

## LYMPHOID NEOPLASIA

Clinical impact of clonal and subclonal *TP53*, *SF3B1*, *BIRC3*, *NOTCH1*, and *ATM* mutations in chronic lymphocytic leukemia

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## Key Points

- Clonal and subclonal mutations of *NOTCH1* and *TP53*, clonal mutations of *SF3B1*, and *ATM* mutations in CLL have an impact on clinical outcome.
- Clonal evolution in longitudinal samples occurs before and after treatment and may have an unfavorable impact on overall survival.

Genomic studies have revealed the complex clonal heterogeneity of chronic lymphocytic leukemia (CLL). The acquisition and selection of genomic aberrations may be critical to understanding the progression of this disease. In this study, we have extensively characterized the mutational status of *TP53*, *SF3B1*, *BIRC3*, *NOTCH1*, and *ATM* in 406 untreated CLL cases by ultra-deep next-generation sequencing, which detected subclonal mutations down to 0.3% allele frequency. Clonal dynamics were examined in longitudinal samples of 48 CLL patients. We identified a high proportion of subclonal mutations, isolated or associated with clonal aberrations. *TP53* mutations were present in 10.6% of patients (6.4% clonal, 4.2% subclonal), *ATM* mutations in 11.1% (7.8% clonal, 1.3% subclonal, 2% germ line mutations considered pathogenic), *SF3B1* mutations in 12.6% (7.4% clonal, 5.2% subclonal), *NOTCH1* mutations in 21.8% (14.2% clonal, 7.6% subclonal), and *BIRC3* mutations in 4.2% (2% clonal, 2.2% subclonal). *ATM* mutations, clonal *SF3B1*, and both clonal and subclonal *NOTCH1* mutations predicted for shorter time to first treatment irrespective of the immunoglobulin heavy-chain variable-region gene (IGHV) mutational status. Clonal and subclonal *TP53* and clonal *NOTCH1* mutations predicted for shorter overall survival together with the IGHV mutational status. Clonal evolution in longitudinal samples mainly occurred in cases with mutations in the initial samples and was observed not only after chemotherapy but also in untreated patients. These findings suggest that the characterization of the subclonal architecture and its dynamics in the evolution of the disease may be relevant for the management of CLL patients. (*Blood*. 2016;127(17):2122-2130)

## Introduction

The clinical course of patients with chronic lymphocytic leukemia (CLL) is highly heterogeneous.<sup>1,2</sup> The mutational status of the immunoglobulin heavy-chain variable-region genes (IGHV) and deletions/mutations of 11q/*ATM/BIRC3* and 17p/*TP53* are important determinants of the clinical outcome of the disease.<sup>1</sup> In recent years, next-generation sequencing (NGS) studies have provided a complete profile of somatic mutations in CLL.<sup>3-9</sup> Few genes have mutations with mid/low frequencies around 11% to 15%, whereas a larger group of genes are mutated at much lower frequencies (2%-5%), highlighting a striking interpatient heterogeneity.<sup>10</sup> The most commonly altered genes cluster

in a limited number of pathways, including DNA damage response and cell cycle control, the nuclear factor- $\kappa$ B signaling pathway, messenger RNA processing, and NOTCH signaling among others.<sup>8,9,11</sup> Multiple studies on population-based or clinical trial cohorts have demonstrated the adverse prognostic value of *TP53*, *ATM*, *SF3B1*, *NOTCH1*, and *BIRC3* mutations.<sup>6,12-14</sup>

Combined copy number analysis<sup>13,15-20</sup> and NGS<sup>11,12,21</sup> have shown that CLL cases may be composed of heterogeneous tumor cell populations with subclonal mutations that may evolve over the course of the disease and influence its biological behavior. The acquisition and

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The sequencing data reported in this article have been deposited in the European Nucleotide Archive (ENA, <http://www.ebi.ac.uk/ena>; accession number ERP013384).

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

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selection of genomic aberrations over the disease course may be critical to understanding the progression and resistance to treatment.<sup>22</sup> In addition, the presence of subclonal driver mutations may influence a more aggressive evolution of the disease.<sup>14</sup> The high sensitivity of ultra-deep NGS allows for the study of the clonal heterogeneity of tumors and the detection of very small mutated subclones.<sup>23,24</sup> Recent studies have shown the clinical relevance of the detection of *TP53* mutation at very low allele frequency.<sup>12,24</sup> However, the presence and prognostic impact of minor mutated subclones of other genes with prognostic impact in CLL and their clonal dynamics in the evolution of the disease are not well known. The goals of this study were to explore the presence of clonal and subclonal mutations of *TP53*, *ATM*, *SF3B1*, *NOTCH1*, and *BIRC3* in CLL using an ultra-deep NGS strategy, define the evolution of these subclones at different time points of the disease, and determine their influence in the outcome of patients.

## Materials and methods

### Patients and samples

Samples from 406 untreated CLL patients were included in this study (Table 1). In 48 patients, longitudinal samples obtained at different time points of the disease, including stable phase, progression before treatment, or relapse, were also examined. Tumor cells were purified from fresh or cryopreserved mononuclear cells using a cocktail of magnetically labeled antibodies as described (AutoMACS; Miltenyi Biotec).<sup>10</sup> The median final fraction of tumor cells determined by flow cytometry was 98% with 85% samples having a tumor purity >90%. The frequency of mutant alleles detected by ultra-deep NGS was corrected for the specific tumor cell content of each tumor. DNA from purified normal blood cells from the same patients was also obtained. The study was approved by the Hospital Clínic ethics committee. All patients gave informed consent according to the International Cancer Genome Consortium guidelines.<sup>25</sup>

### Molecular and genetic characterization

Copy number alterations (CNAs) were investigated as described (supplemental Methods, available on the *Blood* Web site).<sup>9</sup> Targeted ultra-deep NGS of *TP53* (exons 4-10), *ATM* (exons 2-63), *BIRC3* (exons 2-9), *SF3B1* (exons 14-16 and 18), and *NOTCH1* (exons 26, 27, 34, and 3' untranslated region [UTR]) was performed. Specific primers for *TP53*, *ATM*, *BIRC3*, and *SF3B1* were designed with the D3 Assay Design web-based tool (<https://www.fluidigm.com/assays>) (supplemental Table 1). Amplicon libraries were generated using the Access-Array system (Fluidigm), pooled, and paired-end sequenced in MiSeq equipment (Illumina). A mean coverage >3000x was obtained for each gene. Across the whole target region, a coverage >1000x was obtained in >85% of the sequence in 95% of the samples (supplemental Figure 1). *NOTCH1*-specific primers were designed using the Primer3 program (supplemental Table 2).<sup>26,27</sup> Long polymerase chain reaction (PCR) amplifications were performed using the KAPA HiFi DNA Polymerase HotStart ReadyMix (Kapa Biosystems) and normalized with the SequalPrep Normalization Plate kit (Invitrogen).<sup>28</sup> Libraries were generated with the Nextera XT DNA Library Preparation kit (Illumina) and sequenced in a MiSeq. The average sequencing coverage was 2310x; a coverage >250x and >500x was obtained in >85% of the target region in 87% and 71% of the samples, respectively (supplemental Figure 1).

