CME/SAM

High Levels of Vascular Endothelial Growth Factor Protein Expression Are Associated With an Increased Risk of Transfusion Dependence in Myelodysplastic Syndromes

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Key Words: Myelodysplastic syndrome; Vascular endothelial growth factor; International Prognostic Scoring System; WHO classification–based prognostic scoring system; Prognosis

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Upon completion of this activity you will be able to:
- compare the International and World Health Organization prognostic scoring systems currently used in myelodysplastic syndromes.
- list the main functions of the vascular endothelial growth factor (VEGF)/VEGF receptor system in the hematopoietic bone marrow.
- explain how VEGF affects prognosis in myelodysplastic syndromes.

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Questions appear on p 399. Exam is located at www.ascp.org/ajcpcme.

Abstract

*We evaluated the prognostic significance of vascular endothelial growth factor (VEGF) protein expression in 79 bone marrow biopsy specimens of patients with myelodysplastic syndromes (MDS). VEGF levels normalized for bone marrow cellularity (VEGF index [VEGFi]) were higher in the World Health Organization (WHO) classification–based prognostic scoring system (WPSS) "very high risk" than in the "very low risk" group (*P = .009*) and in patients with MDS with a poor karyotype than in the other cytogenetic risk groups (*P = .015*). High VEGFi (>75th percentile) predicted transfusion dependence (adjusted odds ratio, 10.38; 95% confidence interval, 1.02-106), and were correlated with leukemia-free survival and overall survival. The inclusion of VEGFi in the International Prognostic Scoring System and WPSS maintained its significant prognostic role in predicting leukemia-free and overall survival; it also seemed to improve the discrimination of the different prognostic classes, especially WPSS low-risk classes. Our findings support the clinical relevance of VEGFi expression in the bone marrow biopsy specimens of patients with MDS.*

Accumulating evidence suggests that, in addition to their role in angiogenesis and vasculogenesis, the vascular endothelial growth factor (VEGF) and VEGF receptors participate in the physiologic and/or pathologic regulation of cell proliferation, differentiation, and activation in many types of normal and tumoral tissues.[1-8] This receptor system plays an important role in the development and differentiation of normal hematopoietic cells,[9] and in the promotion of neoplastic diseases[10] such as multiple myeloma,[11] lymphomas,[12] myeloproliferative neoplasms,[13] and myelodysplastic syndromes (MDSs).[14]

Using different methods, a number of studies found higher levels of VEGF expression in patients with MDS than in normal controls.[14-17] Bellamy et al[14] detected an intense immunohistochemical coexpression of VEGF and its receptors in the myeloblasts and immature myeloid cells of bone marrow biopsy specimens of patients with MDS, thus suggesting the presence of an autocrine loop that may be involved in leukemia progenitor self-renewal. Wimazal et al[15] demonstrated correlations between the expression of VEGF protein, transcript, percentage of immature myeloid cells, and FAB categories in the bone marrow of patients with MDS. Although Aguayo et al[16] did not find that plasma VEGF levels played any prognostic role, Verstovstek et al[17] demonstrated that high VEGF expression in bone marrow was associated with decreased survival.

We evaluated VEGF protein expression in the bone marrow hematopoietic and stromal cells of a well-characterized series of patients with MDS. The aims of the study were: (1)

to assess whether VEGF was associated with prognostically relevant clinical variables such as transfusion dependence and leukemia-free or overall survival; and (2) to verify whether adding VEGF protein expression to the International Prognostic Scoring System (IPSS)[18] and the World Health Organization (WHO) classification–based prognostic scoring system (WPSS)[19] better discriminates the different prognostic categories.

Materials and Methods

Patients

The study population consisted of 79 patients with MDS (48 men and 31 women) with a median age of 71 years (range, 40-86 years) who were diagnosed between January 1991 and May 2010. According to the 2008 WHO classification,[20] patients were classified as follows: refractory anemia (RA, n = 10), MDS associated with isolated del(5q) (n = 7), RA with ring sideroblasts (RARS, n = 6), refractory cytopenia with multilineage dysplasia (RCMD, n = 17), RA with excess blasts type 1 (RAEB-1, n = 25), and RA with excess blasts type 2 (RAEB-2, n = 14). Using the IPSS,[18] the patients were stratified as being at low (n = 27), intermediate-1 (n = 30), intermediate-2 (n = 17), and high (n = 5) risk, and using the WPSS[19] calculated within 4 months from diagnosis, they were stratified as very low (n = 14), low (n = 17), intermediate (n = 12), high (n = 24), and very high (n = 12) risk.

