# *TP53* mutations with low variant allele frequency predict short survival in Chronic Lymphocytic Leukemia

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## Abstract

**Purpose.** In chronic lymphocytic leukemia (CLL), *TP53* mutations are associated with reduced survival and resistance to standard chemo-immunotherapy (CIT). Nevertheless, the clinical impact of subclonal *TP53* mutations below 10-15% variant allele frequency (VAF) remains unclear.

**Experimental Design.** Using a training/validation approach, we retrospectively analyzed the clinical and biological features of *TP53* mutations above (high-VAF) or below (low-VAF) the previously reported 10.0% VAF threshold, as determined by deep next-generation-sequencing (NGS). Clinical impact of low-VAF *TP53* mutations was also confirmed in a cohort (n=251) of CLL treated with FCR or FCR-like regimens from two UK trials.

**Results.** In the training cohort 97/684 patients bore 152 *TP53* mutations while in the validation cohort 71/536 patients had 109 *TP53* mutations. In both cohorts, *TP53* mutated patients experienced significantly shorter overall survival (OS) than *TP53* wild-type (wt) patients, irrespective of the *TP53* mutation VAF. By combining *TP53* mutation and 17p13.1 deletion (del17p) data in the total cohort (n=1,220), 113 cases were *TP53* mutated only (73/113 with low-VAF mutations), 55 del17p/*TP53* mutated (3/55 with low-VAF mutations), 20 del17p only, and 1,032 (84.6%) *TP53*wt. A model including low-VAF cases outperformed the canonical model, which considered only high-VAF cases (c-indices 0.643 vs. 0.603, *P<0.0001*), and improved the prognostic risk stratification of CLL-IPI. Clinical results were confirmed in CIT-treated cases (n=552) from the retrospective cohort, and the UK trials cohort.

**Conclusion.** *TP53* mutations impacted OS irrespective of VAF. This finding can be used to update the definition of *TP53* mutated CLL for clinical purposes.

## Translational Relevance

Evaluation of the *TP53* mutational status is a central pillar of the prognostic algorithms used for the clinical management of CLL patients, predicting both disease progression and sub-optimal response to therapy. Next-generation sequencing allows detection of *TP53* mutations at a level far below the conventional sequencing threshold recommended by international guidelines. Using a training/validation approach, we demonstrate that in the chemo-immunotherapy setting, *TP53* mutations are associated with short overall survival irrespective of variant allele frequency (VAF); low-VAF mutations (<10.0% VAF) maintained the same deleterious impact as high-VAF mutations (≥10.0% VAF). The clinical impact of small *TP53* mutated subclones was confirmed in an additional cohort of CLL patients treated with FCR-based regimens in two UK CLL trials. These findings can be used to re-define the classification of *TP53* mutated CLL patients and imply that the prognostic scoring systems, which include *TP53* mutation evaluation, e.g., the CLL-IPI, should be updated accordingly.

## Introduction

Disruption of the *TP53* gene, either by deletion at chromosome 17p13.1 (del17p) or mutations, represents the most important biomarker in chronic lymphocytic leukemia (CLL) (1–4) given its inclusion in algorithms with proven prognostic relevance both in the context of chemo-immunotherapy (CIT; e.g. CLL International Prognostic Index, CLL-IPI) and novel target therapies (5,6). Its importance as predictive factor is underscored by the fact that CIT is contra-indicated for patients harbouring such lesions at the time of therapeutic choice (7–9).

Recent studies based on ultra-deep-next generation sequencing (NGS) have shown that *TP53* mutations can be present in tumor cell populations at very low variant allele frequency (VAF), far below the detection limit of Sanger sequencing (10–15). However, the actual clinical impact of these subclonal *TP53* mutations is still a matter of debate (10–15). In fact, while some studies showed that patients bearing low frequency *TP53* mutated subclones experience similar outcome to patients with more evident clonal mutations of *TP53* (10,12), other studies failed to confirm these findings (13,15,16). In addition, the TP53 Network of the European Research Initiative on CLL (ERIC) still recommends the threshold of 10%VAF due to concerns about the possibility of false negative or false positive results below the 10% VAF (12).

In this study, by applying a training/validation approach in a large CLL cohort, we analyzed *TP53* mutations using ultra-deep NGS coupled with a rigorous bioinformatics pipeline. Here we provide evidence that *TP53* mutations impact on overall survival (OS) even if detected at very low subclonal levels. The clinical impact of small *TP53* small subclones was confirmed in an independent cohort of CLL patients treated with fludarabine-cyclophosphamide-rituximab (FCR) or FCR-like regimens in the context of two UK CLL trials (17,18).

## Material and Methods

### CLL cohorts

The REMARK criteria were followed throughout this study (Table S1). The main body of this study is represented by a retrospective analysis of 1,220 CLL cases (552 treated with CIT as first-line therapy, 93 of them treated with new agents as second-line therapy) from a cohort of 1,736 CLL patients, all diagnosed and treated according to iwCLL guidelines (1,19). All cases were referred to a single institution (Clinical and Experimental Onco-Hematology Unit, Centro di Riferimento Oncologico, I.R.C.C.S., Aviano, Italy) for molecular and cytogenetic analyses between 2003 and 2019 (Figure 1; details of the referral policy are given in Supplemental Information). Clinical outcome data were updated as of December 2019. The median follow-up from CLL diagnosis was 77 months (interquartile range, IQR, 39-120 months), while the median follow-up from sampling was 46 months (IQR 22-84 months). In the case of patients undergoing first treatment, they were all analyzed prior to treatment initiation (median time from sampling to first treatment, 4 months, IQR 1-18 months); all samples collected after first-line treatment (n=174) were excluded from the final cohort (Figure 1). No difference was found in terms of OS by comparing patients whose sample was obtained within the first year of diagnosis (659 cases, 299 treated) and those from whom samples were obtained after the first year (561 cases, 253 treated; Figure S1A).

For the purposes of the present study, the whole cohort was split in two separate cohorts: a training cohort (684 patients, all referred from a single center) that was utilized to set-up the NGS approach and bioinformatics pipeline for *TP53* mutation detection, and a validation cohort (536 patients, referred from four different centers). Table S2 summarizes the demographics of the whole cohort, and of the separate training and validation cohorts. A similar median OS was observed in the training cohort compared to the validation cohort (both not reached; P=0.9048, Figure S1B).

As further validation, a random splitting of the whole cohort according to a 70:30 ratio was also performed (see Supplementary Information “Set-up of alternative training and validation cohorts”).

