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Impact of red blood cell transfusion dose density on progression-free survival in patients with lower-risk myelodysplastic syndromes

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ABSTRACT

Progression-free survival (PFS) of patients with lower-risk myelodysplastic syndromes (MDS) treated with red blood cell transfusions is usually reduced, but it is unclear whether transfusion dose density is an independent prognostic factor. The European MDS Registry collects prospective data at 6-monthly intervals from newly diagnosed lower-risk myelodysplastic syndromes patients in 16 European countries and Israel. Data on the transfusion dose density - the cumulative dose received at the end of each interval divided by the time since the beginning of the interval in which the first transfusion was received - were analyzed using proportional hazards regression with time-varying co-variates, with death and progression to higher-risk MDS/acute myeloid leukemia as events. Of the 1,267 patients included in the analyses, 317 died without progression; in 162 patients the disease had progressed. PFS was significantly associated with age, EQ-5D index, baseline World Health Organization classification, bone marrow blast count, cytogenetic risk category, number of cytopenias, and country. Transfusion dose density was inversely associated with PFS

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($P<1x10^{-4}$): dose density had an increasing effect on hazard until a dose density of 3 units/16 weeks. The transfusion dose density effect continued to increase beyond 8 units/16 weeks after correction for the impact of treatment with erythropoiesis-stimulating agents, lenalidomide and/or iron chelators. In conclusion, the negative effect of transfusion treatment on PFS already occurs at transfusion densities below 3 units/16 weeks. This indicates that transfusion dependency, even at relatively low dose densities, may be considered as an indicator of inferior PFS. This trial was registered at *www.clinicaltrials.gov as #NCT00600860.*

Introduction

Red blood cell transfusions (RBCT) are the major component of the supportive care of patients with myelodysplastic syndromes (MDS). The life expectancy of MDS patients treated with RBCT is usually shorter than that of untransfused patients,[1,2] but whether the impaired outcome is a result of intrinsic deterioration of the underlying disease or a result of external factors related to transfusion per se (for example the iron toxicity induced by RBCT) remains an open question. Since 2007, the European MDS (EUMDS) Registry has prospectively collected observational data on patients with MDS classified as low and intermediate-1 risk according to the International Prognostic Scoring System (IPSS),[3] collectively defined as lower-risk MDS.[4] The majority of lower-risk MDS patients become transfusion dependent (51% in the EUMDS Registry),[4] usually within 6 months after diagnosis. With an expected median survival of 2.4 to 11.8 years, these patients might be prone to long-term accumulation of iron due to RBCT.[3,5-8] The toxic effects of iron overload in other iron-loading diseases, such as hereditary hemochromatosis[9] and the thalassemia syndromes,[10] are well known, but the consequences in MDS patients require further clarification. MDS patients are generally older than patients with other iron-loading disorders.[11] Their exposure to RBCT may not be long enough to develop classical tissue damage due to iron overload, but they may suffer from oxidative stress caused by toxic iron species, including non-transferrin bound iron (NTBI) and labile plasma iron (LPI), which have been suggested to serve as early indicators of iron toxicity in iron-loading anemias, such as thalassemia syndromes.[8,12] Biomarkers of oxidative stress have been found to be increased in patients with MDS and iron overload.[4,13-16] Data from a recently completed study of the EUMDS Registry[17] showed that elevated LPI levels - in contrast to elevated NTBI levels and transferrin saturation - are associated with decreased survival. The risk of dying prematurely in patients with detectable LPI levels occurred too early in this study to be explained by classical iron overload with organ toxicity (lungs, liver and heart) after long term transfusions, and this may suggest a direct effect associated with elevated LPI levels.

The aim of this analysis was to assess the effect of RBCT dose density on progression-free survival (PFS) of patients with lower-risk MDS. The hypothesis is that transfusional iron may be toxic and associated with oxidative stress, which may lead to bone marrow failure, genetic damage, increased risk for progression or premature death. Two countervailing forces may play a role in this analysis: (i) patients with symptomatic anemia are more likely to receive more frequent RBCT; and (ii) higher RBCT doses may lead to faster deterioration of lower-risk MDS or to a higher risk of complications by co-morbidities.

