TO THE EDITOR:

The extent of residual WT HSPCs is associated with the degree of anemia in patients with *SF3B1*-mutated MDS-RS

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Myelodysplastic syndrome with ring sideroblasts (MDS-RS) constitutes a unique entity mainly driven by mutations in the *SF3B1* splice factor gene. Studies of patients with lower-risk MDS show that distinct MDS stem cells propagate the disease, and we recently identified the hematopoietic stem cell (HSC) as the exclusive cell of origin in *SF3B1*-mutated MDS-RS.[1-3]

Although patients with MDS-RS rarely develop leukemia, a large proportion of patients develop progressive anemia, which frequently leads to a chronic need for transfusions.[2,4,5] There is limited biological understanding as to why a particular patient shifts from stable to deteriorating erythropoiesis; consequently, specific preemptive strategies are not available. First-line treatment with erythropoietin-stimulating agents (ESAs) leads to clinical response in 45% to 75% of patients, and median duration of response is 1.5 to 3 years.[6-8] A large prospective observational study found more durable effects if treatment was initiated before the onset of transfusion need.[9] Current predictive models for response to ESAs encompass transfusion intensity (TI), serum erythropoietin (S-EPO), and International Prognostic Scoring System (IPSS) and Revised IPSS (IPSS-R) scores. However, patients with MDS-RS are now diagnosed earlier and rarely display high TI or elevated S-EPO, which limits the usefulness of these models.[10,11]

Earlier studies indicated that treatment with ESAs in MDS induces erythroid differentiation and reduces bone marrow (BM) apoptosis.[12-14] However, our knowledge of how clonal involvement in hematopoietic stem and progenitor cells (HSPCs) correlates with progression of anemia and response to treatment remains limited. Furthermore, it is not known to what degree ESAs target mutant and wild-type (WT) BM cells.

Our study used diagnostic and longitudinal samples from patients with MDS-RS to address these questions. The primary aim was to analyze cellular and genetic parameters associated with treatment need (TN) status. Secondary aims encompassed the assessment of potential effects induced by successful ESA treatment on HSPCs and the status of ESA refractory patients. We selected *SF3B1*-mutated MDS-RS as a clinical model system because the MDS-RS phenotype is frequently driven by a mutation only in *SF3B1*, which facilitates clonal tracking.

Fifty-seven patients with *SF3B1*-mutated MDS-RS who were diagnosed and treated according to European guidelines[6] (supplemental Table 1) were included in the study. The criteria for stable asymptomatic anemia (no treatment need [NTN]) and symptomatic anemia with or without transfusions (TN) were stable during the inclusion period (2000 to 2019) (supplemental Table 2). The study was approved by the Swedish Ethical Review Authority 2010/427-31/3 and 2017/4:6, and patients provided informed consent. Clinical and morphological information, including mutational status and *SF3B1* variant allele frequency (VAF) in BM aspirates, was obtained at diagnosis using routine next-generation sequencing (NGS); 27 patients had NTN, whereas 30 had symptomatic anemia and received treatment with ESAs.

SF3B1 VAF in clinical samples was assessed during response to ESAs in 16 patients (Table 1). HSPC sorting and analysis were performed for 21 patients, as previously described (supplemental Methods),[1,3,15] 8 of whom underwent paired HSPC analyses encompassing pre-treatment and response to ESAs stages.

NGS was performed as described previously.[3] Droplet digital polymerase chain reaction (ddPCR) analysis, PCR reaction mixture preparation, droplet generation procedures, and droplet analysis for emission in the HEX or FAM fluorescent signal channels using a QX200 Droplet Digital PCR System (Bio-Rad, Berkeley, CA) and QuantaSoft version 1.15 (Bio-Rad) are described in detail in the supplemental Methods.

Statistical analysis was performed by using GraphPad Prism v. 9. Unpaired testing was performed with the Mann-Whitney U test, and paired testing of values from the same donor at different time points was performed with the Wilcoxon matched-pairs signed-rank test in GraphPad Prism.

