The Journal of Pathology: Clinical Research
J Pathol Clin Res January 2024; **10:** e351
Published online 21 November 2023 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.351

ORIGINAL ARTICLE

Low-burden *TP53* mutations represent frequent genetic events in CLL with an increased risk for treatment initiation

Tamás László[1], Lili Kotmayer[1], Viktória Fésüs[1,2], Lajos Hegyi[1], Stefánia Gróf[1], Ákos Nagy[1], Béla Kajtár[3], Alexandra Balogh[4], Júlia Weisinger[4], Tamás Masszi[4], Zsolt Nagy[4], Péter Farkas[4], Judit Demeter[5], Ildikó Istenes[5], Róbert Szász[6], Lajos Gergely[6], Adrienn Sulák[7], Zita Borbényi[7], Dóra Lévai[8], Tamás Schneider[8], Piroska Pettendi[9], Emese Bodai[9], László Szerafin[10], László Rejtő[10], Árpád Bátai[11], Mária Á Dömötör[11], Hermina Sánta[11], Márk Plander[12], Tamás Szendrei[12], Aryan Hamed[13], Zsolt Lázár[13], Zsolt Pauker[14], Gáspár Radványi[14], Adrienn Kiss[15], Gábor Körösmezey[15], János Jakucs[16], Péter J Dombi[17], Zsófia Simon[17], Zsolt Klucsik[18], Mihály Gurzó[18], Márta Tiboly[19], Tímea Vidra[20], Péter Ilonczai[21], András Bors[22], Hajnalka Andrikovics[22], Miklós Egyed[2], Tamás Székely[1], András Masszi[4,8], Donát Alpár[1], András Matolcsy[1,23] and Csaba Bödör[1]*

[1]*HCEMM-SE Molecular Oncohematology Research Group, Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary*
[2]*Kaposi Mór University Teaching Hospital of County Somogy, Kaposvár, Hungary*
[3]*Department of Pathology, University of Pécs Medical School, Pécs, Hungary*
[4]*Department of Internal Medicine and Hematology, Semmelweis University, Budapest, Hungary*
[5]*Department of Internal Medicine and Oncology, Semmelweis University, Budapest, Hungary*
[6]*Division of Hematology, Department of Internal Medicine, University of Debrecen, Debrecen, Hungary*
[7]*2nd Department of Internal Medicine and Cardiology Center, University of Szeged, Szeged, Hungary*
[8]*Hematology and Lymphoma Unit, National Institute of Oncology, Budapest, Hungary*
[9]*Hetényi Géza Hospital and Clinic of County Jász-Nagykun-Szolnok, Szolnok, Hungary*
[10]*Hospitals of County Szabolcs-Szatmár-Bereg and University Teaching Hospital, Nyíregyháza, Hungary*
[11]*Fejér County Szent György University Teaching Hospital, Székesfehérvár, Hungary*
[12]*Markusovszky University Teaching Hospital, Szombathely, Hungary*
[13]*Petz Aladár University Teaching Hospital, Győr, Hungary*
[14]*Borsod-Abaúj-Zemplén County Hospital and University Teaching Hospital, Miskolc, Hungary*
[15]*Military Hospital – State Health Centre, Budapest, Hungary*
[16]*Békés County Central Hospital, Békéscsaba, Hungary*
[17]*St. Borbála Hospital, Tatabánya, Hungary*
[18]*Bács-Kiskun County Teaching Hospital, Kecskemét, Hungary*
[19]*Hospital of Keszthely, Keszthely, Hungary*
[20]*Soproni Erzsébet Teaching Hospital and Rehabilitation Institute, Sopron, Hungary*
[21]*Markhot Ferenc Teaching Hospital, Eger, Hungary*
[22]*Central Hospital of Southern Pest – National Institute of Hematology and Infectology, Budapest, Hungary*
[23]*Department of Laboratory Medicine, Karolinska Institute, Solna, Sweden*

Correspondence to: Csaba Bödör, HCEMM-SE Molecular Oncohematology Research Group, Department of Pathology and Experimental Cancer Research, Semmelweis University, Üllői Street 26, Budapest, Hungary. E-mail: bodor.csaba1@semmelweis.hu

Abstract

TP53 **aberrations predict chemoresistance and represent a contraindication for the use of standard chemoimmunotherapy in chronic lymphocytic leukaemia (CLL). Recent next-generation sequencing (NGS)-based studies have identified frequent low-burden *TP53* mutations with variant allele frequencies below 10%, but the clinical impact of these low-burden *TP53* mutations is still a matter of debate. In this study, we aimed to scrutinise the subclonal architecture and clinical impact of *TP53* mutations using a sensitive, NGS-based mutation analysis in a 'real-world' cohort of 901 patients with CLL. In total, 225 *TP53* mutations were identified in 17.5% (158/901) of the patients; 48% of these alterations represented high-burden mutations, while 52% were low-burden *TP53* mutations. Low-burden mutations as sole alterations were identified in 39% (62/158) of all mutated cases with 82% (51/62) of these being represented by a single low-burden *TP53* mutation. Patients harbouring low-burden *TP53* mutations had significantly lower time to first treatment compared to patients with wild-type *TP53*. Our study has expanded the knowledge on the frequency, clonal architecture, and clinical impact**

of low-burden *TP53* mutations. By demonstrating that patients with sole low-burden *TP53* variants represent more than one-third of patients with *TP53* mutations and have an increased risk for treatment initiation, our findings strengthen the need to redefine the threshold of *TP53* variant reporting to below 10% in the routine diagnostic setting.

Keywords: chronic lymphocytic leukaemia; *TP53*; NGS

Received 26 July 2023; Revised 21 September 2023; Accepted 25 October 2023

No conflicts of interest were declared.

Introduction

Chronic lymphocytic leukaemia (CLL), the most frequent type of leukaemias in adults, is characterised by significant clinical heterogeneity, with some patients pursuing an indolent disease course and others presenting with progressive disease requiring treatment [1]. Disruptions of the *TP53* gene including *TP53* mutations and deletions of the chromosomal region 17p (del(17p)) represent well-established prognostic and predictive biomarkers of resistance to standard chemoimmunotherapy (CIT) and poor survival in CLL [2].