Methods

Patients with lower-risk MDS (IPSS risk: low or intermediate-1)[3] from 16 European countries and Israel were included in the EUMDS Registry, after providing signed informed consent, within 100 days of their initial diagnosis of a MDS which was made according to the World Health Organization (WHO) 2001 criteria.[18] Patients with an IPSS intermediate-2 or high risk, or with therapy-related MDS were excluded, but MDS-specific treatment, started before registration within 100 days after diagnosis, was not a reason for exclusion. Data were collected at baseline and at each 6-monthly outpatient routine follow-up visit. Clinical information was collected on: demographics, anthropometrics, co-morbidities, performance status, quality of life (EQ-5D), concomitant medication, laboratory parameters, diagnostics including information on bone marrow morphology, histology, cytogenetics, RBCT episodes, total number of transfused units and simultaneous therapeutic interventions. All subjects were followed prospectively by full reports every 6 months until death, progression to high risk MDS or leukemia, loss to follow-up or withdrawal of informed consent. The Registry was approved by each institution's ethics committee in accordance with national legislation.

Transfusion data available in the EUMDS Registry consists of the number of units received between each reported visit, usually at 6-month intervals. In order to assess the association between transfusions received and PFS, proportional hazards regression with time-varying covariates was employed, adjusting the effect of transfusions by appropriate baseline and time-varying variables. For the purposes of the time-to-event analyses, time was measured from the date of diagnosis with MDS to the date of disease progression or date of death. Progression is defined as an increase to either refractory anemia with excess blasts-2 or to acute leukemia. Patients without disease progression and still alive at the time of the analyses were censored at the date of their last visit.

In order to avoid problems with simultaneity of cause and effect assumed by the proportional hazards approach to survival analysis a "dose density" variable was defined, in the following way, for blood transfusions received. The cumulative total of units of blood received at the end of each inter-visit time interval was calculated. This was then divided by the time since the beginning of the time interval in which the first post-diagnosis transfusion was received, giving a dose-density measurement. This dose-density was then assigned to each time interval. The value of this variable at each point in time represents the average rate at which the patient has been receiving units of blood since they started transfusions.

Adjusted baseline variables included age at diagnosis, number of cytopenias and number of units of blood received before diagnosis. Adjusted time-varying variables (with the intention of adjusting for the condition of the patient over time) were bone marrow blast count, EQ-5D index, revised IPSS cytogenetic category, and platelet and neutrophil counts. Additional analyses were adjusted for the effect of treatment with erythropoiesis-stimulating agents (ESA), iron chelation therapy and lenalidomide, taking these treatments to be confounding factors. Finally, a sensitivity

analysis was performed in the survival regressions to take into account that the population was not homogeneous but distributed over different centers in several countries, using a random effects frailty term. The random effect, called "frailty", is the term that describes the common risk or the individual heterogeneity, acting as a factor on the hazard function. Missing values in adjustment variables were imputed with last observation carried forward or next observation carried backward.

Results

Patients' characteristics

The EUMDS Registry contained data from 2,192 patients diagnosed between December 3, 2007 and March 14, 2017 of whom 1,504 patients had data recorded from three or more visits (visit 3 = landmark at the 1-year follow-up). Two patients with refractory anemia with excess blasts-2 were excluded, resulting in the inclusion of 1,502 patients. An additional 235 patients were excluded, as one or more of the following variables had never been measured or the test failed throughout the study: cytogenetics (n=112), EQ-5D (n=101), blast count (n=60), platelet count (n=1), and neutrophil count (n=2). The final cohort consisted of 1,267 patients, unselected for any type of treatment. In 162 patients the disease had progressed to higher-risk MDS or acute myeloid leukemia and 317 patients had died without progression. The median survival after disease progression was 5.3 months [95% confidence interval (95% CI); 3.2- 9.8 months]. Full details of the

Table 1. Baseline characteristics of the included patients from time of diagnosis and progression-free survival, stratified according to transfusion status at the visit 3 landmark.

