High *PDGFRA* expression does not serve as an effective therapeutic target in *ERG*-deleted B-cell precursor acute lymphoblastic leukemia

Several oncogenic aberrations of receptor tyrosine kinases have been reported in B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Herein we describe a subgroup of pediatric patients lacking common chromosomal aberrations ("B-other") but with high expression of platelet-derived growth factor receptor alpha (*PDGFRA*). Oncogenic fusions involving the tyrosine kinase domain of *PDGFRA* are recurrent in eosinophilic leukemia and are responsive to kinase inhibitors.[1] One case with a *FIP1L1-PDGFRA* fusion was reported in adult BCP-ALL.[2] We, therefore, tested for genomic aberrations underlying the overexpression of *PDGFRA* and evaluated the inhibition of PDGFRα as a treatment option in pediatric BCP-ALL. We found that high *PDGFRA* expression was not caused by structural aberrations of *PDGFRA*, was associated with *ERG* deletions, and that the *in vitro* and *ex vivo* response to PDGFRα inhibitors was limited in Nalm6 and primary BCP-ALL cells, respectively.

Genetic aberrations that define distinct subtypes are essential for diagnosis and risk evaluation of ALL. Gene expression microarray analysis of 654 pediatric ALL cases (data deposited in GSE87070) identified high *PDGFRA* expression (2log expression probe-set 203131_at >5.9) in 26 of 574 BCP-ALL cases (4.5%). High *PDGFRA* expression was found exclusively in cases belonging to the "B-other" (n=22) and *BCR-ABL1*-like (n=4) subtypes, and was not present in *ETV6-RUNX1*, high hyperdiploid (51-65 chromosomes), *TCF3-PBX1*, *BCR-ABL1* and *MLL*-rearranged BCP-ALL subtypes or T-ALL. (Figure 1A). *PGFRA* expression was confirmed by quantitative reverse transcriptase polymerase chain reaction analysis in 23 of 25 tested cases, was maintained in xenograft-derived cells of a *PDGFRA*-high case, and also found in the BCP-ALL cell line Nalm6 (*Online Supplementary Figure S1*).

Since high *PDGFRA* expression was mainly found in the "B-other" subtype, we analyzed clinical characteristics within this group. Age, white blood cell count, gender, risk stratification arm, and the frequency of relapse, death, and non-response (together defining "events") were not significantly different between *PDGFRA*-high and -low "B-other" cases (Table 1). However, cells from patients with high *PDGFRA* expression levels were more sensitive to prednisolone than were cells from patients with low *PDGFRA* expression levels, as measured with an *ex vivo* MTT assay previously described[3] (*P*=0.03, see also *Online Supplementary Figure S2*). *Ex vivo* sensitivity towards L-asparaginase or vincristine was not different.

Oncogenic fusions involving the tyrosine kinase domain of *PDGFRA* are recurrent in adult myeloproliferative neoplasms, but have not been reported in pediatric BCP-ALL. Using fusion gene-specific reverse transcriptase polymerase chain reactions, we did not detect any of the six *PDGFRA* fusions reported to date, which involve *FIP1L1, STRN, BCR, CDK5RAP2, ETV6,* and *KIF5B* (see *Online Supplementary Table S1* and *Online Supplementary Methods* for the primers).[1,4-7]

Novel translocations or other structural aberrations affecting the *PDGFRA* locus may be responsible for high *PDGFRA* expression. We performed fluorescence *in situ* hybridization (FISH; Cytocell, Cambridge, UK) with probes located upstream of *FIP1L1*, between *FIP1L1* and *PDGFRA*, and downstream of *PDGFRA* (Figure 1B). The cell line EOL1 served as a positive control, revealing one wildtype allele and two alleles with an interstitial deletion as reported (DSMZ website July 2017, *Online Supplementary Figure S3*). None of the 36 tested primary BCP-ALL samples and cell lines with high or low *PDGFRA* expression showed interstitial deletions between *FIP1L1* and *PDGFRA* or split FISH signals (Figure 1B and *Online Supplementary Figure S3*).

