 24.5% CLL belonging to the very low- and low-risk genetic subgroups had developed new \( TP53, NOTCH1, SF3B1, BIRC3 \) or del11q22-q23 lesions due to clonal evolution, and therefore switched to a higher risk category of the genetic model. Clinical features at presentation predicting the development of new high risk genetic lesions were age >65 years (\( p=0.0192 \)), high LDH (\( p=0.0093 \)), and unmutated \( IGHV \) genes (\( p=0.0087 \)) (Fig. 4A-C).

Consistent with their inferior OS probability, cases belonging to the low-risk subgroup according to the genetic model (i.e. cases harboring +12 or a normal genetics) showed a significantly higher probability of developing poor risk genetic lesions and of switching to intermediate- or high-risk subgroups compared to very low-risk CLL harboring del13q14 only (\( p=0.0435 \)) (Fig. 4D). The increased risk of clonal evolution in cases harboring +12 or a normal genotype may be explained by the higher prevalence of unmutated \( IGHV \) genes (Table S5) and the higher risk of treatment requirement (Fig. 2) observed in these cases compared to del13q14 only CLL.

Impact of high risk clonal evolution on CLL survival

In traditional Kaplan-Meier or Cox regression analysis, usually a risk factor measured at baseline is related to mortality thereafter. However, the development of poor risk factors during disease course may substantially modify the patient's outcome, and estimates of prognosis should improve if such time-dependent changes are taken into account. Thus, we investigated whether the assessment of clonal evolution contributes to improve OS prediction by using a time-dependent Cox regression analysis. This analysis included variables considered “fixed” in time (i.e. sex and \( IGHV \) mutation status) and time-varying variables (i.e. age, Rai stage and status of the genetic lesions) that
were repeatedly assessed at clinically relevant time points (i.e. disease progression or last follow-up).

Time-varying genetic lesions associated with short OS were *TP53* disruption (p<0.0001), *BIRC3* disruption (p=0.0166), and *NOTCH1* mutations (p=0.0247). *SF3B1* mutations were of borderline significance (p=0.0766) (Table S10). Dynamic changes of the genetic model due to clonal evolution retained a statistically significant impact on OS (p_trend=0.0003), independent of modifications affecting other time-varying factors, such as patients’ age and disease stage (Table S11). Consistently, the genetic model stratified CLL OS at 1, 2 and 4 years from diagnosis by landmark analysis (Fig. 5).

These results document the prognostic relevance of developing high-risk genetic lesions during CLL course, and show that the genetic model maintains its prognostic impact at any time from diagnosis.
DISCUSSION

We provide an integrated mutational and cytogenetic model for CLL prognostication that: i) allows to segregate four patient subgroups with distinct risks of death in a fashion that is reproducible and independent from other well established prognosticators; ii) takes into account clonal evolution; and iii) maintains its independent prognostic relevance at any time point from diagnosis.

In terms of discriminating the patients' course, the integrated mutational and cytogenetic model significantly adds to the model based solely on FISH karyotype.\textsuperscript{3} Due to the co-occurrence of unfavorable mutations, ~20\% of patients belonging to low-risk cytogenetic subgroups are reclassified into one of the two highest risk genetic categories. This refinement allows to segregate a subgroup of CLL harboring del13q14 only that account for a relevant fraction (~25\%) of newly diagnosed patients and show an expected survival only slightly, though not significantly, lower than that of the general population. The small residual excess mortality observed in this subgroup may be due to the inclusion in the case mix of patients harboring poor risk genetic lesions that are currently unknown and/or patients with a complex karyotype that is not captured by FISH approaches.\textsuperscript{30} The very favorable outcome of del13q14 only CLL may be explained by their slow progression rate (~4\% per year), documenting that del13q14 only CLL is a highly stable clinical entity. Notably, the progression rate of del13q14 only CLL is in the order of magnitude of that described in other conditions considered as pre-malignant.\textsuperscript{31,32}