	Total N. (%)	Hazard ratio (95% CI)	Adjusted hazard ratio* (95% CI)	Transfusion status at landmark	
				No	Yes
Total	1267 (100.0)			751 (100.0)	516 (100.0)
Median age at diagnosis, years (range)	73.0 (18.0 - 95.0)	1.03 (1.02 - 1.04)	1.03 (1.02 - 1.04)	73.0 (18.0 - 91.0)	73.0 (21.0 - 95.0)
Sex					
Male	757 (59.7)	1	1	445 (59.3)	312 (60.5)
Female	510 (40.3)	0.84 (0.70 - 1.01)	0.76 (0.62 - 0.92)	306 (40.7)	204 (39.5)
WHO diagnosis:					
RA	218 (17.2)	0.84 (0.64 - 1.10)	0.78 (0.59 - 1.03)	139 (18.5)	79 (15.3)
RARS	214 (16.9)	0.73 (0.56 - 0.96)	0.59 (0.45 - 0.78)	123 (16.4)	91 (17.6)
RCMD	492 (38.8)	1	1	296 (39.4)	196 (38.0)
RCMD-RS	86 (6.8)	1.03 (0.72 - 1.46)	0.91 (0.64 - 1.30)	47 (6.3)	39 (7.6)
RAEB-1	133 (10.5)	1.58 (1.20 - 2.07)	1.86 (1.41 - 2.46)	78 (10.4)	55 (10.7)
MDS-U	41 (3.2)	0.64 (0.34 - 1.22)	0.68 (0.36 - 1.29)	27 (3.6)	14 (2.7)
Deletion 5q	83 (6.6)	0.61 (0.40 - 0.92)	0.54 (0.35 - 0.83)	41 (5.5)	42 (8.1)
MDS Comorbidity Index					
Low	782 (61.7)	1	1	482 (64.2)	300 (58.1)
Intermediate	411 (32.4)	1.24 (1.02 - 1.50)	1.08 (0.88 - 1.31)	232 (30.9)	179 (34.7)
High	71 (5.6)	1.55 (1.08 - 2.22)	1.30 (0.90 - 1.89)	35 (4.7)	36 (7.0)
Not known	3 (0.2)	-	-	2 (0.3)	1 (0.2)
Karnofsky status					
80-100	881 (69.5)	1	1	543 (72.3)	338 (65.5)
50-70	210 (16.6)	1.72 (1.38 - 2.15)	1.40 (1.10 - 1.77)	93 (12.4)	117 (22.7)
10-40	10 (0.8)	2.04 (0.76 - 5.48)	1.89 (0.69 - 5.15)	3 (0.4)	7 (1.4)
Not known	166 (13.1)	1.08 (0.80 - 1.45)	0.99 (0.73 - 1.34)	112 (14.9)	54 (10.5)
Quality of life					
Visual analog score, mean (SD)	70.5 (19.7)	0.99 (0.98 - 0.99)	0.99 (0.99 - 1.00)	73.1 (18.9)	66.8 (20.2)
IPSS category					
Low	680 (53.7)	1	1	460 (61.3)	220 (42.6)
Intermediate	557 (44.0)	1.95 (1.62 - 2.34)	1.71 (1.39 - 2.11)	274 (36.5)	283 (54.8)
Cytogenetics not done	30 (2.4)	0.83 (0.43 - 1.62)	0.74 (0.38 - 1.45)	17 (2.3)	13 (2.5)
Revised IPSS category					
Very low	386 (30.5)	1	1	310 (41.3)	76 (14.7)
Low	571 (45.1)	1.80 (1.41 - 2.29)	1.85 (1.45 - 2.37)	309 (41.1)	262 (50.8)
Intermediate	204 (16.1)	3.19 (2.41 - 4.22)	3.40 (2.55 - 4.52)	89 (11.9)	115 (22.3)
High	39 (3.1)	4.27 (2.72 - 6.71)	4.59 (2.91 - 7.22)	11 (1.5)	28 (5.4)
Very high	3 (0.2)	3.15 (0.78 - 12.82)	4.65 (1.13 - 19.15)	1 (0.1)	2 (0.4)
Not known	64 (5.1)	1.69 (1.07 - 2.68)	1.76 (1.11 - 2.80)	31 (4.1)	33 (6.4)

*Hazard ratio adjusted for all other variables in the table. 95% CI: 95% confidence Interval; WHO: World Health Organization; RA: refractory anemia; RARS: refractory anemia with ring sideroblasts, RCMD: refractory cytopenia with multilineage dysplasia; RCMD-RS: refractory cytopenia with multilineage dysplasia & ring sideroblasts; RAEB: refractory anemia with excess blasts; MDS-U: myelodysplastic syndrome, unclassifiable; MDS: myelodysplastic syndrome; SD: standard deviation; IPSS: International Prognostic Scoring System.

exclusions are provided in the *Online Supplementary Data*. Table 1 and *Online Supplementary Table S1* show the patients' baseline demographics. For the landmark analysis patients were defined as untransfused if they had never received a transfusion from diagnosis until the end of the study period (death or progression), or if they had received transfusion only once (n=751). Patients were defined as transfused if they had received multiple transfusions (n=516) within the first year of follow-up (visit 3 = landmark). Regular transfusions were usually initiated during the first 6 months. Using visit 3 as the landmark ensured that the majority of patients who received more than one transfusion were correctly identified.