### Bioinformatic workflow

Sequencing reads were mapped to the human reference genome (GRCh37) using the Burrows-Wheeler Aligner-MEM algorithm (version 0.7.10).<sup>29</sup> Coverage along the targeted regions was analyzed using SAMtools (version 1.1)<sup>30</sup> and custom scripts. Variant calling was performed using the VarScan2 (version 2.3.6).<sup>31</sup> Moreover, the entire pipeline established on the MiSeq Reporter software (MSR; version 2.4.60) was run in parallel. All variants detected by

**Table 1. Patients' baseline characteristics at the time of sampling**

Parameter	Category	CLL, n = 406
Sex	% Male/Female	57/43
Age, y	Median (range)	66 (19-94)
Time from diagnosis to sampling, mo	<12	206
	>12	200
Binet stage	A	313
	B	52
	C	15
	Unknown	26
Rai stage	0	253
	I-II	109
	III-IV	17
	Unknown	27
CNAs	Trisomy 12	52/376 (13.8%)
	Del13q	163/376 (43.4%)
	Del17p	19/398 (4.7%)
	Del11q	36/398 (9.0%)
IGHV mutational status	Mutated	218/382 (57.1%)
Patients treated during follow-up	n (%)	208/406 (51.2%)
Follow-up from sampling, mo	Median (range)	35 (6-224)

Del, deletion; IGHV unmutated, ≥98% identity with germ line.

any of these 2 algorithms were combined and annotated using ANNOVAR (version 2014Jul14)<sup>32</sup> as well as custom scripts. Two additional callers, UnifiedGenotyper and HaplotypeCaller (Genome Analysis Toolkit [GATK], version 3.3-0),<sup>33</sup> were tested but no additional variants were detected by these 2 algorithms. Variant calling was executed after performing the indel realignment and the base quality score recalibration steps defined in the GATK Best Practice recommendations.<sup>34,35</sup> All programs were executed following the authors' recommendations. The complete bioinformatic pipeline is shown in supplemental Figure 2, and a comparison of variant callers in supplemental Figure 3. Synonymous variants and polymorphisms described in the Single Nucleotide Polymorphism Database (dbSNP138) with a European population frequency higher than 1% (1000 Genomes Project database) were removed. *TP53* and *SF3B1* variants were considered as somatic mutations when, in addition to fulfilling the previous criteria, they were truncating, affected splicing sites, or were identified as somatic mutations in COSMIC (<http://cancer.sanger.ac.uk/cosmic>), the International Agency for Research on Cancer *TP53* database (<http://p53.iarc.fr/>), or in our CLL-genome project database.<sup>9</sup> *NOTCH1* truncating mutations were considered somatic whereas all nontruncating variants were confirmed to be in the germ line by sequencing the respective normal DNA sample. All *ATM* and *BIRC3* variants were investigated in the germ line DNA of the patient. All truncating *BIRC3* mutations were identified as somatic whereas the missense variants were present in the germ line and not considered for further studies. *ATM* mutations identified in the germ line were classified as rare polymorphisms, mutations of unknown significance, rare missense mutations, and likely/definitively pathogenic according to previous criteria (supplemental Table 3).<sup>36-39</sup> To assess the sensitivity of this methodology to detect low-frequency mutations, we performed 4 independent dilution experiments using DNA from 4 cases with *TP53*, *ATM*, and *NOTCH1* mutations in >90% of the cells. Our approaches were able to call these mutations down to a variant allele frequency (VAF) <1% (supplemental Figure 4).

### Verification of clonal and subclonal mutations

Sanger sequencing was used to verify a selected number of mutations. As in previous studies, our VAF threshold to detect mutant alleles by Sanger was 12% (supplemental Figure 5).<sup>24</sup> Therefore, mutations were considered subclonal (ie, low-allele frequency) when VAF was <12% and clonal (ie, high-allele frequency) when VAF was ≥12%. To verify clonal mutations, Sanger sequencing was performed on 69 high-frequency mutations and in 233 unmutated regions/genes. All results obtained by our pipeline were confirmed by Sanger (supplemental Methods; supplemental Table 4). In addition, 43 cases carrying high frequency mutations were subjected to a second round of NGS showing concordant results in all cases. All subclonal mutations (VAF <12%) were

verified by a second independent NGS experiment and/or confirmed by allele-specific PCR, as described (supplemental Table 5; supplemental Figure 6).<sup>24,40</sup> According to the verification step, the specificity of our analysis on calling low-frequency mutations was 73%.

### Statistical methods

Primary end points were overall survival (OS) and time to first treatment (TTT). OS was calculated from the date of sampling to the date of death or last follow-up. TTT was calculated from the date of sampling to the date of first treatment or last follow-up, considering disease-unrelated deaths as competing events. The log-rank test was used to compare Kaplan-Meier curves of OS; the Gray test was used to compare cumulative incidence curves of TTT. Multivariate analyses of prognostic factors were modeled using Cox and Fine-Gray regression models as previously described.<sup>41</sup> Thresholds for VAF that offered the best prediction in terms of TTT or OS were calculated for every gene using maximally selected rank statistics and receiver operating characteristic curves (supplemental Results). All calculations were performed using R, version 3.2.2. Double-sided *P* values < .05 were considered significant. A detailed explanation of the statistical methods is available in supplemental Methods.

## Results

### Clonal and subclonal mutations

The minimal mutant allelic fractions observed in our cases were 0.3% for *TP53* and *NOTCH1*, 0.5% for *BIRC3*, 1% for *SF3B1*, and 2% for *ATM*. Missense mutations were the most frequent aberration in *SF3B1* and *TP53*, whereas all *BIRC3* and *NOTCH1* mutations were truncating. *ATM*-truncating variants accounted for half of the variants detected. Clonal and subclonal mutations had similar molecular features and gene distribution (Figure 1). Convergent evolution (acquisition of independent genetic mutations in the same gene) was observed in 30 cases (*NOTCH1*, 13; *ATM*, 12; *TP53*, 9; *BIRC3*, 5; *SF3B1*, 4).

