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The clinical impact of *IKZF1* deletions in paediatric B-cell precursor acute lymphoblastic leukaemia is independent of minimal residual disease stratification in NOPHO treatment protocols used between 1992 and 2013

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Running head: *IKZF1* in paediatric BCP ALL

Summary

Paediatric B-cell precursor acute lymphoblastic leukaemias (BCP ALL) with *IKZF1* deletions (ΔIKZF1) are associated with a poor outcome. However, there are conflicting data as to whether ΔIKZF1 is an independent risk factor if MRD and other copy number alterations also are taken into account. We investigated 334 paediatric BCP ALL, diagnosed 1992-2013 and treated according to NOPHO ALL protocols, with known *IKZF1* status based on either SNP array (*N*=218) or MLPA (*N*=116) analyses. ΔIKZF1, found in 15%, was associated with inferior 10-year probabilities of event-free (60% *vs*. 83%; *P*<0.001) and overall survival (pOS; 73% *vs*. 89%; *P*=0.001). Adjusting for known risk factors, including WBC count and MRD, ΔIKZF1 was the strongest independent factor for relapse and death. ΔIKZF1 was present in 27% of cases with non-informative cytogenetics ("BCP-other") and a poor 10-year pOS was particularly pronounced in this group (58% *vs*. 90%; *P*<0.001). Importantly, neither MRD nor WBC count predicted events in the ΔIKZF1-positive cases. Co-occurrence of pseudoautosomal region 1 (PAR1) deletions in Xp22.33/Yp11.32 (*P2RY8-CRLF2*) and ΔIKZF1 increased the risk of relapse (75% *vs*. 30% for cases with only ΔIKZF1; *P*=0.045), indicating that BCP-other ALL with both *P2RY8-CRLF2* and ΔIKZF1 constitutes a particular high risk group.

Keywords: Paediatric B-cell precursor acute lymphoblastic leukaemia; *IKZF1* deletion; *P2RY8-CRLF2*, *ERG* deletion, minimal residual disease; risk-stratifying factors.

Deletions of the *IKZF1* gene (ΔIKZF1), encoding the transcription factor IKAROS (Georgopoulos *et al*, 1992), occur in approximately 15% of paediatric B-cell acute lymphoblastic leukaemia (BCP ALL) (Olsson & Johansson, 2015). In recent years, several studies have shown that ΔIKZF1 is significantly associated with decreased event-free survival (EFS) and overall survival (OS) both in the non-high risk (NHR) and high risk (HR) groups. Of particular interest is the fact that ΔIKZF1 confers a poor prognosis in cases with non-specific cytogenetic aberrations, normal karyotypes, or karyotypic failures, *i.e.*, "BCP-other" ALL (Mullighan *et al*, 2009; Kuiper *et al*, 2010; Waanders *et al*, 2011; Dörge *et al*, 2013; Öfverholm *et al*, 2013; van der Veer *et al*, 2013; Olsson *et al*, 2014). However, the clinical impact of ΔIKZF1 may be modified by other genetic aberrations, such as rearrangements of *CRLF2, e.g.*, pseudoautosomal region 1 (PAR1) deletions in Xp22.33/Yp11.32 resulting in *P2RY8-CRLF2*, and deletions of the *ERG* gene in 21q22.2, as well as by minimal residual disease (MRD) stratification (Chen *et al*, 2012; Palmi *et al*, 2013; Volejnikova *et al*, 2013; Clappier *et al*, 2014; Zaliova *et al*, 2014; Olsson *et al*, 2015). Some studies have included MRD data when addressing the prognostic impact of ΔIKZF1 in paediatric BCP ALL, but the results have been conflicting as to whether ΔIKZF1 is an independent risk factor (Mullighan *et al*, 2009; Waanders *et al*, 2011; Dörge *et al*, 2013; van der Veer *et al*, 2013) or not (Chen *et al*, 2012; Volejnikova *et al*, 2013). Considering that several ongoing treatment protocols use MRD findings for risk stratification (Borowitz *et al*, 2008; Brüggemann *et al*, 2010; Yamaji *et al*, 2010; Vora *et al*, 2013; Frandsen *et al*, 2014), it is crucial to analyse the relationship between ΔIKZF1 and MRD findings before implementing analyses of ΔIKZF1 in clinical routine.

The rationale for the present study, which is based on 334 Swedish paediatric BCP ALL cases with known *IKZF1* status diagnosed between 1992 and 2013 and treated according to the NOPHO ALL-1992, -2000, and -2008 protocols (Schmiegelow *et al*, 2010; Frandsen *et al*,

2014), was two-fold: first, to ascertain if ΔIKZF1 is an independent risk factor also in the context of MRD and second, to investigate further the prognostic impact of ΔIKZF1 in different patient/cytogenetic groups and in relation to co-occurring *ERG* and PAR1 deletions.

Patients and methods

Patient cohort

The *IKZF1* status, *i.e.*, deleted or not deleted, was ascertained in a total of 354 BCP ALL cases diagnosed morphologically, immunophenotypically, and genetically as requested by the NOPHO ALL-1992, -2000, and -2008 protocols (Schmiegelow *et al*, 2010; Frandsen *et al*, 2014). Of these, 13 were infant ALL, none of which harboured ΔIKZF1, and seven were *BCR-ABL1*-positive cases, three of which had ΔIKZF1; these 20 BCP ALLs were excluded from further analysis. The remaining 334 patients were all treated according to the above-mentioned NOPHO protocols. Patients stratified into standard risk/intensity or intermediate risk/intensity groups in the 1992, 2000, and 2008 protocols were in this study grouped together as standard risk (SR; *N*=136) and intermediate risk (IR; *N*=140), respectively. Patients stratified into high- or very high-risk (1992), intensive, very intensive, or extra intensive (2000), or high-risk-chemo or high-risk-SCT (2008) were here combined into HR (*N*=58). The median age was 4.0 years (range 1 – 17 years), the male/female ratio 1.1, and the median white blood cell (WBC) count 9.7 x 10^9/l (range 0.9 – 1161 x 10^9/l). The basic clinical features are given in Table SI. The genetic investigations were approved by the Research Ethics Committees at the participating centres and informed consent was obtained according to the Declaration of Helsinki.

MRD analysis

Response to therapy was assessed by MRD analysis at day 29, as previously described (Schmiegelow *et al*, 2010; Toft *et al*, 2013). Of the 334 BCP ALL cases, MRD data were available for 219 (66%) patients, of whom 61 (28%) were MRD positive (≥0.1%) and 158 (72%) were MRD negative (<0.1%). In accordance with the NOPHO ALL 2008 protocol, MRD measurements by flow cytometry were used in most instances (*N*=212; 97%), but for the seven cases where this was not feasible (no aberrant immunophenotypic markers), MRD analyses were performed by polymerase chain reaction (PCR). In the current 2008 protocol, MRD is used as a risk stratifying factor, directly influencing the therapy given (Toft *et al* 2013).

Cytogenetic analyses

Chromosome banding analyses were performed using standard methods in four cytogenetic laboratories in Sweden. All abnormal karyotypes have been centrally reviewed each year since 1996 by the Swedish Childhood Leukaemia Cytogenetics Group. Fluorescence *in situ* hybridisation (FISH) or reverse-transcription PCR analyses were used to screen for the translocations/gene fusions t(1;19)(q23;p13) [*TCF3-PBX1*], t(9;22)(q34;q11) [*BCR-ABL1*], and t(12;21)(p13;q22) [*ETV6-RUNX1*], whereas FISH or Southern blot analyses were used to identify 11q23/*KMT2A* **(**a.k.a *MLL***)** rearrangements. These targeted analyses have been performed prospectively from 1996, with several additional cases prior to this time having been ascertained retrospectively. In the NOPHO ALL-2008 protocol, screening for intrachromosomal amplification of chromosome 21 (iAMP21) and dic(9;20)(p13;q11) became mandatory (Frandsen *et al*, 2014); however, many cases diagnosed before 2008 have been identified in retrospective analyses. The genetic findings are summarized in Table SI.

SNP and MLPA analyses

Of the 334 cases, 218 (65%) had been analysed by single nucleotide polymorphism (SNP) arrays and 116 (35%) by multiplex ligation-dependent probe amplification (MLPA). The *IKZF1* status of 286 cases has been reported previously (Öfverholm *et al*, 2013; Olsson *et al*, 2014). PAR1 deletions were ascertained by SNP array or MLPA analyses in all cases, whereas the *ERG* status could be determined in 246 cases (apart from the 218 cases analysed by SNP arrays, array-CGH data on *ERG* were available in 28 of the 116 cases analysed by MLPA).

The platforms used for SNP array analyses were HumanOmniExpress BeadChip (Illumina, San Diego, CA, USA), Human 610-Quad BeadChip (Illumina), HumanOmni1-Quad BeadChip (Illumina), Human1M-Duo BeadChip (Illumina), or Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA). The copy number (CN) state was determined using the GenomeStudio 2011.1 (Illumina), Chromosome Analysis Suite (Affymetrix), or the Nexus Copy Number (BioDiscovery, Hawthorne, CA, USA) software. The criterion to "call" a deletion by SNP arrays was a minimum of three consecutive probes deleted. For cases analysed by the Nexus Copy Number software, the confidence threshold was set to >35. Deletions seen in remission samples or that overlapped with copy number polymorphisms listed in the Database of Genomic Variants (http://projects.tcag.ca/variation/) were excluded.

For the MLPA analyses, the probe kits P335 and P202 (MRC-Holland, Amsterdam, The Netherlands) were used. The P335 probe mix contains one probe each for all eight exons of the *IKZF1* gene, seven probes for *PAX5*, six probes for *ETV6*, five probes for *RB1*, four probes for *BTG1* and the *BTG1* downstream region, four probes for *EBF1*, three probes for *CDKN2A*/*B*, and five probes for the PAR1 region (*SHOX*, *CRLF2*, *CSF2RA*, *IL3RA*, and *P2RY8*). Peaks were normalised against controls to generate relative CN states of the exons in the sample. Relative peak heights between 0.75 and 1.35 were considered normal (CN 2),

whereas values below 0.75 and above 1.35 were considered to represent losses (CN 1) and gains (CN 3), respectively. Values below 0.25 indicated biallelic loss (CN 0). Samples with deletions in *IKZF1* were further analysed with the probe kit P202, which contains two probes per exon of this gene. *IKZF1* deletions that affected single probes in the P335 kit were only regarded as positive if validated by the P202 kit.

