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# Tyrosine kinase inhibitor resistance in de novo *BCR*::*ABL1*–positive BCP-ALL beyond kinase domain mutations

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**Key Points**

- Imatinib resistance at diagnosis was higher in cells of patients who relapsed vs those in remission after imatinib and chemotherapy.
- Low levels of BCR::ABL1 and deletions of B-cell development genes including *IKZF1* and *PAX5* are associated with tyrosine kinase resistance.

A better understanding of *ABL1* kinase domain mutation–independent causes of tyrosine kinase inhibitor (TKI) resistance is needed for *BCR*::*ABL1*–positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Although TKIs have dramatically improved outcomes, a subset of patients still experiences relapsed or refractory disease. We aimed to identify potential biomarkers of intrinsic TKI resistance at diagnosis in samples from 32 pediatric and 19 adult patients with *BCR*::*ABL1*–positive BCP-ALL. Reduced ex vivo imatinib sensitivity was observed in cells derived from newly diagnosed patients who relapsed after combined TKI and chemotherapy treatment compared with cells derived from patients who remained in continuous complete remission. We observed that ex vivo imatinib resistance was inversely correlated with the amount of (phosphorylated) BCR::ABL1/ABL1 protein present in samples that were taken at diagnosis without prior TKI exposure. This suggests an intrinsic cause of TKI resistance that is independent of functional BCR::ABL1 signaling. Simultaneous deletions of *IKZF1* and *CDKN2A/B* and/or *PAX5* (*IKZF1*plus), as well as deletions of *PAX5* alone, were related to ex vivo imatinib resistance. In addition, somatic lesions involving *ZEB2*, *SETD2*, *SH2B3*, and *CRLF2* were associated with reduced ex vivo imatinib sensitivity. Our data suggest that the poor prognostic value of *IKZF1*(plus) deletions is linked to intrinsic mechanisms of TKI resistance other than *ABL1* kinase domain mutations in newly diagnosed pediatric and adult *BCR*::*ABL1*–positive BCP-ALL.

## Introduction

*BCR*::*ABL1*–positive B-cell precursor acute lymphoblastic leukemia (BCP-ALL), also known as Philadelphia chromosome–positive BCP-ALL, is characterized by the t(9;22)(q34;q11) translocation. The BCR::ABL1 tyrosine kinase protein generated because of the translocation is constitutively active, resulting in downstream signaling that promotes transcription, proliferation, and survival, while inhibiting apoptosis.[1] The *BCR*::*ABL1* fusion gene is present in 2% to 5% of pediatric and 25% of adult BCP-ALL cases.[2,3]

In the pre–tyrosine kinase inhibitor (TKI) era, *BCR*::*ABL1*–positive BCP-ALL exhibited an aggressive phenotype characterized by its resistance to standard chemotherapy and a high relapse rate.[4-6] The

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incorporation of TKIs in the standard treatment for patients with *BCR*::*ABL1*–positive BCP-ALL has represented a significant scientific breakthrough and dramatically improved the prognosis for these patients.[7] Despite the introduction of TKIs, 25% to 30% of children and adults with *BCR*::*ABL1*–positive BCP-ALL still experience relapse or have refractory disease when treated with chemotherapy and a TKI.[8-17]

Several prognostic factors for poor response to TKIs have been identified in *BCR*::*ABL1*–positive leukemia. The most well-known TKI resistance mechanism is caused by mutations in the *ABL1* kinase domain–hindering TKI binding. In adults, kinase domain mutations are reported in >80% of patients who relapsed,[18] whereas in children only ~10% of relapses harbor kinase domain mutations.[19,20] These resistance mutations are thought to be present at subclonal level and/or arise during TKI therapy providing a growth advantage due to selective pressure.[19-26] Imatinib resistance caused by kinase domain mutations can be overcome by next-generation TKIs such as dasatinib and bosutinib. Imatinib is classified as a type 2 TKI because it binds to the inactive conformation of the ABL1 kinase domain.[27] Dasatinib and bosutinib are classified as type 1 TKIs because they can bind to both the active and inactive conformations of the ABL1 kinase domain.[28,29] Although ABL1 kinase domain–related mechanisms of resistance are well understood, kinase-independent mechanisms are less well documented. The presence of *IKZF1* deletions in *BCR*::*ABL1*–positive BCP-ALL at diagnosis is predictive of poor outcome after combined TKI and chemotherapy treatment, but whether these lesions are directly associated with TKI resistance is unknown.[30,31] Overall, limited knowledge exists regarding intrinsic TKI resistance in *BCR*::*ABL1*–positive BCP-ALL that exist before treatment initiation.

To gain a better understanding of intrinsic mechanisms of TKI resistance, we aimed to study potential biomarkers at diagnosis. We measured ex vivo TKI sensitivity in a unique set of samples from 32 pediatric and 19 adult patients with *BCR*::*ABL1*–positive BCP-ALL taken at initial diagnosis (before TKI exposure) and observed considerable variability in the intrinsic response of these cells to TKIs. Ex vivo TKI resistance at diagnosis was associated with the occurrence of relapse in patients treated with TKIs. Remarkable, ex vivo resistance was also correlated with lower *BCR*::*ABL1* transcript and BCR::ABL1/ABL1 protein expression in these TKI therapy–naïve samples, suggesting reduced dependence on BCR::ABL1 signaling in intrinsically resistant samples. The B-cell development and JAK/STAT pathways were candidates for activated alternative pathways in newly diagnosed *BCR*::*ABL1*–positive BCP-ALL with ex vivo TKI resistance. Moreover, our data suggest that the poor prognostic value of *IKZF1*(plus) deletions is linked to intrinsic mechanisms of TKI resistance other than *ABL1* kinase domain mutations in newly diagnosed pediatric and adult *BCR*::*ABL1*–positive BCP-ALL.