Recursive partitioning allowed to define that SF3B1 and NOTCH1 mutations are hierarchically classified after TP53 and BIRC3 lesions. Consistently, patients harboring NOTCH1 or SF3B1 mutations, but purged of TP53 and BIRC3 abnormalities, show an intermediate-risk profile similar to del11q22-q23 CLL.\textsuperscript{3,33} TP53 abnormalities play a central role in our understanding of the poor prognosis of high-risk CLL, but fail to explain the molecular basis of a substantial fraction of high-risk patients.\textsuperscript{3,4,33} According to the model proposed in this study, BIRC3 abnormalities
complement TP53 disruption in the identification of high-risk patients. This observation is consistent with the fact that BIRC3 abnormalities occur in ~40% chemorefractory, but TP53 wild type CLL, while are absent in chemosensitive cases.11

NOTCH1, SF3B1 and BIRC3 lesions, even though absent at presentation, may emerge during disease course, thus expanding the spectrum of genetic events currently associated with high-risk clonal evolution that were so far limited to TP53 abnormalities and del11q22-q23.12–22 Overall, the probability of developing new high-risk genetic lesions is substantial (~25% at 10-years), and the acquisition of high-risk genetic lesions over time affects survival in a manner that is independent of modifications of other time-varying factors, such as patient age and disease stage. On these bases, one additional goal of the study was to establish a dynamic model accounting for all prognostically meaningful genetic lesions and their modifications during disease course due to clonal evolution. According to this dynamic approach, the integrated mutational and cytogenetic model retains its prognostic relevance at any time point during the clinical course, and the hazard of death increases any time a CLL patient shifts to a higher risk category of the genetic model. These results point to the relevance of sequentially reassessing the parameters of the genetic model when an updated genetic status is required for redefining the precise prognosis of the patient.

The probability of undergoing clonal evolution is not uniform across CLL patients, but varies according to patients’ age and baseline features of the disease. While IGHV mutation status has been already associated with clonal evolution in CLL,12,34 the relationship between aging and instability of the leukemic clone is an unexpected finding of this study, and might be related to a decline in genomic maintenance mechanisms in older individuals, or a greater propensity of elderly patients to positively select and expand clones harboring high-risk genetic lesions.35–37

Regarding the impact on clonal evolution of the baseline genetics of the clone, low risk cases harboring +12 or a normal genotype at diagnosis are characterized by a ~2 fold higher probability of developing poor risk genetic lesions and of switching to the highest risk genetic subgroups compared to very low risk CLL harboring del13q14 only. The relative instability of the
leukemic clone in CLL with +12 or a normal genotype might explain why these patients experience a shorter survival compared to del13q14 only CLL.

Given the growing number of new targeted agents, the management of CLL will conceivably be revised, and early intervention may also become an option.\textsuperscript{38,39} In this changing scenario,\textsuperscript{38,39} there is increasing interest in the use of prognostic markers that may guide management of patients since the early phases of the disease. Our findings may have relevant implications for the design of clinical trials aimed at testing early intervention approaches. In fact, very low-risk cases harboring del13q14 only may not gain any additional benefit from early treatment because of their indolent course, low risk of clonal evolution and nearly normal life expectancy.

Taken together, these data show that the integrated mutational and cytogenetic model can classify CLL patients into more precise subgroups, advance our understanding of the molecular heterogeneity of CLL, and improve current prognostic algorithms. Future challenges are to design rapid and affordable molecular assays and to prospectively define if specific treatments may overcome the poor prognosis conferred by higher risk lesions.
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Table 1. Time-fixed univariate and multivariate analysis of OS in the CLL training series *

