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## Targeted deep sequencing of CD34<sup>+</sup> cells from peripheral blood can reproduce bone marrow molecular profile in myelodysplastic syndromes

To the Editor:

Myelodysplastic syndromes (MDS) are clonal stem cell diseases of the bone marrow (BM). Acquired somatic mutations account for MDS development and constitute important diagnostic and prognostic markers. Currently, BM aspirates are used for genetic analysis in MDS patients. In this study, we aimed to investigate whether the high sensitivity of targeted deep sequencing (TDS) allows the detection of genetic alterations in peripheral blood (PB) of MDS patients. PB can be obtained by minimally invasive venipuncture and would be ideal for a comprehensive sequential monitoring of MDS over time.

This study includes 48 patients which were analyzed by TDS using either a hybridization probe-based (Supporting Information Table S1) or an amplicon-based TDS sequencing panel (Supporting Information Table S2). We analyzed bone marrow cells (BMC), PB mononuclear cells (PB-MC), and immunomagnetically enriched CD34<sup>+</sup> PB cells (PB-CD34) (Supporting Information Figure S1).

To identify molecular markers of clinical relevance, we screened genes previously reported to be frequently mutated in MDS and other myeloid malignancies.<sup>1,2</sup> The somatic origin of the variants was validated by comparison of the concurrent samples (BMC, PB-CD34, and PB-MC) with a paired germline sample, peripheral CD3<sup>+</sup> cells (PB-CD3).

TDS allowed the detection of potential MDS-related aberrations in 41/48 patients (85%). This was a significant increase of informative cases compared to the results of the classical cytogenetic approaches, chromosome banding analysis (CBA), and fluorescence in situ hybridization (FISH), which yielded an altered karyotype in 17/48 (35%) cases. The application of single nucleotide polymorphism arrays (SNP-A) allowed the detection of aberrations (submicroscopic copy number changes or copy number neutral loss of heterozygosity) in seven additional patients with normal karyotype (Supporting Information Tables S3 and S4).

Overall, the combined application of classical cytogenetics, molecular karyotyping, and TDS identified genetic alterations in 43/48 (90%) patients.

TDS allowed the identification of 105 mutations in 24 genes. The highest mutation frequencies were observed for *TET2* ( $n = 13$ , 27%), *SF3B1* ( $n = 10$ , 21%), *SRSF2* ( $n = 10$ , 21%), *ASXL1* ( $n = 7$ , 16%), *EZH2* ( $n = 6$ , 13%), *ZRSR2* ( $n = 6$ , 13%), *RUNX1* ( $n = 5$ , 10%), *U2AF1* ( $n = 5$ , 10%), and *TP53* ( $n = 4$ , 8%) (Supporting Information Figure S2).

As previously reported we detected an increased number of genetic lesions in high-risk WHO subtypes (Supporting Information Figure S3A) and found a correlation between the average number of genetic aberrations and the IPSS-R prognostic risk score (Supporting Information Figure S3B).<sup>1,2</sup> Interestingly, low risk patients displayed an increased number of somatic mutations compared to the number of lesions detected by CBA or SNP-A. This observation is consistent with data published by Tefferi et al. indicating that TDS can significantly improve prognostication and risk stratification in lower risk MDS patients.<sup>3</sup>

Comparison of the TDS results from BM and PB revealed that TDS allows detection of somatic mutations also in PB samples (Figure 1A). For non-enriched PB, this was previously shown,<sup>4</sup> while in the current study we have added PB-CD34. We were able to detect 93/105 mutations (89%) in all sample types (BMC, PB-CD34, and PB-MC) (Supporting Information Table S5). In BMC, we found 100/105 (95%), in PB-CD34 102/105 (97%), and in PB-MC 95/105 (90%). Thus, enrichment of CD34<sup>+</sup> cells increased the detection rate of mutations in PB.

Besides the qualitative detection of somatic mutations, TDS allows the quantification of the mutation burden by the variant allele frequency (VAF) and the estimation of the tumor clone size.