## Material and methods

### Primary leukemic cells

Peripheral blood and/or bone marrow samples were obtained from patients with newly diagnosed *BCR*::*ABL1*–positive BCP-ALL or T-cell ALL. Written informed consent to use the excess diagnostic material for research purposes was obtained from the patients or the parents or guardians of the children. Patient material of adults treated in HOVON protocols 18, 37, 71, and 100 was used according to the principles outlined in Code Good Conduct of the Federation of Dutch Medical Scientific Societies. The use of patient material was in accordance with the Declaration of Helsinki, as approved by the medical ethics committee of the Erasmus MC Cancer Institute, the Biobank and Data Access Committee of the Princess Máxima Center for Pediatric Oncology, and Medical Ethics Committee of the University Medical Center Hamburg Eppendorf. For all experiments, either primary samples (15 pediatric and 19 adult) or xenograft samples derived (PDX) from patients (17 pediatric) were used with a blast percentage of at least 80%. Ex vivo experiments were performed in primary culture medium as described in the supplemental Material and Methods.

### Drug sensitivity assays

TKI sensitivity of primary and PDX–expanded ALL samples was measured in a coculture with human mesenchymal stromal cells to optimize primary ALL cell viability with flow cytometry–based viability readout. In addition, TKI sensitivity of PDX–expanded ALL samples and PDX cell lines was measured in a metabolic assay using MTT. Survival relative to an untreated control was determined after 3 days of imatinib, dasatinib, or bosutinib exposure. Area under the dose-response curve (AUC) was used as a measure for TKI sensitivity. A drug screen using 200 compounds on *BCR*::*ABL1*–positive PDX cell lines M4A3-BA1 and M4A4-BA1 and *BCR*::*ABL1*–negative M4A1-M2B9 was performed by the high-throughput screening facility at the Princess Máxima Center. Further experimental details can be found in the supplemental Material and Methods.

### (Phosphorylation) flow analysis

Protein expression and/or phosphorylation of ABL1, BCR, STAT5, and CRKL were measured using flow cytometry in PDX–expanded ALL samples and PDX cell lines. Protein expression and phosphorylation levels were measured in thawed PDX–expanded ALL samples without prior TKI treatment. For PDX cell lines, protein expression and phosphorylation levels were measured either after 24 hours exposure to 1.95 μM imatinib or after 24 hours without treatment. Further details including antibodies used can be found in the supplemental Material and Methods.

### Gene expression, mutations, and deletions analysis

RNA sequencing was performed for the analysis of gene expression levels. Real-time quantitative polymerase chain reaction (qPCR) assays designed for *ABL1* and the *BCR*::*ABL1* p190 transcript were performed to determine respective transcript expression levels. Whole-exome sequencing (WES) was performed to determine mutations in leukemia-associated genes. Data from multiplex ligation-dependent probe amplification or SNP array to study copy number alterations were requested at the diagnostic departments of the involved study groups. Further details on sample and data processing can be found in the supplemental Material and Methods.

## Results

### Patient inclusion

We conducted our study on viable frozen cells taken at the time of initial diagnosis from patients with *BCR*::*ABL1*–positive ALL (supplemental Table 1; supplemental Figure 1). The cohort consisted of 32 pediatric and 19 adult patients with

*BCR*::*ABL1*–positive BCP-ALL. For pediatric patients, the diagnosis period ranged from 1994 to 2019. The median age at diagnosis was 9 years (range, 1-15). Among the pediatric group, the median white blood cell count at diagnosis was 25 200 cells/μL, 69% were male, 86% had the p190 fusion, and 55% received imatinib treatment (supplemental Table 2). Regarding adult patients, diagnosis ranged from 1993 to 2014. The median age at diagnosis was 50 years (range, 19-64). Within the adult group, the median white blood cell count at diagnosis was 59 500 cells/μL, 47% were male, 68% had the p190 fusion, and 56% received imatinib treatment (supplemental Table 2). WES or RNA sequencing performed on leukemic cells revealed no evidence for *ABL1* kinase domain mutations at the time of diagnosis in our study patients.

## Ex vivo tyrosine kinase sensitivity

We determined the ex vivo sensitivity to TKIs imatinib, dasatinib, and bosutinib of TKI therapy–naïve *BCR*::*ABL1*–positive primary or PDX–expanded BCP-ALL samples. We did not observe a difference in TKI sensitivity between primary or PDX–expanded BCP-ALL samples (supplemental Figure 2). The ex vivo sensitivity of pediatric and adult samples did not differ and exhibited considerable variability, ranging from high sensitivity (AUC imatinib $<$ 80 AU) to complete lack of response (AUC imatinib $>$ 120 AU) (Figure 1A-C). Cross-resistance to TKIs was observed by a high correlation of the AUC for next-generation TKIs dasatinib and bosutinib with imatinib (Figure 1D). We compared the measurable residual disease (MRD) detected by immunoglobulin/T-cell receptor qPCR at end of induction and the outcome of patients treated with TKI with the corresponding ex vivo response of their diagnostic TKI therapy–naïve cells. The number of patients included in the MRD analysis was limited to patients who started TKI treatment ≤15 days after diagnosis (supplemental Table 1). The ex vivo TKI sensitivity did not correlate with the MRD level at end of induction of *BCR*::*ABL1*-positive patients treated with imatinib, probably due to a good initial response to combined chemotherapy and TKI treatment (Figure 1E). We compared the ex vivo TKI sensitivity of diagnostic samples collected from patients who remained in complete continuous remission (CCR) upon TKI-containing therapy with the sensitivity of diagnostic samples collected from patients who relapsed on TKI-containing therapy. To avoid the use of arbitrary cutoffs for ex vivo TKI sensitivity, we used the z-score of the TKI as continuous variable. Among patients who started TKI treatment ≤50 days after diagnosis, we observed a significantly higher ex vivo imatinib sensitivity in patients who remained in CCR than patients who relapsed after TKI-containing therapy ($P$ = .02; Figure 1F). The same trend was observed for dasatinib and bosutinib, however, the number of samples was insufficient to reach statistical significance (Figure 1F). Although the groups are too small to perform a cumulative incidence of relapse analysis, we illustrated that the patients whose cells from diagnosis are more ex vivo imatinib-resistant have a higher incidence of relapse (supplemental Figure 3). This finding suggests that ex vivo imatinib resistance, measured at the initial diagnosis of the disease and before TKI treatment, is associated with the risk of relapse after TKI-containing therapy.