Karyotypes were classified using the International System for Human Cytogenetic Nomenclature (ISCN 2009).[21] RBC transfusions were administered in accordance with the recommendations of evidence- and consensus-based guidelines.[22,23] Transfusion dependence was defined as requiring at least 1 RBC transfusion every 8 weeks over a period of 4 months, and we also calculated the median number of packed RBCs used per month during the first year after diagnosis.

Each patient was prospectively followed up from the MDS diagnosis until progression to acute myeloid leukemia (AML), to assess leukemia-free survival or death (overall survival). If none of these end points occurred, the follow-up ended on July 1, 2010 (administrative censoring). None of the patients was lost to follow-up. **Table 1** summarizes the clinical and hematologic features of the 79 patients with MDS.

The control group consisted of 20 bone marrow biopsy (BMB) specimens obtained for staging purposes, and found to be free of neoplasia or any other abnormality on histologic and immunohistochemical examination. They were selected among patients in the fifth or sixth decade of life whose clinical and hematologic parameters were within normal ranges.

All of the patients and controls gave their informed consent and the study design was approved by our hospital's institutional review board.

Table 1
Clinical and Hematologic Features of 79 Patients With MDS at the Time of Diagnosis[a]

Variables	Patients (N = 79)
Sex	
M	38 (48)
F	41 (52)
Median (range) age, y	71 (40-86)
2008 WHO classification	
RA	10 (13)
5q– syndrome	7 (9)
RARS	6 (7)
RCMD	17 (21)
RAEB-1	25 (32)
RAEB-2	14 (18)
Peripheral cytopenia risk group	
0-1	51 (65)
2-3	28 (35)
Cytogenetic risk group[b]	
Good	50 (66)
Intermediate	13 (17)
Poor	13 (17)
Bone marrow blasts risk group	
<5%	40 (51)
5%-10%	28 (35)
11%-20%	11 (14)
IPSS	
Low	27 (34)
Int-1	30 (38)
Int-2	17 (22)
High	5 (6)
WPSS	
Very low	12 (15)
Low	19 (24)
Intermediate	12 (15)
High	24 (30)
Very high	12 (15)
RBC transfusion dependence	35 (44)
Mean (range) packed RBCs per month	1.20 (0-12)
Platelet transfusion dependence	11 (14)
Mean (range) packed platelets per month	0.27 (0-7)
AML progression	23 (29)
Deaths	50 (63)

AML, acute myeloid leukaemia; Int, intermediate; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; RA, refractory anemia; RAEB, RA with excess blasts; RARS, RA with ring sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; WPSS, WHO classification–based Prognostic Scoring System.
[a] Data are given as numbers (percentages) unless noted otherwise.
[b] Information not available for 3 patients.

Diagnostic and Laboratory Procedures

BMB specimens were available for all patients. Immunohistochemistry for VEGF (dilution 1:400, rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) and CD34 (dilution 1:100, clone QB-END/10, Dako, Glostrup, Denmark) was performed using an automated staining system (Genomix i-6000, Biogenex, San Ramon, CA). Heat-induced antigen retrieval was obtained using a 0.05 mol/L EDTA solution, pH 8.0, in a thermostat-equipped bath at 95°C for 30 minutes. Double immunostaining was performed to evaluate CD34 and VEGF coexpression. Reactions were revealed using the Novolink Polymer Detection System (Novocastra, Newcastle upon Tyne, England) in accordance with the manufacturer's

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instructions; the negative control slides were incubated with normal goat serum instead of the primary antibody.

VEGF immunohistochemical expression was evaluated by 2 experienced pathologists (U.G. and A.M., blinded to the clinical data) using a double-headed microscope, and determined as the percentage of positive cells among the total number of nucleated marrow cells. To avoid any bias resulting from cellular variations in the BMB specimens, we calculated a VEGF expression index (VEGFi) defined as the percentage of cellularity of the BMB specimen multiplied by the percentage of VEGF-positive cells (% BMB specimen cellularity × % VEGF+ cells)/10^4. The results were expressed as a number between 0 and 1.[13,24]

Statistical Analysis

Continuous variables were expressed as median values and range (minimum and maximum values), categorical variables as counts and frequencies. The between-group differences in VEGFi levels were tested using the Student *t* test or analysis of variance (with Bonferroni post hoc contrast analysis). Continuous variable correlations were examined using the Spearman rank correlation test.