Statistical analyses

The PASW Statistics 20 software for Windows (SPSS Inc., Chicago, IL, USA) was applied for all analyses. The significance limit for two-sided *P*-values was set to <0.05. The Mann-Whitney U-test for continuous variables and the two-tailed Fisher's exact probability test for discrete variables were used to compare clinical and genetic features between cases with known *IKZF1* status (the present cohort of 334 cases) and those with unknown *IKZF1* status (*N*=799) as well as between *IKZF1* deleted (*N*=50) and non-deleted cases (*N*=284). In the EFS analysis, events comprised induction failure, relapse, death in first complete remission, and secondary malignant neoplasm. For OS, death was the only event and in estimations of cumulative incidence of relapse (Rel), only relapses were counted. The 10-year (yr) probabilities of relapse (pRel), EFS (pEFS), and OS (pOS) in cases with and without *IKZF1* deletions were calculated using the Kaplan-Meier method. Multivariate analysis using a Cox regression model was performed to ascertain if ΔIKZF1 had an independent impact on pRel, pEFS, and/or pOS. The median observation time for patients in complete remission 1 (CR1) was 89 months (range 1 – 200 months).

Results

The investigated cohort is representative of paediatric BCP ALL in general

A total of 1133 Swedish children/adolescents (1-17 years) were diagnosed with *BCR-ABL1*-negative BCP ALL and treated in accordance with the NOPHO protocols between 1992 and 2013. Comparing the 334 (29%) cases analysed for ΔIKZF1 presented herein with the 799 (71%) cases with unknown *IKZF1* status revealed no significant differences with regard to age, gender, proportion of patients with Down syndrome, risk stratification, number of patients undergoing allogeneic stem cell transplantation (allo SCT), 10-yr pRel, 10-yr pEFS, 10-yr pOS, and MRD findings at day 29 (Table SII). However, the median WBC count was higher in the investigated cohort: 9.7 x 10^9/l (range 0.9 – 1161 x 10^9/l) *vs.* 7.4 x 10^9/l (range 0.3 – 381 x 10^9/l; $P = 0.001$).

Frequencies and types of ΔIKZF1

Fifty (15%) of the 334 cases harboured ΔIKZF1, comprising 29 focal deletions (only affecting the *IKZF1* gene and ranging from single exons to the whole gene), 19 larger deletions, and 2 cases with monosomy 7 (Table SI); the latter two types of loss are combined below as "non-focal" deletions. All except one of the 50 deletions were hemizygous; one case had a homozygous deletion of exon 1.

Of the 50 *IKZF1* deletions, 31 (62%) were detected with SNP array and 19 (38%) with MLPA analyses. The clinical features and deletion types did not differ significantly between the cases identified by SNP arrays and MLPA (Table SIII). Furthermore, the 29 cases with focal and the 21 with non-focal ΔIKZF1 did not display any significant differences as regards age, gender, MRD, cytogenetic subgroups, proportion of patients with Down syndrome, risk stratification, number of patients undergoing allo SCT, or outcome (Table SIV). The cases with focal ΔIKZF1 had a somewhat higher median WBC count (29 x 10^9/l, range 1.6 – 496 x10^9/l) compared with those with non-focal ΔIKZF1 (14 x 10^9/l, range 1.7 – 191 x 10^9/l); however, this did not reach statistical significance (P=0.06). The positions of the intragenic

deletions could be ascertained in 26 (90%) of the 29 cases with focal ΔIKZF1, revealing that deletions of exons 4-7 (Δ4-7) were the most common (*N*=7; 27%). The ΔIKZF1-positive cases with or without Δ4-7 did not appear to differ with regard to age, gender, WBC count, and frequency of events (data not shown).

ΔIKZF1 is overrepresented in the BCP-other and underrepresented in the t(12;21) groups

The frequencies of ΔIKZF1 among the cytogenetic groups were, in increasing order, 0% of cases with >67 chromosomes (0/2), 6% of t(12;21) (5/83), 7% of t(1;19) (1/14), 10% of high hyperdiploidy (HeH; 51-67 chromosomes; 12/116), 14% of iAMP21 (1/7), 25% of 11q23/*KMT2A* (1/4), 27% of dic(9;20) (3/11), 27% of BCP-other (26/95), and 50% of hypodiploidy (<45 chromosomes; 1/2).

The distributions of the cytogenetic groups hypodiploidy, HeH, dic(9;20), t(1;19), 11q23/*KMT2A*, iAMP21, and >67 chromosomes did not differ between cases with or without ΔIKZF1. In contrast, t(12;21) was significantly less common in the ΔIKZF1-positive group (*P*=0.007), whereas BCP-other was overrepresented (*P*<0.001) (Table SV).

ΔIKZF, high risk features, and outcome

The 50 ΔIKZF1-positive cases had a significantly higher median WBC count (26 x 10^9/l, range of 1.6 – 492 x 10^9/l) than the 284 cases without ΔIKZF1 (8.8 x 10^9/l, range of 0.9 – 1164 x 10^9/l; *P*=0.009). In addition, those with ΔIKZF1 were more frequently stratified as HR (36% *vs.* 14%; *P*<0.001) and were more often treated with allo SCT (8% *vs.* 1.4%; *P*=0.02)**.** The presence of ΔIKZF1 was significantly associated with decreased 10-yr pEFS (60% *vs.* 83%; *P*<0.001) and pOS (73% *vs*. 89%; *P*=0.001) and increased 10-yr pRel (35% *vs.* 12%; *P*<0.001) (Fig 1A-C).

On the other hand, the 14 (28%) patients with ΔIKZF1 who relapsed did not differ with regard to age (*P*=0.134), WBC counts (*P*=0.948), risk group distribution (*P*=0.325), or the frequency of BCP-other (*P*=0.119) compared with the 36 cases (72%) that did not relapse. Among the latter, 32 remain in CR1, one died in CR1, and three died during induction therapy. The presence of ΔIKZF1 was not associated with increased risk of death in first complete remission (1 of 8 cases harboured a ΔIKZF1), secondary malignant neoplasm (none of 4 cases), or with central nervous system involvement (1 of 6 cases).

ΔIKZF1 and pRel in relation to risk stratification, treatment protocols, and cytogenetics

ΔIKZF1 conferred a significantly increased 10-yr pRel in the SR (36% *vs*. 8%; *P*=0.003) and HR (49% *vs*. 17%; *P*=0.012) groups (Fig 1D and E) but not in the IR group (22% *vs*. 12%; *P*=0.237) (Fig 1F). *IKZF1* deletions were significantly associated with increased 10-yr pRel in the NOPHO ALL-1992 (60% *vs*. 10%; *P*<0.001) but not in the -2000 (27% *vs*. 15%; *P*=0.095) and -2008 (18% *vs*. 4%; *P*=0.073) protocols (Fig 1G-I). In contrast, ΔIKZF1 did not significantly alter the pRel for cases with HeH (Fig S1A), t(12;21) (Fig S1B), t(1;19), dic(9;20), and iAMP21 (combined because all three changes are stratified as IR and individually rare; Fig S1C), or *KMT2A* rearrangements and hypodiploidy (combined because they are both stratified as HR and too infrequent to be investigated separately; Fig S1D).

ΔIKZF1 and pRel/pOS of BCP-other ALL

BCP-other with ΔIKZF1 had decreased pOS (58% *vs*. 90; *P*<0.001) and increased pRel (52% *vs*. 4%; *P*<0.001) (Fig 2A and B). The pRel was significantly higher for ΔIKZF1-positive BCP-other than for ΔIKZF1-negative BCP-other in all three treatment protocols [ALL-1992: 83% *vs*. 6%; *P*<0.001, ALL-2000: 33% *vs*. 4%; *P*=0.010, and ALL-2008: 37% *vs*. 0%; *P*=0.009].

ΔIKZF1 and MRD

MRD data were available in 33 (66%) of the 50 ΔIKZF1-positive cases, of which 14 (42%) were MRD-positive and 19 (58%) were MRD-negative at day 29. The IKZF1-positive cases more often had MRD levels ≥0.1% than those without ΔIKZF1 (42% *vs*. 25%); however, this this was not statistically significant (*P*=0.06). MRD-positivity did not predict relapse (*P*=1.000) in the group with ΔIKZF1; in contrast, the presence of ΔIKZF1 conferred a significantly increased 10-yr pRel in both MRD-positive (33% *vs*. 12%; *P*=0.029) and MRD-negative (27% *vs*. 7%; *P*=0.018) cases (Fig 3A and B).

Frequencies and prognostic impact of PAR1 and ERG deletions

PAR1 and *ERG* deletions were found in 12 (3.6%) and eight (3.3%) of the informative cases, respectively. Of the 12 patients with PAR1 deletions, four relapsed and one died, whereas of the eight *ERG*-deleted cases one relapsed and all remain alive.

The 10-yr pRel for cases with both *P2RY8-CRLF2* and ΔIKZF1 and for those with only ΔIKZF1 were 75% and 30%, respectively (*P*=0.045), whereas the presence of *ERG* deletions in ΔIKZF1-positive cases did not affect the pRel (23% *vs*. 26%; *P*=0.832). However, it should be emphasised that only four cases were double positive for *P2RY8-CRLF2* and ΔIKZF1 and only three were double positive for *ERG* and *IKZF1* deletions.

ΔIKZF1 is an independent risk factor

Multivariate Cox regression analyses revealed that ΔIKZF1 was the strongest independent risk factor for relapse when age, WBC count, treatment protocol, MRD, and cytogenetic groups were included as variables in the model (*P*=0.002). The risk groups SR, IR, and HR were not used in the primary analysis because they are partly based on factors already

included. When the component factors were replaced by risk groups in the analysis, ΔIKZF1 was still the strongest independent risk factor for relapse (P<0.001). ΔIKZF1 was also an independent risk factor for decreased EFS and OS when these were used as end-points in the model (P=0.002 and P=0.019, respectively) and for relapse (P=0.033), EFS (P=0.031), and OS (P=0.027) when only patients with available MRD data were included in the analyses (Table I). When BCP-other was analysed separately, ΔIKZF1 remained the strongest risk factor (P <0.001). The same was also true for models including PAR1 deletion as a variable. In these models, ΔIKZF1 remained the strongest risk factor (P<0.001) but PAR1 deletion was also independently associated with an increased pRel (P=0.014).

