

**Keywords:** acute lymphoblastic leukaemia (ALL); next-generation sequencing (NGS); prognosis; outcome; survival; *TP53*; *JAK2*; mutation

# Mutations in *TP53* and *JAK2* are independent prognostic biomarkers in B-cell precursor acute lymphoblastic leukaemia

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**Background:** In B-cell precursor acute lymphoblastic leukaemia (B-ALL), the identification of additional genetic alterations associated with poor prognosis is still of importance. We determined the frequency and prognostic impact of somatic mutations in children and adult cases with B-ALL treated with Spanish PETHEMA and SEHOP protocols.

**Methods:** Mutational status of hotspot regions of *TP53*, *JAK2*, *PAX5*, *LEF1*, *CRLF2* and *IL7R* genes was determined by next-generation deep sequencing in 340 B-ALL patients (211 children and 129 adults). The associations between mutation status and clinicopathological features at the time of diagnosis, treatment outcome and survival were assessed. Univariate and multivariate survival analyses were performed to identify independent prognostic factors associated with overall survival (OS), event-free survival (EFS) and relapse rate (RR).

**Results:** A mutation rate of 12.4% was identified. The frequency of adult mutations was higher (20.2% vs 7.6%,  $P=0.001$ ). *TP53* was the most frequently mutated gene (4.1%), followed by *JAK2* (3.8%), *CRLF2* (2.9%), *PAX5* (2.4%), *LEF1* (0.6%) and *IL7R* (0.3%). All mutations were observed in B-ALL without *ETV6-RUNX1* ( $P=0.047$ ) or *BCR-ABL1* fusions ( $P<0.0001$ ). In children, *TP53*mut was associated with lower OS (5-year OS: 50% vs 86%,  $P=0.002$ ) and EFS rates (5-year EFS: 50% vs 78.3%,  $P=0.009$ ) and higher RR (5-year RR: 33.3% vs 18.6%  $P=0.037$ ), and was independently associated with higher RR (hazard ratio (HR) = 4.5;  $P=0.04$ ). In adults, *TP53*mut was associated with a lower OS (5-year OS: 0% vs 43.3%,  $P=0.019$ ) and a higher RR (5-year RR: 100% vs 61.4%,  $P=0.029$ ), whereas *JAK2*mut was associated with a lower EFS (5-year EFS: 0% vs 30.6%,  $P=0.035$ ) and a higher RR (5-year RR: 100% vs 60.4%,  $P=0.002$ ). *TP53*mut was an independent risk factor for shorter OS (HR = 2.3;  $P=0.035$ ) and, together with *JAK2*mut, also were independent markers of poor prognosis for RR (*TP53*mut: HR = 5.9;  $P=0.027$  and *JAK2*mut: HR = 5.6;  $P=0.036$ ).

**Conclusions:** *TP53*mut and *JAK2*mut are potential biomarkers associated with poor prognosis in B-ALL patients.

B-cell precursor acute lymphoblastic leukaemia (B-ALL) is a malignancy of lymphoid progenitor cells, characterised by large biological and clinical heterogeneity (Roberts and Mullighan,

2015). ALL is the most frequent childhood cancer and also accounts for ~25% of adult acute leukaemias (Bhojwani *et al*, 2015). High-risk B-ALL disease is more likely with adults than

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children, and the long-term disease-free adult survival rates are <40%, despite intensive chemotherapy and/or allogeneic stem cell transplantation treatments. This is in stark contrast to pediatric ALL, in which refined treatment regimens have resulted in cure rates approaching 80% (Paulsson *et al*, 2008; Bhojwani *et al*, 2015). However, in spite of this high cure rate, some children with ALL have a poor outcome, whereby 15% of them die from ALL relapses (Gowda and Dovat, 2013).

To date, the prognosis of B-ALL patients has focused mainly on clinical, haematological and genetic factors, such as age, leukocyte count at diagnosis, percentage of blast in peripheral blood, immunophenotype, central nervous system involvement, cytogenetic and molecular alterations and the presence of minimal residual disease (MRD), the latter two characteristics being most strongly associated with prognosis (Izraeli, 2010; Pui *et al*, 2011; Salari *et al*, 2014; Schrappe, 2014). However, ~30% of pediatric and 50% of adult ALL patients lack defined genetic hallmarks of biological and clinical significance (Bungaro *et al*, 2009; Dawson *et al*, 2011; Forero-Castro *et al*, 2016a; Forero-Castro *et al*, 2016b). Somatic mutations are hallmarks of lymphoid malignancies, and each genetic subtype harbours hidden mutations that are strong independent predictors of outcome (Iacobucci *et al*, 2012).

In recent years, the use of next-generation sequencing (NGS) has greatly increased the ability to identify somatic mutations with clinical impact in both child and adult B-ALL. These mutations involved genes associated with RAS signalling (48%; e.g., *NRAS*, *KRAS*, *PTPN11*, *FLT3*, *BRAF* and *NF1*), B-cell differentiation and development (18%; e.g., *PAX5*, *IKZF1*, *EBF1*, *VPREB1*), JAK/STAT signalling (11%; e.g., *JAK1*, *JAK2*, *IL7R* and *CRLF2*), cell cycle regulation and tumour suppression (6%; e.g., *TP53*, *RB1*, *CDKN2A/B*, *PTEN* and *BTG1*), and non-canonical pathways (9%, e.g., *ETV6*, *CREBBB* and *TBL1XR1*) (Harrison, 2011; Mullighan, 2011; Pui *et al*, 2011; Roberts and Mullighan, 2011; Iacobucci *et al*, 2012; Loh and Mullighan, 2012; Gowda and Dovat, 2013; Inaba *et al*, 2013; Chiaretti *et al*, 2014a; Chiaretti *et al*, 2014b; Woo *et al*, 2014). However, the prognostic impact of these mutations as predictors of clinical course, outcome and response to therapy is still being explored.

Herein, the frequency and clinical relevance of somatic mutations within a selected custom panel of six genes, *TP53*, *JAK2*, *IL7R*, *PAX5*, *LEF1* and *CRLF2* exons, was examined. Targeted exonic regions with known mutational hotspots (Harrison, 2011; Iacobucci *et al*, 2012; Inaba *et al*, 2013; Roberts and Mullighan, 2015), were analysed by amplicon-based NGS in 340 B-ALL patients. We demonstrated that mutations in *TP53* and *JAK2* have a negative impact on the outcome of pediatric and adult patients. This supports their role as prognostic biomarkers, and suggests that if assessed at diagnosis, they might contribute to a better risk stratification of B-ALL patients.