The relationships between VEGFi and other continuous (hemoglobin, WBC, platelets, and age) or categorical (IPSS and sex) predictors at diagnosis were explored using multiple linear regression analysis. The contribution of the model to outcome variability was expressed in terms of the explained variation using R^2 coefficients (for the full model and for each predictor).

The association between VEGFi levels and the risk of transfusion dependence was evaluated in a multiple logistic regression model including IPSS, baseline hemoglobin levels, age, and sex as covariates. VEGFi was considered as a continuous variable and then given a statistically significant nonlinear effect on the log odds, divided into 2 groups across the 75th percentile value of 0.52: VEGFi less than or equal to the 75th (reference group) and higher than the 75th percentile.

Transfusion dependence risk of the latter vs the former group was expressed as odds ratio (OR; 95% confidence interval [CI]). For hemoglobin levels, the OR represented the relative risk associated with every increase of 0.5 g/dL (5 g/L).

Leukemia-free and overall survival was estimated using the Kaplan-Meier method and the between-group differences were investigated using the log-rank test. The effect of VEGFi on the risk of progression to AML and death was explored using a Cox proportional hazard model, including IPSS (or WPSS), age, and sex as covariates. VEGFi levels were treated as in the evaluation of transfusion dependence risk. The risk of developing AML or of dying in the high expression group compared with the reference group was expressed as hazard ratio with 95% CIs. In the case of age, the hazard ratio represented the relative hazard for every 5-year increase.

In all of the regression models, the possible presence of nonlinear relationships between continuous covariates and outcome was evaluated using restricted cubic spline functions with 3 knots, and the effect of each covariate on the outcome was assessed by plotting the model-predicted effects (log odds for logistic regression, log relative hazard for survival function) against the covariate values, and tested using the Wald test.

High IPSS and WPSS scores were very strong risk factors for AML transformation or death, and directly correlated with VEGFi. We therefore explored the possible contribution of VEGFi on these outcomes by including it in the IPSS and WPSS scores, adding 0.5 to the IPSS score and 1 to the WPSS score for patients with a VEGFi greater than the 75th percentile. The classic and modified IPSS and WPSS scoring systems were then compared in a survival analysis to assess whether the addition of VEGFi to the scoring systems better discriminated subjects in different risk categories.

Statistical significance was set at $P < .05$. All of the analyses were made using *R* statistical software (release 2.11.1; R Project for Statistical Computing, Vienna, Austria).

Results

VEGFi and the Prognostic Scoring Systems

The expression of VEGF protein was evaluated in normal bone marrow cells. Some proerythroblasts showed weak cytoplasmic granular positivity, whereas erythroblasts were not immunoreactive. Moderate to marked positivity was shown by myeloid lineage cells, particularly the immature cells. Macrophages and megakaryocytes showed weak and variable granular cytoplasmic positivity, whereas plasma cells were strongly immunoreactive **Image 1A**. We also evaluated the coexpression of CD34 and VEGF by means of double immunostaining in the MDS cases, and found that a fraction of myeloblasts (ranging from 5%-10%) coexpressed both antigens **Image 1B**.

VEGFi was higher in the patients with MDS than in normal controls (median, 0.33 [range, 0.04-0.76] vs 0.23 [0.04-0.48]; $P = .002$]. It did not differ in the various pathologic MDS categories ($P = .23$), but among the different WPSS classes the very high risk group had the highest VEGFi (0.51 [0.18-0.74]) and the very low risk group had the lowest levels (0.29 [0.04-0.53]; $P = .009$). Furthermore, the very high risk group differed from each of the other 4 groups (Bonferroni post hoc comparison from the very low risk to the high risk groups: $P = .010$, $P = .026$, $P = .046$, and $P = .065$, respectively). VEGFi marginally differed among patients stratified on the basis of the IPSS ($P = .061$ for the overall test, with a significant difference between the low risk and high risk groups in Bonferroni post hoc comparison: $P = .051$).