## Phosphorylation and expression of ABL1 as biomarker for imatinib response

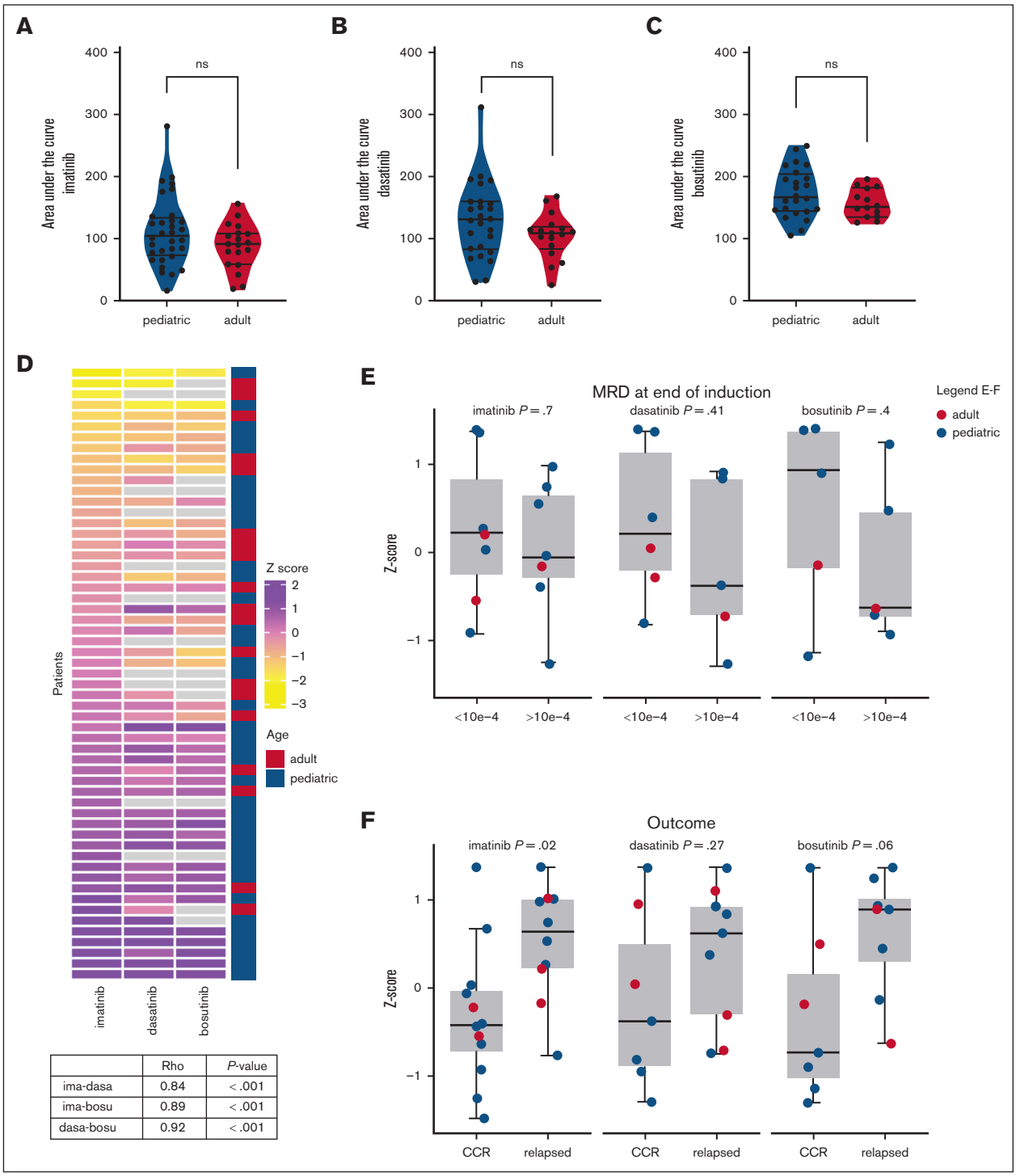
To investigate whether resistant samples were less dependent on the BCR::ABL1 signaling cascade compared with sensitive samples, we conducted (phosphorylation) flow cytometry of BCR::ABL1/ABL1, BCR, and downstream signaling components STAT5 and CRKL on newly generated cell lines from *BCR*::*ABL1*–positive PDXs (PDX cell lines) and thawed PDX samples. The ex vivo imatinib response of the samples used for the flow cytometry experiments was variable, as shown for *BCR*::*ABL1*–positive primary and PDX–expanded BCP-ALL samples (Figure 1A; Figure 2A). After exposure to imatinib, the phosphorylation levels of BCR::ABL1/ABL1 decreased ~50% in both sensitive and resistant cell lines (Figure 2B; supplemental Figure 4). Moreover, we observed that an increase in ex vivo imatinib resistance was correlated with a decreased amount of phosphorylated BCR::ABL1/ABL1 protein (rho = −0.92; $P$ = .0013), total BCR::ABL1/ABL1 protein (rho = −0.82; $P$ = .011), phosphorylated CRKL protein (rho = −0.95; $P$ = .0004), and phosphorylated BCR protein (rho = −0.97; $P$ = .0002), but not phosphorylated STAT5 protein (rho = −0.07; $P$ = not significant), in (long-term cultured) PDX-expanded cells from untreated newly diagnosed samples (Figure 2C-G). This suggests that imatinib inhibits BCR::ABL1 in both sensitive and resistant samples, but resistant cells exhibited lower levels of (phosphorylated) ABL1 protein, indicating reduced dependence on BCR::ABL1 signaling.

## Pathways involving immune response associated with intrinsic TKI resistance

The reduced dependence on BCR::ABL1 signaling in patients resistant to imatinib implies a role of alternative pathways in intrinsic TKI resistance. To decipher the activation of alternative pathways, we conducted a differential gene expression analysis using the AUC as determined in an ex vivo imatinib sensitivity assay as a continuous variable. The differential gene expression analysis resulted in 5 genes with an FDR <0.01 (supplemental Figure 5A). However, all were driven by outliers characterized by expression changes limited to a small subset of samples, and these genes did not demonstrate a correlation with TKI sensitivity (supplemental Figure 5B-F). Gene set enrichment analysis showed that the most prominent pathways associated with imatinib resistance were involved in (lymphocyte) immune response (supplemental Figure 6A-C). This suggests that immune-related genes contribute to intrinsic TKI resistance.