## MATERIALS AND METHODS

**Patients and data collection.** A total of 340 B-ALL patients referred from 22 Spanish centres to the Hematology Service at the Salamanca University Hospital, Spain, between February 1996 and February 2015 were eligible for this study. Two hundred and eleven of the patients were children (62.1%, <18 years) and 129 were adults (37.9%, ≥18 years).

The diagnosis of B-ALL was based on morphological, immunophenotypic and genetic features of leukaemic blast cells, as described previously (Pui and Evans, 1998). Conventional cytogenetic analyses, fluorescent in situ hybridisation, demographic information, clinical characteristics, risk classification, frontline therapy protocol, response to therapy and survival were

recorded. The criteria for defining and categorising the primary chromosomal abnormalities were established according to a previous study (Forero-Castro *et al*, 2016a), and are showed in Supplementary Material file 1. Patients were treated according to PETHEMA (Programa Español de Tratamientos en Hematología) and SEHOP (Sociedad Española de Hematología y Oncología Pediátrica) risk-adapted protocols. The study was approved by the local ethical committee, 'The Comité Ético de Investigación Clínica del área de salud de Salamanca', at the Hospital Universitario de Salamanca. Written informed consent was obtained from each patient or legal guardians before patients entered the study.

**DNA isolation.** Amplicon-based NGS was performed on all 340 samples obtained from untreated patients at diagnosis. Genomic DNA was extracted from frozen bone marrow or fixed peripheral blood cell samples with the QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

**Next-generation amplicon deep-sequencing assay.** An amplicon-based NGS assay was applied, using Titanium amplicon chemistry on a 454-GS Junior DNA Sequencing Platform (454 Life Sciences, Branford, CT, USA). With this approach, preconfigured custom 96-well primer plates containing lyophilised primer pairs (Roche, Branford, CT, USA) were used to prepare the amplicon library following the IRON-II Study procedures from the European Leukaemia Network group (Kohlmann *et al*, 2011). Nineteen hotspot exons were amplified: *TP53* (E4-E11), *JAK2* (E12-E16), *PAX5* (E2-E3), *LEF1* (E2-E3), *CRLF2* (E6) and *IL7R* (E5). The size of amplicons ranged from 304 to 431 bp including the adaptor sequences (see Supplementary Material File 1: Supplementary Table S1 for primer sequences). Information about amplicon library pooling, purification, emulsion PCR, sequencing, validation of variants, data processing and analysis, is presented in the Supplementary Material File 1.

**CRLF2 gene expression analysis.** In a subset of 97 B-ALL patients (81 children and 16 adults) the expression levels of *CRLF2* were evaluated by quantitative RT-PCR assays. *CRLF2*-overexpressed samples were identified as previously described (Yoda *et al*, 2010) (see Supplementary Material file 1 for further details).

**Statistical methods.** Continuous variables were summarised as their median and range; categorical variables were described as the frequency and percentage of subjects in each category. Associations between the presence of mutations and the values of clinical parameters were investigated. Continuous variables were analysed by the non-parametric Mann-Whitney *U*-test. The  $\chi^2$  or Fisher's exact tests were used to identify significant associations between dichotomous variables, as appropriate. All tests were two-sided and values of  $P < 0.05$  were considered to be significant. Kaplan-Meier analysis (log-rank test) was used to assess the relationship between mutations and overall survival (OS), event-free survival (EFS) and relapse rate (RR). Survival rates were given as probabilities of survival at 5 years, with a 95% confidence interval (CI). Pediatric and adult patients were analysed separately. Survival criteria are shown in the Supplementary Material File 1. Clinical and genetic variables were first analysed by univariate analysis, and those with a *P*-value up to 0.05 were included in multivariate analysis. A multivariate Cox proportional hazards regression model was used to estimate the hazard ratio (HR) and 95% CI of risk factors. Specifically simultaneous regression, which SPSS calls the Enter method was used. Analyses were performed using SPSS version 22.0 (IBM).

## RESULTS

**Patient characteristics.** Table 1 shows the clinical characteristics of the pediatric and adult patients with B-ALL included in this

study. The median age was 12 years (range 0–84 years); childhood patients were aged from 0 to 17 years (median 5 years) while adult patients were aged from 18 to 84 years (median 45 years). The median percentage of blast counts in bone marrow was 90% (range, 35–100%) and 30.6% of patients showed normal cytogenetics. The presence of poor prognosis abnormalities t(9;22) (Ph +), t(v;11q23) and hypodiploidy, were more frequent in adult than in childhood B-ALL (48.8% vs 8.1%,  $P < 0.0001$ ). In addition, there was a higher prevalence of highly hyperdiploid cases ( $> 50$  chromosomes) in the pediatric compared with the adult group (15.6% vs 3.9%,  $P = 0.001$ ). The median follow-up of the whole series was 60 months (range, 2–186 months). The children had higher rates of 5-year OS (85.3% vs 40.5%,  $P < 0.0001$ ), EFS (77.7% vs 28.6%,  $P < 0.0001$ ) and relapse (RR) (16.1% vs 48.1%,  $P < 0.0001$ ) compared with adults.

**Frequency and characterisation of molecular mutations in B-ALL.** Thirty different mutations were observed in 42 out of the 340 patients at diagnosis (12.4%). *TP53* was the most frequently mutated gene, being present in 4.1% of patients, followed by *JAK2* (3.8%), *CRLF2* (2.9%), *PAX5* (2.4%), *LEF1* (0.6%) and *IL7R* (0.3%). The mutations were more frequent in adults than in children (20.2% vs 7.6%,  $P = 0.001$ ). The detailed frequency of mutations in children and adults was *TP53* (2.4% vs 7.0%,  $P = 0.038$ ), *CRLF2* (0.5% vs 7.0%,  $P = 0.001$ ), *JAK2* (2.4% vs 6.2%,  $P = 0.086$ ), *PAX5* (1.4% vs 3.9%,  $P = 0.162$ ), *LEF1* (0.5% vs 0.8%,  $P = 1.0$ ) and *IL7R* (0.5% vs 0%,  $P = 1.0$ ) (Supplementary Material File 1: Supplementary Figures S1 and S2).

The clinical characteristics, cytogenetic subgroups, somatic mutations with their respective mutational burden, risk classification, frontline therapy protocol used, response to therapy, survival and clinical status of each pediatric and adult patient are summarised in Supplementary Material File 1: Supplementary Table S2. Most mutations (88.1%) were detected in B-ALL cases lacking recurrent fusion genes. In fact, the presence of mutations in patients with normal cytogenetics was more frequently observed in both childhood (37.5%) and adult (46.2%) B-ALL cohort ( $P = 0.002$ ) (Supplementary Material File 1: Supplementary Table S3). It should be noted that only one pediatric patient (ID10) with *TP53*mut harboured the *TCF3(E2A)-PBX1* fusion and four patients (one child, ID13 and three adults, ID25, ID26 and ID34) with *PAX5*mut, *TP53*mut and/or *CRLF2*mut carried *KMT2A(MLL)-R*.