Identification of *TP53* disruptions plays a pivotal role in molecular characterisation and clinical management of CLL as patients harbouring these alterations should be treated with novel targeted therapies [1,3]. Traditionally, *TP53* disruptions have routinely been analysed using standard technologies including fluorescence *in situ* hybridisation (FISH) and Sanger sequencing with a sensitivity of approximately 10–15% [4–6]. However, the recent introduction of next-generation sequencing (NGS) technologies has provided a novel opportunity to perform in-depth genomic analysis of the disease and led to the identification of a profound subclonal heterogeneity in CLL affecting the majority of driver genes including *TP53* [7,8]. In recent NGS studies of various CLL cohorts, subclonal (also referred to as 'low-burden') *TP53* mutations were identified in 5–35% of CLL patients [7,9–14]. These low-burden *TP53* mutations are restricted to a subpopulation of the tumour cells with variant allele frequencies (VAFs) far below (down to 0.1–1%) the sensitivity of Sanger sequencing. Although the first study reported that patients with subclonal *TP53* mutations showed similar outcomes to patients with clonal *TP53* mutations [9], these findings were not confirmed by others and the clinical implications of the low-burden *TP53* mutations remain still controversial [10,12,13]. The two most recent studies reported adverse effects of the low-burden *TP53* mutations on overall survival (OS) [11,12], but it is becoming apparent that these findings need to be interpreted in the context of disease stage and type of therapy [12]. Given the strong benefit derived by *TP53* mutant patients from targeted therapies including the BTK inhibitor ibrutinib and BCL2 inhibitor venetoclax, defining a clinically relevant threshold for *TP53* variant reporting is of paramount importance.

In this study, we aimed to scrutinise the subclonal architecture and clinical impact of *TP53* mutations using a sensitive, NGS-based mutation analysis approach in a large, multi-centric, 'real-world' cohort of CLL patients.

Materials and methods

Patients and samples

Peripheral blood samples from 901 patients with CLL were collected from 20 oncohaematological units in Hungary within the framework of a real-world study composed of clinically heterogenous CLL populations (Table 1 and supplementary material, Table S1). Patients were diagnosed according to the International Workshop on CLL-National Cancer Institute (iwCLL-NCI) criteria between 1985 and 2022 [15]. Median age at diagnosis was 64 years (range: 27–92) with a female:male ratio of 1:1.35. Twenty-one percent (189/901) of the samples were obtained at the time of diagnosis, 38.3% (345/901) before treatment initiation (excluding samples at diagnosis), and 29% (261/901) post-treatment. Treatment details reflect every treatment modality during the course of the disease of a patient, irrespective of the time when the patient was enrolled in the study. Of all patients, 55.3% (498/901) required treatment during the course of the disease while in 267 cases no treatment was needed. No information was available regarding treatment in 136 cases. Among patients requiring treatment, 33.1% (165/498) were treated with CIT regimens while targeted therapy

Table 1. Patient characteristics

Parameter	Category	Result
Sex	%Male/female	57/43 ($n = 901$)
Age at diagnosis	Median (range)	64 (range: 27–92 years)
Age at sample collection	Median (range)	69 (range: 28–95 years)
Follow-up from diagnosis (months)	Median (range)	51 (range: 0–434 months)
Follow-up from sample collection (months)	Median (range)	12 (range: 0–43 months)
IGHV status	Unmutated	55% (439/792)
	Mutated	39% (311/792)
	Borderline	5% (42/792)
Copy number alterations	del(17p)	9% (73/795)
	del(11q)	22% (116/537)
	Trisomy 12	60% (15/25)*
	del(13q)	66% (44/67)
No. of *TP53* mutations	No *TP53* mutation	82% (743/901)
	1	13% (113/901)
	2	3% (30/901)
	>2	2% (15/901)
No. of patients treated	%	65% (498/765)
Treatment category	No treatment	35% (267/765)
	C(I)T	22% (165/765)
	C(I)T + targeted	28% (213/765)
	Targeted	16% (120/765)
No. of therapy lines	Median (range)	1 (range: 1–7)

*Plausible bias due to lack of adequate information regarding the frequency of negative cases.

(BTK inhibitor, PIK3 inhibitor, BCL2 inhibitor monotherapy, or in combination) was the treatment of choice in 24.1% (120/498) of the patients. 42.8% (213/498) of treated patients received both CIT and targeted therapy during the course of the disease. Fifty-one % (379/743) of patients with wild-type *TP53* required treatment, of whom 38.8% (147/379) were treated exclusively with CIT, 21.9% (83/379) with targeted therapy, whereas 39.9% (149/379) received both CIT and targeted treatment. In comparison, 79% (49/62) of patients harbouring solely low-burden *TP53* mutations required therapeutic intervention. The majority of these patients (59.2%, 29/49) were also treated with CIT and targeted treatment, whereas 24.5% (12/49) and 16.3% (8/49) were treated only with targeted therapy or CIT, respectively. Similarly, 50% (35/70) of patients harbouring at least one high-burden *TP53* mutation were treated with both CIT and targeted therapy while 35.7% (25/70) and 14.3% (10/70) were exclusively treated with targeted therapy or with CIT, respectively. Clinical outcome data were available in 797 cases with median follow-up of 12 months (0–43 months). Written informed consent was obtained from all participants, the study was approved by the Hungarian Medical Research Council (ID: TUKEB: IV/5495-3/2021/EKU) and it was conducted in accordance with the Declaration of Helsinki.

Analysis methods

Genomic DNA was extracted from PBMCs isolated after Ficoll/Histopaque density-gradient centrifugation in all 901 patients. The proportion of CLL cells in the samples was assessed by flow cytometry using CD5/CD19/CD23/CD45 staining. Deletions of chromosomal region 17p were screened as part of the routine diagnostic characterisation by interphase FISH using dual-colour Vysis probe sets (Abbott Molecular, Des Plaines, IL, USA). Samples above the 5% detection limit were considered positive for del(17p) evaluating at least 100 cells according to our institutional policy.

Targeted NGS was performed using the Multiplicom SureMASTR *TP53* Panel (Agilent, Santa Clara, CA, USA) covering the whole coding sequence of the *TP53* gene. Libraries were prepared according to the manufacturer's recommendations and sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) with 150 bp paired-end chemistry with a mean read depth of 16,395 reads (range: 4,356–39,131). Data processing and analysis were performed using the Sequence Pilot 5.1.0 (JSI Medical Systems, New York, NY, USA) workflow. Variants in the *TP53* coding region were annotated using the *TP53*-specific UMD_TP53 (Universal Mutation_Database, Seshat), the TP53 Database (formerly known as IARC database), and COSMIC databases [16–19]. Functional and supertrans missense variants were excluded based on the overall transcriptional activity on eight promoters according to the TP53 Database [19]. *TP53* mutations with <10% VAF were defined as low burden, with ≥10% VAF variants defined as high burden as published recently [12]. *TP53* mutations were analysed and reported according to the most recent ERIC recommendations [20].

Data validation

All *TP53* variants below the 10% cut-off value, along with 33.6% (37/110) of the clonal mutations were confirmed by either NGS or droplet digital polymerase chain reaction (ddPCR).

