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BRIEF REPORT

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Frequency and prognostic impact of *PAX5* p.P80R in pediatric acute lymphoblastic leukemia patients treated on an AIEOP-BFM acute lymphoblastic leukemia protocol

Mareike Jung[1] | Maximilian Schieck[1] | Winfried Hofmann[1] | Marcel Tauscher[1] | Jana Lentes[1] | Anke Bergmann[1] | Marie Stelter[1] | Anja Möricke[2] | Julia Alten[2] | Brigitte Schlegelberger[1] | Martin Schrappe[2] | Martin Zimmermann[3] | Martin Stanulla[3] | Gunnar Cario[2] | Doris Steinemann[1]

[1]Department of Human Genetics, Hannover Medical School, Hannover, Germany

[2]Department of Pediatrics, University Hospital Schleswig-Holstein, Kiel, Germany

[3]Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany

Correspondence
Doris Steinemann, Department of Human Genetics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany.
Email: steinemann.doris@mh-hannover.de

Abstract

PAX5 is a member of the paired box (PAX) family of transcription factors involved in B-cell development. *PAX5*^{P80R} has recently been described as a distinct genetic B-cell precursor (BCP) acute lymphoblastic leukemia (ALL) subtype with a favorable prognosis in adults. In contrast, an unfavorable outcome has been observed in children. Our aim was to determine the frequency of *PAX5*^{P80R} in childhood BCP-ALL treated according to the Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster (AIEOP-BFM) ALL 2000 protocol and to evaluate its clinical significance within this study cohort. The analyses included 1237 patients with ALL treated in the AIEOP-BFM ALL 2000 trial with complete information for copy number variations (CNVs) of *IKZF1*, *PAX5*, *ETV6*, *RB1*, *BTG1*, *EBF1*, *CDKN2A*, *CDKN2B*, and *ERG*. A customized TaqMan genotyping assay was used to screen for *PAX5*^{P80R}. Sanger sequencing was used to confirm *PAX5*^{P80R}-positive results as well as to screen for second variants in *PAX5*. Agilent CGH + SNP arrays (e-Array design 85 320; Agilent Technologies) were performed in *PAX5*^{P80R}-positive patients to verify additional CNVs. Almost 2% (20/1028) of our BCP-ALL cohort were *PAX5*^{P80R}-positive. White blood cell counts higher than 50 000/μl as well as male sex were significantly ($P < .05$) associated with *PAX5*^{P80R}. Most of the *PAX5*^{P80R}-positive cases were 10 years of age or older. *PAX5*^{P80R}-positive samples were enriched for deletions affecting *PAX5*, *IKZF1*, *CDKN2A*, and *CDKN2B*. Compared to *PAX5*^{P80R}-wildtype BCP-ALL, *PAX5*^{P80R}-positive patients showed a significantly reduced 5-year overall survival ($P = .042$). Further studies should evaluate the interaction of *PAX5*^{P80R} with other genetic aberrations to further stratify intermediate risk pediatric BCP-ALL.

KEYWORDS

acute lymphoblastic leukemia, AIEOP-BFM ALL, B-cell precursor ALL, PAX5, pediatric acute lymphoblastic leukemia

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy of childhood and adolescence.[1] Improvement of therapeutic strategies, including application of risk-adapted treatment employing prognostic markers such as minimal residual disease (MRD), has increased long-term survival rates of pediatric ALL to over 80%.[2,3] In addition to treatment response a broad spectrum of genetic aberrations is increasingly being used for patient stratification.[4] High-resolution genome- and transcriptome-wide analyses have led to the description of new biological subgroups and advanced treatment strategies in pediatric ALL.[5,6] Improved definitions of alterations or signatures associated with a high relapse risk may help to further improve patients' outcome. Potential targets for therapy de-escalation might be able to reduce late therapeutic effects.