Supplementary Material File 1: Supplementary Table S4 details the frequency of secondary somatic mutations in hotspot regions of *TP53*, *JAK2*, *PAX5*, *LEF1*, *CRLF2* and *IL7R* genes classified by primary chromosomal abnormalities in children and adult patients with B-ALL. In the entire childhood cohort of B-ALL, all 16 mutations were exclusively detected in the subgroup of patients without *ETV6-RUNX1 (TEL-AML1)* translocation ( $P = 0.047$ ). Moreover, in the entire adult cohort of B-ALL, all 26 mutations were exclusively detected in the subgroup of patients without *BCR-ABL1* translocation ( $P < 0.0001$ ). Thus, none of the cases with secondary somatic mutations showed *ETV6-RUNX1 (TEL-AML1)* or *BCR-ABL1* translocations as primary chromosomal abnormalities. There were no other correlations between the primary chromosomal abnormality and the spectrum of mutations observed.

Supplementary Material File 1: Supplementary Table S5 details the primary chromosomal abnormalities evaluated by conventional and molecular cytogenetics, and describes the secondary somatic mutations according to the number of mutations per case, their mutational burden, gene-exon mutated and type of mutation observed in children and adult patients with B-ALL. The median mutational burden was 24.5% (range, 2–97%) (16.5% children and 28% adults,  $P = 0.730$ ). It is of particular note that 25.9% of mutations detected showed mutation loads of  $\leq 10\%$  (23.5% children and 27% adults,  $P = 1.0$ ), so these variants would

not have been detected by the Sanger capillary sequencing method (Supplementary Material File 1: Supplementary Table S2).

Twenty-two of the 30 different mutations found were previously described in the COSMIC database (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic>) and/or the IARC *TP53* database (<http://p53.iarc.fr/p53Sequences.aspx>) (Leroy *et al*, 2014) but we identified eight undescribed mutations in those genes (one in each gene under study and three novel missense mutations in *PAX5*). Supplementary Material File 1: Supplementary Table S6 showed the protein domain affected by these mutations. The sequence analysis revealed 24 missense mutations, three deletion-insertions, two splicing mutations and one frameshift mutation. As shown in Supplementary Material File 1: Supplementary Figures S1 and S2, *TP53* mutations were generally distributed across several exons, with missense mutations being predominantly found in evolutionarily conserved regions of this gene. The other mutated genes showed recurrent mutations: *CRLF2* (p.F232C, nine patients), *JAK2* (p.R683G, nine patients and p.R683S, nine patients) and *PAX5* (p.P80R, four patients).

**NGS enabled identification of the co-occurrence of mutations and prediction of the presence or absence of distinct subclones harbouring different mutations.** Eleven out of the 42 patients with mutations (26.2%) concomitantly harboured more than one mutation, which were more frequently observed in adult than in pediatric patients (38.5% vs 6.3%,  $P = 0.030$ ). Ten cases had two and one adult case had three mutations in the genes investigated. All of them were orthogonally validated by other methods such as Sanger sequencing, re-sequencing run and/or the IonTorrent sequencer system. Supplementary Material File 1: Supplementary Table S7 shows the distribution of mutations identified in these 11 patients and Supplementary Material File 1: Supplementary Table S2 details their clinical characteristics, frontline therapy and outcome. In four cases (child: ID5 and adults: ID40, ID26 and ID27), the mutations occurred in the same gene and in the same amplicon (*JAK2*, *PAX5* or *TP53*), while one adult patient (ID39) showed two different mutations within two amplicons of the same gene (*TP53*). In five adult cases, mutations occurred in different genes: three patients (ID17, ID21 and ID42) harboured concomitant *JAK2* and *CLFR2* mutations, while one patient (ID25) had *TP53/PAX5*, and other (ID19) *CRLF2/LEF1* mutations. Finally, one adult patient (ID31) harboured three concomitant mutations in *JAK2* and *CRLF2*, of which two mutations were detected in the same *JAK2* amplicon.

The amplicon-NGS allowed separate subclones to be distinguished in two adult patients (ID31 and ID40, Supplementary Material File 1: Supplementary Figure S3 and Supplementary Table S2) with the same combination of *JAK2* mutations located in exon 16 (*JAK2*-E16, p.R683G and p.R683S). In these cases the mutated codon 683 was not found concomitantly within the same sequencing read, but was separated across distinct individual reads. In accordance with the differential mutational loads between the two *JAK2* mutations, the sequencing analysis demonstrated that the dominant clone harboured the p.R683G mutation (c.2047A > G), whereas the minority clone harboured the p.R683S mutation (c.2049A > T). In both cases, the c.2047A > G mutation was always present at a higher percentage with respect to the c.2049A > T mutation (patient ID31: 26% and 2%; and patient ID40: 38% and 4%) and the minority subclone was present with a mutational burden of  $< 5\%$  of leukaemic cells. Finally, one of these patients (patient ID31) harboured a third low-percentage mutation in the *CRLF2* gene (p.F232C in 10% of leukaemic cells).

In addition, amplicon-NGS made it possible to identify clonal heterogeneity in three patients (one child and two adults) who carried a double mutation within the same amplicon. In the first