TP53 variants previously detected by NGS were confirmed using an alternative *TP53* NGS panel (CleanPlex, Paragon Genomics, Fremont, CA, USA) allowing for the detection of somatic mutations with a frequency as low as 1%. Libraries were prepared according to the manufacturer's instructions and

sequenced on a Miseq platform using V2 chemistry (macro 300 cycle). Data analysis was carried out as detailed above.

Ultrasensitive validation of certain *TP53* variants (R196*, Y220C, R248W, R248Q, Y234C) previously detected by NGS was performed by ddPCR using custom assays (dHsaCP2000121, dHsaCP2500536, dHsaCP2000107, dHsaCP2000127, and dHsaCP2506900 respectively) designed for the sensitive discrimination of mutant and wild-type alleles. The ddPCR was performed according to the manufacturer's recommendations on a QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using 100 ng of input DNA. Fractional abundance (FA) was calculated from the ratio of the droplets containing mutant DNA molecules (a) and the total number of mutant (a) plus wild-type (b) DNA molecules detected (FA $= a/(a + b)$).

Statistical analysis

Various measures were considered relative to the time of sample collection. Only samples collected prior to first treatment initiation were considered for the survival analysis. OS was derived from death or last follow-up, treatment-free survival (TFS) obtained from the time of first treatment, death of any cause or last follow-up, whereas time to first treatment (TTFT) was calculated based on the time of treatment initiation or last follow-up. Kaplan–Meier survival curves and log-rank tests were performed to compare survival times between groups using the GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA). Categorical variables were compared by Fisher's exact test. p values equal to 0.05 or below were considered statistically significant.

Results

TP53 mutation status and subclonal architecture

In total, we identified 225 *TP53* mutations in 17.5% (158/901) of the patients using NGS with an average VAF of 22.99% (range: 1.0–92.0%) (Figure 1 and supplementary material, Figure S1 and Table S2). Forty-eight percent (48%; $n = 109$) of these alterations were high-burden mutations, while 52% ($n = 116$)

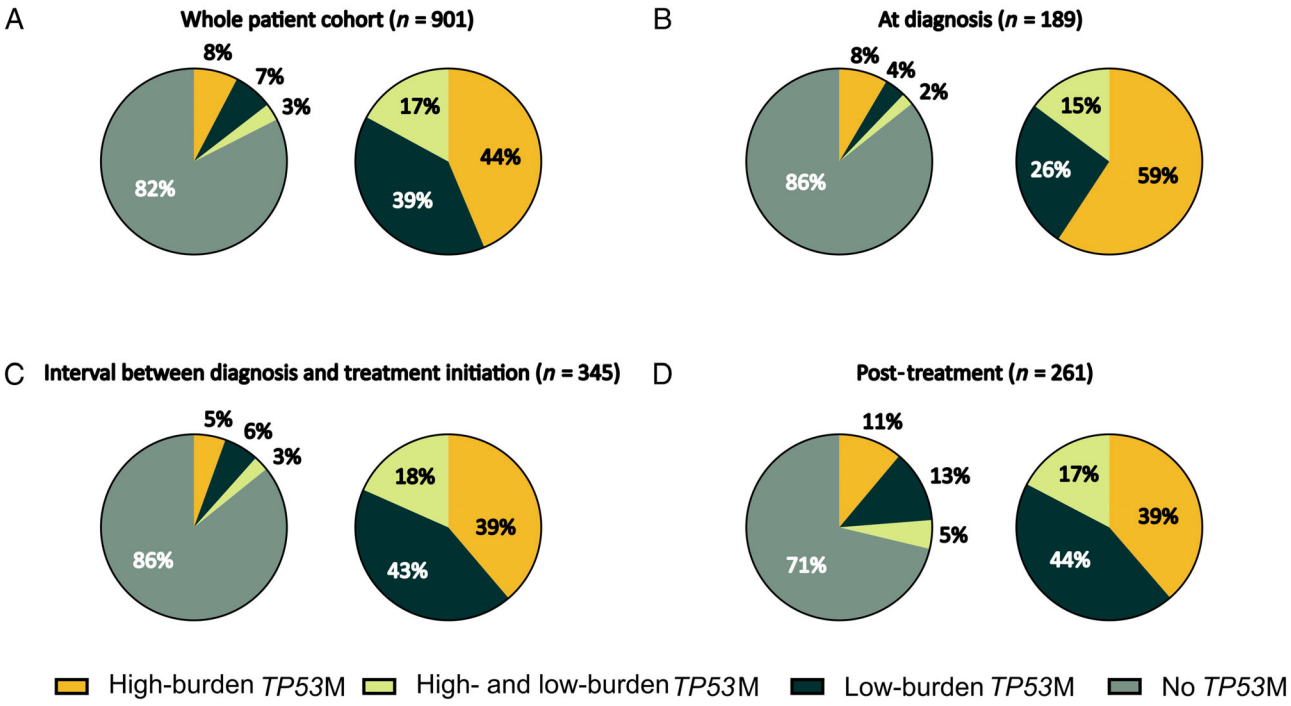

Figure 1. Frequency and distribution of *TP53* mutations. (A) *TP53* mutation was identified in 17.5% of the patients with the illustrated frequency and distribution of low-burden and high-burden mutations. (B–D) The frequency and distribution of *TP53* mutations in samples collected at the time of diagnosis, before treatment initiation, and post-treatment respectively. The first pie chart of each pair (on the left) includes the total number of patients analysed in the respective subgroup, whereas the second pie chart (on the right) includes only patients carrying *TP53* mutations in the respective subgroup. M, mutation.

represented low-burden mutations (Figure 2C). Multiple *TP53* mutations were detected in 28.5% (45/158) of all mutated cases with an average of 2.5 mutations (2–5) per patient (supplementary material, Table S2). Low-burden *TP53* mutations as sole alterations were identified in 39% ($n = 62$) of all mutated cases with 82.3% (51/62) of these being represented by a single low-burden *TP53* mutation. The frequency of patients harbouring solely low-burden *TP53* mutations was 4%, 6%, and 13% considering samples collected at diagnosis, before treatment initiation, and post-treatment, respectively (Figure 1). Solely high-burden *TP53* mutations were detected in 44% (69/158) of the mutant patients, while in 17% (27/158) of the *TP53* mutant cohort, the high-burden mutations coexisted with low-burden mutations (Figure 1). Regarding mutated patients, the frequency of high-burden *TP53* mutations was 59% of samples collected at the time of diagnosis compared with only 39% in both pre-treatment and post-treatment samples.

While both high-burden and low-burden mutations appeared in most cases (71.5%) as sole alterations [39.2% (62/158) and 32.3% (51/158), respectively], we detected multiple mutations in 28.5% (45/158) of *TP53* mutated cases with heterogenous subclonal architecture. The co-occurrence of multiple high-burden mutations was relatively rare; however, they were frequently accompanied by 1–4 low-burden mutations with or without additional 1 or 2 high-burden mutations. Multiple low-burden *TP53* variants with 2–4 concurrent low-burden mutations occurred less frequently.