We studied the *PDGFRA* locus at high resolution by targeted locus amplification in 17 samples (Cergentis BV, Utrecht, the Netherlands). This technique allows amplification and sequencing of genomic regions that are in close proximity to a region of interest and is described in more detail in the *Online Supplementary Methods*. Two viewpoints were selected in intron 2 and exon 15 of *PDGFRA*. In EOL1, we confirmed an interstitial deletion on chromo-

Table 1. Clinical characteristics of patients with "B-other" B-cell precursor acute lymphoblastic leukemia with high *PDGFRA* expression.

Clinical feature	PDGFRA high			PDGFRA low			*P*	Odds ratio (95%-CI)
	Total (n)	Cases (n)	Frequency (%)	Total (n)	Cases (n)	Frequency (%)		
Age ≥10 years	22	9	41%	90	30	33%	0.62	
WBC ≥50/nl	22	4	18%	90	25	28%	0.43	
Male	22	15	68%	90	50	56%	0.34	
Treatment group HR	22	6	27%	87	39	45%	0.15	
Relapse	22	2	9%	90	19	21%	0.24	
Event	22	3	14%	90	26	29%	0.18	
Death	22	2	9%	90	21	23%	0.24	
Prednisolone LC_{50} ≥ 0.1 μg/mL	7	1	14%	26	18	69%	**0.03**	0.08 (0.002 -0.83)
Vincristine LC_{50} ≥ 0.39 μg/mL	7	4	57%	25	15	60%	1.00	
L-asparaginase LC_{50} ≥ 0.033 μg/mL	7	6	86%	25	18	72%	0.64	

"Total" indicates the number of cases for which data were available, "cases" indicates the number of cases in which the respective feature was identified. Frequencies were compared using the Fisher exact test and odds ratios are given with a 95% confidence interval (95% CI). *P*-values <0.05 are printed in bold. WBC: white blood cell count. Treatment group HR: high risk treatment arm of the respective protocol. Event: relapse, non-response after consolidation phase, or death. LC_{50}: 50% lethal concentration of *in vitro* drug sensitivity testing as described previously.[3]