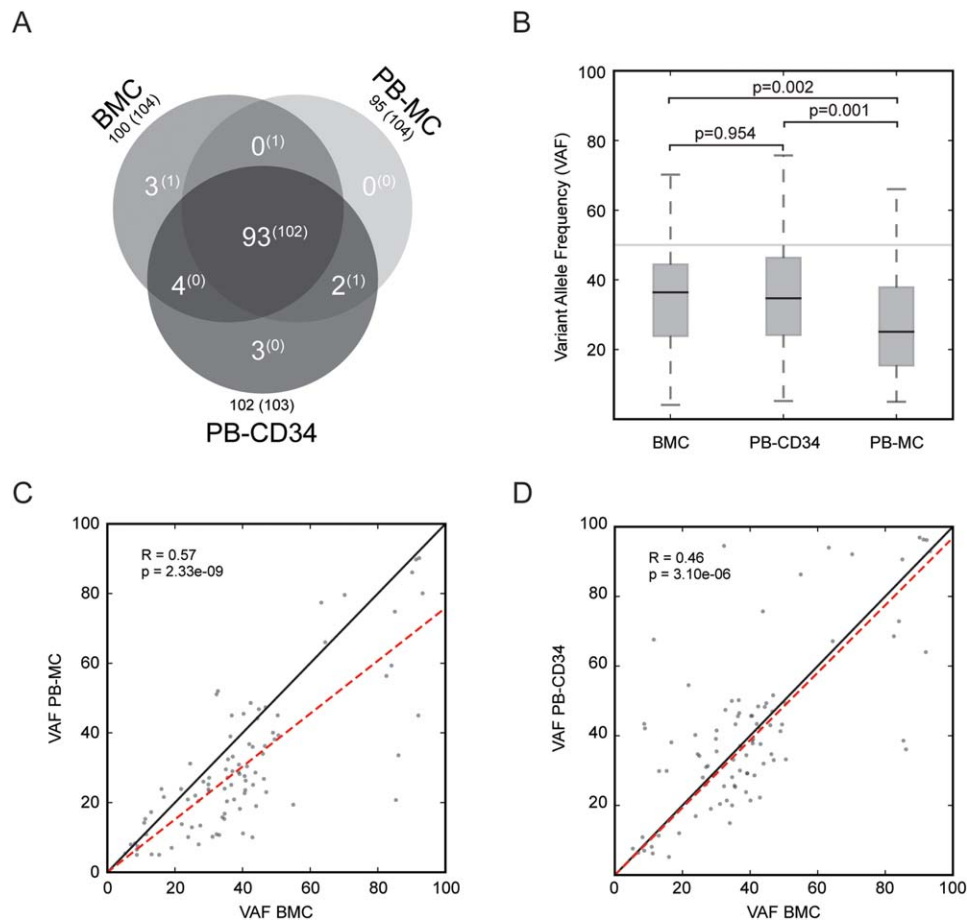
The measured VAF values were significantly lower for PB-MC (median = 25.1%) compared to BMC (median = 36.4%,  $P = .002$ ) (Figure 1B,C). Decreased VAF values in PB were also reported by Mohamedali et al.<sup>4</sup> However, the differences were not statistically significant. The significant deviation that we observed potentially resulted from a more stringent removal of SNPs in our dataset due to the analysis of the germline sample (CD3<sup>+</sup>).

The decreased VAF values measured in PB-MC are consistent with the lower detection rates in PB-MC samples since low frequency mutations drop below the detection limit of the applied TDS analysis (5%) in PB (Supporting Information Table S5 and Figure S4). Including mutations below the detection threshold of 5% VAF allowed the recovery of nearly all mutations (102/105) in BMC, PB-CD34 as well as PB-MC and resulted in the discovery of two additional mutations that might have clinical relevance (data not shown). The currently ongoing optimization of NGS sample preparation kits will facilitate the detection of mutations with VAF values below 5%, improving the reliability of the method especially for challenging sample types such as PB.

In order to overcome the limitations of PB in the quantification of somatic mutations, we additionally tested CD34<sup>+</sup>, which were immunomagnetically enriched from PB. Although the yield and purity of CD34<sup>+</sup> cells depends on physiological conditions such as disease state or therapy and technical challenges, we were able to obtain sufficient