One of the genes most commonly affected by deletions, amplifications, translocations, or point mutations in ALL is *PAX5*.[7-9] *PAX5* is a member of the paired box (*PAX*) family of transcription factor genes and encodes a B-cell lineage-specific activator protein, which controls the identity of B lymphocytes throughout B-cell development from the pro-B to the mature B-cell.[10] A germline *PAX5* variant conferring susceptibility to pre-B-cell ALL has lately been described[11] and intragenic amplification of *PAX5* has been shown to be related to poor outcome and relapses.[12] A nucleotide exchange at aminoacid codon 80 (c.239C > G; NM_016734.2) of the *PAX5* gene (*PAX5*^{P80R}) causes a proline to arginine exchange in the DNA-binding domain of the PAX5 protein (Figure 1A).[13] This somatic *PAX5*^{P80R} variant has been observed with a frequency of 0.4% in standard ALL and 3.1% in high-risk pediatric B-cell precursor (BCP) ALL.[14] Importantly, in nearly all cases a biallelic alteration has been observed.[14] Due to its accumulation in the intermediate to poor outcome group,[14] we aimed to investigate if *PAX5*^{P80R} biallelic alteration represents a prognostic marker in pediatric ALL as observed in adult ALL.[15]

2 | MATERIALS AND METHODS

The present study included 1237 ALL patients diagnosed between August 1999 and May 2009 who have been enrolled in the international multicenter trial Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster ALL 2000 on treatment of pediatric ALL in Germany and had complete information available for copy number alterations of *IKZF1*, *PAX5*, *ETV6*, *RB1*, *BTG1*, *EBF1*, *CDKN2A*, *CDKN2B*, Xp22.33/Yp11.31 (PAR1 region; *CRLF2*, *CSF2RA*, and *IL3RA* genes), and *ERG*.[16] In addition, included patients had to have diagnostic DNA available for further molecular analyses (1237 out of 1323). A customized TaqMan genotyping assay (PAX5_P80R.ANH6G97; ThermoFisher) was used to screen for *PAX5*^{P80R} and positive results were confirmed by Sanger sequencing (Supplementary Table S1).

3 | RESULTS

We identified 20 *PAX5*^{P80R}-positive cases (1.9% of 1028 BCP-ALL, Supplementary Figure S1). As expected, none of the 197 patients with T-ALL were *PAX5*^{P80R}-positive (and none of the 12 patients with unknown immunophenotype as well [Supplementary Figure S2]). Only B-ALL cases were considered for the following statistical analyses. For 11 of the 20 *PAX5*^{P80R}-positive patients, cytogenetic examinations were available; all of these 11 patients belonged to the B-other group,[17-19] none showed *BRC-ABL*, t(4;11), *TCF3-PBX1* or *ETV6-RUNX1* fusions nor high hyperdiploidy or hypodiploidy. Yet, they were designated as B-other throughout the remaining document. For the remaining nine patients who lacked information on *ETV6/RUNX1*, *BCR/ABL1* and *MLL/AF4* no clear statement can be made regarding their classification to the B-other collective.

Next, we were interested to determine whether additional mutational events were present in the 20 *PAX5*^{P80R}-positive samples. MLPA data indicated a deletion of one *PAX5* allele in nine patients, leading to loss of heterozygosity. In confirmation with an allelic loss, the TaqMan assay showed a hemizygous *PAX5*^{P80R} genotype in eight cases (one case was addressed as heterozygous). Sequencing of the entire gene in the remaining 11 samples with no loss showed a second nucleotide variant in 10 cases. One of them appeared to be homozygous for *PAX5*^{P80R} and nine showed a second *PAX5* variant suggesting compound heterozygosity. Of those nine additional variants, five resulted in a frameshift and two were splice site variants. In addition, we identified one missense and one nonsense variant (Figure 1A). Four of the additional variants have previously been described as somatically acquired variants,[14] the other five have not been previously described. All variants were classified as loss of function variants. In summary, only one *PAX5*^{P80R}-positive patient had no detectable second variant, whereas 19 presented a second loss of function variant leading to *PAX5* biallelic alteration (and most likely complete loss of function) (Figure 1B and Supplementary Figure S2). For 18 of the *PAX5*^{P80R}-positive patients, follow-up material was collected during remission, which was used as a germline surrogate. In none of the available remission samples *PAX5*^{P80R} was detectable, showing that *PAX5*^{P80R} is not a germline variant but rather somatically acquired. This confirms previous observations.[15]