Discussion

In 2008, *IKZF1* deletions were for the first time associated with relapse of paediatric *BCR-ABL1*-negative BCP ALL (Yang *et al*, 2008). Since then, several studies have confirmed such an association, also in *BCR-ABL1*-positive cases (Martinelli *et al*, 2009; Mullighan *et al*, 2009; Kuiper *et al*, 2010; Waanders *et al*, 2011; Asai *et al*, 2013; Dörge *et al*, 2013; Dupuis et al, 2013; Öfverholm *et al*, 2013; Schwab *et al*, 2013; van der Veer *et al*, 2013, 2014; Yamashita *et al*, 2013; Olsson *et al*, 2014). More recently, however, the clinical relevance of ΔIKZF1 has been questioned, either as a risk-stratifying change as such or as an independent prognostic marker when MRD data are taken into account (Chen *et al*, 2012; Palmi *et al*, 2013; Qazi *et al*, 2013; Uckun *et al*, 2013; Volejnikova *et al*, 2013). In order to address this issue further, we ascertained all 334 Swedish paediatric BCP ALL cases, diagnosed and treated according to NOPHO ALL protocols, with data on ΔIKZF1. The investigated cohort did not differ from the 799 cases with unknown *IKZF1* status diagnosed during the same time period with regard to age, sex ratio, MRD, proportion of patients with Down syndrome, risk group distribution, treatment with allo SCT, and outcome; the analysed cases, however, had

somewhat higher WBC counts (Table SII). Despite the latter, we deem the present series to be representative of childhood BCP ALL in general. Support for this conclusion is the fact that 15% of the cases harboured ΔIKZF1, a frequency in accord with previous studies (Kuiper *et al*, 2010; Waanders *et al*, 2011; Chen *et al*, 2012; Caye *et al*, 2013; Dörge *et al*, 2013; Öfverholm *et al*, 2013; Schwab *et al*, 2013; van der Veer *et al*, 2013; Yamashita *et al*, 2013; Olsson *et al*, 2014).

Because most ΔIKZF1 are submicroscopic, targeted analyses are needed to identify them. Most often, SNP array or MLPA analyses are used, but more recently multiplex PCR-based methods have been developed (Mullighan *et al*, 2007, 2009; Yang *et al*, 2008; Kuiper *et al*, 2010; Schwab *et al*, 2010, 2013; Caye *et al*, 2013; Dörge *et al*, 2013; Dupuis et al, 2013; Meyer *et al*, 2013; Öfverholm *et al*, 2013; van der Veer *et al*, 2013, 2014; Olsson *et al*, 2014). All these three methods have their limitations. For example, deletions may well escape detection by SNP arrays and MLPA if they are present in smaller (<10% and <50%, respectively) subclones and large, *i.e.*, non-focal, deletions may be missed by multiplex PCR (Kuiper *et al*, 2007; Schwab *et al*, 2010; Li *et al*, 2011; Caye *et al*, 2013; Dupuis *et al*, 2013). Consequently, the frequencies of ΔIKZF1 may vary depending on the technique used, something that could also influence the perceived prognostic impact of ΔIKZF1. In the present study, there was no significant difference in the frequencies of ΔIKZF1 observed by SNP array (14%) and MLPA (16%) analyses, nor did the clinical features and deletion types differ between SNP- and MLPA-identified cases (Table SIII). Of the ΔIKZF1, 58% were focal and 42% were non-focal deletions. There were no significant differences in sex ratio, WBC counts, MRD, and risk group stratification between these two deletion types, and in contrast to a previous study (Dörge *et al*, 2013) that reported an association between high age (>10 years) and focal deletions, we did not find such a correlation. Additionally, and in agreement with prior findings (Dörge *et al*, 2013; Dupuis *et al*, 2013), the prognostic impact

of the deletion types did not differ significantly (Table SIV). Thus, all ΔIKZF1 could be combined, irrespective of detection method and extent of the deletions, in all subsequent analyses in the present study.

ΔIKZF1 influenced the prognosis negatively in the entire patient cohort, being significantly associated with decreased pEFS and pOS and increased pRel (Fig 1A-C). However, the impact varied among the different NOPHO protocols and risk groups. The pRel was significantly higher for ΔIKZF1-positive cases in the 1992 protocol (Fig. 1G) but not in the 2000 and 2008 protocols (Fig. 1H and I). This may well be due to more efficient therapy in these latter protocols. As regards risk groups, ΔIKZF1 was associated with higher relapse rates in the SR and HR groups, but not in the IR group (Fig 1D-F). The reasons for this are presently unclear, but probably reflect both differences in treatment intensity and biological differences among the groups.

IKZF1 deletions were seen in all cytogenetic subgroups except the one with >67 chromosomes, with most subtypes being equally distributed among the ΔIKZF1-positive and -negative cases (Table SV). However, BCP-other was clearly overrepresented in cases with *IKZF1* deletions and there was a negative association between t(12;21) and ΔIKZF1; only 6% of the cases with t(12;21) had *IKZF1* deletions. A similarly low frequency (3-5%) of ΔIKZF1 in BCP ALL with t(12;21) has previously been described (Dörge *et al*, 2013; Enshaei *et al*, 2013). It has been reported that ΔIKZF1 is present in approximately 20% of HeH cases and that it confers an unfavourable outcome in this subgroup (van der Veer *et al* 2013). This could, however, not be confirmed herein. We found ΔIKZF1 in 10% of the HeH cases and there was no significant difference in 10-yr pRel between *IKZF1*-deleted and non-deleted HeH (Fig S1A). In fact, there was no evidence for a prognostic impact of ΔIKZF1 in any of the cytogenetic subtypes (Fig S1), except for BCP-other (Fig 2A and B), in line with prior

studies (Mullighan *et al*, 2009; Dörge *et al*, 2013; Öfverholm *et al*, 2013; Schwab *et al*, 2013; Olsson *et al*, 2014).

BCP-other comprises approximately 25% of all BCP ALL cases and constitutes a substantial proportion of cases that subsequently relapse (Pui *et al*, 2011). In the present study, 95 cases, representing 28% of the entire patient cohort, were grouped as BCP-other. Of these, ΔIKZF1 was seen in 27%, comprising 52% of all ΔIKZF1-positive cases (Table SV). The presence of ΔIKZF1 was strongly associated with increased risk of relapse and decreased OS; only two of the 12 cases that relapsed in the BCP-other group did not have an *IKZF1* deletion (Fig 2). Further support for the impact of ΔIKZF1 in BCP-other is the fact that although the 10-yr pRel for ΔIKZF1-positive cases in general was only significantly increased in the NOPHO ALL-1992 protocol (Fig 1G-I), pRel for BCP-other was increased also in the 2000 and 2008 protocols. In addition, multivariate analysis showed that ΔIKZF1 was the strongest independent risk factor in this group. Hence, it is particularly important to identify ΔIKZF1 in BCP-other in order to improve risk stratification.

The presence of ΔIKZF1 was significantly associated with high WBC counts and ΔIKZF1-positive cases were more often grouped as HR, in agreement with previous studies (Den Boer *et al*, 2009; Martinelli *et al*, 2009; Mullighan *et al*, 2009; Dörge *et al*, 2013; Schwab *et al*, 2013; Yamashita *et al*, 2013, van der Veer *et al*, 2014). Despite this association between ΔIKZF1 and high risk factors, it could not explain the increased risk of relapse in the ΔIKZF1-positive group, nor could the MRD findings. In fact, more than one third of all relapses occurred in the ΔIKZF1-positive group, with the majority of these (71%) being associated with BCP-other, again strongly suggesting that BCP-other with *IKZF1* deletions comprises a specific group characterized by high relapse risk and poor outcome.

Today, MRD is considered to be the most powerful indicator for the outcome of paediatric ALL (Brisco *et al*, 1993; Cavé *et al*, 1998; van Dongen *et al*, 1998; Coustan-Smith

et al, 2002; Borowitz *et al*, 2008; Björklund *et al*, 2009). Hence, a major aim of the present study was to ascertain the impact of ΔIKZF1 in the context of MRD, an issue that has been debated (Mullighan *et al*, 2009; Waanders *et al*, 2011; Chen *et al*, 2012; Dörge *et al*, 2013; van der Veer *et al*, 2013; Volejnikova *et al*, 2013). It may be noteworthy that MRD did not seem to be a strong prognostic factor in univariate analysis of our cohort, only being borderline significant (*P*=0.084; Table I). However, it should be stressed that the majority of the cases (53%) with known MRD status were treated by the current NOPHO-2008 protocol in which MRD is risk stratifying and hence acted upon. Nonetheless, ΔIKZF1 predicted high risk of relapse in MRD-positive as well as in MRD-negative cases (Fig 3) and in multivariate analyses, with age, WBC count, MRD, treatment protocol, cytogenetic subgroups, and *IKZF1* status as variables, ΔIKZF1 was the strongest independent risk factor for relapse (Table I), as well as for inferior EFS and OS.

In some studies, the poor outcome associated with ΔIKZF1 has been suggested to be caused by an underlying genomic instability rather than by the ΔIKZF1 as such; however, this suggestion is based on MLPA data on only a limited set of genes (Palmi et al, 2013; Qazi & Uckun, 2013). In fact, in a recent SNP array analysis of 191 BCP ALL cases no significant difference in the frequencies of CN alterations between ΔIKZF1-positive and ΔIKZF1-negative cases was observed (Olsson *et al*, 2015). Instead, the presence of certain specific cooperative genetic changes seem to modify the prognostic impact of ΔIKZF1, such as PAR1 and ERG deletions.

PAR1 and *ERG* deletions were found in 3.6% and 3.3% of the cases, respectively; frequencies on a par with previous studies (Harvey *et al*, 2010; Palmi *et al*, 2012; Yamashita *et al*, 2013; Clappier *et al*, 2014; Zaliova *et al*, 2014). The presence of *ERG* deletions has been associated with a favourable outcome, also in the context of ΔIKZF1 (Clappier *et al*, 2014; Zaliova *et al*, 2014). We did not detect a significant impact of *ERG* deletions on the pRel of

ΔIKZF1-positive cases; however, it should be stressed that this analysis was based on only a few patients. Interestingly, all eight patients with an *ERG* deletion are alive. In contrast, PAR1 deletions have been associated with a poor outcome (Harvey *et al*, 2010; Chen *et al*, 2012; Palmi *et al*, 2012), particularly in case double positive cases for PAR1 deletion and ΔIKZF1 (Harvey *et al*, 2010; Moorman *et al*, 2014; Olsson *et al*, 2015). The present finding of an increased pRel for double positive cases hence agrees well with previously published data.