Finally, an independent validation cohort of 251 CLL samples from two UK trials, ARCTIC and ADMIRE (Table S3) were included (17,18), in which patients were randomized to receive either FCR or FCR-like regimens without significant difference both in progression-free survival (PFS) and OS (P=0.5923 and P=0.6745, respectively; Figure S1CD). The median follow-up was 83.9 months with 150 progressions and 76 deaths, all deaths were preceded by disease progression. In this cohort, 61/251 cases were treated with new agents as salvage therapy.

Further information is available in Supplemental Information.

### Specimen characteristics

In accordance with the ERIC recommendations for *TP53* disruption,(20) mutation analyses were always carried out on peripheral blood (PB) samples containing >80% tumor cells. When the percentage of leukemic cells in the PB was below the threshold of 80%, as determined by flow cytometry (CD5+/CD19+), CD19-guided positive selection, using an autoMACS Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany), was performed (Figure S3).

### *TP53* mutations and bioinformatics pipeline of analysis

A detailed description of the procedures for *TP53* mutational status determination, including functional evaluation and the applied bioinformatics pipeline, is available in the Supplemental Information, and summarized in Figure S2. In particular, the bioinformatics pipeline can be retrieved from the GitHub website (details in Supplemental Information). All the samples from the retrospective Italian cohort, and the prospective UK cohort were sequenced and analyzed with the same pipeline at the Clinical and Experimental Onco-Hematology Unit (Aviano, Italy).

Briefly, analysis of *TP53* mutations was performed by NGS with an amplicon-based strategy, covering exons 2-11, in keeping with the ERIC recommendations (20). Specific primers were designed with the Primer3 program, and modified according to the Illumina (San Diego, CA) protocol (Table S4). Amplicon libraries were generated using a modified Illumina protocol starting from 40 ng of DNA (~6,000 diploid genomes), a quantity capable to successfully detect mutations below the 1% VAF in the context of our procedures (Figure S3). Multiplex PCR products were generated using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Milan, Italy) and subsequently tagged with specific index according to modified procedures for NexteraXT (DNALibrary Preparation kit, Illumina), as previously reported (21,22). Purified libraries were pooled, and paired-end sequenced in a MiSeq instrument (Illumina).

FASTQ files were aligned to the Hg19 reference with Burrows-Wheeler Aligner (BWA)-MEM algorithm, and allele variants were called by FreeBayes with non-stringent parameters (Figure S2) (23–26). A coverage ≥2,000X was obtained for each sample in 100% of the analyzed sequences (Figure S4). To calculate random/systematic errors, a database utilizing a subset of *TP53* wild-type (wt) cases (n=362) was generated from the training cohort and utilized for comparisons. *TP53* mutations were accepted if both of the following conditions were fulfilled: i) with a VAF that outdistanced for at least 2.75 standard deviations the mean of the transformed VAF distribution related to any single nucleotide position of the *TP53* sequence (Figure S5); ii) validated by Fisher's exact test after Bonferroni correction ($P<0.01$). The minimal allelic fraction for *TP53* mutation calling was 0.4% (Figure S6).

The IARC TP53 Database (http://p53.iarc.fr/) (27) was used to annotate *TP53* mutations (Figure S3), and to functionally evaluate *TP53* missense mutations for their capability to transcribe the *CDKN1A* gene (27,28). *TP53* mutated cases with less than 2% VAF were all confirmed by a second independent NGS run starting from DNA. In selected cases, *TP53* mutation VAF values below 2% were validated by a different experimental approach, i.e., PCR with amplification-refractory mutation system (ARMS-PCR; Figure S7) (12).

### FISH analysis

Interphase FISH was performed to detect del17p, 11q22.3 deletion (del11q), 13q14 deletion (del13p), and trisomy 12 (tris12) (29,30), as detailed in the Supplemental Information.

### Other CLL characterizations

CLL patients were also characterized for age, sex, Rai/Binet staging, Immunoglobulin-heavy-variable (IGHV) gene mutational status, *NOTCH1* mutations (retrospective cohort only) and CD49d expression (Table S2 and S3), as previously reported (21,31–34). Figure S8 summarizes the clinical

impact (OS) for all these variables in the retrospective cohort (training/validation cohorts and composite cohort).

**Statistical analysis**

All statistical analyses were performed by using standard methods (35–37); details are reported in Supplemental Information. OS was computed from date of blood sampling to death (events) or last follow-up (censoring); analysis of OS from first treatment was measured from date of first treatment to date of death (event) or last follow-up (censoring). PFS was calculated from the date of treatment initiation to progression (event) or last follow-up (censoring). Molecular studies were blinded to the study end points.

## Results

### Clinical impact of *TP53* mutations

The clinical impact of *TP53* mutations was investigated in a single-institution training cohort (n=684) and in a multicenter validation cohort (n=536), whose clinical and biological features are summarized in Table S2 and Figures S1AB and S8.

In the training cohort, the set-up of our NGS approach, coupled with a robust bioinformatics pipeline of analysis, led to the identification of a total of 152 *TP53* mutations in 97 patients (range of mutations/patient: 1-11). By identifying *TP53* mutated patients according to the VAF of the most prevalent *TP53* mutation, the VAF range for *TP53* mutated cases was 0.53-95.24% (Table S5 and Figure S9A). When the same approach was applied in the validation cohort, 109 *TP53* mutations were identified in 71 cases (range mutation/patient: 1-6; VAF range of the prevalent mutation: 0.53-92.47%; Table S5 and Figure S9B), with no significant difference between the two cohorts (P=0.3824, Table S2).

Regarding OS, *TP53* mutated patients experienced significantly shorter OS when compared with *TP53*wt patients both in the training cohort (median OS: 80.0 months *versus* not reached; *P<0.0001*; Figure S9C), and in the validation cohort (median OS: 73.0 months *versus* not reached; *P<0.0001*; Figure S9D).

Given the impact of *TP53* disruption in the prognostic stratification and therapeutic choice for CLL patients (3,5,19,20), the current ERIC guidelines suggest that only the variants with ≥10% VAF by NGS, should be reported (20). Here we demonstrate that *TP53* mutations with low VAF, i.e., below the 10% VAF threshold, had a similar clinical impact when compared with *TP53* mutations detected at higher VAF. In this regard, 56 out of 97 cases (57.7%), and 36 out of 71 cases (50.7%) were identified with "high-VAF" (i.e., with ≥10.0% VAF) for *TP53* mutations in the training and the validation cohort, respectively. The remaining 41 and 35 cases were classified as "low-VAF" cases (i.e., with <10.0% VAF) (Table S2). Both in the context of the training and validation cohorts, cases with high-VAF or low-VAF *TP53* mutations experienced shorter OS than *TP53*wt cases, with no difference between high-VAF and low-VAF *TP53* mutated cases (Figure S9E and F). A similar result was obtained following random splitting of the total cohort of 1,220 cases according to a 70:30 ratio.