Types and distribution of *TP53* mutations

TP53 mutations were mainly missense substitutions (74.7%; 168/225), followed by indel mutations (15.6%; 35/225), nonsense (6.2%; 14/225) alterations, and splice site (3.6%; 8/225) (Figure 2B). Splice-site variants were detected exclusively in samples collected after treatment-initiation of any kind. Missense mutations recurrently affected classical hot-spot codons for single base substitutions either resulting in 'DNA-contact mutants' (residues 248 and 273; 29/225, 12.9% of all *TP53* variants) or 'conformational mutants' (residues 175, 245, 249, and 282; 20/225, 8.9% of all variants). In 25.7% (9/35) of indel mutations, codon 209 was affected by the deletion of two nucleotides (c.626_627del) highly specific for CLL [21]. Comparing the molecular characteristics of low-burden and high-burden alterations, we noticed no significant differences in the type of *TP53* mutations regardless of the interval of sample collection (data not shown).

With regard to the localisation, 93.8% (211/225) of the mutations occurred in the DNA-binding domain (residues 94–292) (Figure 2A). A small proportion (6.2%; 14/225) of mutations occurred outside the DNA-binding domain, affecting other important parts of the p53 protein, including the oligomerisation domain (residues 319–357) located in the C-terminus of the protein and the transactivation domain (residues 1–64) located in the N-terminus [17,22]. Another critical element of the p53 protein, the nuclear localisation signal (residues 305–322), was almost entirely affected by nonsense mutations. Interestingly the transactivation domain was involved exclusively in post-treatment samples. Comparing the domain distribution of high-burden and low-burden *TP53* mutations, the oligomerisation domain was solely affected by low-burden alterations, while every other domain was affected by high-burden and low-burden variants as well (Figure 2A).

Deletion of 17p region (del(17p)) and *TP53* mutations

The del(17p) status as determined by FISH was available in 795 patients with this alteration present in 9.2% (73/795) of the cases (Figure 3B). Of these patients, 75.3% (55/73) carried a concurrent *TP53* mutation (12 low burden and 43 high burden) indicating a biallelic *TP53* disruption (Figure 3C). Among the *TP53*-mutated cases, 39.9% (55/138) carried concomitant del(17p) (Figure 3D). Notably, 16 patients carried multiple ($n = 2$–5) *TP53* mutations with concomitant del(17p), suggesting a heterogenous subclonal architecture. 17p deletion was more frequent among patients carrying high-burden *TP53* mutations than among patients with low-burden mutations (52% versus 22%, $p = 0.0007$) (Figure 3A). The vast majority (78.2%, 43/55) of patients with sole low-burden *TP53* mutations presented without del(17p) (Figure 3A). Altogether, we identified at least one form of *TP53* disruption in 19.6% (156/795) of the cases (supplementary material, Figure S2).

Immunoglobulin heavy-chain variable region gene (IGHV) mutational status

The IGHV mutational status was available in 792 patients with the following distribution: mutated (IGHV-M) 39.3% (311/792), borderline (IGHV-B) 5.3% (42/792), and unmutated (IGHV-U) in 55.4% (439/792) of the cases. Low-burden *TP53* mutations were present in 8.2% (36/439), 3.9% (12/311), and 7.1% (3/42), whereas high-burden *TP53* mutations

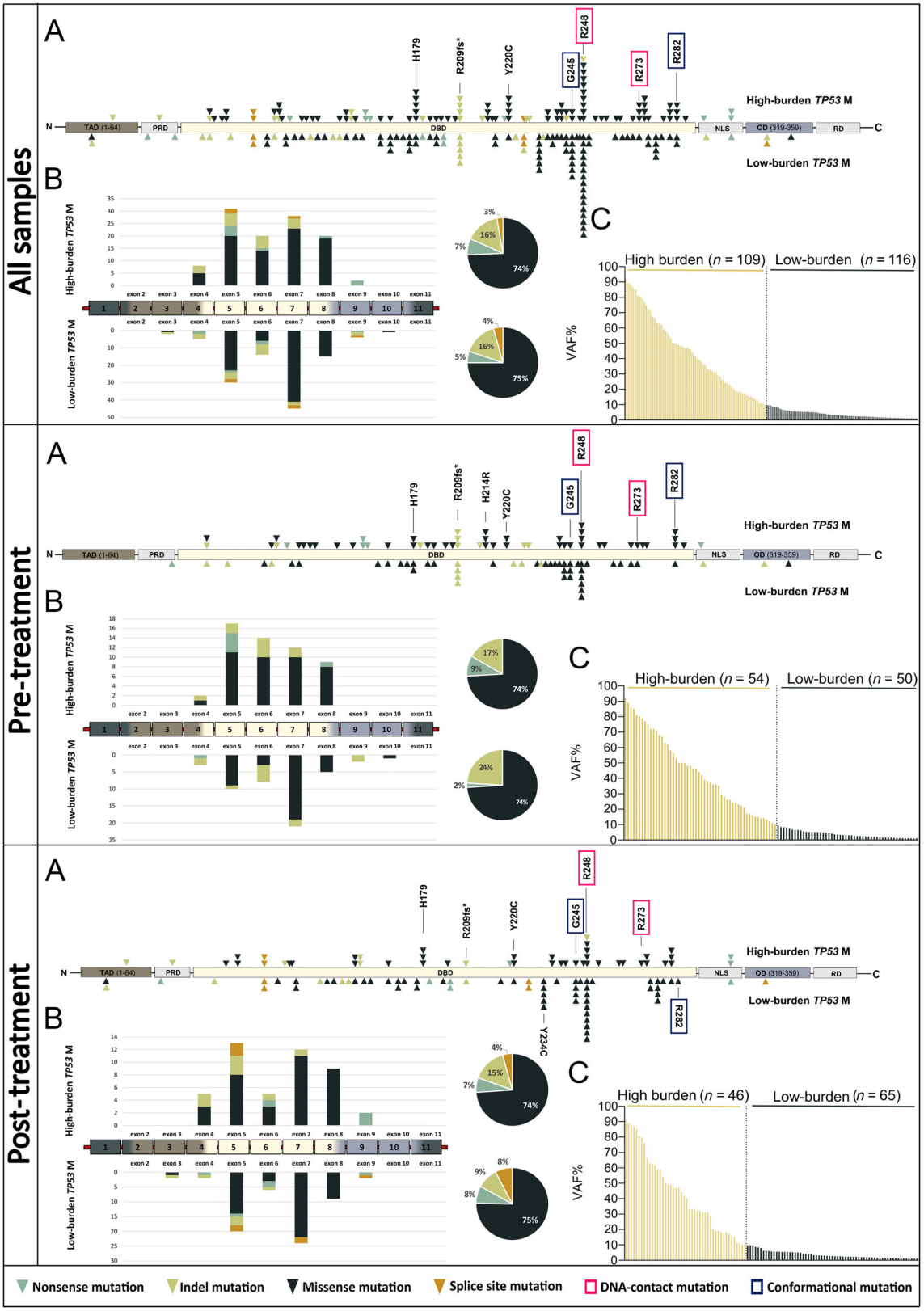

Figure 2. Legend on next page.

occurred in 13.2% (58/439), 8% (25/311), and 7.1% (3/42) of IGHV-U, IGHV-M, and IGHV-B cases, respectively (Figure 3F–H). *TP53* mutation frequency was significantly higher among patients with IGHV-U compared with the IGHV-M subgroup ($p = 0.0009$). The majority of patients with low-burden and high-burden *TP53* mutations had IGHV-U status (70.6% and 67.4%, respectively), while IGHV-U was less common (52.7%) in the subgroup with no *TP53* mutations (Figure 3E).