In conclusion, analyses of *IKZF1* and PAR1 provide essential information pertaining to risk stratification in NOPHO treatment protocols, with available data indicating that it is particularly important to screen for ΔIKZF1 in cases presently grouped as BCP-other.

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Author contributions

LO and IIÖ performed experiments, analysed the data, and wrote the manuscript, UNN and MH provided clinical data and performed statistical analyses, VZ analysed data, JN performed experiments, HS and IG provided patient samples and data on *IKZF1* status, KP and AN analysed data, and GB and BJ designed the study, analysed the data, and wrote the manuscript. All the authors approved the final version of the manuscript.

Conflict-of-interest disclosure

The authors declare no conflict of interest.

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Figure legends

Fig 1. Kaplan-Meyer estimates of pEFS, pOS, and pRel of BCP ALL cases with and without *IKZF1* deletions in the total patient cohort (A-C) and of pRel in relation to risk groups (D-F) and treatment protocols (G-I). In each plot, the curve representing cases with ΔIKZF1 is denoted in blue and the one representing non-deleted cases in green. In the entire cohort, the 10-yr pEFS (A) was 0.60 (standard error 0.08) for ΔIKZF1-positive and 0.83 (0.03) for ΔIKZF1-negative cases, the 10-yr pOS (B) 0.73 (0.07) and 0.89 (0.03), and the 10-yr pRel (C) 0.35 (0.08) and 0.12 (0.02). In relation to risk group, the 10-yr pRel for SR (D) was 0.36 (0.15) for ΔIKZF1-positive and 0.08 (0.04) or ΔIKZF1-negative cases, for HR (E) 0.49 (0.14) and 0.17 (0.06), and for IR (F) 0.22 (0.11) and 0.12 (0.03). In relation to treatment protocol, the 10-yr pRel for NOPHO ALL-1992 (G) was 0.60 (0.16) for ΔIKZF1-positive and 0.10 (0.04) for ΔIKZF1-negative cases, for NOPHO ALL-2000 (H) 0.27 (0.10) and 0.15 (0.04), and for NOPHO ALL-2008 (I) 0.18 (0.12) and 0.04 (0.02).

Fig 2. Kaplan-Meyer estimates of pOS and pRel in the BCP-other group. In each plot, the curve representing cases with ΔIKZF1 is denoted in blue and the one representing non-deleted cases in green. (A) The 10-yr pOS was 0.58 (0.10) for ΔIKZF1-positive and 0.90 (0.06) for ΔIKZF1-negative cases. (B) The 10-yr pRel was 0.52 (0.12) for ΔIKZF1-positive and 0.04 (0.03) for ΔIKZF1-negative cases.

Fig 3. Kaplan-Meyer estimates of pRel in the 132 MRD-positive and 87 MRD-negative BCP ALL cases. In each plot, the curve representing cases with ΔIKZF1 is denoted in blue and the one representing non-deleted cases in green. (A) In the MRD-positive group, the 10-yr pRel was 0.33 (0.12) for ΔIKZF1-positive and 0.12 (0.04) for ΔIKZF1-negative cases. (B) In the

MRD-negative group, the 10-yr pRel was 0.27 (0.17) for ΔIKZF1-positive and 0.07 (0.03) for ΔIKZF1-negative cases.

Supporting information

Additional supporting information is found in the online version of this article:

Table SI. Clinical and genetic features of the 334 BCP ALL cases with known *IKZF1* status.

Table SII. Comparisons between BCP ALLs with and without known *IKZF1* status.

Table SIII. Comparisons between ΔIKZF1-positive cases detected with SNP array and MLPA analyses.

Table SIV. Comparisons between focal and non-focal ΔIKZF1-positive cases.

Table SV. Distribution of cytogenetic subgroups in the *IKZF1* deleted and non-deleted cases.

Fig S1. Kaplan-Meyer estimates of pRel of BCP ALL cases positive and negative for *IKZF1* deletions in relation to cytogenetic features.

Table I. Multivariate Cox regression analyses of pRel.[*]

Risk factor	*N*	Univariate RR (95% CI)	Multivariate RR (95% CI)	*P*-value[†] univ/multiv
Age (years)				
10-17	37	2.13 (0.83-5.49)	1.79 (0.58-5.47)	0.118/0.310
1-9	181	1.0		
WBC (x 10^9/l)				
≥50	38	2.92 (1.18-7.26)	2.76 (1.026-7.44)	**0.021/0.044**
<50	180	1.0		
MRD				
≥0.1%	60	2.14 (0.90-5.09)	1.52 (0.60-3.85)	0.084/0.383
<0.1%	158	1.0		
Cytogenetic group[±]				
Group 1	133	1.0		
Group 2	19	1.18 (0.26-5.32)	0.50 (0.10-2-60)	0.830/0.407
Group 3	6	8.92 (1.90-41.83)	2.70 (0.43-16.94)	**0.006**/0.289
Group 4	60	1.13 (0.42-3.05)	0.62 (0.20-1-93)	0.812/0.408
Treatment protocol				
ALL-2000	103	1.0		
ALL-2008	115	0.48 (0.17-1.35)	0.47 (0.16-1.38)	0.165/0.171
IKZF1 deletion				
Yes	32	3.85 (1.60-9.29)	3.06 (1.09-8.54)	**0.003/0.033**
No	186	1.0		

CI, confidence interval; MRD, minimal residual disease; N, number; pRel, cumulative incidence of relapse; RR, relative risk; univ/multiv, univariate/multivariate; WBC, white blood cell count.

[*]Based on 218 patients with available MRD data (one MRD-positive case was censored), including 21 that relapsed and 32 that had *IKZF1* deletions.

[†]Significant *P*-values are indicated in bold type.

[±]Group 1: HeH, t(12;21), and >67 chromosomes; group 2: t(1;19), dic(9;20), and iAMP21; group 3: hypodiploidy and *KMT2A* rearrangements; and group 4: BCP-other.

Fig 1A

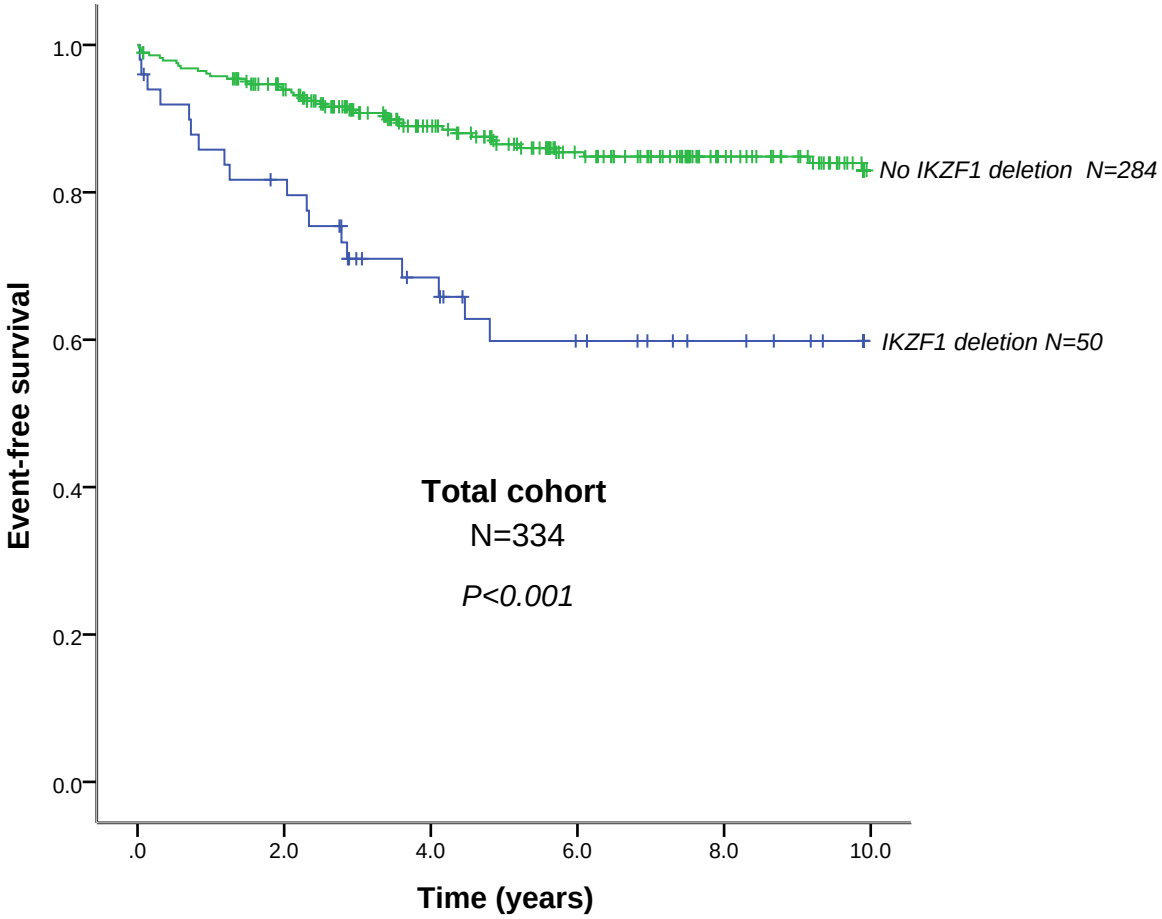

Event-free survival
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)
No IKZF1 deletion N=284
IKZF1 deletion N=50
Total cohort
N=334
P<0.001