When considering the combined cohort of 1,220 cases (Table S2), a total of 261 *TP53* mutations (Table S5) in 168 cases were found (13.8%; mutations per patient: 1-11; Figure 2A), 92 classified as high-VAF cases (VAF range: 10.8-95.2%, median VAF=53.0%), and 76 as low-VAF cases (VAF range: 0.53-9.6%; median VAF=2.6%) (Figure 2A). Again, *TP53*wt CLL cases showed significantly longer OS when compared with both of the *TP53* mutated categories (*P<0.0001*), with no significant difference between high-VAF and low-VAF *TP53* mutated cases (P=0.0712; Figure 2B, left panel). This observation was confirmed by limiting the analysis to newly diagnosed CLL, i.e., sampled within 6 months from diagnosis (n=539), although in this setting low-VAF cases fared similar to *TP53*wt cases during the first 5 years (Figure S10).

No major differences were found by comparing the clinical and biological features of cases with high-VAF *versus* low-VAF *TP53* mutations (Table S6). Notably, the clinical consequence of *TP53* mutations was similar when CLL patients with low-VAF *TP53* mutations were stratified into three subclasses with different VAF, i.e., <1% VAF (22 cases), 1-5% VAF (42 cases), 5-10.0% VAF (12 cases) (Figure S11A). Moreover, the capacity of *TP53* mutations to identify cases with shorter OS was also confirmed by separately considering cases that presented with single or multiple *TP53* mutations, either classified as high-VAF or low-VAF (Figure S11B).

These observations were further validated by Harrell's c-indices comparison of *TP53*wt *versus* *TP53* mutated CLL models. In particular, a model combining *TP53* mutated cases with both high-VAF *TP53* mutations and low-VAF *TP53* mutations significantly outperformed (c-index 0.643, range 0.606-0.686) a model where only cases with high-VAF *TP53* mutations were considered as mutated (c-index 0.603, range 0.572-0.638; $P<0.0001$) according to current recommendations (20).

Finally, the presence of *TP53* mutations, as evaluated by combining low-VAF and high-VAF mutations, remained an independent OS predictor in multivariate analysis even after adjusting for possible confounders, including sex, age, Rai staging, and other biological factors (i.e. IGHV status, CD49d expression, *NOTCH1* mutations, del11q and del17p. The same held true when the training and the validation cohorts were separately considered (Table S7).

**Molecular profile of *TP53* mutations**

By considering all 261 mutations found in the combined cohort (VAF range: 0.4-95.2%; median VAF=3.2%), 100 (38.3%) were classified as high-VAF (VAF range: 10.8-95.2%; median VAF=48.9%), while 161 (61.7%) were low-VAF (VAF range: 0.4-9.8%; median VAF=1.3%) (Table S5 and Figure 2A). According to the needle plot graphs (Figure 2C), no distribution differences were observed along the *TP53* coding sequence between high-VAF and low-VAF *TP53* mutations. When mutations were analyzed for their effect on the p53 protein in terms of amino acid changes, according to previous reports (38–40), the most common mutations in *TP53* coding region were missense mutations, followed by nonsense, frameshift, and splicing mutations, again without any distribution difference between high-VAF and low-VAF mutations (P=0.65 Figure 2D). In this context, the residual capacity of missense mutations to transcribe the *CDKN1A* gene, as retrieved from the IARC TP53 database (27,28), was comparably low in both high-VAF (13.04%, 95% CI 4.72-16.92) and low-VAF (13.74%, 95% CI 6.93-20.10; P=0.20) mutations. Consistently, by splitting patients into different categories according to the effect on protein of the mutation with the highest VAF, a significantly shorter OS was observed in all instances compared to *TP53*wt cases, regardless of the different types of mutations (Figure 2B, left panel). This observation was confirmed in the context of the separate training and validation cohorts (Figure S12AB).

***TP53* mutations and del17p**

In the context of training (n=684), validation (n=536) and total (n=1,220) cohorts, cases bearing del17p accounted for 41, 34, and 75, respectively (Table S2), these cases experienced significantly shorter OS according to the conventional hierarchical Dohner classification (41) (Figure S8AB).

Combining FISH data on del17p with *TP53* mutation data (Table S2 and Table S6) in the total cohort, we obtained: 1,032 cases without any *TP53* aberration, 20 cases with del17p only, 113 *TP53*

mutated only cases (73/113 with low-VAF *TP53* mutations), and 55 cases bearing both del17p deletion and *TP53* mutation (3/55 with low-VAF *TP53* mutations; Figure 3A). The reason behind the underrepresentation of low-VAF cases in the del17p/*TP53*-mutated category remains obscure; in some cases, especially with borderline VAF values, it could be due to a relative VAF overestimation caused by chromosome 17 loss. Patients with *TP53* mutations alone (median OS: 80.0 months) or concomitant *TP53* mutations and del17p (median OS: 67.0 months) had significantly shorter OS than *TP53*wt cases (median OS: not reached; *P<0.0001*; Figure 3B). Conversely, CLL cases bearing del17p only (median OS: 107.0 months) were too few (n=20) and had relatively short follow-ups to be able to draw any definitive conclusions (Figure 3B).

Finally, by splitting cases according to the presence of a single-hit (i.e. either *TP53* mutations or del17p), or a double-hit (i.e. both *TP53* mutations and del17p) *TP53* disruption, both groups experienced significantly shorter OS intervals than *TP53*wt cases, without difference by sub-dividing these categories into low-VAF and high-VAF *TP53* mutated cases (Figure S13AB); similar results were obtained by separately considering the training and the validation cohort (Figure S13C-F).