Clinical outcome

Considering samples collected exclusively prior to first treatment initiation ($n = 522$), we found no significant differences in OS between the *TP53* mutant and wild-type cohorts (supplementary material, Figure S3A). Regarding TFS, patients with high-burden ($n = 46$), as well as patients with low-burden ($n = 28$) *TP53* mutations had significantly shorter TFS than patients with wild-type ($n = 431$) *TP53* gene (high burden versus wt *TP53*: $p < 0.0001$; low burden versus wt *TP53*: $p = 0.0012$) (Figure 4A). We observed no significant differences in TFS between patients harbouring high-burden and low-burden mutations ($p = 0.9778$). Considering TTFT, patients with high-burden mutations ($n = 43$), along with patients harbouring low-burden *TP53* variants ($n = 26$) had significantly higher rates of treatment initiation (high burden versus wt *TP53*: $p < 0.0001$; low burden versus wt *TP53*: $p = 0.0031$) compared to patients harbouring wild-type *TP53* ($n = 407$) (Figure 4B). Similarly to TFS, we detected no significant differences between patients with high-burden and low-burden mutations ($p = 0.6993$).

By extending the analysis with IGHV mutational data, regarding OS we only found significant differences between patients harbouring wild-type *TP53* with IGHV-U ($n = 197$) and IGHV-M ($n = 180$) status ($p = 0.0086$) (supplementary material, Figure S3B). Patients with IGHV-U status and *TP53* mutation showed significantly lower TFS and TTFT compared to patients with wt *TP53* regardless of the IGHV mutational status (Figure 4C,D). Along with these findings, the above-mentioned cases had significantly lower TFS and TTFT compared to patients harbouring high-burden *TP53* mutations and IGHV-M status; however, we found no significant differences compared to patients with low-burden *TP53* and IGHV-M (Figure 4C,D).

Discussion

TP53 mutations represent well-established adverse prognostic biomarkers in CLL [23]. According to international guidelines, in patients with *TP53* mutations treatment with CIT should be avoided and they should instead be treated with targeted therapies including BTK and BCL2 inhibitors [2]. The most recent ERIC recommendations on *TP53* mutation analysis and interpretation propose NGS as the gold standard for mutation detection with a threshold of 10% VAF for clinical reporting [20]. However, the clinical impact of the low-burden *TP53* mutations with <10% VAF is still a matter of debate with some studies suggesting lowering the VAF reporting threshold below 10% [11,12].

In this study, we performed a comprehensive analysis of *TP53* alterations in a large, multi-centric, 'real-world' cohort of CLL patients using a sensitive NGS-based mutation analysis approach. All variants below the 10% cut-off value were confirmed by using either a highly sensitive ddPCR approach or an alternative NGS panel (supplementary material, Table S2). Considering the entire patient cohort, 68% (153/225) of the detected *TP53* variants were subjected to independent confirmation (all low-burden ($n = 116$) and 33.9% (37/109) of the high-burden variants). Overall, 85.0% (153/180) of the re-tested mutations were successfully verified. All re-evaluated high-burden *TP53* mutations were confirmed ($n = 37$), while low-burden alterations could be verified in 81.1% (116/143) of the cases examined. In line with recently published data by Pandzic *et al*, all low-burden *TP53* mutations between 5% and 10% VAF range were confirmed ($n = 38$), whereas 74.3% (78/105) of *TP53* variants with lower than 5% VAF could be validated [14]. Considering *TP53* variants with VAF values between 2% and 5% (≥2% and <5%; $n = 47$), 96% (45/47) of the detected variants were confirmed using NGS ($n = 39$) or ddPCR ($n = 6$), whereas only 57% (33/58)

Figure 2. Distribution and type of *TP53* mutations along the *TP53* gene and protein in the high-VAF and low-VAF *TP53* mutation context. (A) Mutational distributions along the p53 protein. Each triangle represents an individual *TP53* mutation with a colour referring to mutation type. (B) Mutational distributions along the coding sequence of the *TP53* gene with pie charts showing the frequency of each mutational type. (C) VAFs of *TP53* mutations. Each column represents the VAF of an individual *TP53* mutation. DBD, DNA-binding domain; M, mutation; OD, oligomerisation domain; PRD, proline-rich domain; TAD, transactivation domain. *Pre-treatment samples include samples collected at the time of diagnosis.