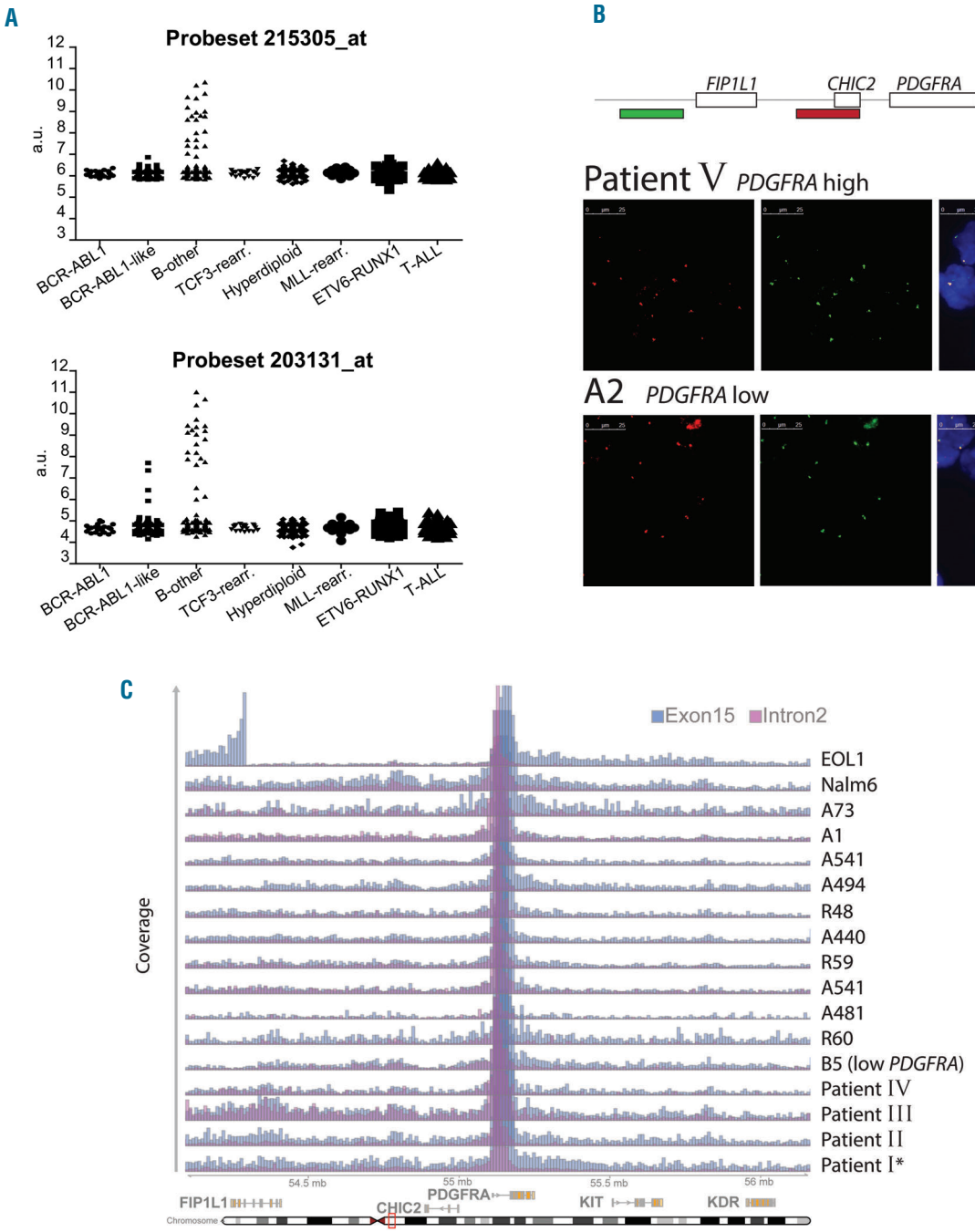

Figure 1. Genomics of *PDGFRA* in pediatric B-cell precursor acute lymphoblastic leukemia. (A) Gene expression of *PDGFRA* in the genetic subtypes of pediatric BCP-ALL. Expression was measured on Affymetrix U133 plus 2.0 GeneChips using two different probe sets. (B) Schematic overview and representative results of interphase FISH probing the *FIP1L1-PDGFRA* locus in one case with high and one case with low *PDGFRA* expression. Red: probe spanning the *CHIC2* gene; green: probes upstream of *FIP1L1* and downstream of *PDGFRA*, respectively. Merge: overlay of red and green signals, blue: DNA stained by DAPI. (C) Targeted locus amplification performed from two viewpoints within the *PDGFRA* locus. Bars represent the mean coverage over a 10 kb sliding window plotted on the y-axis, while chromosomal position is indicated on the x-axis. Coverage around the viewpoint was capped for data visualization.[7] Fourteen cases with high *PDGFRA*, one case with low *PDGFRA*, and two cell lines were tested. In case of translocations or interstitial deletions (e.g. causing the *FIP1L1-PDGFRA* fusion gene in EOL1), sequencing coverage drops steeply and rises again on a distant site. Samples marked by roman numerals (patients I to V) were used in *ex vivo* cytotoxicity assays in Figure 2. (D) Frequencies of secondary aberrations detected by means of multiplex ligation-dependent probe amplification and array comparative genomic hybridization in "B-other" patients with high or low *PDGFRA* expression. chr21 gain: partial or complete gain of chromosome 21, including germline trisomy. High *CRLF2*: high expression of *CRLF2* by gene expression array as described above. Frequencies were compared using the Fisher exact test. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$. For details see also *Online Supplementary Methods*.

some 4 which rearranges *FIP1L1* intron 12 to *PDGFRA* exon 12 (Figure 1C). The coverage pattern and sequence information obtained from Nalm6 and primary BCP-ALL samples did not indicate genomic aberrations in *PDGFRA*.