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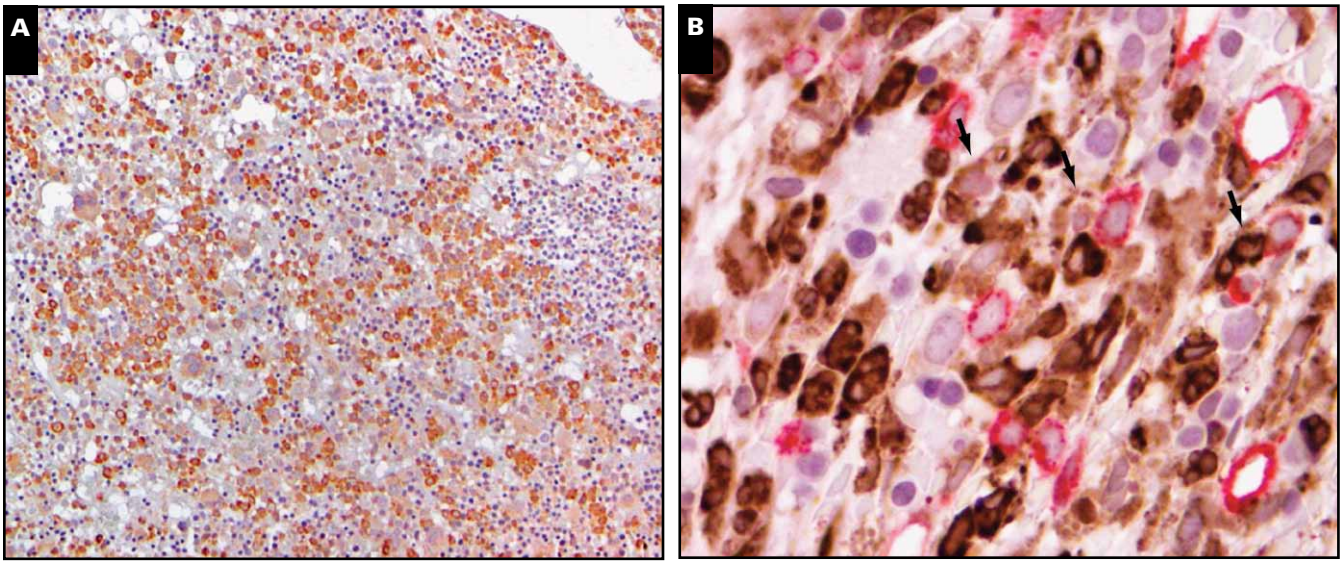

Image 1 **A**, Refractory anemia with excess of blasts (×20). Example shows marked vascular endothelial growth factor (VEGF) protein expression. **B**, High-power field view (×60) of the same case showing the coexpression of CD34 and VEGF in a fraction of myeloblasts (arrows).

VEGFi was higher in the patients with a poor karyotype (0.49 [range, 0.18-0.76]) vs good (0.32 [0.04-0.64]) or intermediate karyotype (0.30 [0.14-0.68]; $P = .015$), whereas no differences were scored for blast percentages and cytopenias, as codified based on IPSS criteria.

At multiple linear regression analysis, the variables that correlated most with VEGFi were IPSS (β estimate = 0.45 [95% CI = 0.24-0.66] for highest risk vs lowest risk; partial R^2: 0.2) and WBC levels (β estimate = 0.195 [95% CI = 0.07-0.324] for every 1 unit increase in the log(WBC), excluding the nonlinear component; partial $R^2 = 0.12$).

VEGFi and Transfusion Dependence

Thirty-five (44%) of the 79 patients with MDS became transfusion dependent during follow-up. Multiple logistic regression analysis showed a significant nonlinear relationship between the VEGFi and the risk of transfusion dependence (log odds; Wald test for nonlinearity: $\chi^2 = 4.19$, $P = .04$). At univariate analysis, individuals with a VEGFi greater than the 75th percentile had a 5-fold greater risk of developing transfusion dependence than those with a VEGFi less than or equal to the 75th percentile (OR = 5.20; 95% CI = 1.65-16.43). In the multiple logistic model, this risk was 10-fold greater (adjusted OR = 10.38; 95% CI = 1.02-106) **Table 2**. For every 0.5 g/dL (5 g/L) increase in baseline hemoglobin levels, the relative risk of transfusion dependence was significantly reduced by about 70% (adjusted OR = 0.32; 95% CI = 0.18-0.59).