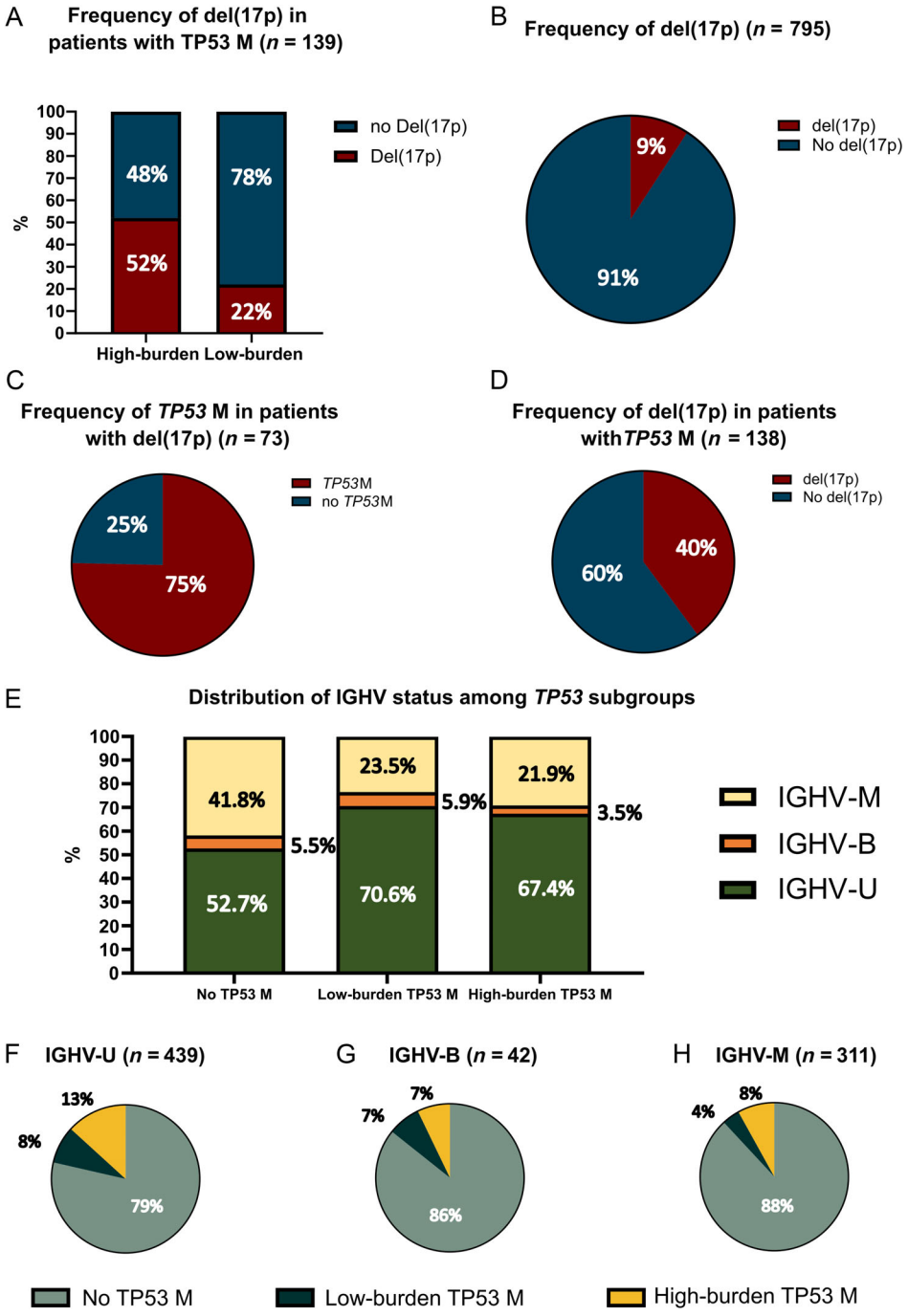

Figure 3. Frequency and distribution of del(17p) as well as IGHV status in the cohort. (A) Frequency of del(17p) in the high-VAF and low-VAF *TP53* mutation context. (B) Frequency of del(17p) in the cohort, and in patients harbouring *TP53* mutation (D). (C) Frequency of *TP53* mutations in patients harbouring del(17p). (E) Distribution of IGHV status among *TP53* subgroups. (F–H) The frequency of *TP53* mutations among IGHV subgroups.

of *TP53* variants with VAF values below 2% were successfully confirmed by validation experiments (supplementary material, Figure S4). Our data strengthen the supposition that *TP53* variants in the 5–10% VAF range can be reliably detected by NGS, in the routine diagnostic setting. Based on our results, the 10% cut-off value for *TP53* variant reporting in routine diagnostics might be worth reconsidering.

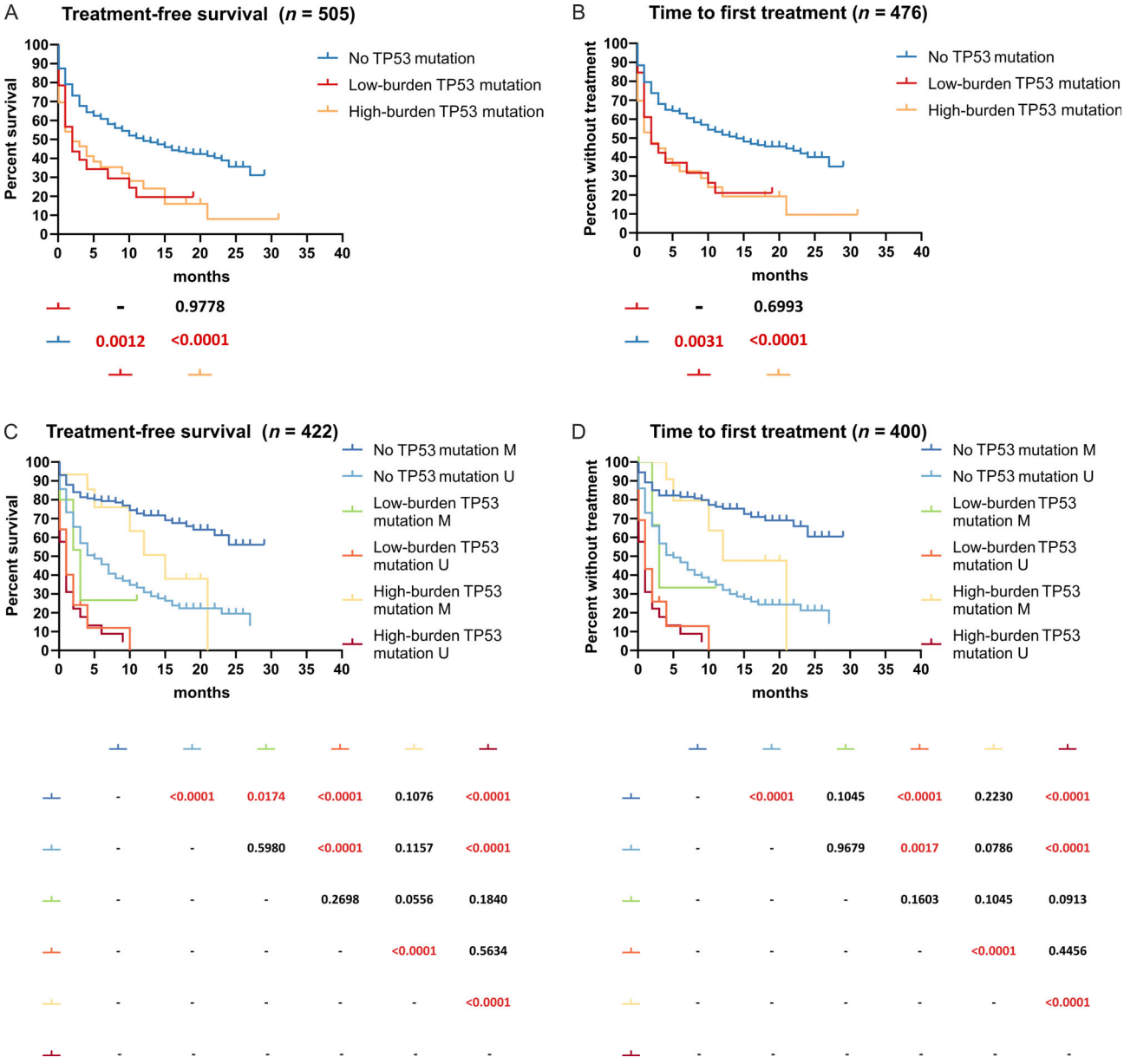

Figure 4. TFS (A and C) and TTFT (B and D) computed as time (months) from sample collection. *p* values are represented in the tables below the panels. M, mutated IGHV status; *TP53*M, *TP53* mutation; U, unmutated IGHV status.