***TP53*.** A total of 55 *TP53* mutations were found in 43 of the 405 patients (10.6%) studied: 28 clonal (51%) and 27 subclonal (49%) (Figure 1A; supplemental Table 6). Mutations were mainly located at the DNA-binding domain of the protein (Figure 1B), and around 70% were missense (Figure 1C). Subclonal mutations were the only *TP53* aberration in 16 of 405 patients (4%) and co-occurred with other abnormalities in 5 of 405 patients (1%): 4 *TP53* clonal mutations and 1 17p deletion (Figure 2). In contrast, 14 of 22 patients (64%) with *TP53* clonal mutations also had a 17p deletion. Isolated 17p deletions were only observed in 4 of 405 patients (1%) (Figure 2B).

***ATM*.** *ATM* had 126 variants in 95 patients. To determine whether these variants were somatic, we sequenced the germ line DNA of all mutated patients. Fifty-three mutations were classified as somatic (supplemental Table 7) and 73 as germ line variants. The latter were 67 missense (92%) and 6 truncating (8%) mutations. These germ line variants were classified as definitely (*n* = 8) or likely (*n* = 2) pathogenic (2 of these 10 were present in the same patient), rare missense (*n* = 33), variants of unknown significance (*n* = 12), or polymorphisms (*n* = 18) (supplemental Table 8). Interestingly, 4 of 9 cases (44%) with germ line pathogenic but only 3 of the 53 (6%) with nonpathogenic variants acquired 11q deletions (*P* < .01), suggesting a possible role of these germ line variants in the progression of the disease through deletion of the remaining allele (supplemental Figure 8).<sup>37</sup> On the other hand, most somatic mutations (40 of 53, 75%) were clonal and 27% (13 of 53) subclonal (Figure 1A). Mutations were detected in 32 of 63 exons, with no hotspot regions (Figure 1B).

Combining both *ATM* somatic mutations and pathogenic germ line variants, 63 mutations were identified in 44 of 398 patients (11%). All but 1 subclonal alterations were detected in cases that already carried a

clonal mutation (4 patients), 11q deletion (4 patients), or clonal mutation, and 11q deletion (4 patients) (Figure 2). Among the remaining 31 patients, 17 carried isolated clonal *ATM* mutations whereas in the other 14 cases, clonal *ATM* mutations coexisted with 11q deletions. Isolated 11q deletion without mutations was observed in 12 patients (Figure 2B; supplemental Figure 7). Both *ATM* mutations and 11q deletions mainly occur in IGHV-unmutated CLL (87%). Coexistence of *TP53* and *ATM* mutations was only observed in 2 of 87 cases (2%).

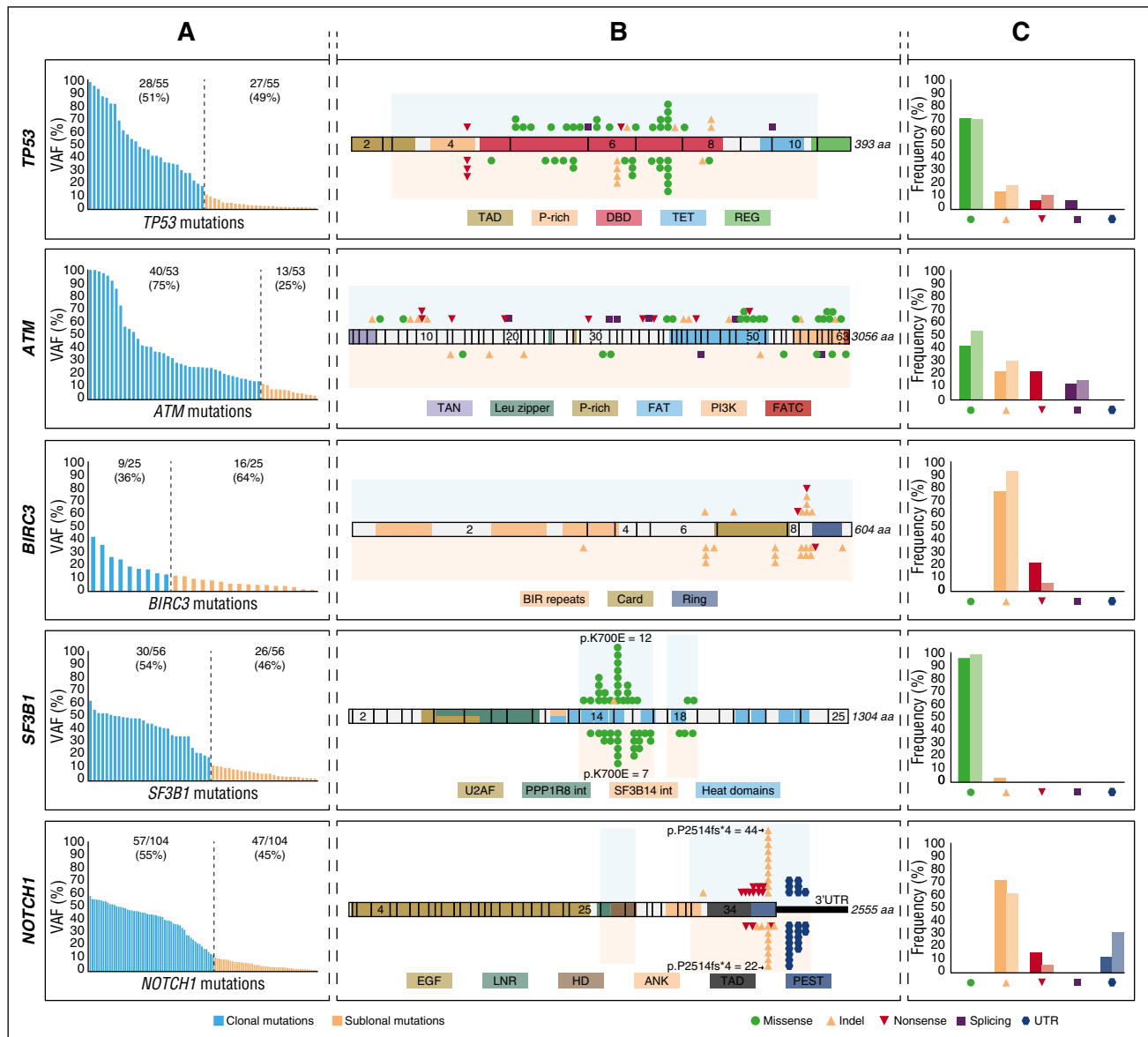
***BIRC3*.** We found 25 *BIRC3*-truncating mutations in 17 of 399 patients (4%), 9 clonal (36%), and 16 subclonal (64%) (Figure 1A; supplemental Table 9). All but 1 mutation were located in exons 6 to 9 (Figure 1B). Nine of the 17 patients (53%) had subclonal mutations, 3 of which were associated with 11q deletions. Eight patients (47%) had clonal *BIRC3* mutations, 3 coexisting with a subclonal mutation (Figure 2).