**Low-VAF *TP53* mutations and CLL-IPI risk categories**

We also tested whether low-VAF *TP53* mutations had an impact in the context of the risk categories identified by the CLL-IPI (17,18). Complete data to enable CLL cases to be scored according to the CLL-IPI were available on 652 patients from the retrospective cohort. Again, cases with low-VAF *TP53* mutations had shorter OS than *TP53*wt cases, with no difference with cases bearing high-VAF *TP53* mutations (Figure S14A). In this cohort, the CLL-IPI was computed by considering cases to be *TP53* mutated only if they harbored high-VAF *TP53* mutations (standard CLL-IPI), or by combining both high-VAF and low-VAF *TP53* mutations (CLL-IPI revisited). In particular, in standard CLL-IPI, 322, 183, 115, and 32 cases were assigned to the low, intermediate, high, and very high-risk categories, respectively (Figure S14B). Including low-VAF *TP53* mutated patients in the *TP53* mutated group resulted in an additional 40 cases being assigned into the high/very high risk categories. Previously, these cases were considered to assign to better categories (low risk category, 17 cases; intermediate risk category, 11 cases; high risk category, 12 cases). Importantly, all of these cases demonstrated a lower 10-year OS than the other in their original CLL-IPI category (Figure S14B). Although the CLL-IPI showed strong prognostic resolution in both configurations, the CLL-IPI revisited, with the inclusion of low-VAF *TP53* mutated cases (c-index 0.730), significantly outperformed the standard CLL-IPI (c-index 0.721; *P<0.0001*).

***TP53* mutations and del17p in treated CLL**

By focusing on treated patients from the retrospective cohort (n=552), and using as a clinical readout the OS from the start of therapy, *TP53* mutated CLL (64 cases; median OS: 54.0 months) or *TP53* deleted/mutated CLL (39 cases; median OS: 57.0 months) again experienced significantly shorter OS when compared with *TP53*wt cases (median OS: not reached; *P<0.0001* in both comparisons; Figure 3C). In this context, CLL cases bearing high-VAF and low-VAF *TP53* mutations (61 and 42 cases, respectively) had similar OS intervals (median OS: 47.0 months, or 62.0 months, respectively; P=0.3170), significantly shorter than *TP53*wt cases (median OS: not reached; *P<0.0001*; Figure 3D). Superimposable results were obtained by separately considering

the training and the validation cohorts (Figure S15AB), as well as by limiting the analyses to patients (n=368) whose samples were collected less than 12 months from treatment (Figure S15C).

In the context of the ARCTIC/ADMIRE cohort, a total of 65 *TP53* mutations (VAF range: 0.57-86.8%; 38.5% high-VAF, 61.5% low-VAF; Table S8 and Figure 4A) were distributed in 40 out of 251 cases (15.9%; 1-8 mutations per patient), 18 of them (45.0%) with a VAF<10.0% (Table S8). No distribution differences along the *TP53* coding sequence were observed between high-VAF and low-VAF *TP53* mutations, and the most common mutations were missense mutations, both in the high-VAF and low-VAF *TP53* mutation categories (P=0.3153; Figure 4B and 4C) (38–40).

Again, CLL cases bearing high-VAF and low-VAF *TP53* mutations had OS (Figure 4D) and PFS (Figure S16A) intervals, significantly shorter than *TP53*wt cases (median OS: 107.9 months; *P<0.0001* and *P=0.0058 versus* high-VAF and low-VAF *TP53* mutation cases, respectively; median PFS: 69.3 months; *P<0.0001* and *P=0.0045 versus* high-VAF and low-VAF *TP53* mutation cases, respectively).

Also in this setting, *TP53* mutations (high-VAF and low-VAF combined) remained independent predictors of OS/PFS after adjustment for possible confounders (Table S9), and both single-hit and double-hit *TP53* disruption identified patient subsets with worse OS compared to *TP53*wt cases, irrespective to low-VAF/high-VAF *TP53* mutations (Figure 4E and Figure S16B).

**Evolution of *TP53* mutated clones upon treatment**

The evolution of *TP53* mutated clones was assessed by longitudinal NGS analysis of sequential PB samples collected from 13 patients before first treatment (median time of sampling from first treatment, -1 month; range –68/0 months, Figure S17A) and at relapse (median time from treatment, 27 months, range 2.5-76.0 months, Figure 17B) corresponding to 14 *TP53* mutations (8 low-VAF and 6 high-VAF *TP53* mutations; Table S10). In these cases, the *TP53* mutated clone identified before treatment invariably increased at the time of second analysis (*P=0.0001*; Table S10), which was performed in close proximity to the 2$^{nd}$- or 3$^{rd}$-line therapy in 10/13 cases (Figure S17B). In particular, in the 3 out of 8 cases with low-VAF *TP53* mutations, the small *TP53* mutation identified in the pre-treatment sample crossed the 10% threshold and became predominant at relapse (Table S10 and Figure S17A).

## Discussion

The present study took advantage of different retrospective and prospective CLL cohorts (17,18), all analyzed for *TP53* mutations using a highly controlled and sensitive NGS approach. We were able to demonstrate that: i) *TP53* mutations can be reliably detected at low subclonal level in a significant fraction of CLL cases; ii) these low level *TP53* mutations, even if they represent the sole *TP53* aberration, have clinical relevance as they identify CLL cases with OS shorter than cases bearing *TP53*wt configuration; iii) cases harboring low-VAF *TP53* mutations experienced similar OS to cases bearing a higher *TP53* mutational burden, although high-VAF cases may display a trend to worse outcome. The clinical impact of small *TP53* mutated subclones was confirmed in a separate analysis of CIT-treated patients, including those from the prospective UK trials (17,18). Even after adjustment with possible confounders, low-VAF *TP53* mutations retained prognostic significance in both the retrospective and ARCTIC/ADMIRE cohorts. Altogether, these results suggest that CLL patients, even if affected by a disease bearing low-VAF *TP53* mutations, should not be given CIT as 1$^{st}$-line therapy (10,12). In the case of patients treated with novel agents after 1$^{st}$-line therapy (93/1,220 in the retrospective cohort, 61/251 in the ARCTIC/ADMIRE cohort), the lack of *TP53* mutation re-testing at the time of starting of novel agent treatments does not allow any conclusions to be drawn regarding the impact of low-VAF *TP53* mutations in this setting.

As a cutoff to discriminate between high-VAF and low-VAF *TP53* mutations, we relied upon the 10 % VAF cutoff, a threshold consistent with ERIC recommendations and in line with the increasing use of NGS rather than Sanger in most centers nowadays (12,20). The frequency of *TP53* mutated cases observed here was 14%, including cases with high-VAF and low-VAF *TP53* mutations, this was reduced to 7.5% if we considered only the cases bearing high-VAF mutations as *TP53* mutated cases. So, about 6.5% of cases (76 cases in our series) would have been misclassified as *TP53*wt, in keeping with other studies (10,12).