We identified *TP53* mutations in 17.5% of the cohort with increasing frequency along the disease progression (14.3% at diagnosis, 14.2% before treatment initiation, and 28.7% post-treatment) in accordance with previously published data (Figure 1) [9,13,24–26]. Sole low-burden *TP53* mutations were identified in 7% of the patients, accounting for 39% of the *TP53* mutant patient cohort. Since 78% of the cases with sole low-burden mutations presented without concurrent del(17p), these patients would have been misinterpreted as *TP53* wild-type according to the currently recommended 10% VAF cut-off [20]. Considering the recent evidence on clonal expansion of the low-burden *TP53* mutations leading to relapse under the selective pressure of standard CITs with no clonal expansion observed upon targeted treatment, identification of these subclonal *TP53* variants might have important clinical implications [11,12]. Comparing the molecular characteristics and distribution of *TP53* mutations across the gene, no significant differences

were observed between the high-burden and low-burden mutations, supporting the genuine nature of the subclonal *TP53* variants identified. Of note, splice-site mutations occurred exclusively in post-treatment samples. Similarly, the TAD domain was solely affected in samples collected after treatment initiation. Although these findings may be of interest, given the fact that a relatively high number of splice variants has already been described in the literature in samples collected prior to treatment initiation, and the relative low frequency of these alterations in our cohort, the biological relevance of these findings remains unknown [21].

With regard to the clinical impact of *TP53* mutations, we demonstrated no significant impact of *TP53* variants on the OS of patients. This may be explained by the short follow-up time, or the preferential use of novel targeted agents such as BTK or BCL2 inhibitors. However, we noticed significant differences in TFS, regarding both *TP53*-mutated cohorts compared with *TP53* wild-type patients. To eliminate the possible bias caused by the CLL-unrelated death of the patients, we have calculated TTFT, which also showed significant differences between patients harbouring low-burden or high-burden *TP53* mutations compared to cases with wt *TP53*. Since, according to the most recent iwCLL guideline, treatment initiation is only indicated at progression or at the emergence of novel symptoms, this finding suggests that low-burden *TP53* variants have the same unfavourable clinical impact on CLL as high-burden mutations. Indeed, contradictory results on the clinical impact of the low burden *TP53* variants in terms of their effect on OS have been published in the literature. While the studies by Brieghel *et al* [13] and Blakemore *et al* [10] failed to demonstrate the inferior impact of the subclonal *TP53* mutations, other studies including the most recent findings by Bomben *et al* [11] and Malcikova *et al* [12] reported that the low-burden *TP53* mutations have similar effect on survival to that of the high-burden *TP53* mutations. It is becoming apparent that these seemingly contrasting findings are largely influenced by the different patient cohort composition, immunogenetic characteristics i.e. the IGHV status, and the types of therapies applied [27]. Considering both *TP53* and IGHV status we demonstrated significant differences between patients harbouring wild-type *TP53* with unmutated and mutated IGHV. Regarding TFS and TTFT, synergy was detected between unmutated IGHV status and the presence of *TP53* mutations, with patients harbouring both markers having significantly lower TFS and TTFT. Our data are in line with the most recent publication by ERIC, where the authors observed a significant difference in TTFT between patients harbouring *TP53* aberration with IGHV-U status and patients with IGHV-U without associated *TP53* deficiency [28]. To the best of our knowledge, our study provides the largest real-world dataset regarding the clinical impact of low-burden *TP53* mutations with adjustment to the IGHV status in CLL. The fact that, in the real-world setting, *TP53* mutation analysis is mostly done at the time of treatment initiation may lead to some possible bias in survival analysis relative to the date of sample collection. To overcome this potential bias, we have reanalysed the cohort considering TFS, OS, and TTFT relative to the time of diagnosis restricting the analysis to samples obtained within 6 months from diagnosis leading to the same correlations, suggesting that low-burden variants indeed have an important clinical significance (supplementary material, Figure S5).

Recent reports clearly indicate that switching to targeted therapies in patients with *TP53* aberrations prolongs their survival [12,29], which also represents a confounding effect for these types of survival analyses. More importantly, as demonstrated by several longitudinal studies [7,11,12,30] analysing the clonal dynamics of *TP53* mutations under the selective pressure of CIT, low-burden *TP53* mutations are positively selected and eventually undergo clonal expansion and become dominant clones leading to chemoresistance. This observation provides a strong argument for the routine screening of *TP53* mutations using sensitive technologies below the currently recommended 10% VAF cut-off value. Although some studies have reported *TP53* variants with VAFs as low as 0.1% [8,9,12,13], considering the need for advanced bioinformatic algorithms and other pre-analytical factors, reporting cut-offs lower than 1% may not be feasible in the routine diagnostic setting. Indeed, Malcikova *et al* reported that the risk of rapid expansion to a dominant clone was higher in patients with >1% VAF compared to patients with a VAF of <1% [12] suggesting that a cut-off >1% may be of biological/clinical relevance. In summary, our study expands the knowledge on frequency, clonal architecture, and clinical impact of low-burden *TP53* mutations. By demonstrating that patients with sole low-burden *TP53* variants represent more than one-third of patients with *TP53* mutations, having increased risk for treatment initiation compared to cases with wild-type *TP53*, our findings strengthen the need to redefine the threshold of *TP53* variant reporting to below 10% in the routine diagnostic setting.

Acknowledgements

This work was funded by the EU's Horizon 2020 Research and Innovation Program under grant agreement No. 739593, by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the K21_137948, FK20_134253, TKP2021-EGA-24, and TKP2021-NVA-15 funding schemes, the EFOP-3.6.3-VEKOP-16-2017-00009 and ÚNKP-22-3-II-SE-21 grants, the János Bolyai Research Scholarship program (BO/00125/22) of the Hungarian Academy of Sciences, and Elixir Hungary.

Author contributions statement

CB and TL designed the study. BK, AB, JW, TM, ZN, PF, JD, II, RS, LG, AS, ZB, DL, TS, PP, EB, LS, LR, ÁB, MAD, HS, MP, TS, AH, ZL, ZP, GR, AK, GK, JJ, PJD, ZS, ZK, MG, MT, TV, PI, AB, HA, ME, TSz (Székely), AM, DA and AM (Matolcsy) provided patient samples and/or annotations. TL, SG and LH performed the experiments. TL, LK, LH, ÁN and FV performed data analysis. TL and CB wrote the paper. All authors read and critically reviewed the final version of the manuscript.