Distribution of transfusion dose density

The distribution of non-zero dose densities at the third visit (the landmark visit) is shown in Figure 1. Mean dose density among those who had received a transfusion at 1 year of follow-up was 1.24 units per month, with a median of 0.88 units per month (interquartile range, 0.31 – 1.85). Dose densities of the transfused patients declined on approach to the final recorded interval, if the patient died or progressed to higher-risk MDS during the last interval (*Online Supplementary Figure S1*). This implies that patients received fewer transfusions per month in the interval during which death occurred than in the preceding intervals. Presumably, the treatment focus switches to palliative care at home on the approach to death. Patients alive at the last recorded visit and with no signs of progression did not show an increase of transfusion density over time (*Online Supplementary Figure S1*).

Outcome of patients stratified according to transfusion status at the landmark 1 year after registration

The patients' characteristics at the time of the landmark visit 3 stratified according to transfusion status are shown in *Online Supplementary Table S2*. One hundred forty-five subjects untransfused at visit 3 went on to have transfusions after the landmark visit. Out of 516 transfused by the time of the landmark, 288 subjects were not reported to have received any further transfusions, but of these 288, 125 subjects did not have any further visits and another 91 had only one additional visit. Of the 163 subjects who had one or more additional visits (91+72, respectively), 73 received treatment with ESA, 19 with lenalidomide, ten with hypomethylating agents, two with hydroxycarbamide, and three with iron chelators. Unadjusted PFS stratified by transfusion status (transfused n=516, untransfused n=751) at the third visit is presented in Figure 2A. The overall PFS of the untransfused patients at visit 3 was significantly better (P<0.0001) than that of the transfused patients.

Transfused patients were divided into those receiving above (high density) or below (low density) the median value (0.87 units per month) of non-zero dose densities. Unadjusted PFS stratified by transfusion status and dose density (untransfused n=751, low dose density n=258, high dose density=258) at the third visit is presented in Figure 2B. The overall PFS of the three groups of patients, stratified according to the dose density at visit 3, was significantly different (P<0.0001). We evaluated the time to progression in the three groups of patients by censoring those who died before progression (Figure 2C). The hazard ratios for the patients in the low and high density groups were 1.85 (95% CI: 1.24-2.76), and 3.79 (95% CI: 2.65-5.42) relative to the non-transfused group. The recently revised International Working Group (IWG) hematologic response criteria for patients with MDS refined RBCT burden by dividing patients into three categories (non-transfused patients, patients with a low transfusion burden (0.75-2 units per month) and those with a high transfusion burden (≥2 units per month).[19] We therefore repeated the analysis, subdividing the patients into four groups: no transfusions, >0 to <0.75 (low transfusion burden), 0.75 to 1.75 (mid transfusion burden) and >1.75 (high transfusion burden). The results are shown in Figure 2D. The main effect occurred for low dose densities, such that the outcomes of the mid and high transfusion density groups were similar. The low transfusion burden group of Figure 2D (density >0 - <0.75 units per month) is almost identical to the low burden group (density <0.89 units pwer month) of Figure 2B. MDS-related causes of death increased from 28% in the non-transfused group to 39% and 48% in the mid and high transfusion burden groups, respectively (*data not shown*).

Impact of individual prognostic factors

The univariate effect of various covariates on outcome was investigated in order to discover the appropriate functional form for the covariates (i.e., to discover whether a linear or non-linear form was best) and to discover appropriate ways of adjusting for confounding covariates. Increasing RBCT dose density was associated with inferior PFS (P<1x10^{-4}). The functional form is shown in Figure 3A. The effect of the dose density increased until a dose density of about 1 unit per month; thereafter, the effect was flat. Baseline age (as a continuous variable) was strongly associated with PFS (P<1x10^{-4}) in univariate regression analyses, as were baseline MDS diagnosis (P<1x10^{-4}), quality of life measured by the EQ-5D Index (P<1x10^{-4}), country of origin (P=0.002), bone marrow blast count (P<1x10^{-4}), number of cytopenias (P<1x10^{-4}), revised IPSS cytogenetic category (P<1x10^{-4}), hemoglobin concentration (P<1x10^{-4}), neutrophil count (P<1x10^{-4}) and platelet count (P<1x10^{-4}). No difference in