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<tr>
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<td>1.19</td>
<td>2.77</td>
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<td>&lt;0.0001</td>
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<tr>
<td><strong>IGHV homology &gt;98%</strong></td>
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<td><strong>Integrated mutational and cytogenetic model</strong></td>
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<tr>
<td><strong>Very-low risk</strong></td>
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<td></td>
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<tr>
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<td>2.50</td>
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<td>1.20</td>
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<td><strong>High-risk</strong></td>
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<td>6.79</td>
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*OS, overall survival; HR, hazard ratio; LCI, 95% lower confidence interval; UCI, 95% upper confidence interval; IGHV, immunoglobulin heavy variable gene

**Analyzed as a continuous variable**

*p for trend

Discrimination: bias-corrected c-index: 0.762; optimism: 0.005
Calibration: bias-corrected calibration slope: 0.965; optimism: 0.035

Shrinkage coefficient: 0.97
FIGURE LEGENDS

Fig 1. Decision tree resulting from recursive partitioning analysis and amalgamation in the training series. Disruption of TP53 and BIRC3, mutations of SF3B1 and NOTCH1, as well as del11q22-q23 were the factors selected by the algorithm to split the patient population in six terminal nodes. Presence or absence of TP53 disruption independent of co-occurring genetic lesions was the most significant covariate for the entire study population. Among patients lacking TP53 abnormalities, the most significant covariate was BIRC3 disruption. Among patients lacking both TP53 and BIRC3 abnormalities, the most significant covariate was SF3B1 mutation status. Among patients lacking TP53, BIRC3, and SF3B1 lesions, the most significant covariate was NOTCH1 mutation status. Among patients lacking TP53, BIRC3, SF3B1 and NOTCH1 lesions, the most significant covariate was del11q22-q23. Based on the application of the amalgamation algorithm to the terminal nodes, cases harboring TP53 abnormalities and cases harboring BIRC3 abnormalities were grouped into a single category, as well as cases harboring NOTCH1 mutations, SF3B1 mutations or del11q22-q23. Genetic lesions are represented from right to left according to their hierarchical order of relevance in splitting the parent node into daughter nodes with significantly different survival probabilities. The p value corresponds to the log-rank test adjusted for multiple comparisons. The right branch of each split represents the presence of the lesion. The left branch of each split represents the absence of the lesion. The Kaplan-Meier curves estimate the overall survival (OS) of patients belonging to each terminal node. N=number of patients in the node, M=mutation, DIS= disruption.

Fig 2. Kaplan-Meier estimates of overall survival and treatment free survival according to the integrated mutational and cytogenetic model in the training series. Panel A. Overall survival (OS). Panel B. Probability of progressive disease requiring treatment.
according to IWCLL-NCI guidelines as indicated by treatment free interval. Cases harboring \( TP53 \) and/or \( BIRC3 \) disruption (\( TP53 \) DIS/\( BIRC3 \) DIS) independent of co-occurring genetic lesions are represented by the red line. Cases harboring \( NOTCH1 \) mutations (\( NOTCH1 \) M) and/or \( SF3B1 \) mutations (\( SF3B1 \) M) and/or del11q22-q23 in the absence of \( TP53 \) and \( BIRC3 \) disruption are represented by the yellow line. Cases harboring +12 in the absence of \( TP53 \) disruption, \( BIRC3 \) disruption, \( NOTCH1 \) mutations, \( SF3B1 \) mutations and del11q22-q23, and cases wild type for all genetic lesions (Normal) are represented by the green line. Cases harboring del13q14 as the sole genetic lesion are represented by the blue line. Nr=not reached.