MLPA data were used to further analyze the copy number profile of the *PAX5*^{P80R}-positive patients (Figure 1B). As mentioned before, nine *PAX5*^{P80R}-positive samples showed an additional *PAX5* deletion due to 9p deletion including *CDKN2A/B*. *CDKN2A* was deleted biallelically in three and *CDKN2B* deleted biallelically in five cases. In addition, four samples had a monoallelic, intragenic *IKZF1* deletion (located at 7p12.2). Two of the four *IKZF1* deleted patients fulfilled the recently described *IKZF1*^{plus} criteria.[20] The *IKZF1*^{plus} criteria are fulfilled if in addition to an *IKZF1* deletion at least one additional deletion in *CDKN2A and/or CDKN2B* (homozygous deletion only), a deletion in *PAX5* or the *PAR1* region is present and an *ERG* deletion (*ERG*$_{del}$) is absent.[20] *ETV6* was deleted in one patient, as was *BTG1*. There were no alterations in *ERG*, *CRLF2*, *EBF1*, and *RB1* in *PAX5*^{P80R}-positive patients.

Agilent CGH + SNP arrays (e-Array design 85 320; Agilent Technologies, Supplementary Table S2) were used to screen for additional copy number alterations in five *PAX5*^{P80R} patients, who experienced an event (Figure 2A). This included the patient that appeared to be