We compared recurrent copy number aberrations, detected by multiplex ligation-dependent probe amplification and array comparative genomic hybridization (as reported by Boer *et al.*[5]), between *PDGFRA*-high and -low "B-other" BCP-ALL cases (Figure 1D and *Online Supplementary Table S2*). The frequency of deletions in *IKZF1, EBF1, RB1, BTG1*, and *TCF3* did not differ significantly between "B-other" patients with high or low *PDGFRA* expression. *PAX5* and *CDKN2A* and/or B deletions were rare in patients with high *PDGFRA* (*P*=0.01 and

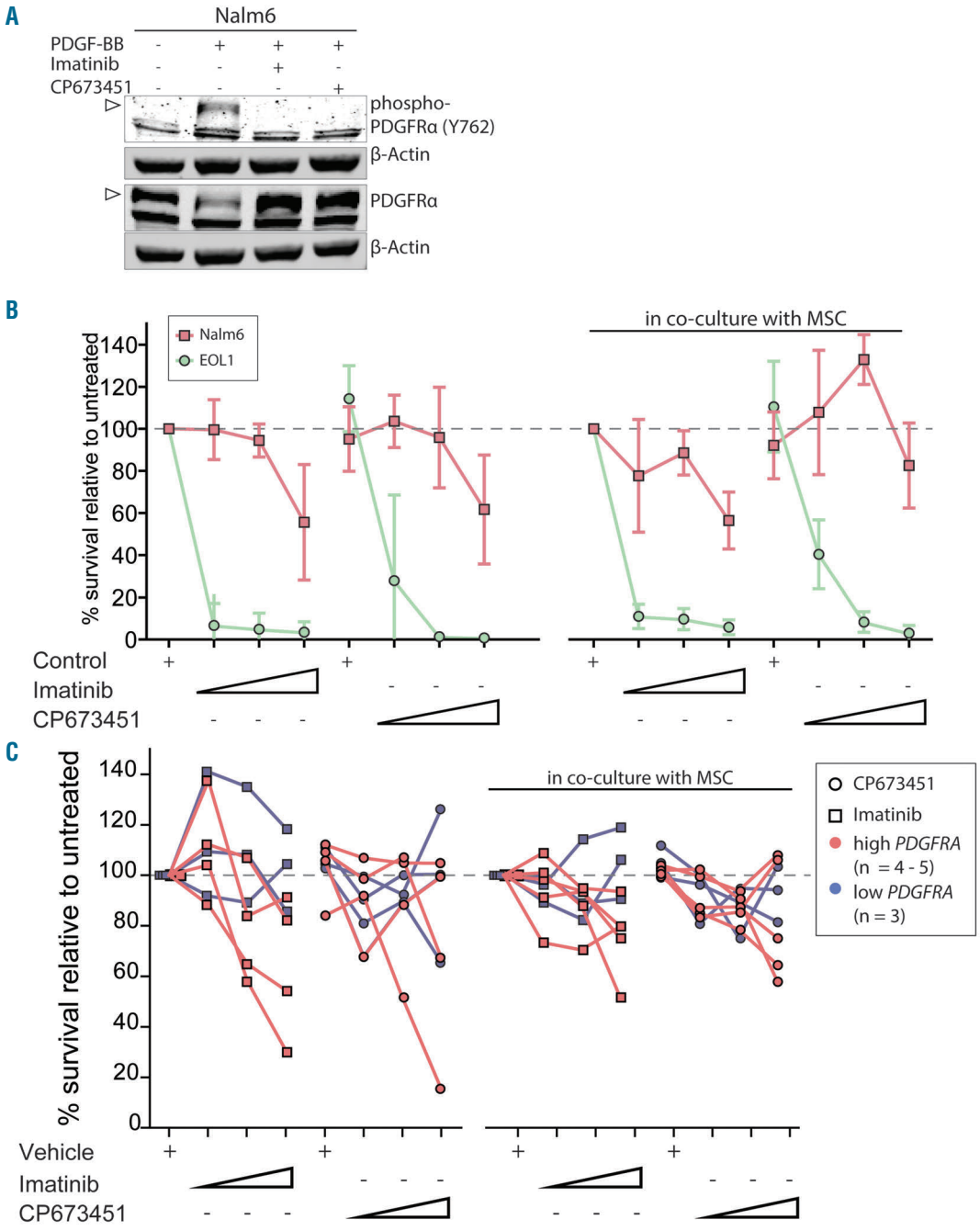

Figure 2. PDGFRα signaling and inhibition in pediatric B-cell precursor acute lymphoblastic leukemia cells. (A) Western blot for phosphorylated (Y762) and total PDGFRα protein in the cell line Nalm-6. Cells were treated or not with 10 μM imatinib or 1 μM CP673451 and 10 ng/mL recombinant human PDGF-BB for 10 min. Arrow heads indicate the expected band. Total protein load was measured by staining for β-actin. It should also be noted that the closely related *PDGFRB* is not expressed in cases with high *PDGFRA* expression. (B) and (C) *Ex vivo* sensitivity of cell lines and primary leukemic cells to PDGFRα inhibitors in an *ex vivo* co-culture model. Cell lines (4 to 6 replicates) (B) and primary leukemic cells (C) were cultured with or without mesenchymal stromal cells (MSC) and exposed to a range of imatinib conv (0.2 μM – 1.6 μM – 12.5 μM) or CP673451 (0.02 μM – 0.16 μM – 1.25 μM) for 4 days. Cell survival was analyzed by flow cytometry and is depicted relative to the untreated control. Vehicle represents the respective vehicle control for imatinib (water) or CP673451 (DMSO) treated conditions. For details see the *Online Supplementary Methods*. The results of individual samples are plotted and connected by lines.