**Table 1. Characteristics of pediatric and adult B-ALL patients included in the study**

Characteristics	Whole cohort		Children < 18 years		Adults ≥ 18 years		P
	N	%	n	%	n	%	
All patients	340	100	211	100	129	100	NA
Age at diagnosis (years), median (range)	12	(0–84)	5	(0–17)	45	(18–84)	NA
Sex							
Male, n (%)	168	49.4	94	44.5	74	57.4	<b>0.022</b>
Female, n (%)	172	50.6	117	55.5	55	42.6	
Counts and other parameters							
Bone marrow blast <sup>a</sup> , median (range)	90	(35–100)	90	(35–100)	88	(35–98)	<b>0.011</b>
WBC count ( $\times 10^9 l^{-1}$ ), median (range)	15	(1–634)	15	(1–634)	25	(1–575)	<b>0.011</b>
WBC $\geq 30 \times 10^9 l^{-1}$ (%)	112	38.4	59	32.2	53	48.6	<b>0.005</b>
Hb count ( $g l^{-1}$ ), median (range)	90	(26–172)	76	(26–144)	103	(39–172)	<b>&lt;0.0001</b>
Platelet count ( $\times 10^9 l^{-1}$ ), median (range)	53	(2–580)	64	(3–556)	50	(2–580)	0.251
Elevated LDH ( $U l^{-1}$ ) level <sup>b</sup> , n (%)	204	85.7	122	84.1	82	88.2	0.386
ECOG score $\geq 2$ , n (%)	22	33.3	5	23.8	17	37.8	0.262
Down syndrome, n (%)	8	2.4	6	2.9	2	1.6	0.715
Cytogenetics							
Normal, n (%)	104	30.6	68	32.2	36	27.9	0.401
Abnormal, n (%)	236	69.4	143	67.8	93	72.1	
Cytogenetic risk groups							
Poor risk <sup>c</sup> , n (%)	80	23.5	17	8.1	63	48.8	<b>&lt;0.0001</b>
Others, n (%)	260	76.5	194	91.9	66	51.2	
Risk group <sup>d</sup>							
Low risk, n (%)	57	19.3	57	33.9	0	0	<b>&lt;0.0001</b>
Standard (intermediate) risk, n (%)	69	23.3	54	32.1	15	11.7	
High risk, n (%)	170	57.4	57	33.9	113	88.3	
MRD at the end of induction <sup>a</sup>							
MRD $< 0.01\%$ , n (%)	165	69.6	119	73.5	46	61.3	0.059
MRD $\geq 0.01\%$ , n (%)	72	30.4	43	26.5	29	38.7	
Outcome data							
SCT performed in first CR, n (%)	48	68.6	21	67.7	27	69.2	0.894
Relapse, n (%)	76	27.7	34	18.1	42	48.8	<b>&lt;0.0001</b>
Very early relapse <sup>e</sup> , n (%)	41	56.2	13	40.6	28	68.3	0.06
Patients alive in first CR, n (%)	174	65.4	144	84.2	30	31.6	<b>&lt;0.0001</b>
Deaths, n (%)	107	33.5	32	16.3	75	61	<b>&lt;0.0001</b>
Median follow-up (range), months	60	(2–186)	66	(2–186)	39	(3–171)	<b>0.002</b>
5-year OS rates % (95% CI)	68.8	(NR, 60.0–71.5)	85.3	(NR, 77.1–88.5)	40.5	(17, 9.1–24.8)	<b>&lt;0.0001</b>
5-year EFS rates % (95% CI)	60.1	(NR, 50.7–62.7)	77.7	(NR, 81.1–68.0)	28.6	(10, 5.3–14.6)	<b>&lt;0.0001</b>
5-year RR % (95% CI)	26.1	(NR, 25.1–38.0)	16.1	(NR, 13.2–26.0)	48.1	(70, 50.6–89.5)	<b>&lt;0.0001</b>

Abbreviations: B-ALL = B-cell precursor acute lymphoblastic leukaemia; CI = confidence interval; CR = complete remission; ECOG = Eastern Cooperative Oncology Group; EFS = event-free survival; HR = hazards ratio; LDH = lactate dehydrogenase; MRD = minimal residual disease; NA = not applicable; NR = not reached; OS = overall survival; RR = relapse rate; SCT = stem cell transplantation; WBC = white blood cell.

Probabilities highlighted in bold indicate statistically significant results ( $P < 0.05$ ).

<sup>a</sup>By flow cytometry.

<sup>b</sup>Normal range: 135–214  $U l^{-1}$ .

<sup>c</sup>Includes patients with t(9;22), t(v;11q23) and hypodiploidy.

<sup>d</sup>Risk group stratification established according to PETHEMA protocols, based on age, WBC and cytogenetic subgroup.

<sup>e</sup>Time of relapse criteria: very early, earlier than 18 months after initial diagnosis and less than 6 months after cessation of frontline treatment; early, more than 18 months after initial diagnosis, but less than 6 months after cessation of frontline treatment; late, more than 6 months after cessation of frontline treatment.

patient (ID27-adult) who harboured two distinct mutations in the same amplicon (*PAX5*-E03, c.215A > G and c.239C > G), 454 deep-sequencing allowed the presence of four lines to be discriminated; one minority and germline (*PAX5*wt: 8.8%), one dominant clone with c.215A > G mutation (51.4%), one subclone with c. 239C > G mutation (36.6%) and one minority subclone (3.2%) with a double mutation in the *PAX5*-E03 gene (c.215A > G and c. 239C > G) (Supplementary Material File 1: Supplementary Figure S4). In the second patient (ID5-child) who carried two mutations in the *JAK2*-E16 gene, three lines were identified: one germline (*JAK2*wt: 84%), one subclone with c.2044\_2045insGGACCTCCTCCCTCC mutation (12%) and one minority subclone with c.2049A > T mutation (4%) (Supplementary Material File 1: Supplementary Figure S5). Finally, three independent lines were identified in the third patient (ID26-adult) presenting two mutations in the *TP53*-E08 gene: one germline (*TP53*wt:83.4%) line, and two minority lines,

one subclone with c.841G > A mutation (10%) and one subclone with c.845G > C mutation (6.6%) (Supplementary Material File 1: Supplementary Figure S6).

#### Mutated genes associated with clinical and prognostic features.

Next, mutations were associated with cytogenetic subtypes, clinical features and risk factors commonly used to stratify pediatric and adult B-ALL patients (Supplementary Material File 1: Supplementary Table S8 and Supplementary Table S9). Thus, the presence of mutations in any of the genes analysed was associated with children stratified in the high-risk group ( $P = 0.018$ ). Mutations in the *TP53* gene were associated with poor response to frontline therapy due to refractoriness or relapse events ( $P = 0.032$ ) (Supplementary Material File 1: Supplementary Table S8). Meanwhile, in the adult cohort, all 26 mutated cases were exclusively detected in *BCR-ABL1*-negative cases ( $P < 0.0001$ ). The presence of *JAK2*mut was associated with poor-prognosis