Figure 1A and supplemental Figure 1 summarize the experimental design and methods used to investigate HSPC subsets during stable anemia, at the onset of symptomatic anemia, during successful treatment with ESAs, and at ESA refractoriness and chronic transfusion need. Twenty-seven (47%) of 57 newly diagnosed patients with MDS-RS had asymptomatic stable anemia, and no patients had high TI or S-EPO >100 U/L at onset of TN; hence, no

Table 1. Clinical characteristics of patients with MDS-RS and *SF3B1* mutation with and without TN and during EPO response

| | At diagnosis | | | | | At follow-up | | |
| | NTN | | TN (before EPO) | | | Receiving EPO during response | | |
Variable	No. (%)	Median (IQR)	No. (%)	Median (IQR)	P*	No. (%)	Median (IQR)	P†
Total no. of patients	27		30			16		
Age, y		72 (67-77)		76 (69-82)	.26			
Sex					.79			
Male	15		12					
Female	12		18					
Blood								
Hemoglobin, g/dL‡		10.8 (10.2-11.6)		9.0 (8.0-10.0)	<.001		10.7 (10.3-11.7)	<.001
White blood cell count × 10⁹/L		4.7 (4.1-7.4)		5.3 (4.8-6.7)	.53		4.9 (4.1-6.5)	.50
Absolute neutrophil count × 10⁹/L		2.7 (2.0-5.2)		3.4 (2.2-4.5)	.94		2.7 (1.6-4.1)	.61
Platelet count × 10⁹/L		282 (205-312)		277 (211-376)	.68		305 (238-375)	.79
S-EPO, U/L		32 (16-48)		40 (29-62)	.13		NA	NA
S-ferritin, μg/L		358 (246-519)		480 (281-756)	.43		472 (264-790)	.54
BM								
Cellularity (%)		60 (45-70)		60 (50-70)	.58		70 (70-90)	.03
Blasts (%)		2.0 (1.0-3.5)		2.5 (1.5-3.5)	.09		1.5 (0-2.5)	.04
Erythropoiesis (%)		34 (15-43)		28 (18-40)	.30		32 (26-55)	.54
Ring sideroblasts (%)		40 (29-53)		35 (25-52)	.64		40 (38-55)	.13
IPSS-R (% of known)								
Very low	17 (63)		5 (18)		<.001	NA		NA
Low	10 (37)		21 (75)			NA		
Intermediate	0 (0)		2 (7)			NA		
Not known/missing cytogenetics	0 (0)		2 (NA)			NA		
Mutations (% of known)								
SF3B1 only	17 (63)		16 (53)		.81	11 (69)		.47
SF3B1 + 1	7 (26)		9 (30)			2 (12)		
SF3B1 + ≥2	3 (11)		5 (17)			3 (19)		
SF3B1 VAF		36 (27-43)		35 (30-39)	.85		36 (31-43)	.63

Patients were diagnosed with (1) MDS-RS with single lineage dysplasia or multiple lineage dysplasia (MDS-RS-SLD/MLD) or (2) MDS/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T); all patients had *SF3B1* mutation. Disease progression was rarely observed. Transformation to acute myeloid leukemia (AML) occurred in 1 of 57 patients; this patient had mutations in *SF3B1*, *TET2*, and *RUNX1* at time of diagnosis, responded to EPO for more than 4 years, and developed AML after 7 years. A total of 20 patients were sequenced at 2 time points. In patients re-evaluated while receiving successful EPO treatment (at a median treatment period of 26 months [range, 10-80 months]), only 1 of 9 patients acquired additional mutations (*ZRSF2* and *TP53* in the same patient). In patients re-sequenced at time of loss of response to EPO, 1 of 11 gained an additional mutation (*TET2*).

IQR, interquartile range; NA, not applicable.

*NTN vs TN (before EPO); Wilcoxon rank sum test for continuous variables, Fisher's exact test for categorical variables.

†Receiving EPO during response vs TN (before EPO).