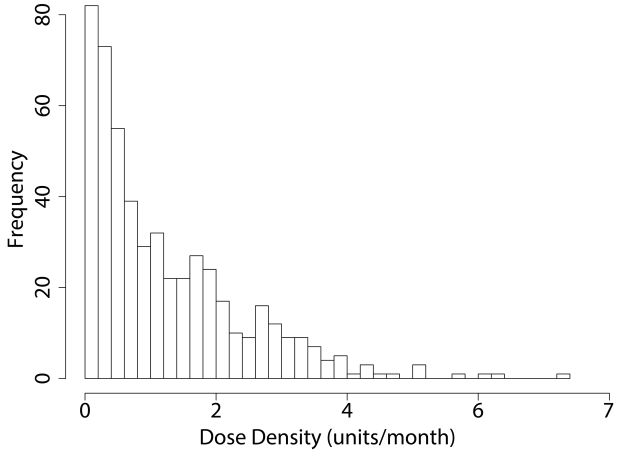

Figure 1. Distribution of dose densities of all transfused patients in the interval preceding the 1-year landmark. Frequency: number of patients in each dose density ranging from >0 to 0.2 units per month to >6 units per mont.

PFS was detected by sex (P=0.1), but PFS in females was superior in the multivariate analyses.

Progression-free survival using time-varying covariates proportional hazards regression analysis

Variables used for adjustment at baseline included age at diagnosis, sex, country of origin, number of cytopenias (and their corresponding blood counts), and number of units of blood received before registration. Time-varying variables measured longitudinally included: dose density, EQ-5D Index, components of the IPSS-R, and receipt of ESA, iron chelators and lenalidomide.

In multivariate analysis, not adjusting for the effects of ESA, iron chelation and lenalidomide therapy, all variables entered in the regression retained statistical significance. The functional form of the dose density effect ($P<10^{-4}$) is shown in Figure 3B. With a frailty term added for the subjects' country of origin, all previously significant variables, including the dose density, retained statistical significance, with a dose density P-value of $<10^{-4}$.

Impact of therapeutic interventions on red blood cell transfusion densities

Treatment with ESA, lenalidomide and iron chelators may improve erythropoiesis and reduce the need for RBCT. Reduction of the RBCT rate results in a gradual decrease of the subsequent RBCT dose densities in intervals during the response period. We therefore investigated how many of the transfused patients had been treated with these interventions and calculated the average treatment duration and the number of patients with reduced transfusion densities after starting the intervention. In our cohort of 1,267 patients, 679 received treatment with an ESA and 151 had reduced transfusion densities in the first