While in accordance with previous studies (10,12), our findings differ from others (17,18), allegedly due to different pre-analytical preparations and/or the bioinformatics pipeline used for analysis. For example, in Blakemore et al (15), the number of cases with low-VAF mutations was only 16 (3.2% of cases) with a minimum VAF of 1.43% (0.43% in the present study). Purification of samples with <80% tumor cell frequency, as routinely performed by us, reduces the possibility of under representation of mutations due to “contaminating” normal DNA. This is clearly important when considering the reliable identification of cases with a very low mutational burden.

On the other hand, Brieghel et al (13), in a cohort of 290 cases, reported about 5% of cases with a *TP53* mutational burden <1% VAF, as compared to the 1.8% of cases detected here; this was probably due to a declared sequencing depth (20,000X) far superior to the 2,000X of our study, which is consistent with the read depth used for *TP53* mutation analyses in routine clinical practice (13). In our study about 0.5% was the lowest VAF value found for a single mutation in *TP53* mutated CLL case. Therefore, further studies are needed to evaluate the clinical impact of mutations with a VAF below 0.5%.

The *ad-hoc* bioinformatics pipeline generated in this study, available on GitHub for possible users, was based on the creation of a matrix dataset of *TP53*wt cases in order to eliminate the background

noise due to random sequencing errors. This approach allowed us to confidently detect *TP53* mutations with a <1% VAF, which were validated by a high-sensitivity extra-assay method like allele-specific PCR (12). Of note, in addition to our pipeline, each CLL sample entering our study was double-tested using the commercially available Miseq-Reporter software, which needs neither deep bioinformatics skills, nor any type of reference database. In keeping with the detection limit of the software, the Miseq-Reporter identified all the mutations called by our pipeline up to the lower limit of 1.3% VAF, leading to the potential misinterpretation of 30 cases out of 1,220 from the retrospective cohort (2.4%), all with VAF below the 1.3% threshold (R.B., unpublished observation).

Low-VAF *TP53* mutations, as reported here, displayed a molecular distribution along the *TP53* gene, and functional features of p53 dysfunction, completely superimposable to those reported for high-VAF *TP53* mutations (12,28). Moreover, in sequential samples from patients subjected to CIT treatment, the minor *TP53* mutated clones were positively selected and became the dominant leukemic population at relapse in some cases. This effect is in keeping with the notion that these mutations, although initially occurring in a minority of cells, are selected over time eventually resulting in the dominant population (42). The observation that low-VAF and high-VAF *TP53* mutations had similar negative clinical impact is also in keeping with the known capability of *TP53* mutated tumor cells to enhance the overall tumor cell fitness by influencing the tumor microenvironment and/or the *TP53*wt neoplastic component (43).

Whereas tumor suppressors are commonly inactivated by frameshift or nonsense mutations, the most frequent mutation type of *TP53* in CLL is represented by missense mutations (10,12,13,40,44), an observation confirmed by our findings. Here, we demonstrated that in the context of CLL patients, all the identified *TP53* mutants could be considered functionally equivalent from a clinical point of view. Grouping patients according to the type of *TP53* mutation showed that each group experienced the same shorter OS when compared to *TP53*wt patients. Even if missense *TP53* mutations were split into mutations occurring within the DNA-binding motifs (DBM, 59 cases in our retrospective composite cohort), and mutations occurring outside the DBM (66 cases in our cohort), as reported by Trbusek et al (44), no difference was found in terms of median OS (73 months *versus* 78 months, R.B., unpublished observation). Overall, these data suggest that the effect of *TP53* mutations in CLL seems to be neither related to the percentage of mutations nor to the different types of mutations.

When integrating mutational results for *TP53* with del17p data, a small proportion of “17p deleted only cases” (20/1,220 cases in our total retrospective series, 1.6%) was observed, in keeping with the literature (3,45,46). Although the number of cases was low and with short follow-up intervals, the sole presence of del17p did not seem to associate with a significant negative impact on OS when compared with *TP53*wt. This is in line with previous reports for other lympho-proliferative diseases (14,47,48), while the same was not observed in the so-called *TP53* mutated only cases. In this regard, given the different sensitivity between the FISH *versus* the NGS methods, it cannot be excluded that small subclonal deletions of *TP53*, below the FISH detection limit, may actually have occurred in (some) *TP53* mutated only cases.

In conclusion**,** we have demonstrated that in the CIT setting *TP53* mutations confer a significantly shorter OS, irrespective of VAF percentage. As such, these findings may have important implications for the clinical management of CLL patients bearing *TP53* disruption, and may imply to redefine the threshold used to identify *TP53* mutated cases. In this context, further investigations are needed to revise those prognostic scoring systems, e.g., the CLL-IPI (5), that include *TP53* disruption data in their evaluation. The clinical impact of this finding in the setting of CLL treatment with chemo-free regimens (49,50) remains to be established.

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**Authors' contribution:** R.B., designed the study, interpreted data, and wrote the manuscript F.M.R., F.V., T.D.A., T.B., A.Z., E.T., F.P., E.V., M.D., E.Z., I.C., P.V., P.N., M.B., A.B., J.A.C., G.F., D.R. performed and interpreted molecular studies, and contributed to data interpretation; F.V., J.P., and R.B. generated the bioinformatics pipeline of analysis, and performed statistical analyses; E.S., A.B., M.G., F.M., G.P., G.D.A., J.O., P.B, A.C., F.Z., F.D.R., G.D.P. collected clinical data and contributed to data interpretation; C.P., A.H., A.S., P.H., collected clinical data related to the U.K. trials and contributed to data interpretation; V.G. designed the study, interpreted data, and wrote the manuscript.

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## Figure legends

**Figure 1. Flow-chart representation of the study.** Reported is a schematic representation of the flow-chart of the study with the number of patients analyzed.