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**FIGURE 1** Recovery of mutations in BM and PB samples by TDS. **A**, Most mutations were detectable in samples from BM and PB. Overall, 105 mutations were found. A total of 93 aberrations were recovered in BMC, CD34<sup>+</sup> cells enriched from PB (PB-CD34) as well as in mononuclear PB cells (PB-MC). Ten mutations were not recovered in PB-MC samples. Five mutations were not detected in BMC samples. Three additional aberrations were exclusively found in BMC. Inconsistent recovery was apparent mostly for mutations with low VAF values below 5%. Numbers in parentheses were obtained by including variants detected at <5% VAF values after reanalysis. **B**, Distribution of the measured VAFs for the samples from BMC, PB-CD34, and PB-MC. The VAF values of the second and third quartiles are between 15% and 45% for all sample types. The median VAF (black horizontal lines in the boxes) for BMC and PB-CD34 is 36.4% and 34.7%, respectively. The value for PB-MC is significantly lower (25.1%,  $P = .001$  and  $P = .002$ ).  $P$ -values were determined according to Mann–Whitney–Wilcoxon test. **C, D**, Mutual correlation of the VAF for the individual mutations identified in BMC, PB-CD34, and PB-MC. Dots denote the VAF of individual mutations, bisecting lines are marked in black, and regression lines are shown as dashed red lines.  $R$  denotes the Spearman Correlation Coefficient.  $P$  denotes the Spearman Correlation  $P$ -value. **C**, Values of PB-MC are lower compared to the measured VAF from BMC. Individual mutations with values above 50% indicate potential copy number variations. **D**, In contrast, concordance between the VAF of BMC and PB-CD34 is apparent as most values cluster near the bisecting line

DNA from PB-CD34 for TDS in all cases (see Supporting Information Methods). Regarding the total dataset, we did not observe differences in the VAF distribution between BMC (median = 36.4%) and PB-CD34 (median = 34.7%,  $P = .954$ ) (Figure 1B,D).

A similar experimental setup was previously used by Brulke et al.<sup>5</sup> The authors applied FISH analysis on CD34<sup>+</sup> cells from PB and BM and observed a slight but not significant difference. It is likely that we did not observe such differences due to the analysis of unselected BMC.

It is noteworthy that we also observed apparent variations of the VAF values in PB-MC compared to BMC or PB-CD34 for some mutations. However, in most cases only individual mutations of the patient were affected and in just two cases the differences would have resulted in a changed assessment of the clonal hierarchy that was estimated by the VAF (Supporting Information Table S5).

Taken together, our data indicate that TDS allows the adequate detection of somatic mutations from BM, circulating CD34<sup>+</sup> cells and PB-MC. However, the malignant cell population is less abundant in the PB-MC fraction and therefore the detection and especially the quantification of clonal somatic mutations is more challenging in this sample type. Immunomagnetic enrichment of circulating CD34<sup>+</sup> cells from PB allowed to obtain TDS data that are well comparable to the results from BMC. Therefore, we recommend to use CD34<sup>+</sup> cells from PB in diagnostic TDS testing to reduce invasive BM aspirates especially regarding patient monitoring.

#### CONFLICT OF INTEREST

Nothing to report.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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## Hemoglobin response to ferric citrate in patients with nondialysis-dependent chronic kidney disease and iron deficiency anemia

## To the Editor:

Anemia is a common complication in patients with chronic kidney disease (CKD) and often occurs due to iron deficiency. Causes of iron deficiency in CKD include upregulation of hepcidin (a peptide hormone that reduces iron transport across enterocytes) leading to decreased dietary iron absorption, impaired iron release from body stores associated with heightened inflammation, and gastrointestinal blood loss. All result in insufficient bone marrow iron availability and inefficient erythropoiesis, leading to iron deficiency anemia.<sup>1</sup>

Recent clinical practice guidelines from *Kidney Disease: Improving Global Outcomes* recommend using oral or intravenous iron before erythropoietin-stimulating agents for the treatment of iron deficiency anemia in patients with nondialysis-dependent (NDD)-CKD.<sup>1</sup> Oral iron preparations are typically ineffective and/or poorly tolerated due to gastrointestinal side effects.<sup>1</sup> Intravenous iron is used infrequently in nephrology offices as it requires intravenous infusion in a monitored setting with facilities for resuscitation because of risks of serious adverse drug events, including hypersensitivity reactions.<sup>1</sup>

In a randomized placebo-controlled trial of ferric citrate in 234 patients with NDD-CKD and iron deficiency anemia,<sup>2</sup> ferric citrate-treated patients were significantly more likely to achieve a  $\geq 1.0$  g/dL increase in hemoglobin (52.1% vs. 19.1% with placebo;  $P < .001$ ) during the 16-week randomized phase; the least-squares mean (LSM) relative change in hemoglobin was 0.84 g/dL (95% confidence interval [CI], 0.58–1.10).<sup>2</sup>

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