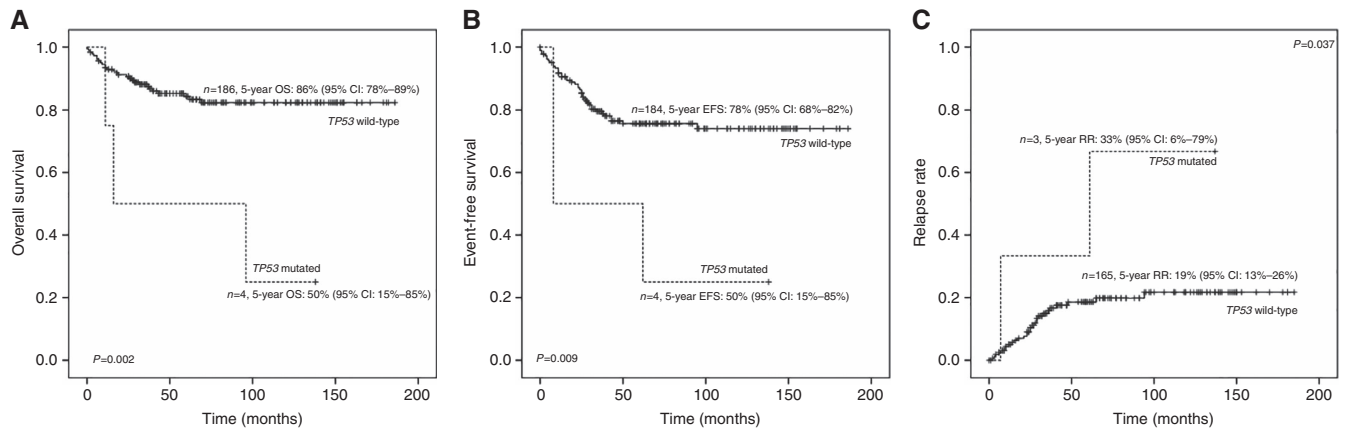


Figure 1. Kaplan-Meier curves for OS, EFS and RR of the whole cohort of children with B-ALL. (A-C) OS, EFS and RR in patients bearing TP53mut.

frontline therapy ( $P=0.031$ ) (Supplementary Material File 1: Supplementary Table S9).

**Gene mutations are related to a worse outcome.** In the survival analysis of the children, significantly lower OS (5-year OS: 50% vs 86%,  $P=0.002$ ) and EFS rates (5-year EFS: 50% vs 78.3%,  $P=0.009$ ) and higher RR (5-year RR: 33.3% vs 18.6%  $P=0.037$ ), were observed in patients with TP53mut compared with patients without TP53mut (Figure 1 and Supplementary Material file 1: Supplementary Table S10). The present study also confirmed in pediatric cohort the well-known associations of particular clinical and biological variables with worse prognosis, such as the high-risk group (OS:  $P<0.0001$ , EFS:  $P<0.0001$  and RR:  $P=0.002$ ), MRD  $\geq 0.01\%$  (OS:  $P=0.004$  and EFS:  $P=0.027$ ),  $WBC \geq 30 \times 10^9 l^{-1}$  (OS:  $P=0.004$ ), poor-risk cytogenetics due to the presence of t(9;22), t(v;11q23) or a hypodiploid karyotype (OS:  $P<0.0001$ , EFS:  $P<0.0001$  and RR:  $P=0.016$ ), age over 10 years (OS:  $P<0.0001$ , EFS:  $P<0.0001$  and RR:  $P<0.0001$ ), and a Pro-B phenotype (OS:  $P<0.0001$  and EFS:  $P=0.004$ ) (Supplementary Material file 1: Supplementary Table S10). Multivariate analysis of the group of children showed that TP53mut was an independent risk factor associated with significantly higher RR (HR=4.5; 95% CI 1.1-19.2,  $P=0.04$ ) (Table 2).

In the group of adults, TP53mut had a negative effect on OS (5-year OS: 0% vs 43.3%,  $P=0.019$ ) and was associated with a higher RR (5-year RR: 100.0% vs 61.4%,  $P=0.029$ ), and JAK2mut was related with a lower EFS (5-year EFS: 0% vs 30.6%,  $P=0.035$ ) and a higher RR (5-year RR: 100% vs 60.4%,  $P=0.002$ ) (Figure 2 and Supplementary Material File 1: Supplementary Table S11). The NGS analysis also confirmed the clinical parameters commonly associated with shorter OS, EFS and RR in adults, such as MRD  $\geq 0.01\%$  (RR:  $P=0.003$  and EFS:  $P=0.023$ ),  $WBC \geq 30 \times 10^9 l^{-1}$  (RR:  $P=0.024$  and EFS:  $P=0.008$ ), poor-risk cytogenetic abnormalities (OS:  $P=0.013$  and EFS:  $P=0.025$ ) and age  $\geq 55$  years (OS:  $P=0.001$ , RR:  $P=0.027$  and EFS:  $P=0.002$ ). Supplementary Material File 1: Supplementary Table S11 shows these clinical parameters commonly associated with shorter survival in adults. Multivariate analysis of the whole cohort of ALL adults indicated that the presence of TP53mut was an independent risk factor associated with significantly shorter OS (HR=2.3; 95% CI 1.1-5.1,  $P=0.035$ ). Moreover, TP53mut (HR=5.9; 95% CI 1.2-28.6,  $P=0.027$ ) and JAK2mut (HR=5.6; 95% CI 1.1-28.1,  $P=0.036$ ) retained their independent prognostic significance in multivariate analysis regarding for RR (Table 3).

Finally, it is worth mentioning that there were no associations between the mutational burden and the cytogenetic, clinical and prognostic parameters of the pediatric and adult cohorts with B-ALL. In the same way, the survival rates of patients who carried

Table 2. Univariate and multivariate survival analysis in children with B-ALL

Multivariate analysis with OS				
Parameter	Univariate analysis P	HR	CI (95%)	P
TP53mut	<b>0.002</b>	3.9	0.7-20.1	0.107
Poor risk cytogenetic	<b>&lt;0.0001</b>	3.4	0.7-16.0	0.129
$WBC \geq 30 \times 10^9 l^{-1}$	<b>0.042</b>	1.1	0.4-3.1	0.836
MRD $\geq 0.01\%$	<b>0.004</b>	3	1.1-8.3	<b>0.039</b>
Pro-B B-ALL	<b>&lt;0.0001</b>	0.3	0.1-0.9	<b>0.026</b>
JAK2mut	0.0174			
Multivariate analysis with EFS				
Parameter	Univariate analysis P	HR	CI (95%)	P
TP53mut	<b>0.009</b>	2.8	0.6-12.8	0.192
Poor risk cytogenetic	<b>&lt;0.0001</b>	2.3	0.7-8.0	0.189
MRD $\geq 0.01\%$	<b>0.027</b>	1.8	0.8-4.0	0.128
Pro-B B-ALL	<b>0.004</b>	0.4	0.2-1.2	0.105
$WBC \geq 30 \times 10^9 l^{-1}$	0.117			
JAK2mut	0.469			
Multivariate analysis with RR				
Parameter	Univariate analysis P	HR	CI (95%)	P
TP53mut	<b>0.037</b>	4.5	1.1-19.2	<b>0.04</b>
Poor risk cytogenetic	<b>0.016</b>	3.6	1.2-10.5	<b>0.018</b>
$WBC \geq 30 \times 10^9 l^{-1}$	0.668			
MRD $\geq 0.01\%$	0.063			
Pro-B B-ALL	0.05			
JAK2mut	0.208			

Abbreviations: B-ALL = B-cell precursor acute lymphoblastic leukaemia; CI = confidence interval; EFS = event-free survival; HR = hazards ratio; MRD = minimal residual disease; OS = overall survival; RR = relapse rate; WBC = white blood cell. The parameters with P-values < 0.05 were considered as statistically significant in the univariate analysis and were included in multivariate analysis. Probabilities highlighted in bold indicate statistically significant results ( $P<0.05$ ).