***SF3B1*.** We detected 56 *SF3B1* mutations in 51 of 401 patients (13%), 30 clonal (54%), and 26 subclonal (46%) (Figure 1A; supplemental Table 10). Mutations were found in the 4 evaluated exons, although the hotspot p.K700E mutation was the most prevalent (19 of 56, 34%) (Figure 1B). All but 1 were missense mutations (Figure 1C). Clonal *SF3B1* mutations were seen in 28 of 51 patients (55%), mutated subclones in 21 of 51 (41%), and only 2 of 51 cases (4%) had both clonal and subclonal mutations (Figure 2). *SF3B1* mutations were found together with *TP53* or *ATM* mutations in 5 and 13 CLL cases, respectively, and none with *BIRC3*.

***NOTCH1*.** We found 104 *NOTCH1* mutations in 86 of 391 patients (22%), 57 clonal (55%), and 47 subclonal (45%) (Figure 1A; supplemental Table 11). All mutations were truncating and detected in exon 34 (82, 79%) or the 3'UTR region (22, 21%) (Figure 1B-C). p.P2514fs\*4 (*n* = 66) and 3'UTR<sup>9</sup> (*n* = 22) mutations accounted for 85% of all *NOTCH1* mutations (Figure 1B). Interestingly, only subclonal *NOTCH1* mutations were observed in 30 of 86 (35%) of the mutated cases, only clonal mutations in 46 cases (53%), and the remaining 10 cases (12%) carried both clonal and subclonal mutations (Figure 2). *NOTCH1* mutations mostly occur in IGHV-unmutated CLL (82%), with no difference between clonal and subclonal alterations.

### Clinical impact

***Time to first treatment.*** The impact of clonal and subclonal mutations was initially evaluated in the 206 patients in whom the sample was obtained within 1 year of diagnosis. *ATM* mutations had a significant effect on TTT independent of the presence of 11q deletions. Seventy-one percent of patients with *ATM* mutations and no 11q deletions had received therapy within 1 year of sampling compared with only 37% of unmutated patients (*P* = .0014). Patients with 11q deletions also had a significantly shorter TTT compared with patients without *ATM* disruption (74% vs 37%, *P* < .0001). There was no significant difference between patients with *ATM* mutations without 11q deletion and patients with 11q deletion (*P* = .93) (Figure 3A). *SF3B1* clonal (*P* < .0001), but not subclonal (*P* = .22), mutations had a significant impact on TTT. At 1 year from sampling, 87% of patients with clonal mutations in *SF3B1* had required therapy compared with 51% with subclonal mutations and 40% with wild-type (WT) *SF3B1* (Figure 3B). In contrast, both clonal (*P* < .0001) and subclonal (*P* = .0001) *NOTCH1* mutations predicted for a shorter TTT compared with patients with a WT *NOTCH1* sequence (Figure 3C). Indeed, 74% of patients with clonal and 69% of patients with subclonal *NOTCH1* mutations had received first-line therapy within 1 year of sampling compared with 34% of WT patients. Other covariates with a significant impact on TTT were IGHV mutational status (*P* < .0001) (Figure 3D) and Rai stage (*P* < .0001) (Figure 3E). All 5 covariates