VEGFi and Leukemia-Free Survival

The median follow-up of the study population was 597 days (range, 13-2,990), for a total of 65,250 patient-days (178.8 patient-years). Twenty-three (29%) of the 79 patients with MDS developed acute myeloid leukemia (AML) during follow-up, corresponding to an annual incidence of 12.9% patient-years (95% CI = 7.6%-18.1%). **Figure 1A** shows a significant difference in leukemia-free survival between the 2 VEGFi groups (VEGFi >75th and ≤75th percentile; log-rank test $\chi^2 = 8.32$ [$P = .004$]). The probability of being AML free after 1,000 days was 0.40 (95% CI = 0.14-0.65) in the group with high VEGFi expression levels and 0.73 (95% CI = 0.60-0.86) in the reference group. In the Cox proportional hazard model, the hazard ratio of AML progression for every 0.1 unit increase in VEGFi was 1.51 (95% CI = 1.02-2.21). At univariate analysis, the patients with high VEGFi had a 3-fold greater

Table 2
Risk of Transfusion Dependence in Patients With MDS Based on Putative Risk Factors

	Transfusion Dependence, No.		
Predictor	**No**	**Yes**	**Odds Ratio (95% CI)[a]**
VEGFi			
≤0.520	39	21	1 (reference)
>0.520	5	14	10.38 (1.02-106)
IPSS			
Low	22	5	1 (reference)
Int-1	15	15	2.19 (0.32-15.04)
Int-2	5	12	13.06 (1.08-158)
High	2	3	119 (1.22-11,541)
Hemoglobin[b]			0.32 (0.18-0.59)

Int, intermediate; IPSS, International Prognostic Scoring System; MDS, myelodysplastic syndrome; VEGFi, vascular endothelial growth factor index.
[a] Each risk estimate is adjusted for age and sex, and for the effect of the other variables in the model.
[b] Odds ratio for each increase of 0.5 g/dL (5 g/L).

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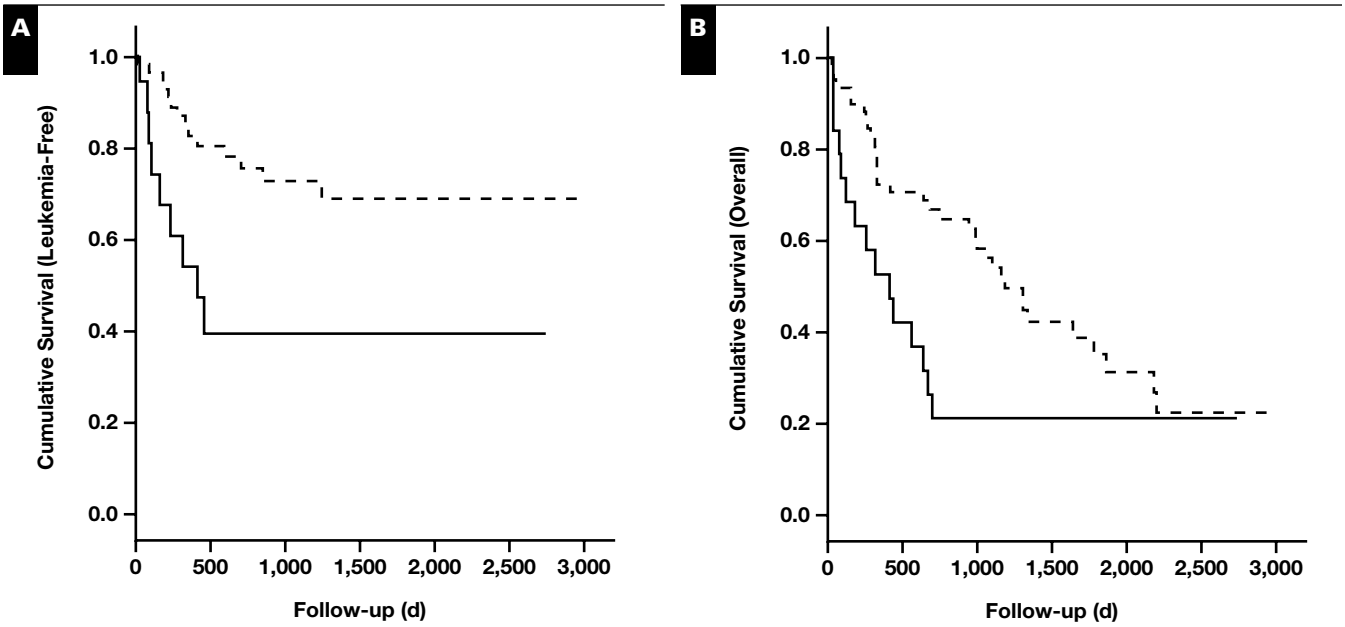