**Figure 2. Molecular profile and clinical impact of *TP53* mutations in the total retrospective cohort.** A) Distribution of *TP53* mutations in the total retrospective cohort. Bar chart graph reports the percentage of VAF identified by NGS analysis for all 261 *TP53* mutations sorted in descending order with regard to the percent of VAF. Black bars indicate *TP53* mutations with the highest VAF in cases with multiple mutations. Gray bars indicate *TP53* mutations with a lower VAF respect to the mutations with the highest VAF in the context of a single case with multiple mutations. According to a cut-off of 10.0% VAF (dotted lines) 100 *TP53* mutations (92 cases) had more than 10.0% VAF (high-VAF *TP53* mutations) and 161 *TP53* mutations (76 cases) had less than 10.0% VAF (low-VAF *TP53* mutations). B) Left-panel. Kaplan-Meier curves comparing OS probabilities of 1,052 *TP53* wild-type cases (wt, green line), 92 cases with high-VAF *TP53* mutations, i.e., more than 10.0% of VAF (red line), and 76 cases with low-VAF *TP53* mutations, i.e., less than 10.0% of VAF (blue line). Right-panel. Kaplan-Meier curves comparing OS probabilities of 1,052 *TP53*wt cases (wt, green line), 26 cases with *TP53* frameshift mutations (blue line), 125 cases with missense mutations (red line), 9 cases with nonsense mutations (black line), and 8 cases with mutations affecting splice sites (splicing; purple line). For cases with more than one mutation the effect of the mutation with the highest VAF is reported. The number of patients in each group is reported; P value refers to log-rank test. C) Needle plot graph of high-VAF and low-VAF *TP53* mutations along the *TP53* coding sequence. Sequences referring to the transactivation domain, the DNA binding domain and the tetramerization domain of the p53 protein are reported in green, red and blue, respectively. D) Pie-chart of mutations effect on the p53 protein in terms of amino acid changes in the high-VAF and low-VAF *TP53* mutation context.

**Figure 3. *TP53* mutations and 17p deletions in the total retrospective cohort.** A) Pie-chart reporting the number of patients classified in four different categories according to the combination of *TP53* mutations and 17p deletions. B) Kaplan-Meier curves comparing OS probabilities of 1,032 *TP53* wild-type cases (wt, green line), 20 cases with del17p only (del17p_only, black line), 113 cases with *TP53* mutations only (Mut_only, red line), and 55 cases with concomitant *TP53* mutation and del17p (Mut&del17p, blue line). C) Kaplan-Meier curves comparing OS after treatment of 441 *TP53* wt cases (wt, green line), 8 cases with del17p only (del17p_only, black line), 64 cases with *TP53* mutations only (Mut_only, red line), and 39 cases with concomitant *TP53* mutation and del17p (Mut&del17p, blue line). D) Kaplan-Meier curves comparing OS after treatment of 441 *TP53*wt cases (wt, green line), 44 cases with *TP53* mutations less than 10.0% of VAF (Mut<10.0%, blue line), and 59 cases with *TP53* mutations more than 10.0% of VAF (Mut≥10.0%, red line). The number of patients in each group is reported; P value refers to log-rank test.

**Figure 4. *TP53* mutations and deletions in the ARCTIC/ADMIRE cohort.** A) Distribution of *TP53* mutations VAF in the ARCTIC/ADMIRE cohort (251 cases). Bar chart graph reports the percentage of VAF identified by NGS analysis for all 65 *TP53* mutations sorted in descending order with regard to the percent of VAF. Black bars indicate *TP53* mutations with the highest VAF in

cases with multiple mutations. Gray bars indicate *TP53* mutations with a lower VAF respect to the mutations with the highest VAF in the context of a single case with multiple mutations. According to a cut-off of 10.0% VAF (dotted lines) 40 *TP53* mutations (22 cases) had more than 10.0% VAF (high-VAF *TP53* mutations) and 25 *TP53* mutations (18 cases) had less than 10.0% VAF (low-VAF *TP53* mutations). B) Needle plot graph of high-VAF and low-VAF *TP53* mutations along the *TP53* coding sequence. Sequences referring to the transactivation domain, the DNA binding domain and the tetramerization domain of the p53 protein are reported in green, red and blue, respectively. C) Pie-chart of mutations effect on the p53 protein in terms of amino acid changes in the high-VAF and low-VAF *TP53* mutation context. D) Kaplan-Meier curves comparing OS, computed as time (months) from first treatment, of 211 *TP53*wt cases (green line), 22 cases with high-VAF mutations, i.e., ≥10.0% VAF (red line), and 18 cases with low-VAF mutations, i.e. <10.0% VAF (blue line). E) Kaplan-Meier curves comparing OS, computed as time (months) from first treatment, of 201 *TP53*wt cases (green line), 31 cases that present either *TP53* mutations only, or del17p only (single, blue line), and 11 cases with a concomitant *TP53* mutation and del17p (double, red line). The number of patients in each group is reported; P value refers to log-rank test.

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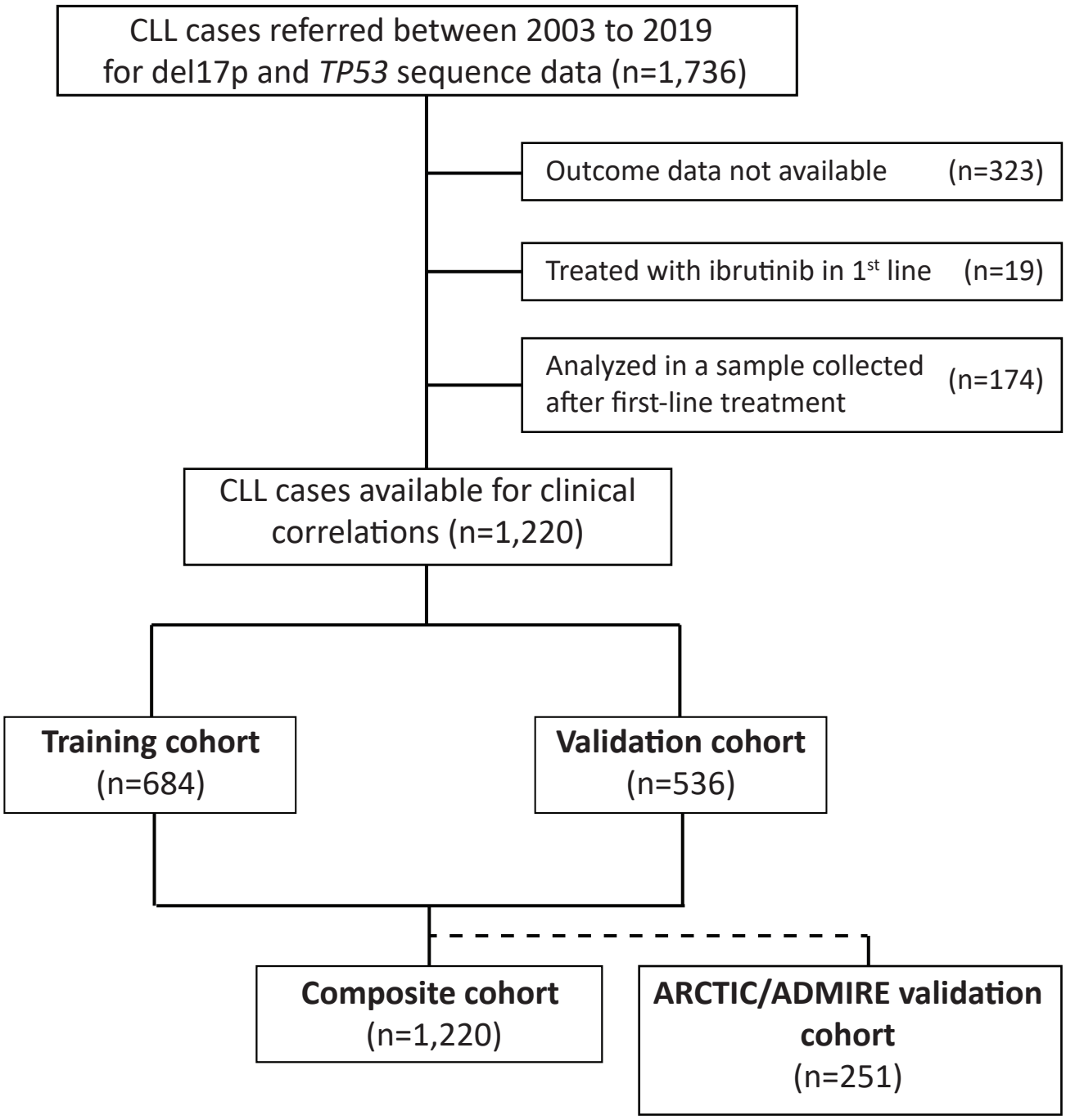
Figure 1