P=0.03, respectively), and no loss of the entire chromosome-arm 9p was observed (*P*=0.02). No *ETV6* deletion (*P*=0.003), high *CRLF2* expression (*P*=0.04), or chromosome 21 gain (*P*=0.006) was found in *PDGFRA*-high patients. In contrast, 63% of patients with high *PDGFRA* expression carried *ERG* deletions (10/16), compared with 0% of *PDGFRA*-low "B-other" cases (0/71, *P*<0.001). Vice versa, all *ERG*-deleted cases expressed high levels of *PDGFRA* (*Online Supplementary Figure S4*). A distinct gene expression profile (referred to as R6 cluster) was described as being characteristic for, but not limited to, *ERG*-deleted cases.[9] Recently, Lilljebjörn *et al*. reported strong overlap of *DUX4* rearrangements with *ERG* deletions and provided evidence that the *DUX4* rearrangement is causal for this exclusive gene expression profile.[10] In this newly described BCP-ALL subtype, a truncated copy of the usually silenced germline transcription factor *DUX4* is inserted into an actively transcribed region and aberrantly expressed.[10,11] Zhang *et al*. showed that aberrant *DUX4* induces the expression of an alternative isoform of *ERG*, thereby directly linking the function of these two transcription factors.[12] Importantly, not all *DUX4*-rearranged cases carried *ERG* deletions, and they can arise at various points during leukemogenesis of *DUX4*-rearranged leukemia.[12,13] The identification of *DUX4* rearrangements at a genomic level is challenging because of varying integration sites, many repeats in the genome, and high GC content. We were unable to confirm *DUX4* rearrangements in cases with high *PDGFRA* expression, but strong evidence supports our conclusion: Yasuda *et al*. have shown that Nalm6 carries a *DUX4* rearrangement.[11] In addition, in the extensive dataset on *DUX4*-rearranged leukemia published by Zhang *et al*.[12], *PDGFRA* is highly expressed in *DUX4*-rearranged leukemia and its transcription start site was directly bound by *DUX4* (*Online Supplementary Tables S3a* and *S14* of reference 12). We therefore regard it as highly likely that the *PDGFRA*-high cases represent this novel *DUX4*-rearranged subtype.