Fig 1B

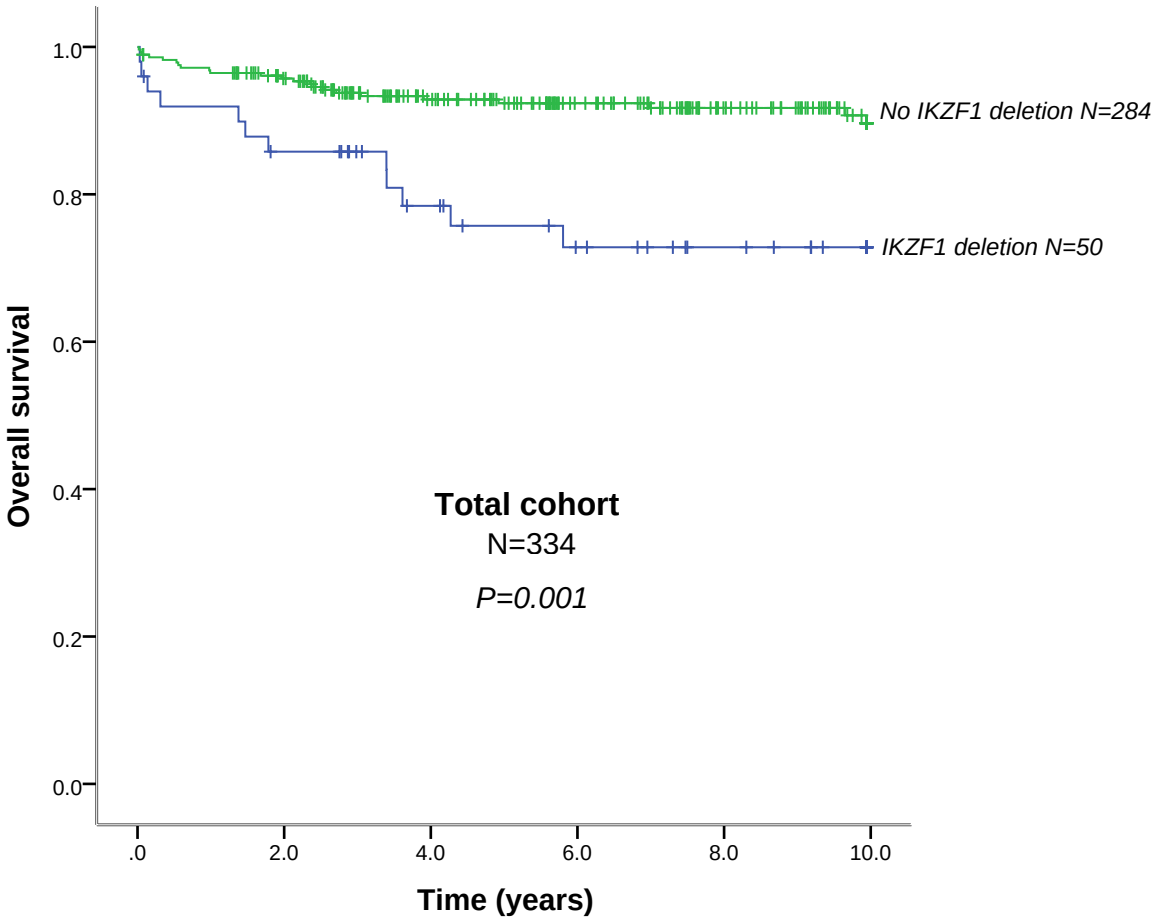

1.0
0.8
0.6
0.4
0.2
0.0
Overall survival
No IKZF1 deletion N=284
IKZF1 deletion N=50
Total cohort
N=334
P=0.001
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 1C

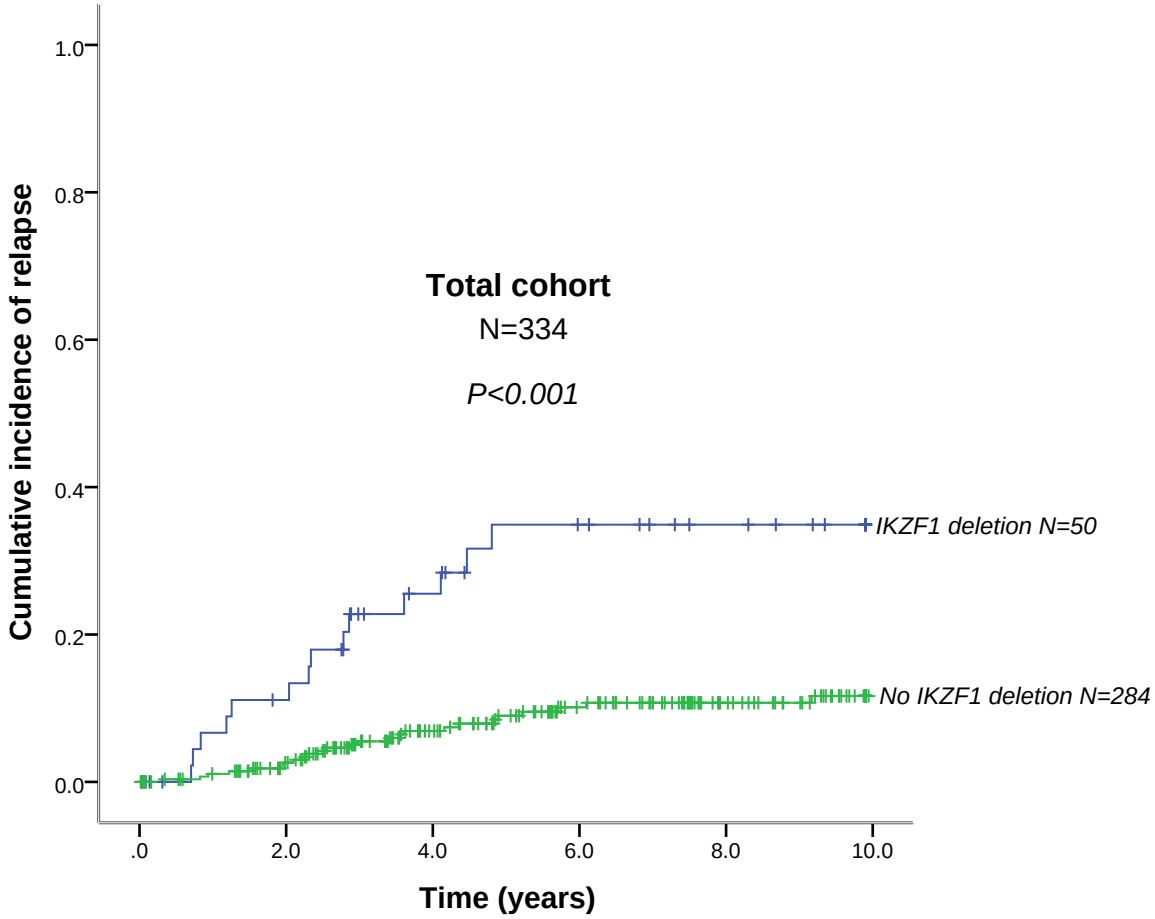

Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
Total cohort
N=334
P<0.001
IKZF1 deletion N=50
No IKZF1 deletion N=284
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 1D

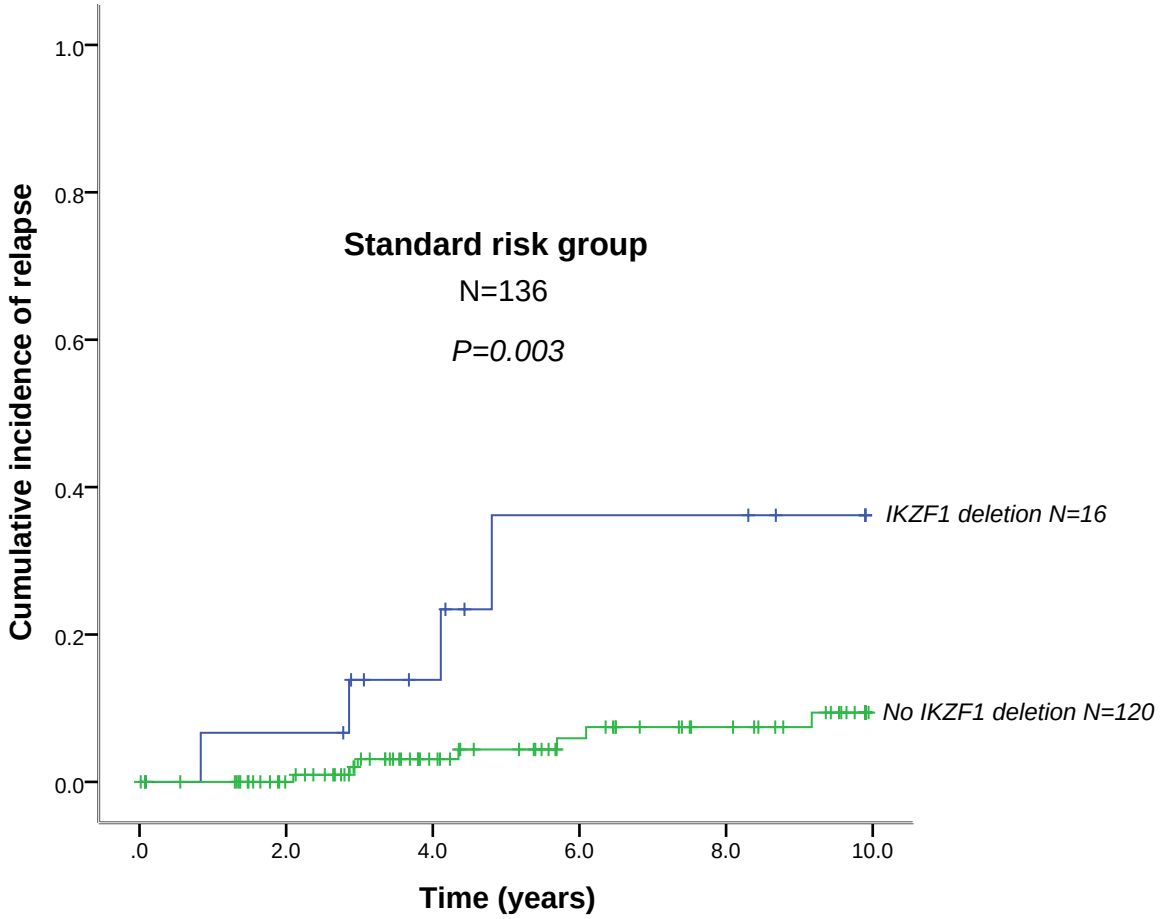

Standard risk group
N=136
P=0.003
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)
IKZF1 deletion N=16
No IKZF1 deletion N=120