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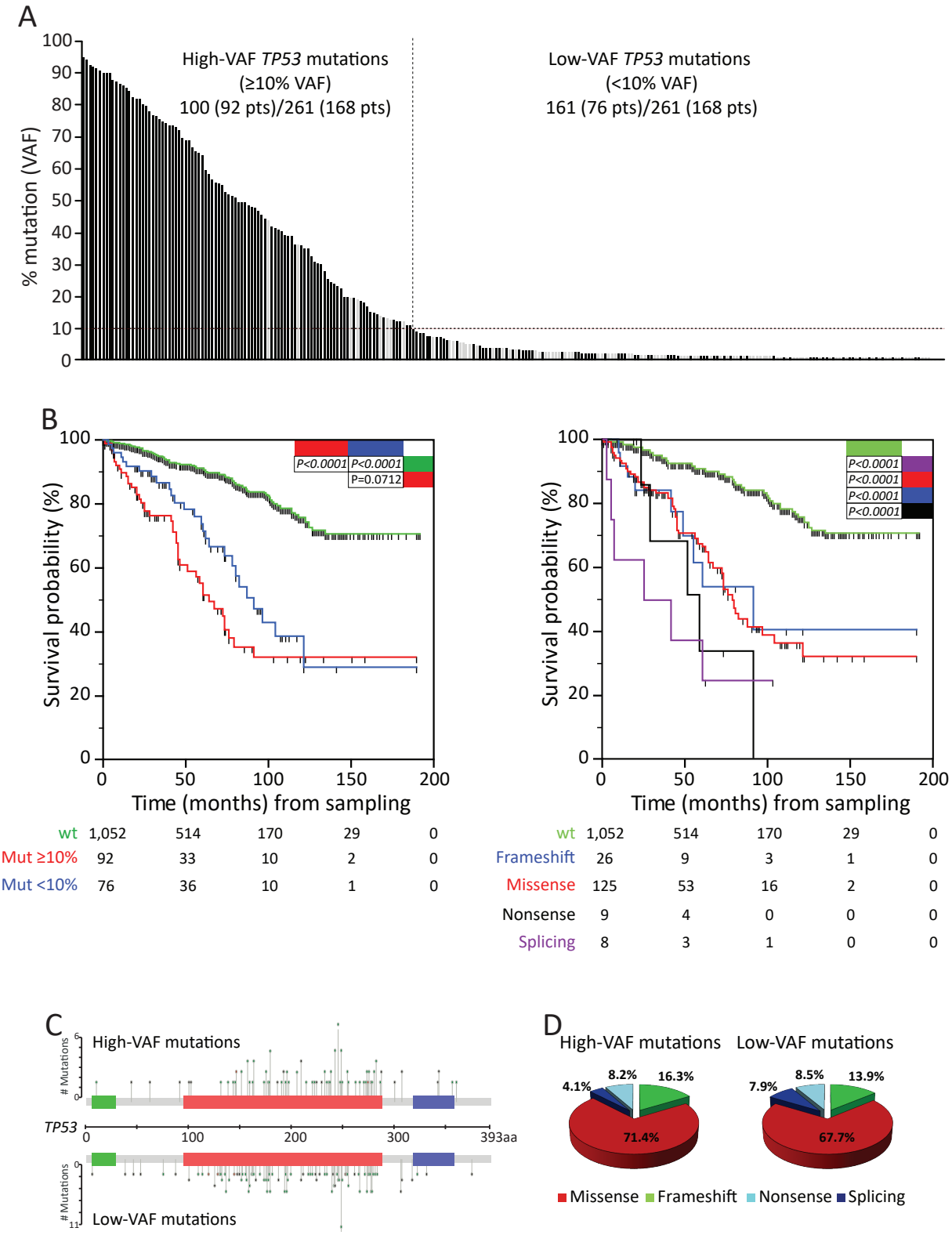
# Figure 2