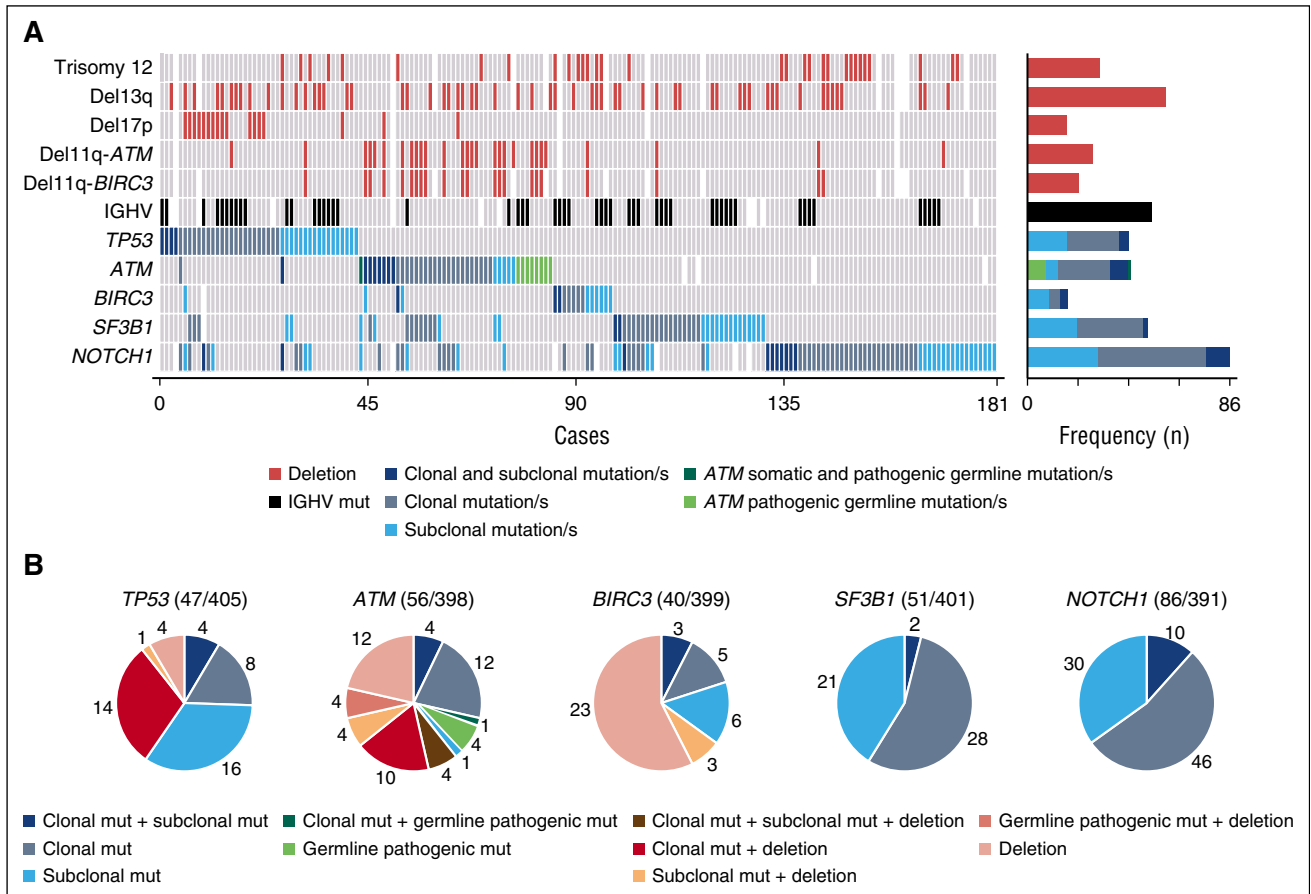


**Figure 1. Molecular profile and schematic diagram of clonal and subclonal *TP53*, *ATM*, *BIRC3*, *SF3B1*, and *NOTCH1* mutations.** (A) VAF of the mutations identified by NGS in each of the studied genes. Blue bars correspond to clonal mutations (VAF  $\geq 12\%$ ) whereas orange bars to the subclonal mutations (VAF  $< 12\%$ ). (B) Schematic diagram of *TP53*, *ATM*, *BIRC3*, *SF3B1*, and *NOTCH1*. Exons are represented by boxes and the main protein domains are colored. Color-coded shapes indicate the position and type of the mutation. Variants represented on the top of the protein correspond to high-frequency mutations (clonal) whereas variants represented under the diagram correspond to low-frequency mutations (subclonal). Shaded area corresponds to the region sequenced. (C) Comparison of the molecular profile of the identified clonal and subclonal mutations. Each pair of bars represent clonal (dark) and subclonal (light) mutations. No statistical differences were observed by the Fisher exact test.

were independently associated with TTT according to the Fine and Gray regression model: IGHV mutational status (hazard ratio [HR] = 1.78; 95% confidence interval [CI], 1.06-2.98;  $P = .028$ ), *SF3B1* mutations (HR = 1.73; 95% CI, 1.05-2.86;  $P = .031$ ), *ATM* aberrations (mutations/deletions) (HR = 1.36; 95% CI, 1.05-1.76;  $P = .021$ ), *NOTCH1* mutations (HR = 1.39; 95% CI, 1.11-1.76;  $P = .0049$ ), and Rai stage (HR = 4.34; 95% CI, 2.71-6.96;  $P < .0001$ ). On the other hand, the presence of *TP53* or *BIRC3* mutations, either clonal or subclonal, was not significantly associated with TTT ( $P = .63$  and  $P = .97$ , respectively) (supplemental Figure 9). The TTT impact of clonal and subclonal mutations was also evaluated in the entire cohort of 406 patients with similar results for all 5 genes (supplemental Results; supplemental Figure 10).

**Overall survival.** The 5-year OS of patients harboring *TP53* mutations was significantly shorter for patients with both clonal (54%)

and subclonal (64%) mutations compared with those with WT *TP53* (82%) ( $P < .0001$  and  $P = .011$ , respectively), with no significant difference between clonal and subclonal mutations ( $P = .44$ ) (Figure 4A). Given the frequent co-occurrence of *TP53* mutations with 17p deletions, we also evaluated the impact of isolated mutations vs 17p deletions. All 3 subgroups (17p deletions, *TP53* clonal mutations without deletions and *TP53* subclonal mutations without deletions) had prognostic impact compared with the WT sequence ( $P < .0001$ ,  $P = .037$ , and  $P = .037$ , respectively [supplemental Figure 11]). Patients harboring clonal, but not subclonal, *NOTCH1* mutations had a significantly shorter OS compared with those having a WT sequence ( $P = .001$  and  $P = .94$ , respectively) (Figure 4B), whereas clonal *BIRC3* ( $P = .049$ ) or *SF3B1* ( $P = .097$ ) mutations had a trend toward a shorter OS compared with the WT cases (supplemental Figure 12). Finally, other covariates with a significant impact on OS by



**Figure 2. Graphical representation of gene aberrations observed in the entire cohort.** (A) CNA, IGHV status, and mutational status of the studied genes are represented. Each column represents an untreated CLL case carrying at least 1 mutation in any of the studied genes. Bar plot on the right represents the number of times that each CNA and IGHV status was observed in all mutated cases. Blue bar plots refers to the number of cases carrying isolated subclonal mutations, only clonal mutations, or both regarding the mutational status of the studied genes. Cases carrying *ATM* definitely/likely pathogenic germ line variants are also shown. (B) Incidence of *TP53*, *ATM*, *BIRC3*, *SF3B1*, and *NOTCH1* alterations classified regarding its clonal representation in the study cohort. Del, deletion; mut, mutation/s.