## Deletions, secondary fusions, and mutations in patients resistant to TKIs

To explore the role of secondary lesions in TKI resistance, deletions in B-cell development and cell cycle genes *IKZF1*, *PAX5*, *BTG1*, *CDKN2A/B*, and the PAR region were assessed in patient samples at the time of diagnosis. Pediatric patients had frequent deletions of *IKZF1* (58%), *PAX5* (56%), *BTG1* (38%), and *CDKN2A/B* (73%), whereas none had PAR deletions (Figure 3A; supplemental Table 2). Adult patients had frequent deletions of *IKZF1* (73%) and *CDKN2A/B* (46%), whereas the other genes were not assessed (Figure 3A; supplemental Table 2). *CDKN2A/B* deletions were found in both sensitive and resistant samples (Figure 3A). Notably, codeletions of *IKZF1* with *PAX5* and/or *CDKN2A/B*, referred to as the *IKZF1*plus profile, were observed in all imatinib-resistant samples with *IKZF1* deletions (Figure 3A). In *IKZF1*-nondeleted samples, *PAX5* deletions were associated with imatinib resistance ($P$ = .03; Dunn test; Figure 3B). Samples with the *IKZF1*plus profile showed a trend of imatinib resistance ($P$ = .18; Dunn test;




| | Rho | *P*-value |
|---|---|---|
| ima-dasa | 0.84 | < .001 |
| ima-bosu | 0.89 | < .001 |
| dasa-bosu | 0.92 | < .001 |

**Figure 1. TKI response of *BCR*::*ABL1*–positive primary or PDX-expanded BCP-ALL samples using an ex vivo coculture assay.** (A-C) Ex vivo response toward imatinib (A), dasatinib (B), and bosutinib (C). Violin plot of the AUC (y-axis) for pediatric (blue) and adult (red) *BCR*::*ABL1*–positive BCP-ALL samples (x-axis) cultured on mesenchymal stromal cells for 3 days. The thick black line represents the median, and the thin black lines represent the first quartile and the third quartile. (D) Correlation between the z-score of the AUC of imatinib, dasatinib, and bosutinib for pediatric and adult *BCR*::*ABL1*–positive BCP-ALL samples. (E) Relation of ex vivo TKI sensitivity with MRD at end of induction in patients started with imatinib treatment within 15 days from diagnosis. The box plots show the median z-score ± 1.5 interquartile range (IQR) in Tukey style. A Mann-Whitney *U* test was used to compare the z-score values between patients with a high MRD (≥10e-4) and a low MRD (<10e-4). (F) Relation of ex vivo drug sensitivity with occurrence of relapse in patients started with imatinib treatment within 50 days from diagnosis and with follow-up of at least 1 year from diagnosis, a relapse within 1 year from diagnosis, or a relapse after >1 year from diagnosis. One pediatric and 7 adult patients who died within 1 year of diagnosis in complete remission were excluded (supplemental Table 1). The box plots show the median z-score ± 1.5 IQR in Tukey style. A Mann-Whitney *U* test was used to compare the z-score values between patients who remained in CCR and those who relapsed. ima-dasa, imatinib-dasatinib; ima-bosu, imatinib-bosutinib; dasa-bosu, dasatinib-bosutinib; ns, not significant.