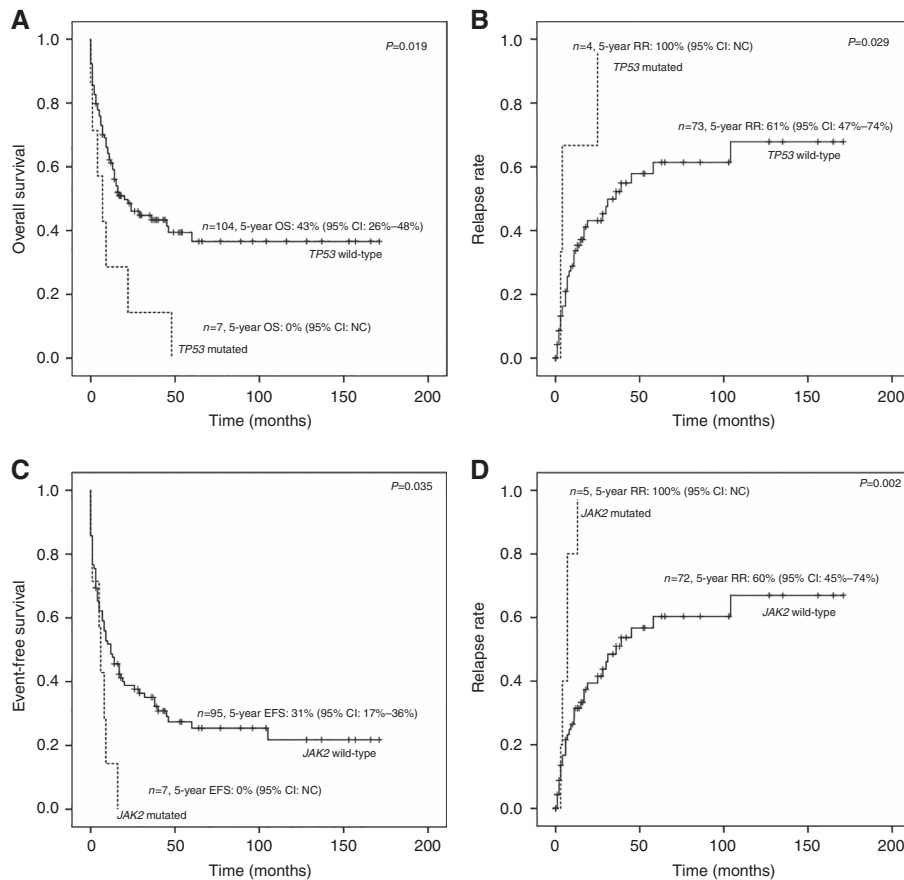


Figure 2. Kaplan–Meier curves for OS, EFS and RR of the whole cohort of adults with B-ALL. (A and B) OS and RR in patients bearing *TP53*mut. (C and D) EFS and RR in patients with *JAK2*mut.

small clones with  $\leq 10\%$  of mutational burden did not have significant differences with respect to those who carried clone sizes  $> 10\%$  (children: 5-year OS: 50% vs 72.7%,  $P = 0.544$ ; 5-year EFS: 50% vs 72.7%,  $P = 0.606$  and 5-year RR: 50.0% vs 20%,  $P = 0.426$ ; adults: 5-year OS: 0% vs 31.3%,  $P = 0.453$  and 5-year EFS: 0% vs 12.5%,  $P = 0.710$ ). Particularly, for *TP53* and *JAK2* mutations, there was no difference of mutational burden on outcome and survival parameters.

***CRLF2* overexpression is associated with *JAK* mutations.** Overexpression of the *CRLF2* gene (*CRLF2*oe) was observed in 17.5% of B-ALL patients by quantitative RT-PCR assays. There were no significant differences in the *CRLF2*oe between child and adult B-ALL patients (17.3% vs 18.8%,  $P = 1.0$ ). *CRLF2*oe was common only in B-ALL cases that lack rearrangements of *TEL-AML1*, *KMT2A(MLL)*, *TCF3(E2A)-PBX1*, and *BCR-ABL* ( $P = 0.007$ ). Supplementary Material File 1: Supplementary Table S2 shows the results obtained from the analysis of *CRLF2* expression in each of the patients with mutations. The presence of *JAK2* mutations was associated with *CRLF2*oe ( $P = 0.023$ ). Furthermore, neither the clinical nor prognostic features were associated with *CRLF2*oe in both childhood and adult B-ALL patients. However, the survival analysis of whole cohort of adults with B-ALL showed that the presence of *CRLF2*oe had a negative effect on OS (5-year OS: 0% vs 53.8%,  $P = 0.005$ ) and EFS rates (5-year EFS: 0% vs 33.3%,  $P = 0.006$ ).

## DISCUSSION

The presence of gene mutations is a hallmark of B-ALL. In this study, we evaluated 340 B-ALL cases at diagnosis (211 children and 129 adults) to address the incidence and prognostic impact of *TP53*, *JAK2*,

*IL7R*, *PAX5*, *LEF1* and *CRLF2* mutations by NGS. The incidence (12.4%) and frequency of mutations were higher in adults than in children according with the better prognosis of pediatric B-ALL. Most mutations (88.1%) were detected in B-ALL cases lacking recurrent fusion genes. The incidence and frequency of mutations detected in our study were similar to observations made in previous ALL studies (Mullighan, 2011; Pui *et al*, 2011; Roberts and Mullighan, 2011; Iacobucci *et al*, 2012; Loh and Mullighan, 2012; Gowda and Dovat, 2013; Inaba *et al*, 2013; Chiaretti *et al*, 2014a, b; Woo *et al*, 2014). The use of the NGS strategy further allowed the identification of clonal heterogeneity in B-ALL patients. A negative impact of the presence of *TP53* and *JAK2* mutations on the OS, RR and EFS in patients with adult and childhood B-ALL was found in the present study. Therefore, deep sequencing may ultimately be a better guide to treatment decisions for B-ALL patients.