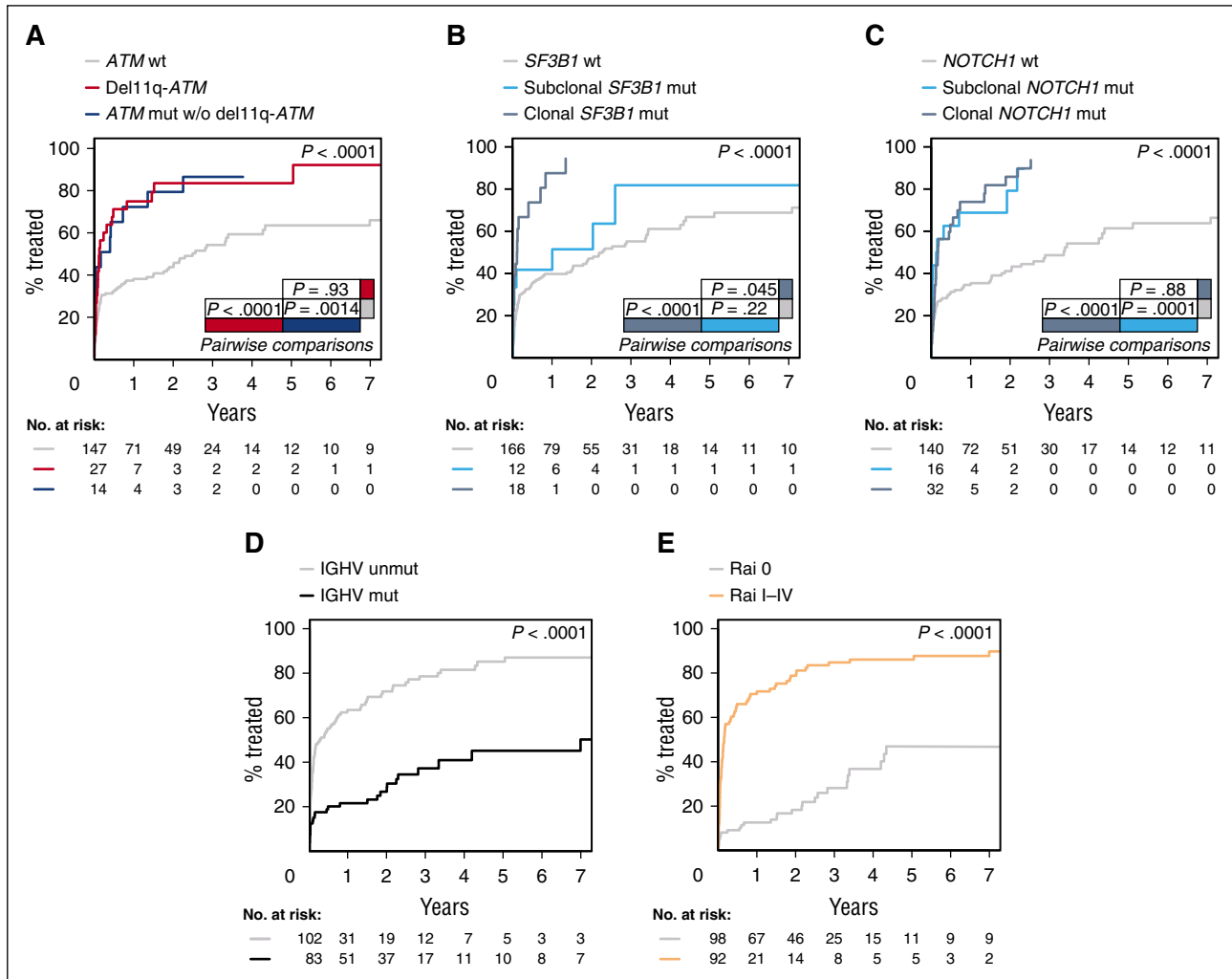
univariate analysis were IGHV mutational status ( $P = .0006$ ) and Rai staging ( $P = .001$ ) (Figure 4C-D). In contrast, neither 11q deletions or *ATM* mutations nor biallelic *ATM* inactivation had a significant impact on OS ( $P = .69$  and  $P = .91$ , respectively) (supplemental Figure 13). A multivariate analysis revealed that CLL patients harboring *TP53* aberrations (clonal and subclonal mutations/deletions) had a 1.71-fold increased risk of death (95% CI, 1.28-2.26;  $P = .0001$ ), and also patients with clonal *NOTCH1* mutations (HR, 1.5; 95% CI, 1.13-1.99;  $P = .0049$ ). Other factors independently associated with a shorter OS were unmutated IGHV (HR = 1.84; 95% CI, 1.05-3.21;  $P = .032$ ) and Rai stage I-IV (HR = 2.33; 95% CI, 1.41-3.85;  $P = .0009$ ). The internal validity of the model was evaluated using bootstrapping, and the 4 covariates were selected for the model in 69% of 1000 replications.

### Clonal evolution

We also performed a longitudinal analysis in 48 patients with sequential samples available (median time between samples [months]: 38, range: 1-198) (supplemental Table 12-13; supplemental Figure 14). Twenty of them lacked mutations in any of the 5 genes analyzed at sampling. Only 4 of the 17 cases (24%) in which the second sample was examined before treatment acquired mutations: 1 in *TP53*, 2 in *NOTCH1*, and the other acquired 2 mutations in *BIRC3* (Figure 5 case 84). This contrasts with patients evaluated after therapy, in which all 3 (100%) acquired mutations in *TP53* (2) or *BIRC3* (1). Eight of the 28 cases (29%) with mutated genes at sampling expanded the mutated clone in the

subsequent study: 5 at the moment of progression (3 *TP53*, 1 *SF3B1*, and 1 *TP53* + *SF3B1*) and 3 at relapse posttreatment (1 *TP53*, 1 *SF3B1*, and 1 *NOTCH1*). Two of the latter 3 cases also acquired additional mutations after treatment (*TP53* and *SF3B1*). Four mutations observed before treatment in 3 patients (1 *TP53*, 1 *SF3B1*, and 2 *BIRC3* in cases 7, 75, and 84, respectively) were not detected in the relapsed sample after treatment. The negative detection was confirmed by allele-specific PCR and/or a second NGS round. More complex patterns of evolution involving several mutated subclones in the same case were also observed in 5 cases: 4 before treatment and 1 at relapse. No evolution was seen before treatment in 4 cases with small mutated subclones (1 *ATM* + *SF3B1*, 1 *NOTCH1* + *ATM* + *TP53*, 1 *ATM*, and 1 *SF3B1* + *NOTCH1*), and in 9 cases (4 before treatment, 5 after treatment) in which virtually all cells carried a driver mutated gene (with a VAF around 50% or 100%).

The allele frequency of *TP53* mutations expanded before treatment in 5 cases. Three samples examined at relapse showed an expansion (Figure 5 case 27), persistence, or disappearance of the initial *TP53*-mutated clone. We also observed that *SF3B1*-mutated subclones also expanded before any treatment in 4 of the 6 cases (Figure 5 case 320). *NOTCH1* mutations appeared before treatment in 3 patients, expanded in one after treatment and remained stable in the others with and without treatment (Figure 5 case 48). On the contrary, no evolution was observed in cases with clonal or subclonal *ATM* mutations (Figure 5 case 48), although the mean time between samples was relatively short in these cases.



**Figure 3. TTT according to gene aberrations.** (A) Comparison of TTT among patients carrying *ATM* mutations without 11q deletion (blue line), 11q deletion (red line), and cases carrying a WT *ATM* gene (gray line) ( $P = .0014$  for *ATM* mutations vs WT;  $P < .0001$  for 11q deletion vs WT;  $P = .93$  for *ATM* mutations vs 11q deletion). (B) Comparison of TTT among cases carrying isolated subclonal *SF3B1* mutations (light blue line), clonal *SF3B1* mutations (dark blue line), and cases carrying a WT *SF3B1* gene (gray line) ( $P < .0001$  for clonal mutations vs WT;  $P = .22$  for subclonal mutations vs WT;  $P = .045$  for clonal vs subclonal mutations). (C) Comparison of TTT among patients carrying subclonal *NOTCH1* mutations (light blue line), clonal *NOTCH1* mutations (dark blue line), or WT *NOTCH1* gene sequence (gray line) ( $P < .0001$  for clonal mutations vs WT;  $P = .0001$  for subclonal mutations vs WT;  $P = .88$  for clonal vs subclonal mutations). (D) Comparison of TTT among patients carrying the mutated (black line) or unmutated IGHV gene sequence (gray line) ( $P < .0001$ ). (E) Comparison of TTT among patients diagnosed with Rai I-IV (orange line) or Rai 0 disease (gray line) ( $P < .0001$ ). *P*, *P* values by Gray test.