Fig 1E

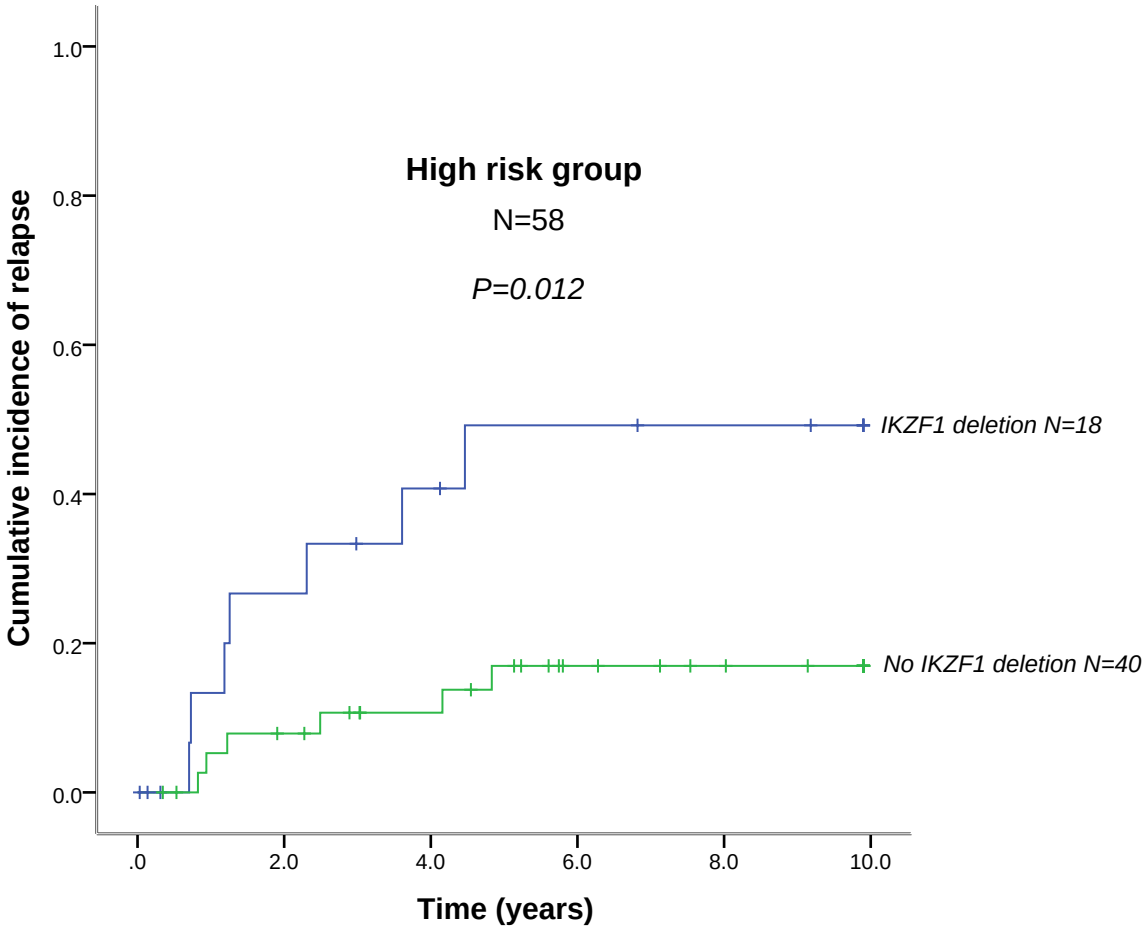

High risk group
N=58
P=0.012
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)
IKZF1 deletion N=18
No IKZF1 deletion N=40

Fig 1F

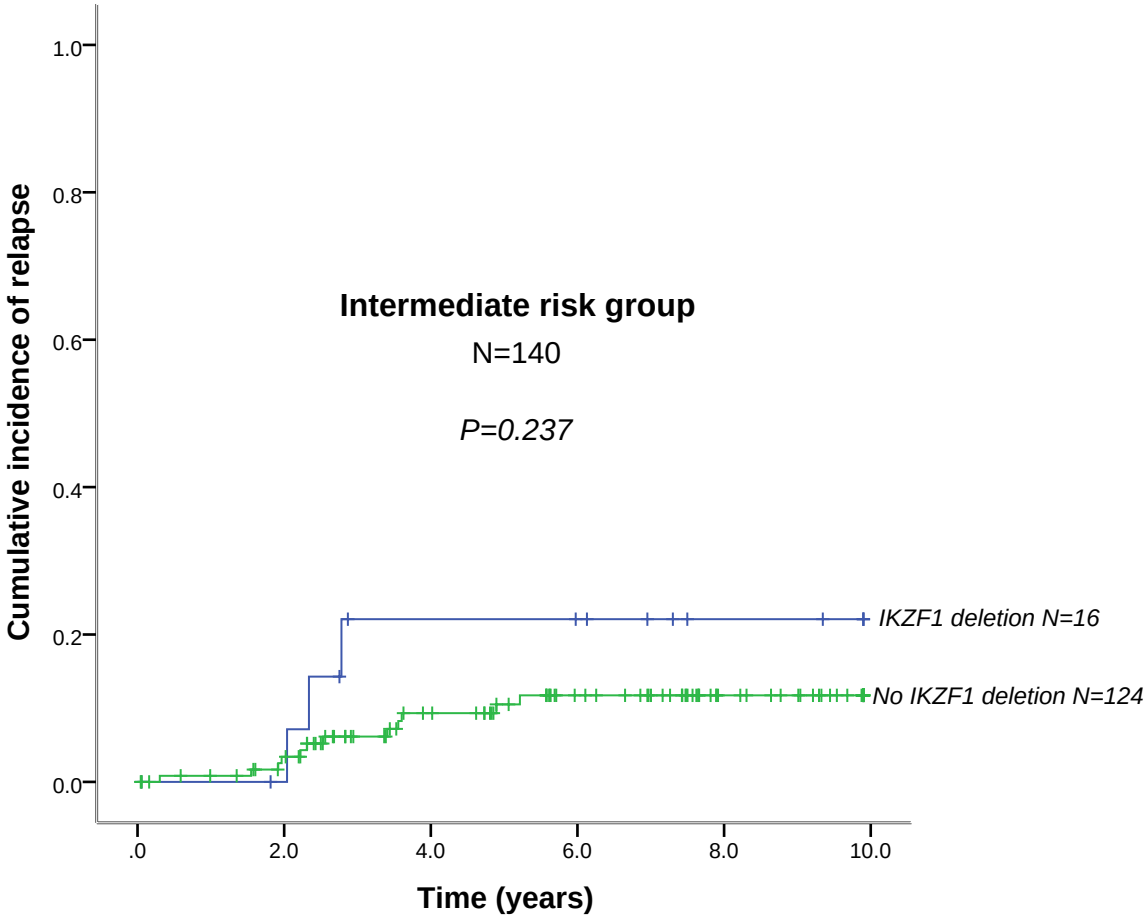

Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
Intermediate risk group
N=140
P=0.237
IKZF1 deletion N=16
No IKZF1 deletion N=124
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 1G

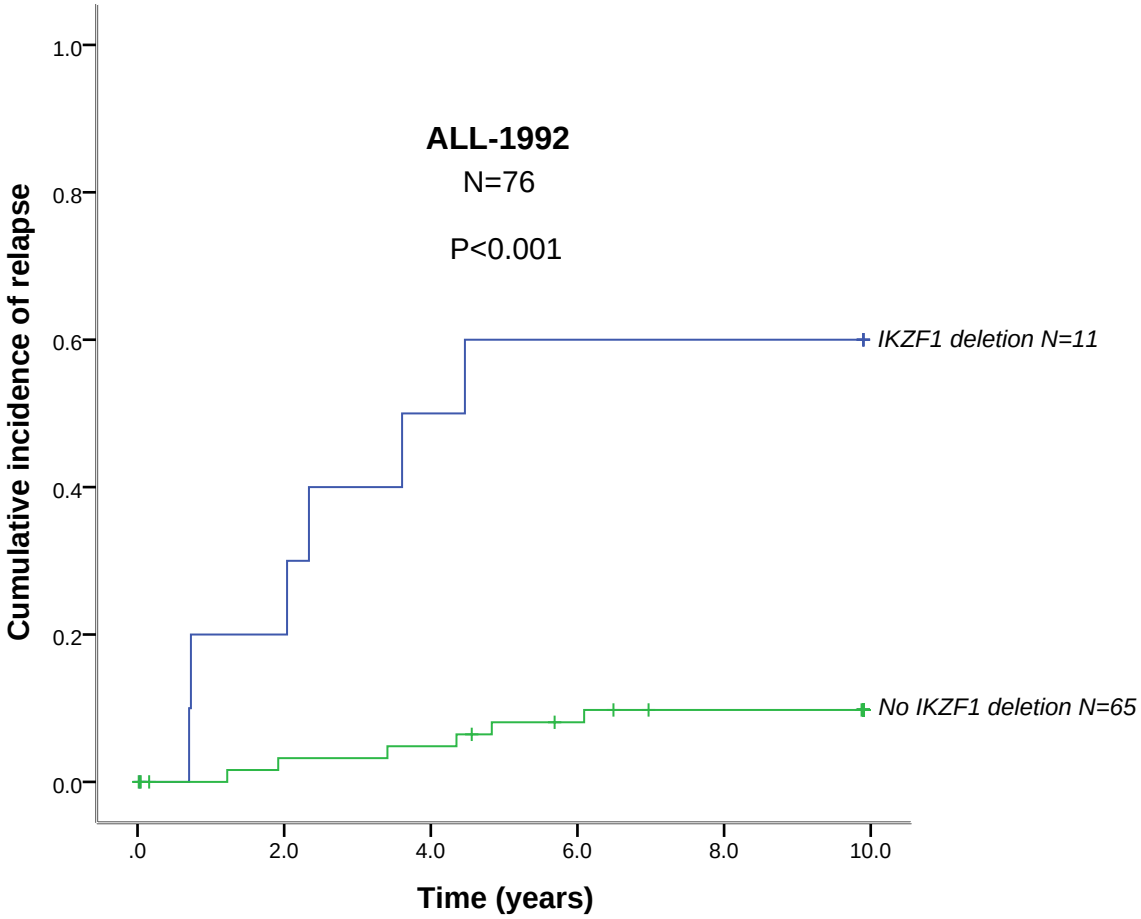

ALL-1992
N=76
P<0.001
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
IKZF1 deletion N=11
No IKZF1 deletion N=65
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 1H