**Fig 3.** Observed overall survival (OS) in patients from the training series compared to the expected OS in the matched general population. OS in CLL patients stratified according to the FISH cytogenetic model (Panel A) and the integrated mutational and cytogenetic model (Panel B) relative to the expected OS in the age-, sex- and calendar year of diagnosis-matched general population (black line). Panel A. Cases harboring del17p13 irrespective of co-occurring cytogenetic lesions are represented by the red line. Cases harboring del11q22-q23 in the absence of del17p13 are represented by the purple line. Cases harboring +12 in the absence of del17p13 and del11q22-q23 are represented by the yellow line. Cases harboring a normal FISH karyotype are represented by the green line. Cases harboring del13q14 deletion in the absence of other cytogenetic abnormalities are represented by the blue line. Panel B. Cases harboring \( TP53 \) and/or \( BIRC3 \) disruption (\( TP53 \) DIS/\( BIRC3 \) DIS) independent of co-occurring genetic lesions are represented by the red line. Cases harboring \( NOTCH1 \) mutations (\( NOTCH1 \) M) and/or \( SF3B1 \) mutations (\( SF3B1 \) M) and/or del11q22-q23 in the absence of \( TP53 \) and \( BIRC3 \) disruption are represented by the yellow line. Cases harboring +12 in the absence of \( TP53 \) disruption, \( BIRC3 \) disruption, \( NOTCH1 \) mutations, \( SF3B1 \) mutations and del11q22-q23, and cases
wild type for all genetic lesions (Normal) are represented by the green line. Cases harboring del13q14 as the sole genetic lesion are represented by the blue line.

**Fig 4.** Cumulative incidence of high risk clonal evolution. Time to high risk clonal evolution was defined as the time elapsed from diagnosis to the date of development of *TP53* abnormalities, *BIRC3* abnormalities, *NOTCH1* mutations, *SF3B1* mutations or del1q22-q23 (events) or last follow-up or death (censoring). Analysis was performed using death as a competing risk. Only patients that did not present high risk abnormalities at diagnosis were included in this analysis. Panels A-D. Cumulative incidence of clonal evolution according to age >65 years (HR: 4.18; 95% CI: 1.26-13.8; 5-year risk: 17.1%, 10-year risk: 32.9%), high LDH (HR: 3.15; 95% CI: 1.32-7.55; 5-year risk: 24.8%, 9-year risk: 62.8%), unmutated *IGHV* genes (HR: 2.89; 95% CI: 1.31-6.39; 5-year risk: 18.5%, 9-year risk: 50.8%), +12 or a normal genetics (HR: 2.29; 95% CI: 1.03-5.10; 5-year risk: 13.8%, 9-year risk: 34.5%).

**Fig 5.** Landmark analysis of the cumulative probability of overall survival (OS) according to the integrated mutational and cytogenetic model. Panel A: diagnosis. Panel B: landmark at 1 year. Panel C: landmark at 2 years. Panel D: landmark at 4 years. Cases harboring *TP53* and/or *BIRC3* disruption (*TP53 DIS/BIRC3 DIS*) independent of co-occurring genetic lesions are represented by the red line. Cases harboring *NOTCH1* mutations (*NOTCH1 M*) and/or *SF3B1* mutations (*SF3B1 M*) and/or del11q22-q23 in the absence of *TP53* and *BIRC3* disruption are represented by the yellow line. Cases harboring +12 in the absence of *TP53* disruption, *BIRC3* disruption, *NOTCH1* mutations, *SF3B1* mutations and del11q22-q23, and cases wild type for all genetic lesions (Normal) are represented by the green line. Cases harboring del13q14 as the sole genetic lesion are represented by the blue line.
Figure 2

A

B

del13q14
Normal/+12
NOTCH1 M/SF3B1 M/del11q22-q23
TP53 DIS/BIRC3 DIS

Events
Total
Median
95% CI

del13q14 vs Normal/+12
Normal/+12 vs NOTCH1 M/SF3B1 M/del11q22-q23
NOTCH1 M/SF3B1 M/del11q22-q23 vs TP53 DIS/BIRC3 DIS

p=0.0406
p=0.0082
p=0.0195

p=0.0031
p<0.0001
p<0.0001

p<0.0001

Figure 2
Figure 3
Normal LDH
Elevated LDH

p=0.0093

Age ≤65y
Age >65y

p=0.0192

IGHV M
IGHV UM

p=0.0087

del13q14 +12/normal

p=0.0435

Figure 4
Figure 5
Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia

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