These patients have a favorable outcome, but the risks of chemotherapy still demand new therapeutic approaches.[13] Because transcription factors are difficult to target with small molecule inhibitors, PDGFRα inhibition could represent a treatment option. First, we confirmed protein expression and activation of PDGFRα by western blotting using Nalm6 cells. These cells were first starved and afterwards briefly stimulated with 10 ng/mL recombinant PDGFR-ligand PDGF-BB. Western blot analysis revealed that the ligand activates the phosphorylation of PDGFRα at position Y762 and reduces total PDGFRα levels (Figure 2A). Exposure to the PDGFR inhibitors imatinib and CP673451 abrogated the ligand-induced activation of PDGFRα, and prevented the phosphorylation of Y762.

The sensitivity of BCP-ALL cells to PDGFRα inhibition was evaluated in an *ex vivo* co-culture model including bone marrow mesenchymal stromal cells (MSC). This model has been previously shown to improve the survival of primary BCP-ALL cells and also serves as a model of microenvironment-mediated drug resistance.[14] The cell line EOL1 was highly sensitive to PDGFR inhibitors (imatinib and CP673451) in culture with and without MSC, while Nalm6 showed reduced viability only at the highest inhibitor concentrations (Figure 2B). Primary BCP-ALL samples with high *PDGFRA* expression were marginally sensitive to imatinib, but not to CP673451, in mono-culture (Figure 2C, left side,) and in co-culture with MSC (Figure 2C, right side). No cytotoxic effect of PDGFR inhibitors was observed in cases with low *PDGFRA* expression (Figure 2C). For Nalm6 and three BCP-ALL samples with sufficient material, the assay was repeated including recombinant human PDGF-BB, but ligand exposure did not sensitize to PDGFR inhibition (*Online Supplementary Figure S5*). We, therefore, conclude that inhibition of PDGFRα signaling with imatinib or CP673451 does not strongly reduce viability in *PDGFRA*-expressing primary cells, nor in the *DUX4*-rearranged *PDGFRA*-high expressing Nalm6 cell line. This is in contrast to a strong reduction in survival seen for the positive control, the *FIP1L1-PDGFRA*-rearranged cell line EOL1.

In summary, we have identified a group of BCP-ALL cases with high expression of the receptor tyrosine kinase PDGFRα. No genomic aberrations affecting the *PDGFRA* locus were detected: fusion-specific polymerase chain reactions to all known translocations were negative, FISH to the *PDGFRA* locus showed no interstitial deletion or translocation, and targeted locus amplification revealed no structural aberrations. However, two-thirds of the cases carried *ERG* deletions and, vice versa, all *ERG*-deleted cases showed high *PDGFRA* expression. Therefore, high *PDGFRA* expression likely characterizes the newly discovered *DUX4*-rearranged subtype with frequent *ERG* deletions.

Western blot experiments in the Nalm6 cell line demonstrated ligand-dependent activation of PDGFRα which was inhibited by imatinib and CP673451. *Ex vivo* exposure to these inhibitors revealed that activation of PDGFRα is not predictive for cytotoxicity in *PDGFRA*-high BCP-ALL cells. The baseline clinical characteristics of *PDGFRA*-high cases were not significantly different from those of "B-other" cases with low *PDGFRA* expression, although the small group size may have limited the analysis. Interestingly, we found a marked *ex vivo* sensitivity towards prednisolone in *PDGFRA*-high cases, in line with the good prognosis reported for *ERG*-deleted/*DUX4*-rearranged leukemia.[10,11,13]

In conclusion, despite high *PDGFRA* expression, PDGFRα signaling was not essential to BCP-ALL cell survival *ex vivo*. Although *in vivo* data are warranted to corroborate these results, we conclude that targets other than PDGFRα should be explored for the cure of *PDGFRA*-high BCP-ALL.

Isabel S. Jerchel,[1] Danai Chatzivasileiou,[1] Alex Q. Hoogkamer,[1] Judith M. Boer,[1] H. Berna Beverloo,[2] Rob Pieters[3] and Monique L. den Boer[1]

[1]Department of Pediatric Oncology, Erasmus Medical Center-Sophia Children's Hospital, Rotterdam; [2]Department of Clinical Genetics, Erasmus Medical Center, Rotterdam and [3]Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands

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Correspondence: m.l.denboer@erasmusmc.nl

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