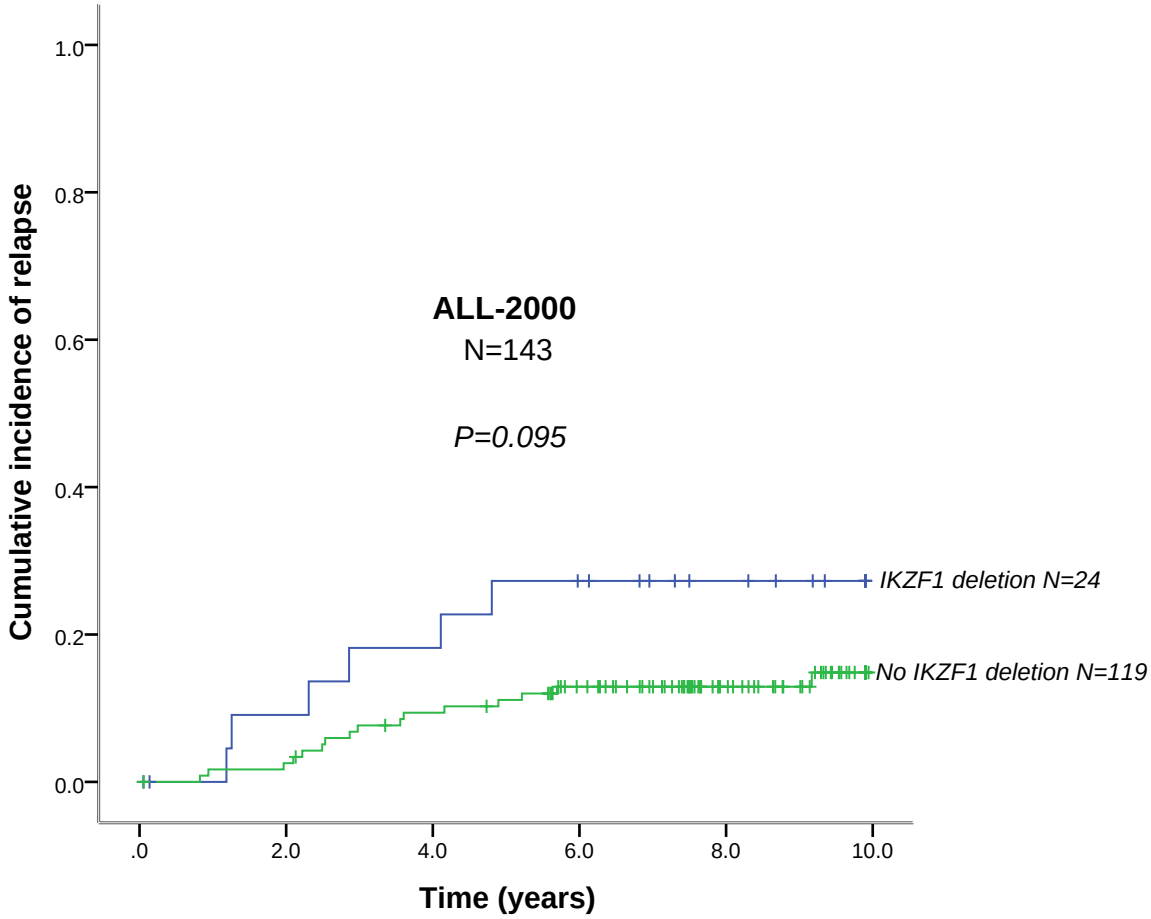

Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
ALL-2000
N=143
P=0.095
IKZF1 deletion N=24
No IKZF1 deletion N=119
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 1I

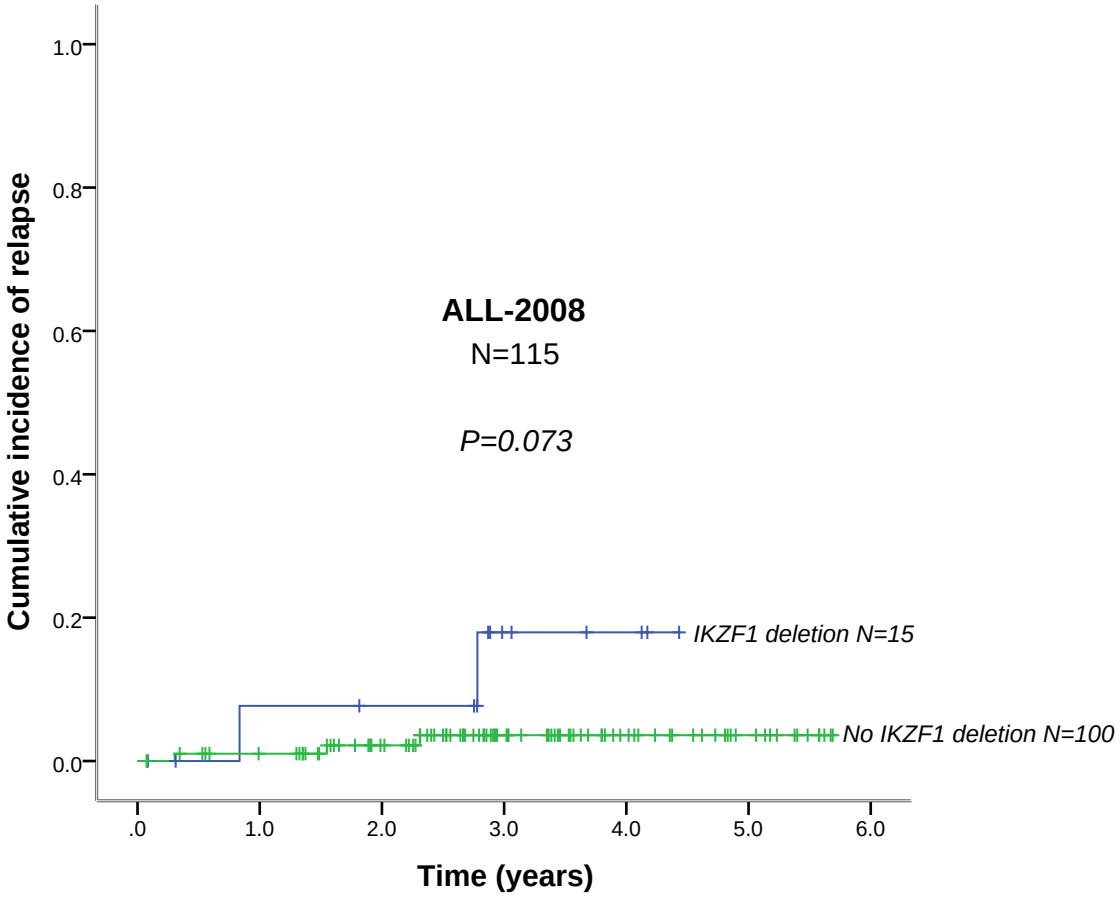

Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
ALL-2008
N=115
P=0.073
IKZF1 deletion N=15
No IKZF1 deletion N=100
.0
1.0
2.0
3.0
4.0
5.0
6.0
Time (years)

Fig 2A

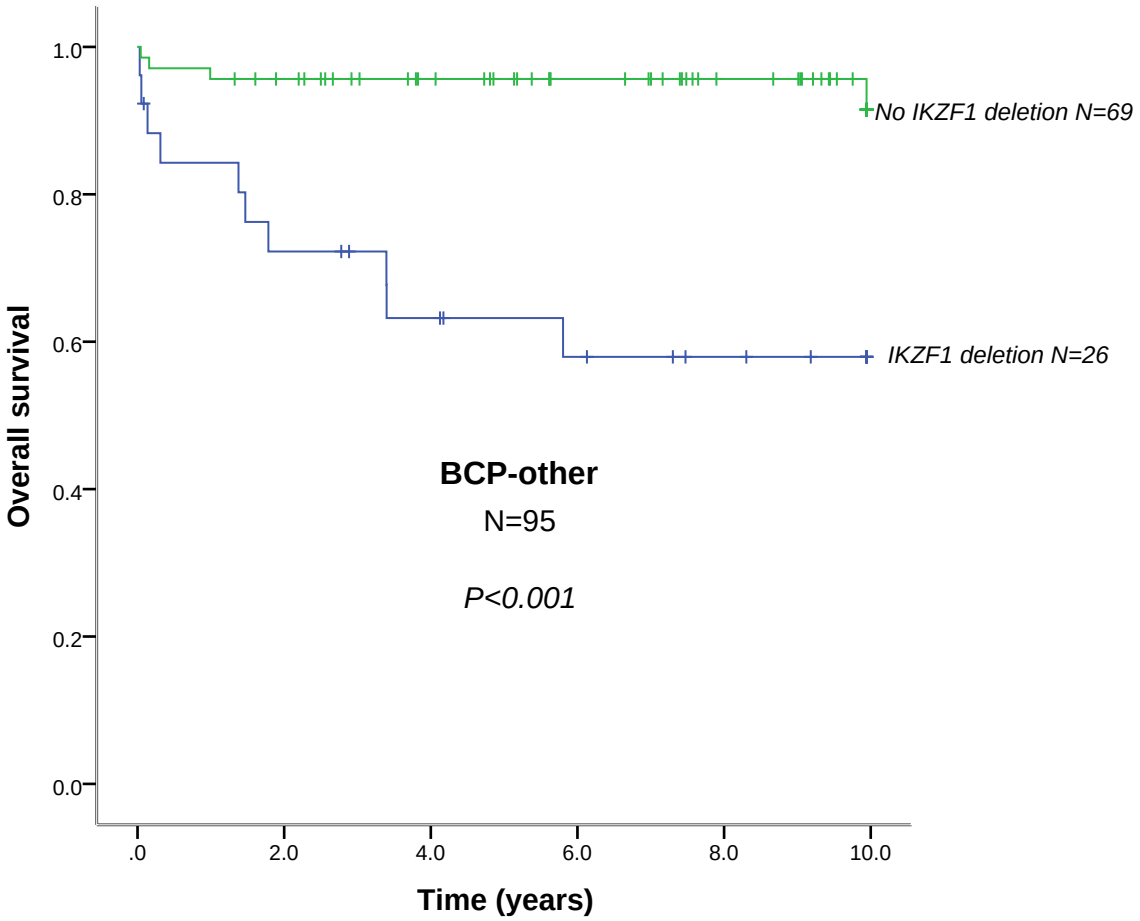

1.0
0.8
0.6
0.4
0.2
0.0
Overall survival
No IKZF1 deletion N=69
IKZF1 deletion N=26
BCP-other
N=95
P<0.001
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 2B