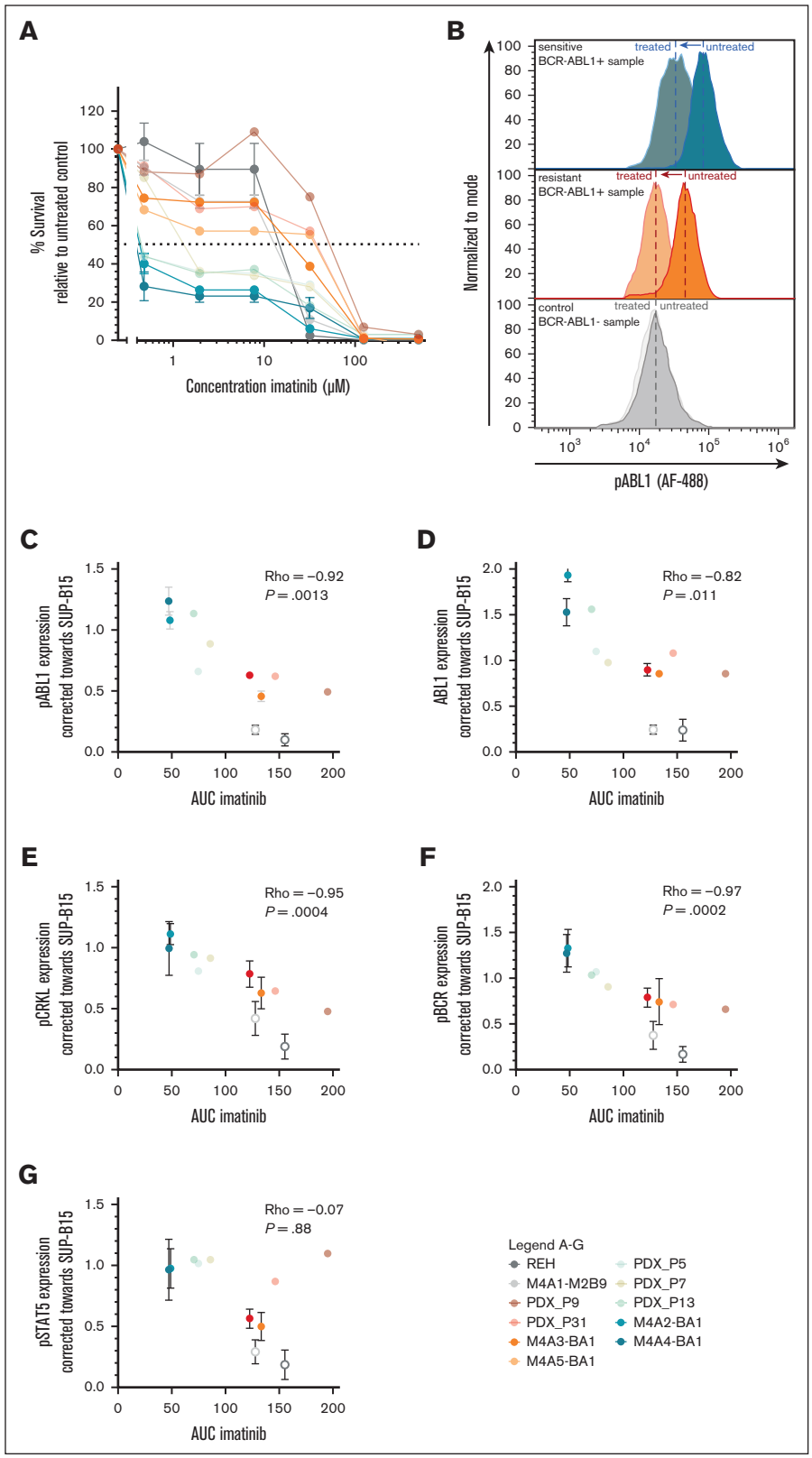



**Figure 2. Correlation of phosphorylation and/or expression of BCR::ABL1 signaling cascade components with imatinib sensitivity ex vivo.** (A) Dose-response curve of PDX cell lines or PDX–expanded ALL samples after 3-day treatment with imatinib. Values are normalized against untreated controls for each cell line and represent mean ± standard error of the mean (SEM; n = 3), except for M4A5-BA1 and the PDX–expanded ALL samples (n = 1). (B) Representative example of median fluorescent intensity (MFI) of phosphorylated ABL1 (pABL1) of an imatinib-sensitive (M4A4-BA1) and an imatinib-resistant PDX cell line (M4A3-BA1) before and after imatinib exposure; x-axis represents MFI/expression of pABL1 measured with AF-488 and y-axis normalized to mode. (C-G) Correlation between imatinib sensitivity and the phosphorylation level of ABL1 (C), expression of ABL1 (D), phosphorylation of CRKL (E), phosphorylation of BCR (F), and phosphorylation of STAT5 (G), respectively; x-axis, AUC of imatinib; y-axis, MFI of corresponding target. Average of 3 experiments ± SEM for PDX cell lines, average of duplicates for PDX samples. PDX–expanded ALL samples are denoted by PDX followed by the corresponding patient number. Negative controls were included to indicate basal expression levels. Correlation was calculated using Spearman correlation excluding *BCR::ABL1*–negative controls REH and M4A1-M2B9.