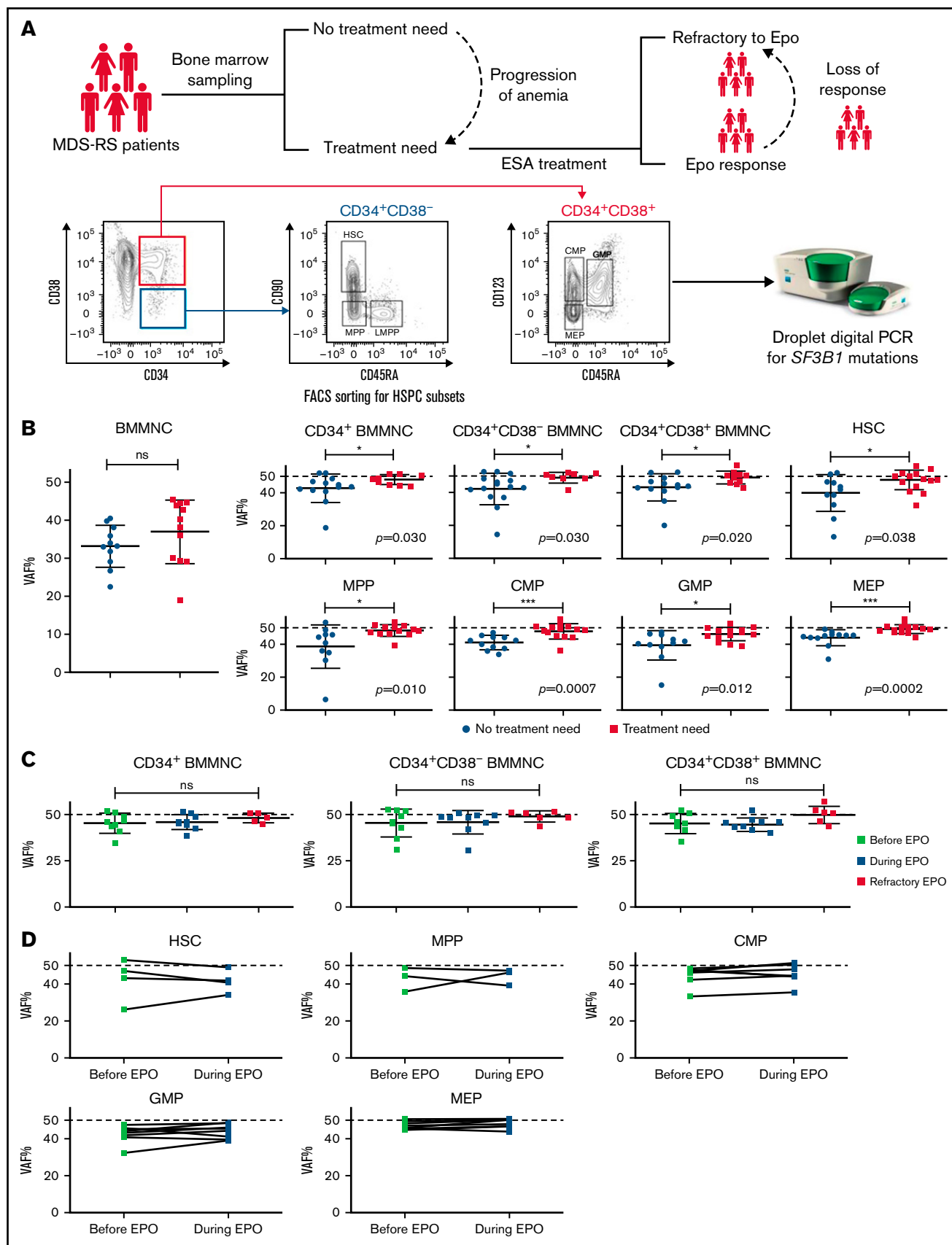‡Hemoglobin for transfused patients entered as 8.0 g/dL.

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Figure 1.

patients had a low probability of response according to published predictive models.[6]

Except for hemoglobin levels, no clinical parameters, including S-EPO levels, number of co-mutations, or *SF3B1* VAF in whole BM were significantly associated with TN (Table 1; supplemental Table 1). An IPSS-R status of "very low" was associated with stable asymptomatic anemia, likely reflecting the trend toward a lower percentage of BM blasts in this group (supplemental Table 2).

We then analyzed the HSPC compartment and measured the VAF in density gradient–separated BM mononuclear cells, total CD34$^+$, and different HSPC subsets (Figure 1B). We previously reported that the frequencies of HSPC populations in MDS-RS are similar to those of normal age-matched controls,[3] and here we show that HPSC frequencies do not differ statistically between patients with and without TN (supplemental Figure 1).

The *SF3B1* VAF in BM mononuclear cells (Figure 1B) did not differ between patients with TN and those without TN, in line with *SF3B1* VAF in BM aspirates (Table 1). However, patients without TN showed significantly lower clonal involvement in CD34$^+$ and CD34$^+$CD38$^+$ sorted progenitors, as well as in immature CD34$^+$CD38$^-$ HSCs. These differences were significant in all HSPC subsets (Figure 1B).