Data availability statement

Raw sequencing data is available from the corresponding author upon reasonable request.

References

1. Delgado J, Nadeu F, Colomer D, *et al*. Chronic lymphocytic leukemia: from molecular pathogenesis to novel therapeutic strategies. *Haematologica* 2020; **105**: 2205–2217.
2. Hallek M, Cheson BD, Catovsky D, *et al*. iwCLL guidelines for diagnosis, indications for treatment, response assessment and supportive management of CLL. *Blood* 2018; **131**: 2745–2760.
3. Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment. *Am J Hematol* 2019; **94**: 1266–1287.
4. Pospisilova S, Gonzalez D, Malcikova J, *et al*. ERIC recommendations on TP53 mutation analysis in chronic lymphocytic leukemia. *Leukemia* 2012; **26**: 1458–1461.
5. Döhner H, Stilgenbauer S, Benner A, *et al*. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000; **343**: 1910–1916.
6. Catherwood MA, Wren D, Chiecchio L, *et al*. *TP53* mutations identified using NGS comprise the overwhelming majority of *TP53* disruptions in CLL: results from a multicentre study. *Front Oncol* 2022; **12**: 909615.
7. Nadeu F, Delgado J, Royo C, *et al*. Clinical impact of clonal and subclonal TP53, SF3B1, BIRC3, NOTCH1, and ATM mutations in chronic lymphocytic leukemia. *Blood* 2016; **127**: 2122–2130.
8. Nadeu F, Clot G, Delgado J, *et al*. Clinical impact of the subclonal architecture and mutational complexity in chronic lymphocytic leukemia. *Leukemia* 2017; **32**: 645–653.
9. Rossi D, Khiabanian H, Spina V, *et al*. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. *Blood* 2014; **123**: 2139–2147.
10. Blakemore SJ, Clifford R, Parker H, *et al*. Clinical significance of TP53, BIRC3, ATM and MAPK-ERK genes in chronic lymphocytic leukaemia: data from the randomised UK LRF CLL4 trial. *Leukemia* 2020; **34**: 1760–1774.
11. Bomben R, Rossi FM, Vit F, *et al*. TP53 mutations with low variant allele frequency predict short survival in chronic lymphocytic leukemia. *Clin Cancer Res* 2021; **27**: 5566–5575.
12. Malcikova J, Pavlova S, Kunt Vonkova B, *et al*. Low-burden TP53 mutations in CLL: clinical impact and clonal evolution within the context of different treatment options. *Blood* 2021; **138**: 2670–2685.
13. Brieghel C, Kinalis S, Yde CW, *et al*. Deep targeted sequencing of TP53 in chronic lymphocytic leukemia: clinical impact at diagnosis and at time of treatment. *Haematologica* 2019; **104**: 789–796.
14. Pandzic T, Ladenvall C, Engvall M, *et al*. Five percent variant allele frequency is a reliable reporting threshold for TP53 variants detected by next generation sequencing in chronic lymphocytic leukemia in the clinical setting. *Hemasphere* 2022; **6**: e761.
15. Hallek M, Cheson BD, Catovsky D, *et al*. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008; **111**: 5446–5456.
16. Tikkanen T, Leroy B, Fournier JL, *et al*. Seshat: a web service for accurate annotation, validation, and analysis of TP53 variants generated by conventional and next-generation sequencing. *Hum Mutat* 2018; **39**: 925–933.
17. Bouaoun L, Sonkin D, Ardin M, *et al*. TP53 variations in human cancers: new lessons from the IARC TP53 database and genomics data. *Hum Mutat* 2016; **37**: 865–876.
18. Tate JG, Bamford S, Jubb HC, *et al*. COSMIC: the catalogue of somatic mutations in cancer. *Nucleic Acids Res* 2019; **47**: D941–D947.
19. de Andrade KC, Lee EE, Tookmanian EM, *et al*. The TP53 Database: transition from the International Agency for Research on Cancer to the US National Cancer Institute. *Cell Death Differ* 2022; **29**: 1071–1073.
20. Malcikova J, Tausch E, Rossi D, *et al*. ERIC recommendations for TP53 mutation analysis in chronic lymphocytic leukemia-update on methodological approaches and results interpretation. *Leukemia* 2018; **32**: 1070–1080.
21. Soussi T, Baliakas P. Landscape of TP53 alterations in chronic lymphocytic leukemia via data mining mutation databases. *Front Oncol* 2022; **12**: 808886.
22. Hainaut P, Pfeifer GP. Somatic *TP53* mutations in the era of genome sequencing. *Cold Spring Harb Perspect Med* 2016; **6**: a026179.

23. Rossi D, Rasi S, Spina V, *et al*. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood* 2013; **121**: 1403–1412.
24. Zenz T, Kröber A, Scherer K, *et al*. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. *Blood* 2008; **112**: 3322–3329.
25. Dicker F, Herholz H, Schnittger S, *et al*. The detection of TP53 mutations in chronic lymphocytic leukemia independently predicts rapid disease progression and is highly correlated with a complex aberrant karyotype. *Leukemia* 2009; **23**: 117–124.
26. Zenz T, Vollmer D, Trbusek M, *et al*. TP53 mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations. *Leukemia* 2010; **24**: 2072–2079.
27. Lazarian G, Cymbalista F, Baran-Marszak F. Impact of low-burden TP53 mutations in the management of CLL. *Front Oncol* 2022; **12**: 841630.
28. Mansouri L, Thorvaldsdottir B, Sutton L-A, *et al*. Different prognostic impact of recurrent gene mutations in chronic lymphocytic leukemia depending on IGHV gene somatic hypermutation status: a study by ERIC in HARMONY. *Leukemia* 2023; **37**: 339–347.
29. Burger JA, Robak T, Demirkan F, *et al*. Up to 6.5 years (median 4 years) of follow-up of first-line ibrutinib in patients with chronic lymphocytic leukemia/small lymphocytic lymphoma and high-risk genomic features: integrated analysis of two phase 3 studies. *Leuk Lymphoma* 2022; **63**: 1375–1386.
30. Landau DA, Carter SL, Stojanov P, *et al*. Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. *Cell* 2013; **152**: 714–726.

SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Codon distribution of *TP53* mutations

Figure S2. Forms of *TP53* disruption among the cohort with both markers available

Figure S3. OS computed as time (months) from sample collection

Figure S4. Confirmation of *TP53* mutations, including relationship to VAF

Figure S5. TFS, TTFT and OS in samples collected within 6 months from diagnosis

Table S1. Patient characteristics

Table S2. *TP53* mutation characteristics