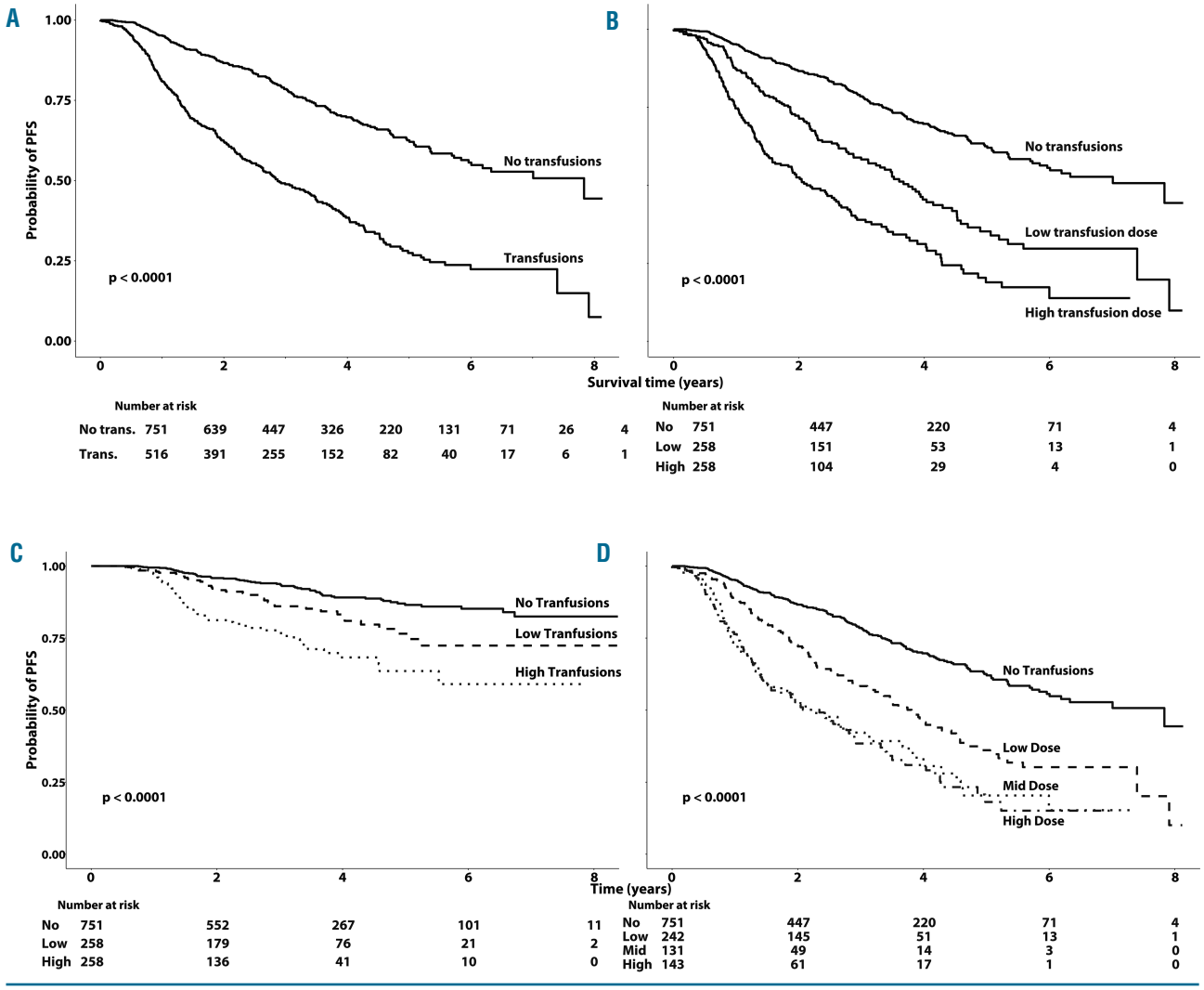

Figure 2. Progression-free survival and risk of progression according to transfusion status at the landmark of visit 3 (1 year after registration). (A) Kaplan-Meier plot of progression-free survival (PFS) of patients who did or did not receive transfusions by the landmark (visit 3). (B) Kaplan-Meier plot of PFS of patients who received transfusions at a low density (<0.87 units/month) or at a high density (>0.87 units/month) by the landmark *versus* PFS of patients who did not receive transfusions; (C) Kaplan-Meier plot of time to progression of patients surviving until progression subdivided according to transfusion burden or not as in panel B; (D) Kaplan-Meier plot of PFS of patients receiving transfusions at densities according to the revised International Working Group criteria: low dose density: >0- <0.75 units per month; mid dose density: 0.75 - 1.75 units per month; high dose density >1.75 units per month.

visit after starting ESA treatment. *Online Supplementary Figure S2* gives the individual dose density over time during ESA treatment of the 151 responding patients. Overall, 100 patients received treatment with lenalidomide: of these, 53 patients had a reduced transfusion density in the first visit after starting lenalidomide treatment; *Online Supplementary Figure S3* shows the individual dose density over time during lenalidomide treatment of the 53 responding patients. Within our study group 186 patients received treatment with iron chelators and 75 patients had a response leading to reduced transfusion densities in the first interval after starting of iron chelation treatment (*Online Supplementary Figure S4*). In contrast to the dose densities over time during ESA and lenalidomide treatment, the longer-term dose densities during iron chelation appeared to show a more stable pattern: subjects receiving a certain level of blood transfusion dose density when they first received iron chelation appeared to maintain that level of dose density. The decline of the dose density was less pronounced, but this might be a reflection of the longer transfusion period before starting chelation treatment when compared with the other two interventions.

The observed patterns of dose density trajectories suggest that receiving ESA, lenalidomide or iron chelation therapy modulates the dose density and we, therefore, included these variables in the regression model. This analysis resulted in an effect for the dose density similar to that of the previous analyses (Figure 3B), with a *P*-value of <0.0001. Indeed all variables entered in the regression retained statistical significance, except for platelet count (*P*=0.47) and neutrophil count (*P*=0.24). However, the dose density effect continued to increase beyond 1 unit per month after correction for the three interventions (ESA, iron chelation and lenalidomide) up until a dose of 6 units per month (Figure 3C).

Some patients received more than one intervention simultaneously, including 25 patients who received chelation and lenalidomide and 88 patients who received ESA and chelation. However, no additional impact could be detected over and above the impact of the two individual interventions.