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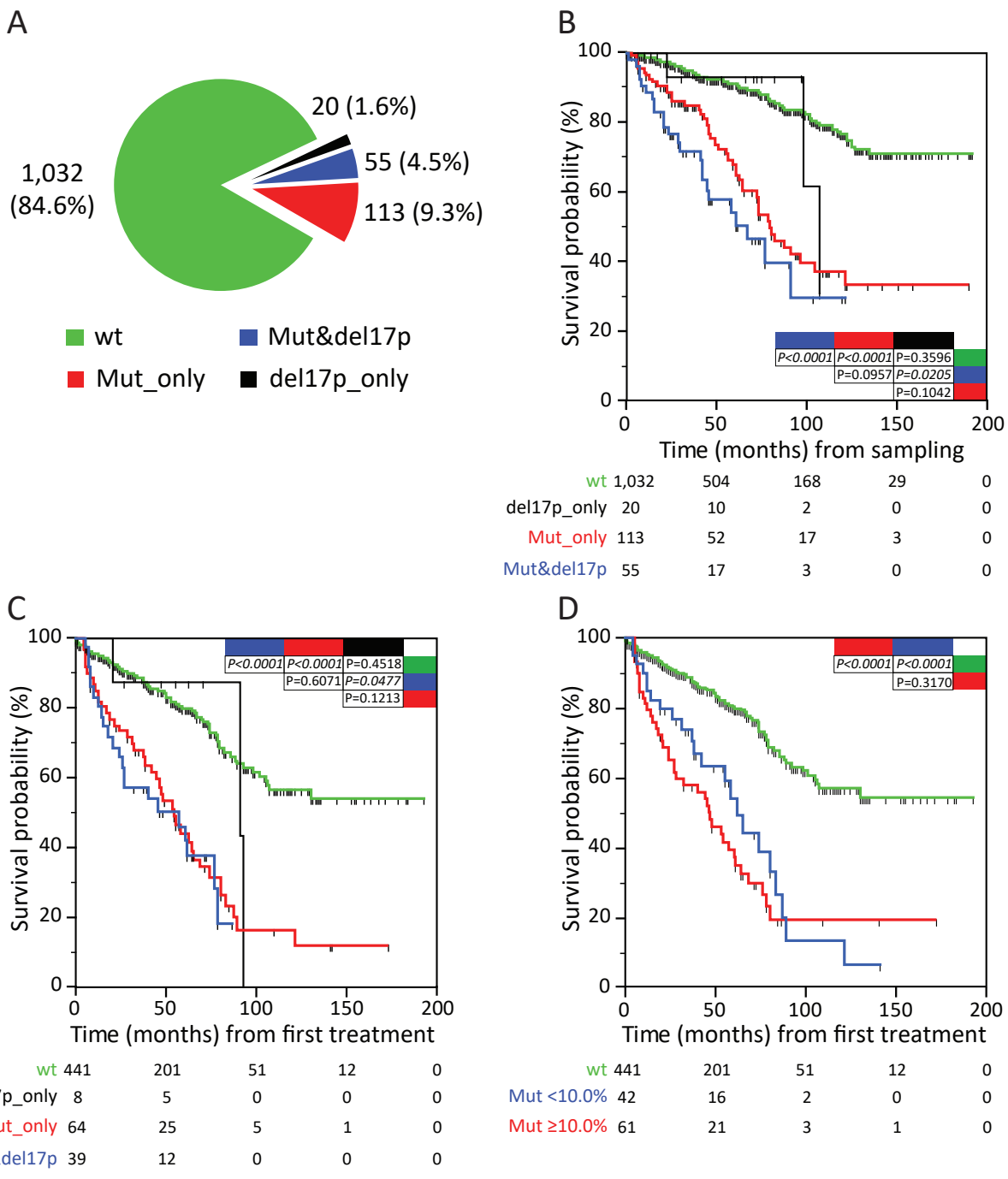
# Figure 3



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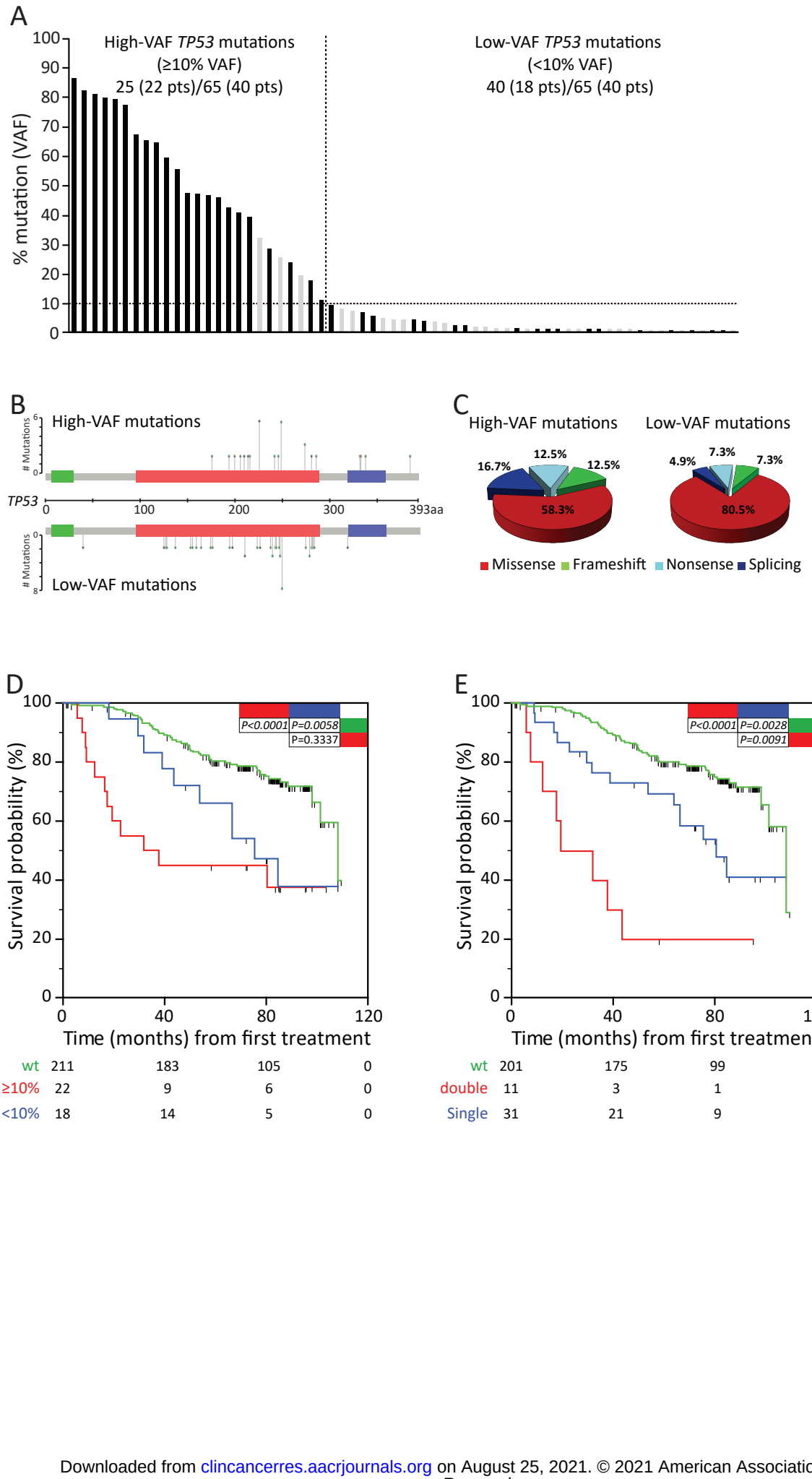
# Figure 4



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# *TP53* mutations with low variant allele frequency predict short survival in Chronic Lymphocytic Leukemia

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