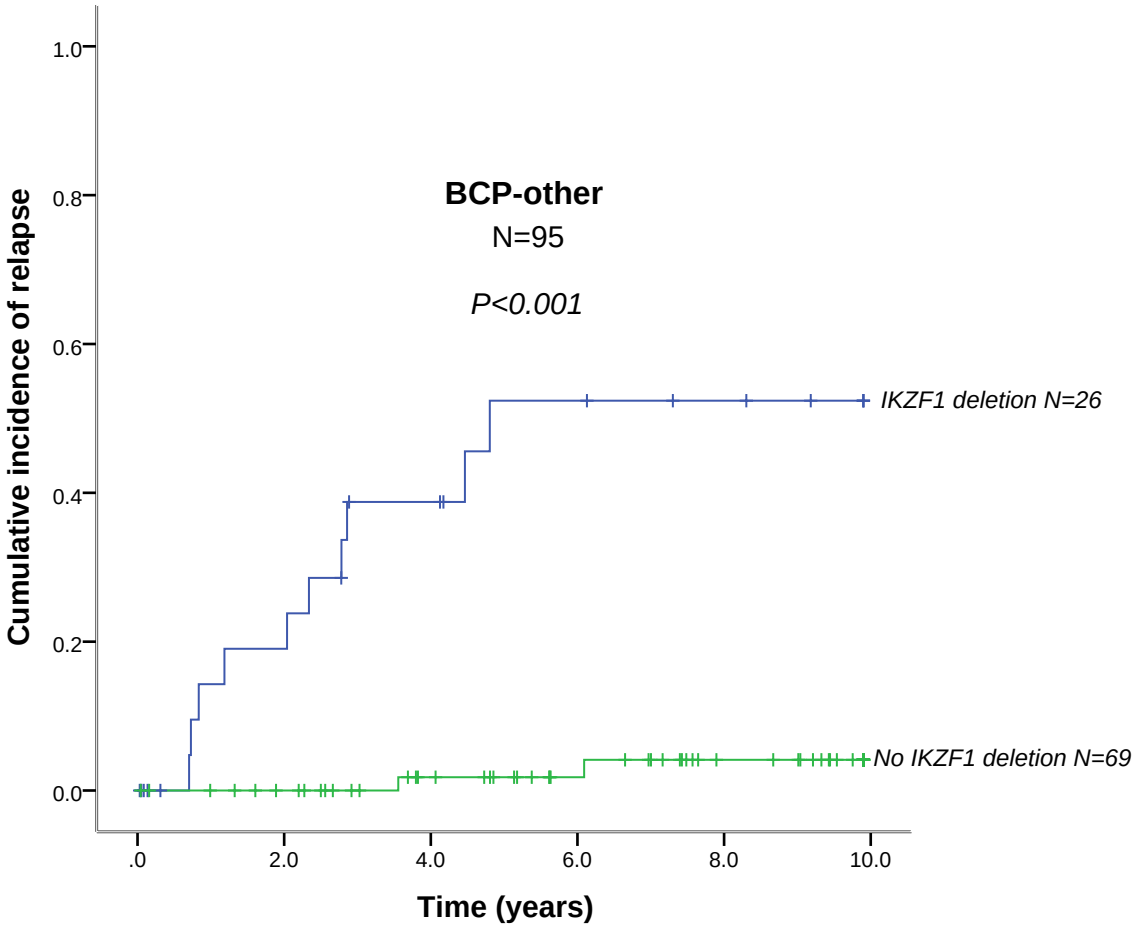

BCP-other
N=95
P<0.001
IKZF1 deletion N=26
No IKZF1 deletion N=69
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)

Fig 3A

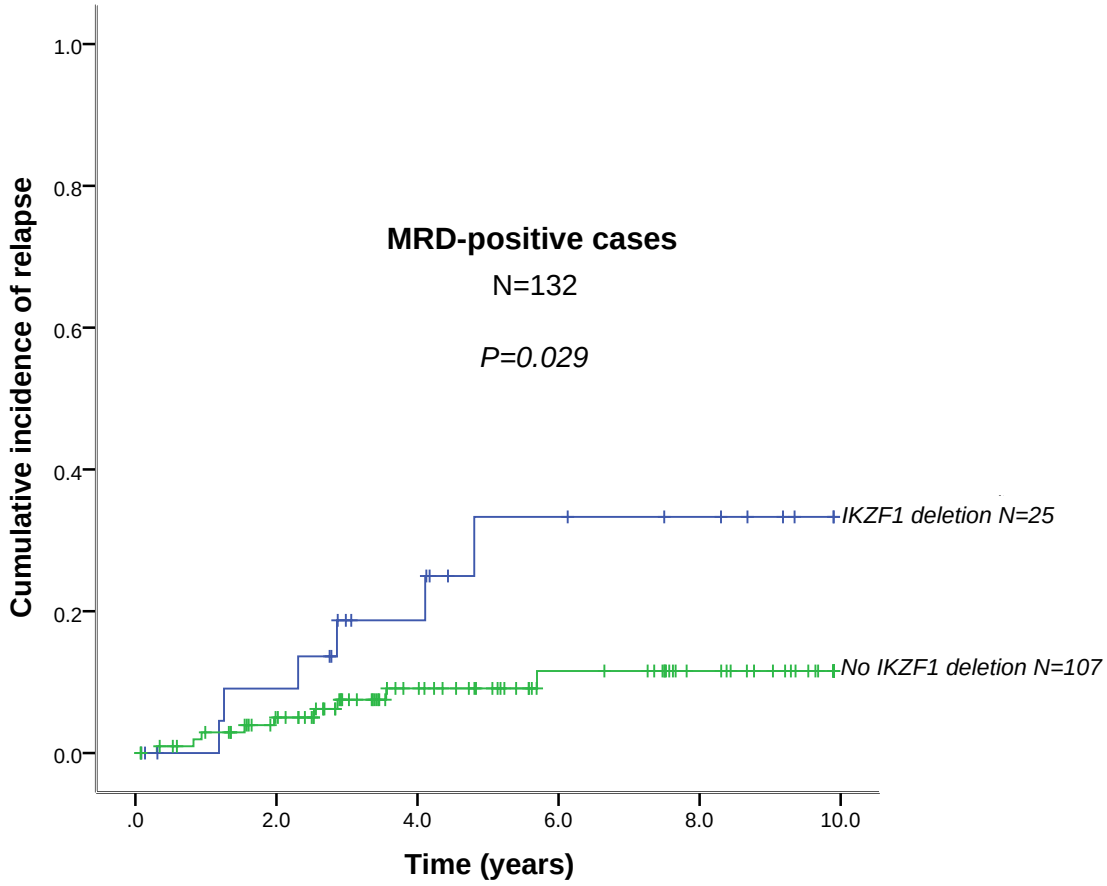
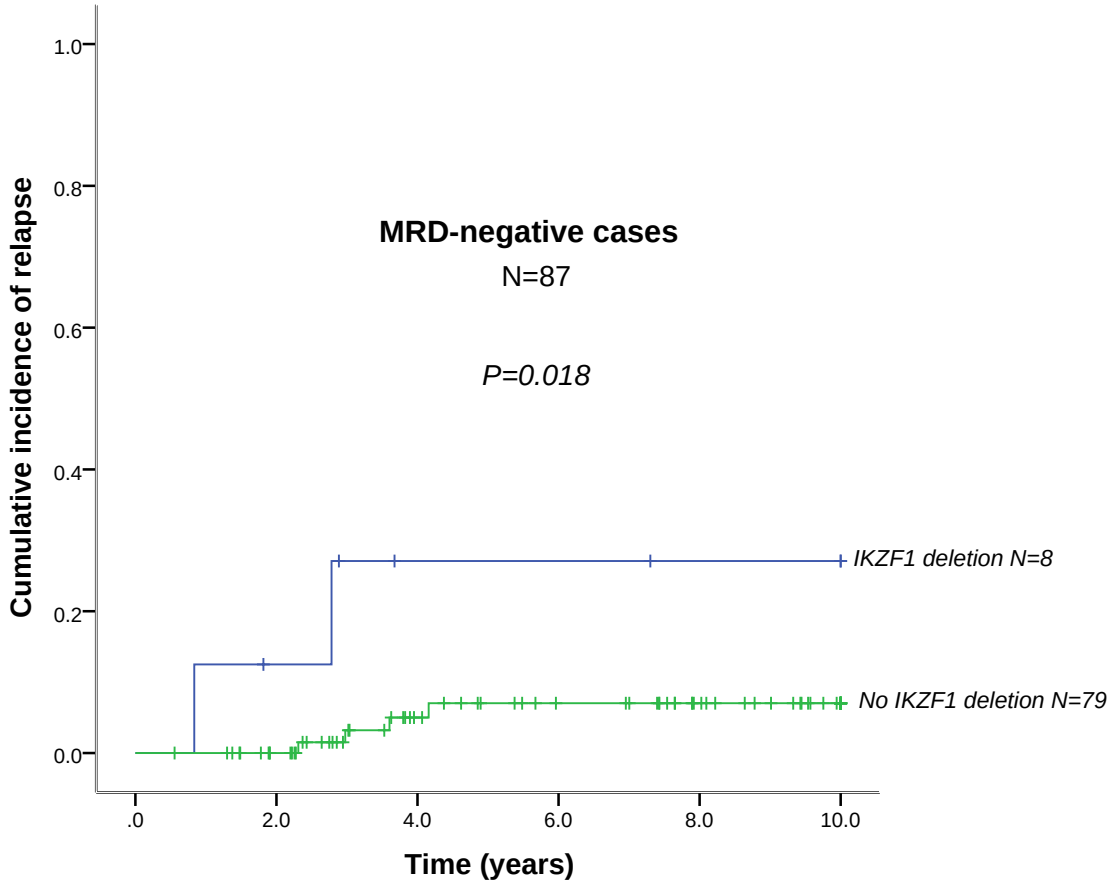

MRD-positive cases
N=132
P=0.029
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)
IKZF1 deletion N=25
No IKZF1 deletion N=107

Fig 3B


MRD-negative cases
N=87
P=0.018
Cumulative incidence of relapse
1.0
0.8
0.6
0.4
0.2
0.0
.0
2.0
4.0
6.0
8.0
10.0
Time (years)
IKZF1 deletion N=8
No IKZF1 deletion N=79