Discussion

This large prospective, observational study confirmed the reported association of transfusion dose density with reduced PFS in patients with lower-risk MDS.[20] More surprisingly, we showed in this study that this negative association already occurred at a low transfusion rate. In addition, we showed that the risk of progression increased both in the low and high transfusion burden groups when compared to the non-transfused patients. We were even able to show that the deleterious effect of transfusions occurred at a very low transfusion burden (<0.75 units per month or <3 units per 16 weeks as defined by the revised IWG criteria), when the patients were subdivided according to the revised IWG hematological response criteria.[19] These patients with a very low transfusion burden are considered as untransfused patients using the revised IWG response criteria.[19]

The main focus of our study was to analyze the association of transfusion rate with outcome, assuming that regularly transfused patients may be exposed to the postulated toxicity of RBCT at a lower transfusion burden than generally accepted. Several studies have addressed this question using various definitions of transfusion rate. The initial publications describing the impact of RBCT on outcome in MDS compared RBCT-dependent patients with RBCT-independent patients, using RBCT dependency as a time-dependent variable.[1,21] These studies were based on various definitions of RBCT dependency,[22,23] including a study using a rigid criterion, which implied a RBCT rate of at least 1 unit per month during a period of 2 months.[24] In this last study, transfusion dependency occurred in a minority of the patients (35% to 44%). The use of this

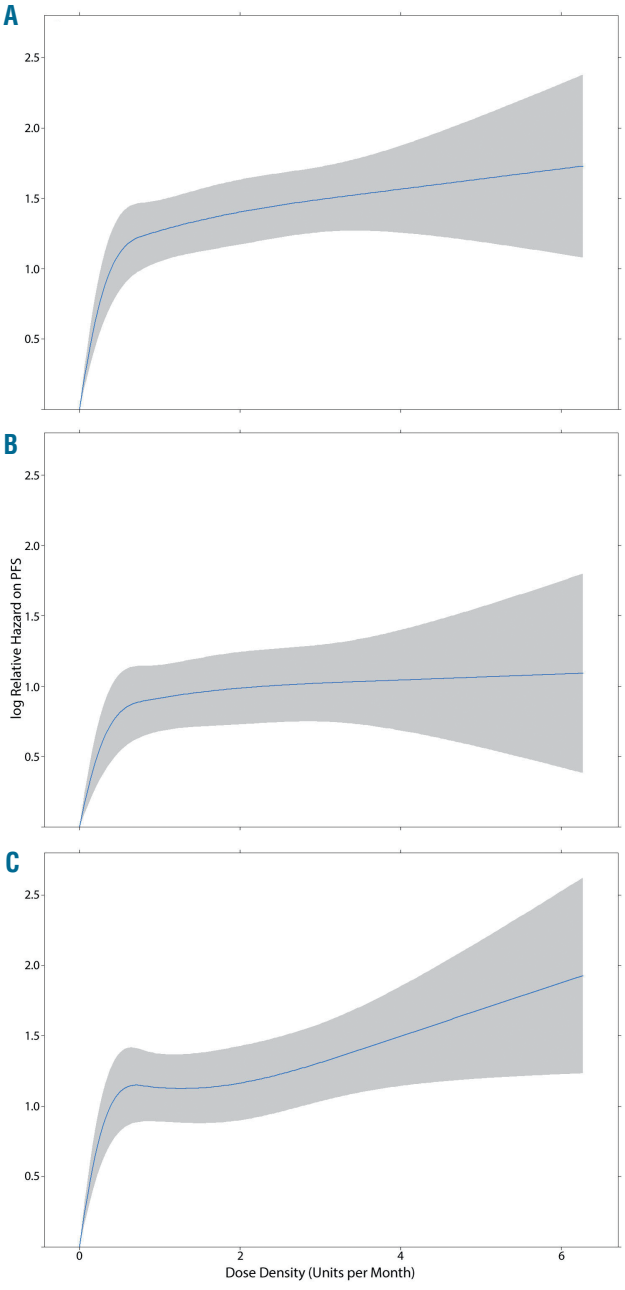

Figure 3. Influence of dose density on progression-free survival. (A) Dose density effect on progression-free survival (PFS) in an univariate analysis. (B) Dose density effect on PFS in a multivariate regression model unadjusted for the three treatment variables. (C) Dose density effect on PFS in a multivariate regression model adjusted for treatment with either erythropoiesis-stimulating agent, Iron chelation or lenalidomide.