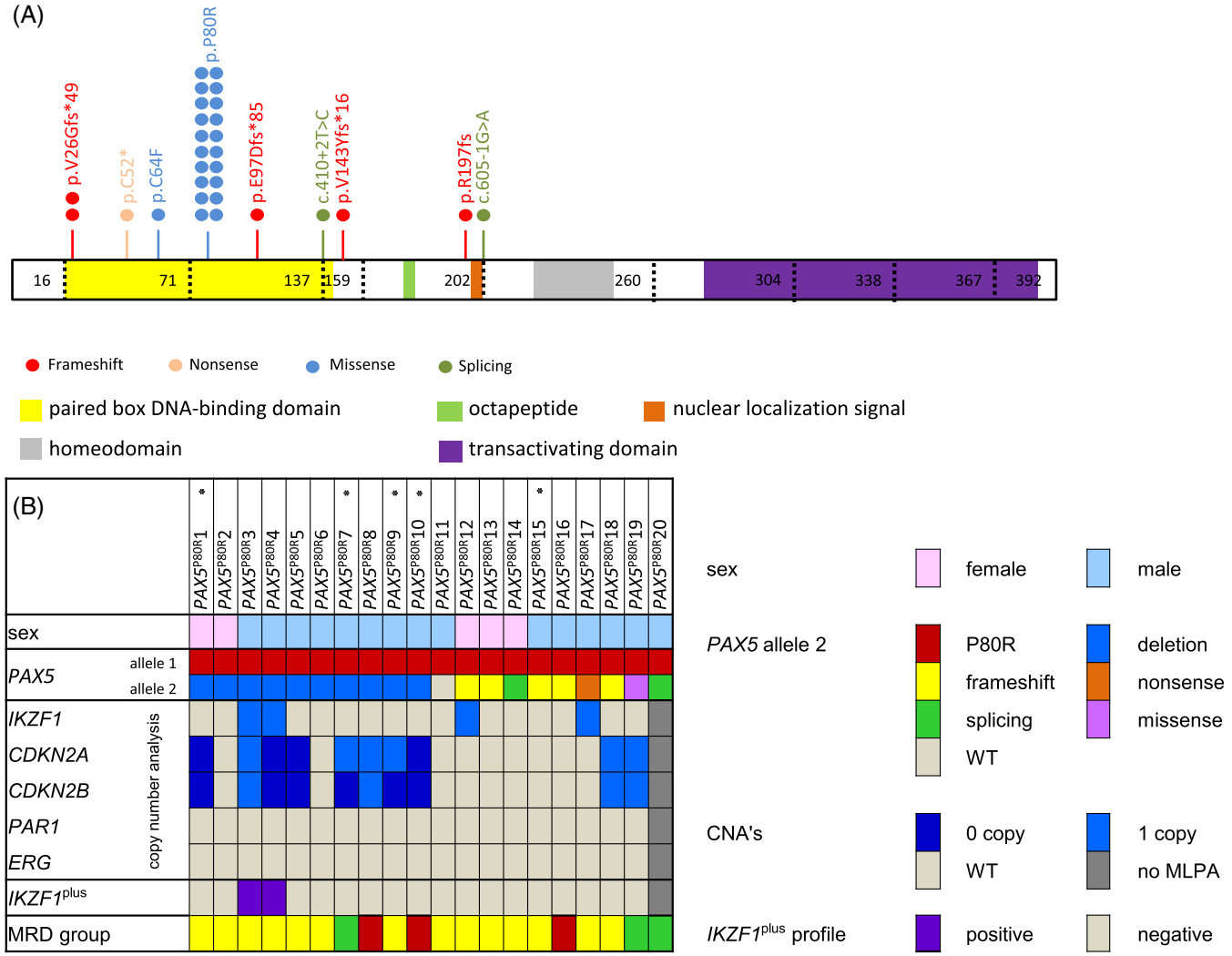

FIGURE 1 Summary of biallelic *PAX5* alteration involving *PAX5*^{P80R} observed in a cohort of 1237 pediatric BCP-ALL cases. A, Protein structure and variants of *PAX5*^{P80R}-positive patients in the Associazione Italiana Ematologia ed Oncologia Pediatrica-Berlin-Frankfurt-Muenster (AIEOP-BFM) ALL 2000 trial. Each dot symbolizes one variant; patients can harbor more than one variant. Variants leading to frameshifts are shown in red, nonsense variants in rose, missense variants in light blue and splice-site variants in dark green. B, Additional CNVs detected in *PAX5*^{P80R}-positive patients. Each column represents one patient; the characteristics examined are listed on the left hand. Sex is shown in rose for female and light blue for male patients. The status of *PAX5* is displayed in red for *PAX5*^{P80R}, blue for deletion of *PAX5*, yellow for variants leading to frameshift and orange for nonsense variants. Green represents splicing variants, pink missense variants and the WT is represented in light gray. The legend for all copy number analyses displays wildtype in light gray, loss of one copy in light blue and loss of two copies in dark blue. Copy numbers were detected via MLPA. Patients without MLPA data are depicted in dark gray. Patients that fulfill the *IKZF1*plus criteria are marked in purple, patients that do not fulfill the criteria are represented in light gray and patients without MLPA data in dark gray. The MRD group is labelled red for high risk patients, yellow for medium and green for standard risk patients. ALL, acute lymphoblastic leukemia; BCP, B-cell precursor; MLPA, multiplex ligation-dependent probe amplification; MRD, minimal residual disease; p.P80R, *PAX5*^{P80R}; WT, wildtype; *, patients that experienced an event and were analyzed by Agilent CGH + SNP arrays

homozygous for *PAX5*^{P80R} at first (#10). Importantly, array analyses showed a 9p deletion leading to hemizygosity of *PAX5* in this case, too (Supplementary Figure S3). Additionally, in this case and in another one, alterations at chromosome 20 (Supplementary Figure S3) were present. All but one of the five patients had a biallelic *CDKN2A/B* deletion, which was in line with the MLPA data (Supplementary Table S2).