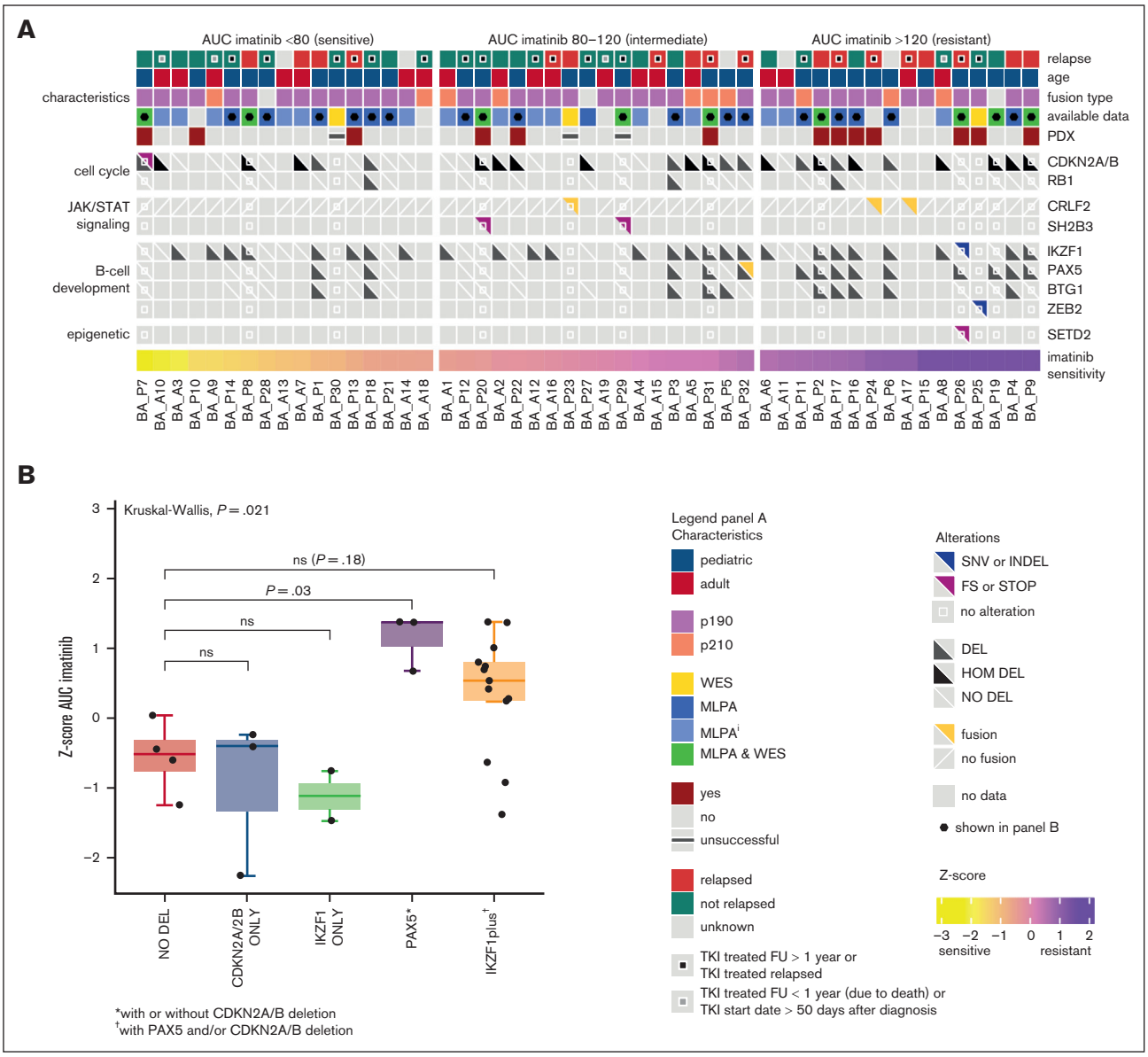



**Figure 3. Relation of secondary lesions with TKI sensitivity in *BCR::ABL1*–positive BCP-ALL samples.** (A) Overview of patients sorted on ex vivo imatinib sensitivity presenting clinical characteristics and secondary lesions of *BCR::ABL1*–positive adult (n = 19) and pediatric (n = 32) BCP-ALL samples. The availability of WES (yellow), MLPA data (dark blue), or limited MLPA data (‡*IKZF1* or *IKZF1* and *CDKN2A/B*, light blue) is depicted in the "available data" row. Samples included in panel B, the ones with full MLPA data available, are depicted with a black hexagon in the "available data" row. If MLPA data were available, the rectangle is divided by a white diagonal line. If WES data were available, but no mutations were identified, this is depicted with a white rectangle. (B) Relation between deletions in B-cell development genes (*IKZF1* and *PAX5*) and cell cycle genes (*CDKN2A/B*) with the z-score for AUC for imatinib. The box plots show the median z-score ± 1.5 IQR in Tukey style. Mean AUC of imatinib was compared using the Kruskal-Wallis test with Dunn post hoc test. DEL, deletion; HOM DEL, homozygous deletion; FS or STOP, frame shift or stop mutation; MLPA, Multiplex Ligation-dependent Probe Amplification; NO DEL, no deletions in *IKZF1*, *PAX5*, *CDKN2A/B*, *RB1*, or *BTG1*; PAX5*, deletion of *PAX5* with or without *CDKN2A/B* deletion; *IKZF1*plus, deletion of *IKZF1* and *CDKN2A*, *CDKN2B*, or *PAX5*; SNV or INDEL, single nucleotide variation, insertion, or deletion.

Figure 3B). In addition, the *IKZF1*plus profile was associated with low phosphorylation levels of ABL1 (Mann-Whitney *U* test; $P = .05$; supplemental Figure 7). None of the samples used in the phosphorylation assay exhibited solely a *PAX5* deletion; they all co-occurred with *IKZF1* deletions. Consequently, it was not possible to evaluate the impact of a *PAX5* deletion alone on the ABL1 phosphorylation level. Our data suggest that the *IKZF1*plus profile and *PAX5* deletions are associated with ex vivo imatinib resistance.

In addition to deletions, we assessed the potential relation between somatic mutations and intrinsic resistance to imatinib in BCP-ALL samples (n = 12) of pediatric patients with WES data. All major somatic mutations in leukemia-associated genes described by Brady et al[2] that were annotated as (likely) pathogenic in the ClinVar database were assessed. This resulted in mutations in 5 genes of interest: *CDKN2A*, *IKZF1*, *SETD2*, *SH2B3*, and *ZEB2* (Figure 3A). One patient with imatinib sensitivity harbored an

inactivating *CDKN2A* mutation and a *CDKN2A/B* deletion but no deletions in *IKZF1* or *PAX5*. This suggests that a deletion or mutations in cell cycle regulator *CDKN2A* alone is not a predictive marker for resistance to imatinib. Mutations in B-cell development genes *IKZF1* and *ZEB2* and epigenetic modifier *SETD2* were present in 2 of 5 resistant cases (AUC imatinib > 120) with WES data available. Moreover, mutations in negative regulator of cytokine signaling *SH2B3* were found in 2 of 4 patients with WES data available with intermediate ex vivo imatinib sensitivity (AUC imatinib between 80 and 120 AU); both patients had wildtype *IKZF1*. We also obtained WES data from 2 diagnosis relapse pairs (BA_P26 and BA_P30) and 1 relapse only (BA_P32). All 3 patients were TKI treated according to the ESPHALL protocol. The variants identified at relapse were largely deviating from the variants at diagnosis (supplemental Figure 8). Moreover, we identified a subclonal *ABL1* kinase domain mutation (H396P) in de relapse material of BA_P32 with a VAF of 4.2%, which was not detectable by RNA sequencing at diagnosis. In addition, imatinib resistance was not associated with a complex karyotype or a high mutational burden (supplemental Figure 9).

We also determined the frequency of secondary fusions in imatinib-resistant and -sensitive primary samples. Secondary fusions were rare in all *BCR*::*ABL1*–positive BCP-ALL samples. However, we identified *CRLF2* to be rearranged in 2 of 16 resistant samples and 1 of 15 intermediately sensitive samples (Figure 3A). Despite incomplete data, in the total cohort, at least 14 of 16 TKI-resistant cases (88%) were characterized by ≥1 of the above-described secondary lesions (8 *IKZF1*plus, 3 *PAX5* deletion, 1 *ZEB2* mutation, and 2 JAK/STAT activation), at least 9 (6 *IKZF1*plus and 3 JAK/STAT activation) of 18 intermediate sensitivity cases (50%), and 3 (3 *IKZF1*plus) of 17 sensitive cases (18%).

### Drug sensitivity screen

We performed a screen using 200 drugs (supplemental Table 3) to identify drugs that inhibit proliferation in *BCR*::*ABL1*–positive PDX cell lines. We compared the resistant *BCR*::*ABL1* cell line M4A3-BA1 with the *IKZF1*plus profile with the sensitive cell line M4A4-BA1 without *IKZF1* and *PAX5* deletions and a *BCR*::*ABL1*–negative PDX cell line M4A1-M2B9 (*MEF2D*::*BCL9*–fused). As expected, the imatinib-resistant M4A3-BA1 exhibited cross-resistance to other TKIs (dasatinib, bosutinib, ponatinib, and nilotinib) compared with the imatinib-sensitive M4A4-BA1 (Figure 4A). Notably, the resistant cell line also showed decreased sensitivity to dexamethasone. None of the 200 tested compounds demonstrated increased sensitivity in the resistant compared with the sensitive *BCR::ABL1*–positive cell line (Figure 4A). Only 1 novel agent, THZ531 (a CDK12/13 inhibitor), exhibited slightly enhanced sensitivity in the imatinib-resistant *BCR*::*ABL1*–positive cell line compared with the *BCR*::*ABL1*–negative cell line (Figure 4B) but not when compared with the imatinib-sensitive *BCR*::*ABL1*–positive cell line. On the contrary, idasanutlin (an MDM2 inhibitor) showed slightly decreased activity. These results confirm the presence of an intrinsic mechanism causing resistance toward all TKIs targeting ABL1.