definition implies that patients regularly receiving fewer than 3 units per 16 weeks are defined as RBCT-independent, but these patients might also be subject to the deleterious association with RBCT. In addition, patients may respond to therapeutic interventions, such as ESA, lenalidomide or iron chelators and become RBCT-independent again. The conclusion was that the severity of anemia was the leading cause of impaired survival rather than RBCT dependency.[24] However, the definition of severe anemia (<9 g/dL in males and <8 g/dL in females) implies that the majority of these patients were regularly transfused, as confirmed in the study.[24] This study also showed that the transfusion rate was significantly associated with an increased risk of cardiac complications. The risk of cardiac complications was significantly higher in patients with a RBCT intensity of >3 units per month compared to that in patients transfused with <1 unit per month.[24] In an open forum discussion RBCT dependency was even defined much higher, at 2 units per month in a 3-month interval.[25] In a Spanish study of 191 transfused patients with MDS, the interval between each transfusion was used to calculate the transfusion intensity.[26] It was concluded that high transfusion intensity was associated with decreased survival and increased risk of development of acute myeloid leukemia, in concordance with our study. Interestingly, the cumulative transfusion burden was not a prognostic factor when the transfusion intensity was included in the model.[26]

The traditional evaluations of the prognostic impact of factors influencing outcome have used standard time-to-event methods based on variables at diagnosis; however, many variables in MDS may change over time. This aspect can be addressed by using proportional hazards regression with time-varying covariates. The EUMDS Registry is collecting its observational data at registration of each new patient (within 100 days after diagnosis) and follow-up data at 6-monthly intervals. This practice leads to regular visit intervals of 6 months. For many patients in this dataset, the value of the recorded transfusion rate varied strongly over time, as shown in the *Online Supplementary Files*. We therefore calculated the RBCT rate at each reported visit during all preceding visit intervals between the date of the first RBCT and the date of the last visit, leading to a "smoothed" variable, defined as dose density. This reflects an average rate of receiving transfusions during the whole observation period with transfusions. The relatively low number of red cell units transfused per month can be explained by the remarkable variation of the transfusion rate over time, even when using interval visit reports of 6 months' duration.

Baseline age, bone marrow percentage category, number of cytopenias, and the EQ-5D Index retained their significant prognostic impact in the proportional hazards regression with time-varying explanatory variables. The non-linear component of the dose density effect was also retained ($P<1x10^{-4}$). The unfavorable effect of the dose density increased until a dose density of about 2 units per month and leveled off thereafter. A similar form and effect was observed when using the cumulative dose of RBCT units over time in an identical multivariate regression model with the same variables (*data not shown*). The negative impact of the cumulative RBCT dose started already at the time of administering the first RBCT and did not increase any further beyond 30 units received (*data not shown*).

Many patients showed a (temporary) decrease of the RBCT dose density, reflecting response to ESA,[27] lenalidomide,[28] and/or iron chelators[12] in 22%, 53% and 40% of the treated patients, respectively. The observed patterns of dose density trajectories suggest that receipt of ESA, lenalidomide and/or iron chelation modulates the dose density and we therefore included these variables as confounding variables in the regression model. This analysis showed that the impact of the dose density remained similar to that in the previous analyses, but in contrast to the previous analyses there is some evidence that the dose density effect continued to increase beyond 2 units per month after correction for the three interventions.

Red blood cells are usually transfused after a certain period of storage, but the survival of stored red blood cells depends on this period.[29,30] Transfusion of stored red cells leads to pro-inflammatory reactions, associated with a higher risk of infection and increased levels of circulating iron and, in particular, NTBI species, which enhance bacterial growth *in vitro*.[31,32] Infusion of autologous red blood cells from healthy volunteers after prolonging storage up to 6 weeks resulted in increased extravascular hemolysis, decreased red cell survival, elevated NTBI and ferritin levels in units transfused after 6 weeks compared to units transfused after shorter shortage.[33] Excess toxic iron species, including NTBI and especially its component LPI,[34] catalyze the cellular generation of reactive oxygen species. Oxidative stress may lead to pro-inflammatory responses and to oxidation of lipids, proteins and DNA causing cell and tissue damage.[35,36] Elevated NTBI levels after a single unit of RBC stored for 6 weeks normalize within 24 hours.[37] However, in multi-transfused patients (cumulative number of units ≥10) with MDS, NTBI and LPI remained elevated until the next transfusion.[17]

In conclusion, the negative association of transfusions on PFS already occurs at low RBCT dose densities below 3 units per 16 weeks. This indicates that the RBCT dependency in patients transfused at relatively low rates, who are usually considered as untransfused patients, may be considered as an indicator of poor prognosis for PFS. This poor prognosis in transfusion-dependent patients might be the result of direct toxicity of iron radicals resulting from the RBCT or the result of concomitant disease progression, including hematopoietic impairment. Data from our group provide support for the direct toxicity of RBCT density on outcome, because patients had a better outcome if treated with chelators, which remove toxic iron radicals effectively. Future studies, including interventional studies, are needed to confirm our observations, which may lead to adaptions of the current recommendations.

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