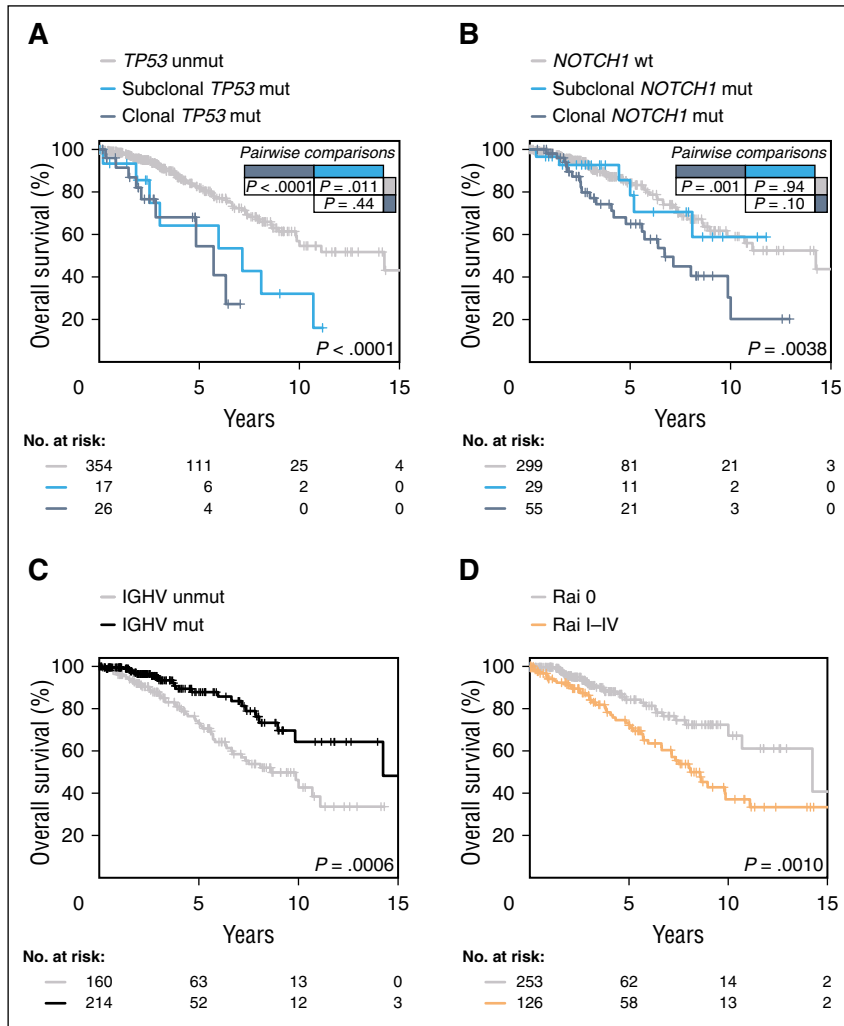
We then evaluated the impact of “clonal evolution” on the OS of these 48 patients with sequential samples. Clonal evolution was observed in 21 patients, 13 of 34 untreated (38%) and 8 of 14 after treatment (57%). These patients had a significant shorter OS than those with no evidence of clonal evolution (HR = 2.95; 95% CI = 1.16-7.5;  $P = .023$ ).

## Discussion

Genomic studies in CLL have recently emphasized the complex heterogeneity of the disease.<sup>11,21</sup> The characterization of the clonal architecture at early and subsequent phases of the disease may provide relevant information to orient management strategies more related to the biology of the tumor.<sup>22</sup> Whole-genome and exome-sequencing studies have revealed a large number of driver genes and the influence of their subclonal heterogeneity in the outcome of the

patients.<sup>9,10,14,42,43</sup> However, these comprehensive approaches have limited power to detect very small subclones of mutated driver genes that can expand over time and influence the evolution of the disease.<sup>14</sup> Recent studies using ultra-deep NGS have confirmed the clinical relevance of low-frequency *TP53*-mutated subclones on the outcome of CLL patients but whether this phenomenon occurs for other drivers is not well known.<sup>12,24</sup>

Using a highly sensitive NGS strategy, we have detected small subclones (down to 0.3% allele frequency) of 5 major CLL drivers (*TP53*, *SF3B1*, *BIRC3*, *NOTCH1*, and *ATM*) in a relative high proportion of patients (93 of 406, 23%). These subclonal mutations have similar molecular characteristics as their respective high-allele frequency mutations supporting a comparable pathogenic effect.<sup>7,24,42,44</sup> In this sense, we have confirmed the unfavorable impact on OS of *TP53* subclonal mutations, which was analogous to that of clonal alterations, even in the absence of deletions of the other allele.<sup>12,24</sup> We have also observed that both clonal and subclonal *NOTCH1* mutations and clonal, but not subclonal, *SF3B1* mutations