We next investigated whether a complete erythroid response to ESAs was associated with an increased fraction of residual *SF3B1* WT HSPCs. We obtained paired samples from 8 patients with TN before the start of treatment with ESAs as well as in complete erythroid response (hemoglobin ≥11.5 g/dL without transfusions). Development of *SF3B1* clonal involvement in HSPCs during response to ESAs showed an inconsistent pattern with no significant changes in any of the subfractions analyzed. Samples from transfusion-dependent patients obtained during transfusion need after failed treatment with ESAs showed fully clonal CD34$^+$, CD34$^+$CD38$^+$, and CD34$^+$CD38$^-$ subpopulations (Figure 1C-D). In addition, clinical and morphological parameters obtained during response did not change in comparison to pretreatment values (Table 1). These data suggest that treatment with ESAs mainly affects more mature cells than HSPCs or acts equally on WT and mutated cells.

Although patients with MDS-RS who have an SF3B1 driver mutation have a high risk of developing anemia and resistant transfusion dependency, this primary patient cohort showed that almost 50% of patients are diagnosed before the onset of symptomatic anemia. It has been demonstrated that the need for transfusions has a negative impact on overall survival and quality of life, and that initiating treatment with ESAs before the onset of a permanent TN is associated with significantly better response rates and duration.[16] A clear goal for MDS-RS management is thus to prevent the development of symptomatic anemia and TN.

This is, to our knowledge, the first study assessing HSPC clonality in relation to the degree of anemia and treatment status. We demonstrate that conventional clinical predictors of response do not explain the degree of anemia in newly diagnosed patients. Instead, the prevalence of residual *SF3B1*-WT HSPCs, although a minority, is associated with freedom from TN. In agreement with this, deteriorating erythropoiesis and, in particular, refractoriness to ESAs is associated with a very high clonal involvement. Our studies indicate that treatment with ESAs does not change this balance within the investigated time frames; however, we suggest that it would be relevant to assess clonal involvement in HSPCs or more practically in total CD34$^+$ BM cells when novel therapeutic approaches are evaluated.[17] Our results also have general implications for studies of clonal hematopoiesis, and we propose that protection of residual WT HSPCs is an important goal for this patient cohort.

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Figure 1 (continued) Mutation burden of CD34$^+$ BM populations is significantly lower in patients with MDS-RS with asymptomatic anemia than in patients with TN. (A) Study overview. BM samples from patients with MDS-RS were taken at diagnosis, at onset of TN, during successful ESA treatment, and in a refractory state. Fluorescence-activated cell sorting (FACS) was used to isolate defined HSPC compartments. VAF was determined in all populations using ddPCR. (B) VAF of *SF3B1* mutations measured in different HPSC subpopulations in patients with NTN (blue) and in patients with TN (yellow): bulk BM mononuclear cells (BMMNCs; n = 13), CD34$^+$ BMMNCs (n = 12 NTN; n = 10 TN), CD34$^+$CD38$^-$ BMMNCs (n = 15 NTN; n = 8 TN), CD34$^+$CD38$^+$ BMMNCs (n = 13 NTN; n = 10 TN), HSCs (n = 11 NTN; n = 14 TN), MPP cells (n = 10 NTN; n = 14 TN), CMP cells (n = 11 NTN; n = 14 TN), GMP cells (n = 11 NTN; n = 14 TN), and MEP cells (n = 12 NTN; n = 14 TN). The numbers of investigated subpopulations in each graph reflect subpopulations with enough cells to allow for statistical comparison. A dotted horizontal line is added at 50% VAF to indicate the theoretical maximum VAF of heterozygous *SF3B1* mutation. Error bars indicate the standard deviation. (C) VAF of *SF3B1* mutations measured in paired samples from patients with TN: before EPO treatment (n = 9; light green), during successful EPO treatment (n = 9; dark green), and in patients who had become refractory to EPO (n = 6; red). The response samples were taken at a median of 21 months (range, 6-71 months) from start of EPO treatment. (D) VAF analysis of *SF3B1* mutations in paired sequential samples from patients (n = 8) taken before and during successful EPO treatment. Analysis was performed in HSCs, and MPP, CMP, GMP, and MEP cells. Significance was tested with paired two-tailed Wilcoxon tests. Mann-Whitney *U* test: *$P < .05$; ***$P < .001$. CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; LMPP, lymphoid-primed multipotent progenitor; MEP, megakaryocyte-erythroid progenitor; MPP, multipotent progenitor; ns, not significant.

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