### *BCR*::*ABL1* gene expression as a biomarker for imatinib sensitivity

We explored the potential of *ABL1* and *BCR*::*ABL1* messenger RNA (mRNA) expression measured by real-time qPCR as an alternative to phosphorylated or total BCR::ABL1/ABL1 protein expression in primary samples. This allowed for a direct comparison between the imatinib sensitivity as determined in the ex vivo TKI sensitivity assay and the mRNA expression level per primary or PDX–expanded ALL sample. This revealed that low *BCR*::*ABL1*/*ABL1* mRNA expression was related to imatinib resistance (rho = –0.49; $P = .001$; Figure 5A), and this effect was mainly caused by the expression level of the fusion gene, because an even stronger negative correlation was observed using the *BCR*::*ABL1* p190 fusion transcript (rho = –0.66; $P < .0001$; Figure 5B). There were not enough samples with the *BCR*::*ABL1* p210 fusion transcript to make a meaningful comparison between p210 transcript expression levels and imatinib sensitivity. This suggests that expression of the *BCR*::*ABL1* p190 transcript could be an alternative to phosphorylated or total BCR::ABL1/ABL1 protein expression and could serve as a potential biomarker for ex vivo imatinib sensitivity.

## Discussion

In this study, we assessed potential biomarkers of intrinsic TKI resistance in patients with newly diagnosed *BCR*::*ABL1*–positive BCP-ALL without *ABL1* kinase domain mutations. In our study, we evaluated the ex vivo TKI sensitivity toward imatinib and dasatinib,

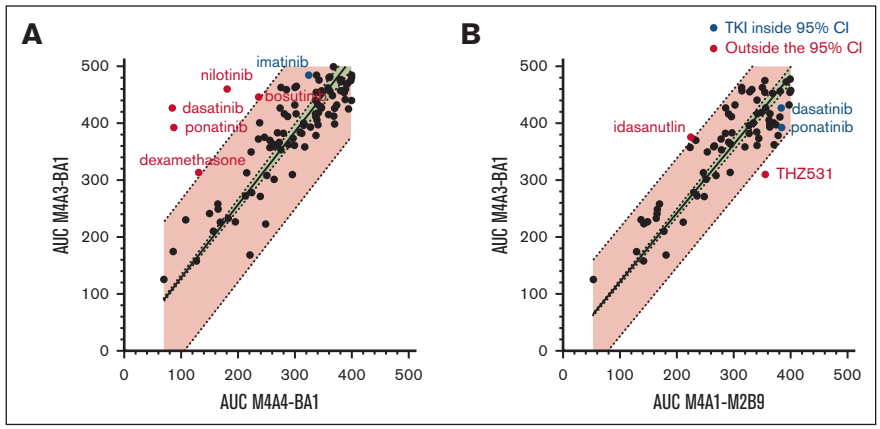



**Figure 4. Comparison of drug sensitivity between *BCR*::*ABL1*–positive imatinib-resistant PDX cell line and imatinib-sensitive or *BCR*::*ABL1*–negative cell line.** (A) Comparison of all tested drugs with an AUC of <400 between *BCR*::*ABL1*–positive imatinib-sensitive cell line (M4A4-BA1) and *BCR*::*ABL1*–positive imatinib-resistant cell line (M4A3-BA1). (B) Comparison of all tested drugs with an AUC of <400 between *BCR*::*ABL1*–negative cell line (M4A1-M2B9) and *BCR*::*ABL1*–positive resistant cell line (M4A3-BA1). The red area shows the 95% confidence interval (CI); dots in red are outside the 95% CI; dots in blue are TKIs inside the 95% CI.

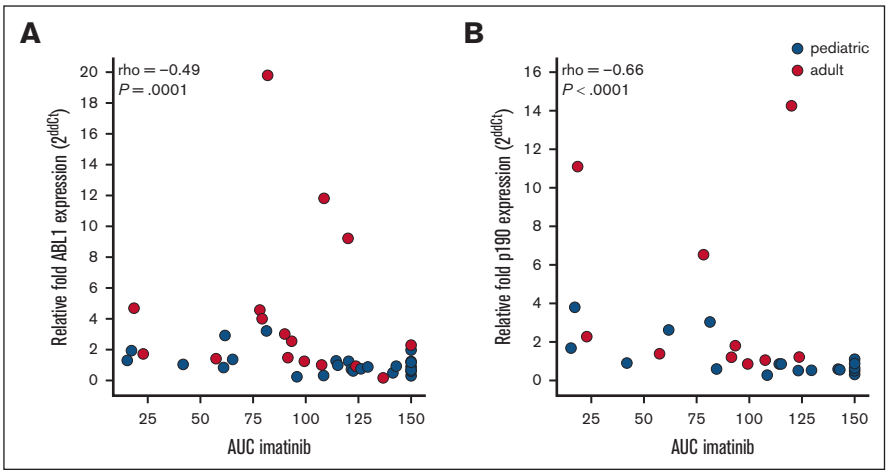



**Figure 5. Relation between *ABL1* mRNA or *BCR*::*ABL1* p190 transcript expression and ex vivo imatinib sensitivity.** (A) Correlation between *ABL1* mRNA expression and the AUC as determined in an ex vivo imatinib sensitivity assay plotted per (PDX–expanded) primary ALL sample. (B) Correlation between *BCR*::*ABL1* p190 transcript expression and the AUC plotted per (PDX–expanded) primary ALL sample. Correlation was calculated using Spearman correlation; AUC values that exceeded the maximum value of 150 AU for imatinib were artificially adjusted to the 150 AU to classify it as nonresponsive.

which are currently approved for firstline treatment of *BCR*::*ABL1*–positive ALL. Moreover, we evaluated the sensitivity to bosutinib, which is recently approved for newly diagnosed pediatric chronic myeloid leukemia (CML)[32] and approved for adult CML.[28,33,34] The ex vivo sensitivity to TKIs was highly variable between samples from patients who were TKI therapy naïve, and in general, the sensitivity to all 3 tested TKIs was highly correlated. We identified an *ABL1* kinase domain mutation in a subclone of one of the relapsed pediatric *BCR*::*ABL1*–positive BCP-ALL samples. The subclonal presence of this mutations implies that this mutation has had limited impact on the development of relapse and illustrates that other nonkinase domain–related lesions may be more important. In correspondence, *ABL1* kinase domain mutations have been infrequently found in pediatric patients who relapsed after TKI therapy.[19,20] This suggests that other mechanisms are underlying relapse in these patients. In this study, we showed that ex vivo imatinib resistance was already present in therapy-naïve *BCR*::*ABL1*–positive BCP-ALL samples without identifiable *ABL1* kinase domain mutations, and patients who relapsed after TKI-containing chemotherapy also displayed ex vivo imatinib resistance in their diagnostic, hence TKI naïve, cells compared with diagnostic samples of patients who remained in CCR. This indicates that intrinsic mechanisms active at diagnosis can cause the imatinib resistance. Setting a cutoff for clinical purposes, for example, by the AUC as determined in an ex vivo imatinib sensitivity assay, is arbitrary and difficult to achieve in prospective studies. Therefore, we studied other potential predictive biomarkers.