Among 30 mutations identified at diagnosis, 22 have previously been described in B-ALL databases (Bercovich *et al*, 2008; Mullighan *et al*, 2009b; Roll and Reuther, 2010; Yoda *et al*, 2010; Mullighan *et al*, 2011; Izraeli *et al*, 2014; Leroy *et al*, 2014), confirming these mutated exons to be hotspot regions in leukaemia. Recurrent mutations in *CRLF2* (p.F232C), *JAK2* (p.R683G and p.R683S) and *PAX5* (p.P80R) have also been observed in other cohorts of patients with ALL (Bercovich *et al*, 2008; Mullighan *et al*, 2009b; Roll and Reuther, 2010; Yoda *et al*, 2010; Mullighan *et al*, 2011; Izraeli *et al*, 2014). It is of particular note that we have found five undescribed mutations in those genes, three of which are missense mutations in *PAX5*. Mutations in the paired box (PAX) domain are predicted as deleterious, probably impairing the DNA-binding capability of this lymphoid transcription factor which is essential for normal B-cell development (Roberts *et al*, 2001). Also, two undescribed mutations were found in either *IL7R* or *LEF1*, the least frequently mutated genes in our series. Somatic gain-of-function mutations in *IL-7R* have been

**Table 3. Univariate and multivariate survival analysis in adults with B-ALL**

Multivariate analysis with OS				
Parameter	Univariate analysis P	HR	CI (95%)	P
TP53mut	<b>0.019</b>	2.3	1.1–5.1	<b>0.035</b>
Poor risk cytogenetic	<b>0.013</b>	1.7	1.1–2.9	<b>0.02</b>
WBC $\geq 30 \times 10^9 l^{-1}$	0.288			
MRD $\geq 0.01\%$	0.314			
Pro-B B-ALL	0.923			
JAK2mut	0.972			
CRLF2mut	0.641			
Multivariate analysis with EFS				
Parameter	Univariate analysis P	HR	CI (95%)	P
JAK2mut	<b>0.035</b>	3.7	0.8–7.1	0.097
Poor risk cytogenetic	<b>0.025</b>	2.3	1.1–4.9	<b>0.028</b>
WBC $\geq 30 \times 10^9 l^{-1}$	<b>0.008</b>	1.2	0.6–2.5	0.66
MRD $\geq 0.01\%$	<b>0.023</b>	2.1	1.0–4.0	<b>0.036</b>
Pro-B B-ALL	0.516			
TP53mut	0.08			
CRLF2mut	0.642			
Multivariate analysis with RR				
Parameter	Univariate analysis P	HR	CI (95%)	P
TP53mut	<b>0.029</b>	5.9	1.2–28.6	<b>0.027</b>
JAK2mut	<b>0.002</b>	5.6	1.1–28.1	<b>0.036</b>
WBC $\geq 30 \times 10^9 l^{-1}$	<b>0.024</b>	2.2	0.9–5.1	0.072
MRD $\geq 0.01\%$	<b>0.003</b>	2.4	1.1–5.3	<b>0.026</b>
Pro-B B-ALL	0.599			
Poor risk cytogenetic	0.988			
CRLF2mut	0.412			

Abbreviations: B-ALL = B-cell precursor acute lymphoblastic leukaemia; CI = confidence interval; EFS = event-free survival; HR = hazards ratio; MRD = minimal residual disease; OS = overall survival; RR = relapse rate; WBC = white blood cell. The parameters with P-values < 0.05 were considered as statistically significant in the univariate analysis and were included in multivariate analysis. Significant parameters are highlighted in bold.

shown to act as oncogenes in T- and B-ALL (Shochat *et al*, 2011; Mazzucchelli *et al*, 2012). (Shochat *et al*, 2011). These *IL-7R* mutations are usually insertions of bases encoding a cysteine and a proline into the 6th exon of the extracellular domain, at the border with the transmembrane region, immediately before or after residue 244 (Mazzucchelli *et al*, 2012). It is well known that cysteines and prolines are essential for the constitutive activation of the receptor causing cytokine independent growth of mouse pro-B cells (Shochat *et al*, 2011).

In the case of *TP53mut*, 11 out of 13 patients were negative for fusion genes, consistent with the findings of a previous ALL cohort at diagnosis (Chiaretti *et al*, 2013). Only one pediatric patient (ID10) with the *TCF3(E2A)-PBX1* fusion and one adult (ID26) with *KMT2A(MLL)-R* harboured *TP53mut*. It should be noted that a significant association between *TP53mut* and *MLL/AFF1* translocations has been reported (Hof *et al*, 2011). Furthermore, two patients with low hypodiploid (ID9 and ID38) and two patients with near

triploid karyotypes (ID22 and ID24) carried *TP53* mutations. The high incidence of *TP53* alterations (mutation, deletion) was previously associated with low hypodiploid/near triploid ALL, making it possible to distinguish this rare subset from other ALL subgroups including near haploid ALL. Furthermore, this high incidence can guide the prognosis of the disease because these alterations are usually associated with worse prognosis (Muhlbacher *et al*, 2014; Stengel *et al*, 2014). Therefore, the use of NGS could enable the prognostic B-ALL subgroups of patients to be refined.

Amplicon-based NGS was also able to detect variants with a low mutational burden, which have been identified in genomic databases (Bercovich *et al*, 2008; Mullighan *et al*, 2009b; Roll and Reuther, 2010; Yoda *et al*, 2010; Mullighan *et al*, 2011; Izraeli *et al*, 2014; Leroy *et al*, 2014) or found in previous studies in ALL patients (Shochat *et al*, 2011). Thus, our study confirmed that NGS is a suitable method for accurately detecting and quantifying a variety of mutations in important genes associated with pathogenesis and prognosis of B-ALL. In fact, 26.4% of variations described in our study had mutation loads of  $\leq 10\%$ , underlining the high sensitivity of amplicon-based NGS for detecting small leukaemic subclones, commonly undetectable by conventional Sanger capillary sequencing. It is well known that mutational screening by bidirectional Sanger sequencing does not reveal the presence of mutant subclones representing less than 10–20% of leukaemic cells (Kastner *et al*, 2014).