In our cohort, white blood cell counts higher than 50 000/µl as well as male sex were significantly ($P < .05$) associated with the presence of *PAX5*^{P80R}. In addition, most of the *PAX5*^{P80R} cases were 10 years of age or older and only 15% were MRD-negative (Supplementary Table S3). Furthermore, 70% of our *PAX5*^{P80R}-positive cases were initially stratified to the intermediate risk (IR) group (Figure 1B), a group of patients still positive for MRD after induction.[3] IR patients neither fulfill criteria for treatment intensification, nor for therapy de-escalation and may benefit from improved treatment stratification.[3]

Compared to *PAX5*^{P80R}-wildtype BCP-ALL, *PAX5*^{P80R}-positive patients showed no significant differences in event-free-survival

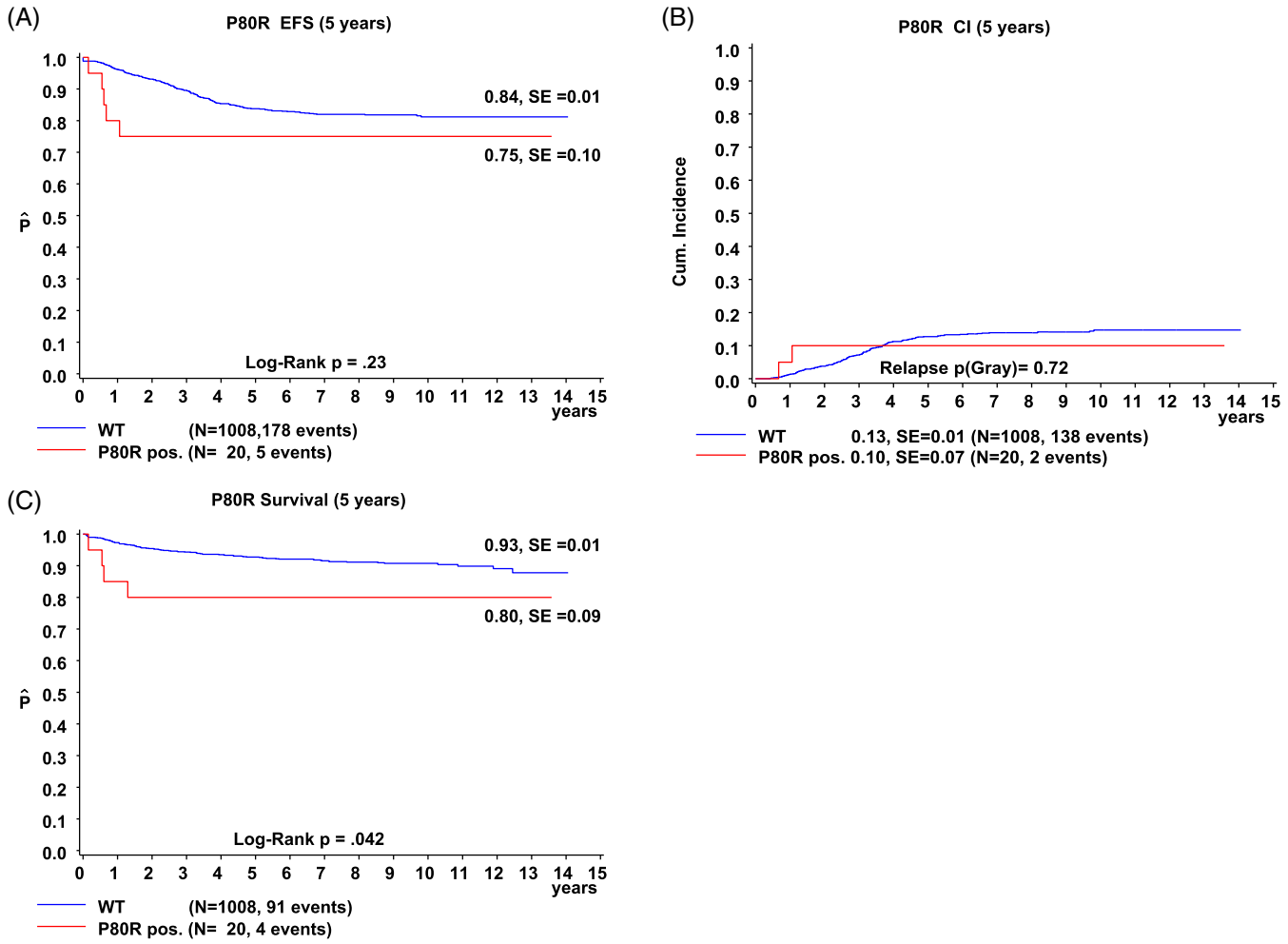

FIGURE 2 Long-term outcome data according to *PAX5*^{P80R} status. *PAX5*^{P80R}-WT patients are represented in blue, *PAX5*^{P80R}-positive patients in red. A, EFS, B, CI, and C, OS. CI, cumulative incidence; EFS, event-free survival; OS, overall survival; P80R, *PAX5*^{P80R}; WT, wildtype

(Figure 2A) and the cumulative incidence of relapse (Figure 2B). During the 5 years of follow-up, events were recorded for five patients: three died treatment-related, one deceased of a relapse and one relapsed but survived (Figure 2A). *PAX5*^{P80R}-positive patients had a significantly poorer 5-year overall survival (P = .042, Figure 2C). Although the overall survival was significantly different in univariate analysis, this was not observed in multivariate analysis (Supplementary Table S4).

4 | DISCUSSION

PAX5^{P80R} has been the focus of interest of several other studies.[13-15,21] While our research was focused on children, others have examined adults[13] or both[14,15] with opposing conclusions regarding outcome. In our BCP-ALL cohort 1.9% of the patients were positive for *PAX5*^{P80R}, whereas other trials have shown higher prevalence (ie, 2.4%,[14] 5.3%,[13] and 6.4%[15] of BCP-ALL). Most of our *PAX5*^{P80R}-positive patients were 10 years of age or older (P < .0001) matching the findings of increased prevalence with increasing age[14] and in young adults.