To understand the cause of intrinsic resistance mechanisms, we studied gene expression, secondary lesions, BCR::ABL1/ABL1 protein phosphorylation and expression, and *BCR*::*ABL1* mRNA expression levels in leukemic cell taken at diagnosis (before treatment). We did not observe an association between differential gene expression and ex vivo imatinib sensitivity. However, we did observe an increase in secondary lesions involving B-cell signaling, JAK/STAT signaling, and epigenic modifications in ex vivo imatinib-resistant samples (at least 88%) compared with imatinib-sensitive samples (at least 18%). We observed 5 patients with either the loss of *SH2B3* or a rearrangement of *CRLF2,* which is expected to lead to increased JAK/STAT signaling, potentially serving as a rescuing mechanism of imatinib inhibition in resistant samples.[35,36]

Moreover, we identified an inactivating lesion in *SETD2* in 1 imatinib-resistant sample, this gene has previously been described as a tumor suppressor gene and has been associated with TKI resistance in CML cell lines.[37] In addition, all imatinib-resistant samples with multiplex ligation-dependent probe amplification or SNP array data available exhibited an *IKZF1*plus profile or harbored *PAX5* deletions, whereas 3 of 8 imatinib-sensitive samples exhibited this *IKZF1*plus profile, and none of these cases harbored a *PAX5* deletion with wildtype *IKZF1*. Deletions of *IKZF1* are known to affect B-cell differentiation and are common in *BCR*::*ABL1*–positive BCP-ALL and associated with poor prognosis in patients treated with imatinib.[30,31,38] *IKZF1* deletions co-occurring with *CDKN2A/B* deletions in *BCR*::*ABL1*–positive ALL models were previously associated with reduced ex vivo and in vitro TKI sensitivity.[39] This suggests a direct relation between *IKZF1* deletions and TKI sensitivity. Our study also suggests a role of *PAX5* deletions in TKI resistance. *PAX5* deletions did not exhibit prognostic relevance in an adult *BCR*::*ABL1*–positive BCP-ALL cohort, in which some patients were treated with imatinib and chemotherapy and others with chemotherapy only.[40] In addition to these lesions, we identified an patient with imatinib resistance with a p.His1038Arg mutation in *ZEB2*; this gene has previously been described as B-cell development gene.[41,42] This specific mutation has been described as recurrent and associated with a poor prognosis in BCP-ALL.[43] Moreover, TKI-resistant samples were characterized by low phosphorylated and total ABL1 protein expression, suggesting less dependence on the BCR::ABL1 signaling cascade, which might be explained by alternative signaling as a consequence of the identified secondary lesions. The relation between ABL1 expression and TKI sensitivity was validated in a larger cohort on mRNA expression level of *ABL1* and the *BCR*::*ABL1* p190 transcript. Our data suggest that lesions affecting B-cell development genes, specifically *IKZF1*plus and *PAX5* deletions, were enriched in imatinib-resistant leukemic cells derived from *BCR*::*ABL1*–positive patients, and these patients would require alternative treatment.

TKI therapy–naïve *BCR*::*ABL1*–positive BCP-ALL samples with intrinsic ex vivo resistance to imatinib exhibit cross-TKI resistance toward the next-generation TKIs dasatinib and bosutinib. Our drug screen data also indicated cross-TKI resistance to nilotinib and

ponatinib. We showed that imatinib effectively inhibited BCR::ABL1 signaling in intrinsic imatinib-resistant samples. This suggests that the use of alternative TKIs, either targeting the ATP pocket or the myristate pocket, such as asciminib,[44] would not be beneficial for patients with intrinsic imatinib resistance. Alternative therapeutic options for patients with relapsed or refractory *BCR*::*ABL1*–positive BCP-ALL after TKI-based therapy are antibody based and cellular immunotherapies targeting CD19 or CD22, such as blinatumomab, inotuzumab, or chimeric antigen receptor T-cell therapy.[45-51] In adult patients, blinatumomab- or inotuzumab-induced remission after relapse is often followed by a hematopoietic stem cell transplantation.[48,51] These approaches might also be beneficial as firstline treatment for patients with TKI-resistant *BCR*::*ABL1*–positive BCP-ALL.

In conclusion, TKIs effectively inhibit BCR::ABL1 signaling; however, we here showed that there are intrinsic resistance mechanisms that may explain why not all patients with newly diagnosed *BCR*::*ABL1*–positive BCP-ALL respond to TKIs. The level of *BCR*::*ABL1* gene or (phosphorylated) BCR::ABL1/ABL1 protein expression could serve as an indicator of intrinsic resistance, which in turn is associated with secondary lesions in B-cell development genes and genes involved in JAK/STAT signaling. Our data suggest that the poor prognostic value of *IKZF1*(plus) deletions is linked to intrinsic mechanisms of TKI resistance other than *ABL1* kinase domain mutations in newly diagnosed pediatric and adult *BCR*::*ABL1*–positive BCP-ALL.

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## Authorship

Contribution: M.L.d.B. conceived this project and conceptualized it together with J.M.B., A.W.R., C.v.d.V., and I.v.O.; I.v.O., C.E.J.R., B.K., C.v.d.V., A.S.-S., and A.B. designed and performed the experiments and analyzed the results; H.A.d.G.-K., G.E., and A.W.R. provided samples and data; the manuscript was drafted by I.v.O., J.M.B., and M.L.d.B.; and all authors reviewed and approved the manuscript.

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