One of the most important tasks in understanding clonal progression in leukaemia is to assess the nature and number of different subclones within an individual cancer (Mullighan *et al*, 2008; Grossmann *et al*, 2011; Landau *et al*, 2014). In agreement with the findings of previous studies (Kohlmann *et al*, 2010; Grossmann *et al*, 2011; Kohlmann *et al*, 2011; Kastner *et al*, 2014; Landau *et al*, 2014), we confirmed the complex clonal architecture of ALL. NGS technology allowed the detection of clonal heterogeneity in some of the analysed patients. This was achieved by identifying distinct subpopulations with a dominant leukaemic clone and their relative proportions in the total B-ALL cell population. The case ID27 (Supplementary Material File 1: Supplementary Figure S4) shows the presence of leukaemia subclones that were derived from a common tumour-initiating cell, whereas cases ID5 and ID26 (Supplementary Material File 1: Supplementary Figures S5 and S6) have clones with divergent mutations (Jan and Majeti, 2013). Further investigation with samples from relapsed ALL is needed to elucidate the effect of these clones and subclones on disease progression and whether they can provide clues to the cause of treatment failure (Meyer *et al*, 2013; Tzoneva *et al*, 2013; Lindqvist *et al*, 2015). Unfortunately these samples were not available in this study.

The present study showed that *TP53* mutations are the mutations most frequently observed in B-ALL patients. The tumour suppressor gene *TP53* has a crucial role in cell cycle regulation and apoptosis after DNA damage, and its role in tumorigenesis is well recognised in solid and haematological malignancies (Chiaretti *et al*, 2013). In our study, *TP53mut* was associated with poor outcome in the whole cohort of patients and when considering adults and children separately. These results are in agreement with those of previous studies in which *TP53mut* was associated with resistance to treatment and worse prognosis in several tumours (Olivier *et al*, 2010; Salmoiraghi *et al*, 2016). In addition, alterations of the *TP53* gene were described as being important at relapse in childhood and adult ALL, in which they independently predict a high risk of treatment failure in a significant number of patients (Hof *et al*, 2011). The presence of *TP53* mutations was also associated with a reduced response rate to induction therapy (Chiaretti *et al*, 2013) and a shorter survival (from time of diagnosis and from time of relapse), even after successful reinduction therapy (Diccianni *et al*, 1994). These lines of evidence, together with our results, highlight the importance of sequencing *TP53* at diagnosis.

*JAK2*mut was also frequent in B-ALL. *JAK2* is an important mediator of cytokine receptor signalling and has key roles in the hematopoietic and immune response. Constitutive signalling through *JAK2* contributes to the proliferation of many cancers, including B-cell leukaemia and lymphomas (Ihle and Gilliland, 2007; Vainchenko and Constantinescu, 2013). The acquired *JAK2*mut (p.R683S and p.R683G) is presumed to be a biomarker for B-ALL (Bercovich *et al*, 2008; Mullighan, 2008; Mullighan *et al*, 2009b). Recent studies suggested that the amino acid residue p.R683 located in the linker between the N and C lobes of JH2 domain is important for maintaining the activity, structural stability and folding of *JAK2* (Li *et al*, 2013). The mutations in p.R683 disrupted the structure of JH2 domain leading to *JAK2* constitutive activation and induced growth factor-independent cell proliferation of the mouse Ba/F3 hematopoietic cell line (Mullighan, 2008; Li *et al*, 2013). In our study, two B-ALL patients with two independent clones each, displayed different mutations in the p.R683 residue (Bercovich *et al*, 2008). Even though the oncogenic effect of p.R683S and p.R683G mutations could be similar or identical, further research will be necessary to fully understand the biological relevance of this clonal heterogeneity. The development of *JAK2* inhibitors that abrogates JAK/STAT activation may be a useful approach for treating patients harbouring these mutations (Mullighan *et al*, 2009b; Roberts and Mullighan, 2011).

We found that *CRLF2*oe is associated with *JAK2* mutations in B-ALL in cases lacking recurrent gene fusions, according to previous reports (Mullighan *et al*, 2009a; Yoda *et al*, 2010; Harvey *et al*, 2010). In our study, the frequency of *CRLF2*oe found in children and adults was around 17%. While in children it was similar to that observed by Chen *et al* (2012), in adults was lower (Chiaretti *et al*, 2016) even though maintained its association with poor outcome in this group of patients. Therefore, *CRLF2* quantification could be an important prognostic marker in adult B-ALL. Evidence from *in vitro* studies suggests that these proteins could cooperate to transform B-ALL cells as *CRLF2* acts as a scaffold for *JAK2*mut signalling (Yoda *et al*, 2010), although the precise molecular mechanism remains undescribed. However, cells dependent on *CRLF2* signalling are also sensitive to *JAK2* inhibitors. Thus, ALL patients with *CRLF2*oe may benefit from future kinase inhibitor approaches (Roll and Reuther, 2010; Yoda *et al*, 2010). Future studies should be conducted to further elucidate *JAK2/CRLF2* association and prognosis in B-ALL. In this sense, further assessment of gene rearrangements involving *CRLF2* as *IGH@-CRLF2* and *P2RY8-CRLF2* should be performed to establish the prognostic significance of *CRLF2*oe and *CRLF2* rearrangements across risk subgroups of B-ALL.

## CONCLUSIONS

The amplicon NGS results indicate that the incidence of mutation in *TP53*, *JAK2*, *IL7R*, *PAX5*, *LEF1* and *CRLF2* is higher in adults than in children. All mutations were frequent in B-ALL cases without recurrent fusion genes. Given the negative influence on outcome, we suggest that *TP53* and *JAK2* status should be investigated at diagnosis, particularly in patients negative for recurrent fusion genes, for whom genetic-based prognostic stratification is still limited. Deep sequencing may ultimately guide treatment decisions better for B-ALL patients bearing *TP53* and *JAK2* mutations and could give rise to alternative therapeutic regimens.

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## DECLARATIONS

Ethics approval and consent to participate: The study was approved by the local ethical committee, the *Comité Ético de Investigación Clínica del área de salud de Salamanca*, at the Hospital Universitario de Salamanca. Written informed consent was obtained from each patient or legal guardians before patients entered the study. Availability of data and material: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

MFC wrote the paper and edited the manuscript. MFC, IBM, SR and EFR recorded and analysed the clinical and biological data. MFC, CR, RB, MA, MHS, JMHS, MQA, IBM, MSV and IR designed, performed and analysed the NGS experiments. IBM, MSV and FAS performed the *CRLF2* expression studies. JLF, MArf, NH, JNR, LH, JR, IR, JMSP, MC, MR, CD, EB, JM, JMR and JMHR provided patient samples and the clinical data. AK provided reagents and supported NGS analysis; all authors participated in discussions and critically reviewed the manuscript; JM, EFR, AK and JMHR analysed and interpreted the data, led and supervised the study and corrected and approved the final version of the manuscript.

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