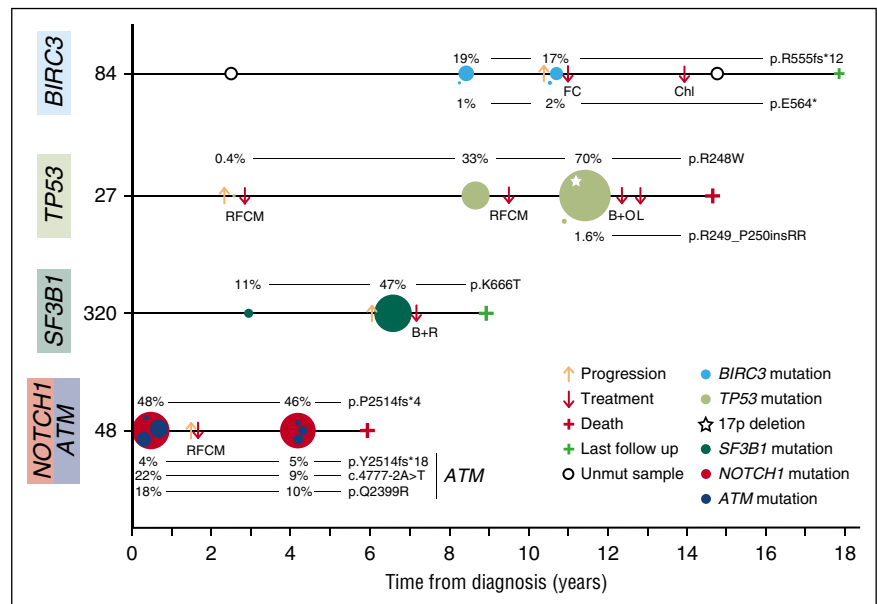


**Figure 4. OS according to gene aberrations.** (A) Comparison of OS among patients carrying subclonal *TP53* mutations (light blue line), clonal *TP53* mutations (dark blue line), and cases harboring an unmutated *TP53* gene (gray line) ( $P < .0001$  for clonal mutations vs WT;  $P = .011$  for subclonal mutations vs WT;  $P = .44$  for clonal vs subclonal mutations). (B) Comparison of OS from date of sampling between CLL patients carrying subclonal *NOTCH1* mutations, clonal *NOTCH1* mutations, and WT *NOTCH1* gene (light blue, dark blue, and gray lines, respectively) ( $P = .001$  for clonal mutations vs WT;  $P = .94$  for subclonal mutations vs WT;  $P = .10$  for clonal vs subclonal mutations). (C) Comparison of OS among patients carrying mutated (black line), and unmutated IGHV genes (gray line) ( $P = .0006$ ). (D) Comparison of OS among patients diagnosed with Rai I-IV (orange line), or Rai 0 disease (gray line) ( $P = .001$ ). *P*, *P* values by log-rank test.

have a significant impact on TTT, independent of IGHV mutations. The unfavorable prognosis of clonal *SF3B1* and *NOTCH1* mutations has been confirmed in several studies but the impact of

subclonal mutations had not been investigated.<sup>7,9,45-48</sup> The prognostic value of the *NOTCH1* subclonal mutations shown here is relevant because deep-sequencing approaches might be able to

**Figure 5. Representative examples of clonal evolution observed in a 48-sample longitudinal analysis.** Illustration of 4 representative CLL cases of clonal evolution showing the decrease or expansion of the *BIRC3*, *TP53*, *SF3B1*, *ATM*, or *NOTCH1*-mutated clone. Time 0 corresponds to the diagnosis time point. Each circle represents a unique mutation and its size is proportional to the VAF of the mutation corrected by the sample's tumor purity. Each mutation is represented at the time point at which a tumor sample was collected. B+O, bendamustine, ofatumumab; B+R, bendamustine, rituximab; Chl, chlorambucil; FC, fludarabine, cyclophosphamide; L, lenalidomide; RFCM, rituximab, fludarabine, cyclophosphamide, mitoxantrone.



identify these high-risk patients that were undetected by classical techniques.

The mutational study of *ATM* is challenging due to its large size and the need to distinguish potential pathogenic mutations already present in the germ line from polymorphisms or nonpathogenic variants. In this study, we have completely characterized the mutational status of *ATM* in a large series of patients. Of note, isolated subclonal *ATM* mutations were uncommon (only 1 case of 44). We found that *ATM* mutations had a significant impact on TTT even in the absence of 11q deletions, suggesting that fluorescence in situ hybridization or CNA are not sufficient for a complete *ATM* characterization. No effect of *ATM* mutations or 11q deletions on OS was observed, as previously described.<sup>38,49,50</sup>

*ATM* germ line variants previously described as definitively/likely pathogenic were frequently associated with 11q deletions, confirming the hypothesis that these germ line variants may influence disease progression through loss of the other allele.<sup>37</sup> Germ line variants considered as nonpathogenic had no impact on outcome and were rarely associated with 11q deletions. The advent of NGS platforms will certainly help to better characterize both somatic and germ line *ATM* mutations. The requirement of germ line DNA may be also relevant for *BIRC3* and *NOTCH1* because missense variants detected in the tumor sample were already present in the germ line. On the contrary, all *TP53* and *SF3B1* mutations detected had been previously confirmed already as somatic or pathogenic, suggesting that germ line DNA may be dispensable in these studies.

Our longitudinal study reveals the complex clonal evolution of this disease. We confirmed the expansion of most *TP53*-mutated clones after therapy observed also in other studies.<sup>12,20,24</sup> However, *TP53*, *SF3B1*, and *NOTCH1* mutations appeared de novo or expanded before any therapy in some patients, indicating that progressive dynamics of these clones are not only dependent on therapy selection. On the contrary, small *ATM*-mutated clones seem to be more stable, although the time between samples in our study was relatively short. We have also observed 2 subclonal (*TP53*, *SF3B1*) and 1 clonal (*BIRC3*) mutations that apparently disappeared under the detection threshold after treatment, suggesting that in some cases therapy may control these small subclones. Although the number of cases is limited, we observed that clonal evolution in longitudinal samples had an unfavorable impact on OS, suggesting that, in addition to the subclonal architecture of the tumor, the study of the clonal dynamics may provide relevant information to understanding the outcome of the patients.

In conclusion, this study shows the presence of a high number of clonal and subclonal mutations and convergent evolution of 5 driver genes in CLL and their impact on the outcome of the patients, as well as their possible patterns of clonal evolution. Particularly, clonal *NOTCH1*, *SF3B1*, and *ATM* mutations had an impact on shorter TTT, whereas clonal *NOTCH1* and *TP53* mutations conferred a shorter OS. Regarding the subclonal mutations detected in this study, only *NOTCH1* subclonal mutations had an impact on TTT,

whereas only subclonal *TP53* mutations influenced OS. Therefore, once validated by prospective studies, targeted ultra-deep NGS may well become a common approach for the assessment of patients' genomic alterations in daily practice and may be relevant for management strategies of CLL patients.

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## Authorship

Contribution: F.N., A.E., and E. Campo designed the study; F.N., M.P., P.J., A.E., X.S.P., C.L.-O., and E. Campo interpreted data; J.D. performed statistical analysis; F.N. performed the bioinformatic analysis; F.N., S.B., I.S., and D.M.-G. performed CNA analysis; F.N., C.R., A.N., and H.S.-C. performed and interpreted molecular studies; T.S., C.O., and E. Campo defined the classification of *ATM* germ line mutations; J.D., T.B., M. Aymerich, M.R., A.L.-G., N.V., D.C., M.G., M. Alcoceba, M.J.T., E. Colado, and E. Campo collected clinical and pathological data; and F.N., J.D., A.E., and E. Campo wrote the manuscript.

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