[15] Bastian et al have described an equal sex distribution,[15] whereas Gu et al have observed a larger proportion of males (65.9%),[14] as we did in our cohort (75%, P < .05). As described previously, *PAX5*^{P80R}-positive samples were enriched for deletions of *PAX5*, *IKZF1*, *CDKN2A*,[13-15] and *CDKN2B*.[15] Bastian et al have recently described *PAX5*^{plus}, a subgroup defined by these enrichments, the frequent occurrence of *RAS* pathway mutations and the lack of assignability to a previously known group.[15] As formerly described none of our patients belonged to one of the known groups as well. However, we cannot confirm the favorable outcome of *PAX5*^{P80R} described in adults.[13,15] In fact, we observed rather intermediate to poor outcome[14] for patients harboring *PAX5*^{P80R}, which is in line with Gu et al (Figure 2).

With a rate of 1.9% in our study and the absence of established genetic risk markers, *PAX5*^{P80R}-positive patients might represent a novel subgroup in BCP-ALL, especially in adolescents and young adults. This subgroup seems to be further characterized by enrichment of copy number variations (CNVs) in specific genes other than

PAX5 and by an intermediate to poor outcome. Although *PAX5*^{P80R} is not a germline variant it may represent an initiating event in the development of B-ALL,[14] and subsequent biallelic loss of *PAX5* function further contributes to neoplastic progression.[22] One important limitation of our study is the rare occurrence of *PAX5*^{P80R} in children, which resulted in a moderate case number despite the large cohort. Nevertheless, our data underline the importance of *PAX5*^{P80R} in pediatric BCP-ALL and that international collaborative approaches with larger cohorts are warranted to further evaluate the interaction of *PAX5*^{P80R} with other genetic aberrations (eg, additional deletions in 9p, or complex aberrations of chromosome 20) and its impact on the patient's outcome.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Mareike Jung https://orcid.org/0000-0003-0728-8695
Maximilian Schieck https://orcid.org/0000-0001-5878-0546
Doris Steinemann https://orcid.org/0000-0001-8797-0816

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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