Figure 1 Kaplan-Meier curves showing the leukemia-free (**A**) and overall survival (**B**) of patients with myelodysplastic syndrome with high levels of vascular endothelial growth factor (VEGF) index expression (VEGF index >75th percentile, solid line) and low levels of VEGF expression (VEGF index ≤75th percentile [reference group], dashed line).

risk of developing AML than those in the reference group (hazard ratio, 3.24; 95% CI = 1.39-7.53), which was reduced to about 2-fold when IPSS, age, and sex were added to the model (adjusted hazard ratio, 1.87; 95% CI = 0.75-4.70). The IPSS score was closely associated with a high risk of developing AML over time: the hazard ratio was 75 times greater in the highest than in the lowest risk category (adjusted hazard ratio, 74.72; 95% CI = 7.88-709). When the WPSS categories were added, the adjusted hazard ratio for AML progression for patients with high VEGFi was 1.38 (95% CI = 0.51-3.75) compared with the reference group; in this model, the risk of AML progression was 15-fold greater in the highest than in the lowest WPSS risk category (adjusted hazard ratio, 14.57; 95% CI = 2.25-94.45).

Table 3
Hazard Ratios of AML Progression and Death by WPSS Category Using the Classic and Modified Scoring Systems

Predictors	AML Progression Hazard Ratio (95% CI)[a]	Death Hazard Ratio (95% CI)[a]
WPSS		
Very low (n = 12)	1 (reference)	1 (reference)
Low (n = 19)	0.20 (0.02-1.89)	1.09 (0.31-3.82)
Intermediate (n = 12)	1.42 (0.19-10.58)	4.68 (1.41-15.50)
High (n = 24)	6.64 (1.29-34.11)	11.21 (3.42-36.78)
Very high (n = 12)	17.15 (2.82-104)	31.23 (8.42-116)
WPSS modified[b]		
Very low (n = 11)	1 (reference)	1 (reference)
Low (n = 15)	0.92 (0.08-10.54)	1.66 (0.46-5.94)
Intermediate (n = 14)	1.14 (0.10-13.43)	3.20 (0.92-11.09)
High (n = 26)	10.47 (1.23-88.87)	10.76 (3.39-34.18)
Very high (n = 13)	33.41 (3.52-317)	21.33 (5.98-76.12)

AML, acute myeloid leukemia; WPSS, WHO classification–based prognostic scoring system.
[a] Adjusted for age and sex.
[b] WPSS modified by adding 1 to the score of patients with a vascular endothelial growth factor index greater than the 75th percentile.

VEGFi and Overall Survival

Fifty (63%) of the 79 patients with MDS died during follow-up, for an annual incidence of 25.6% patient-years (95% CI = 18.5%-32.6%). **Figure 1B** shows the overall survival curves of the group of patients with high VEGFi and the reference group (log-rank χ^2 test, 6.15 [P = .013] for the difference between the 2 groups). The probability of surviving after 1,000 days was 0.21 (95% CI = 0.03-0.39) for the patients with a VEGFi greater than the 75th percentile and 0.58 (95% CI = 0.45-0.72) for those with a VEGFi less than or equal to the 75th percentile. Cox proportional hazard model showed that the hazard ratio of death for every 0.1 unit increase in VEGFi was 1.23 (95% CI = 1.03-1.48). The hazard of death was twice as great in the patients with high expression levels as in the reference group (hazard ratio, 2.13; 95% CI = 1.16-3.93). This risk was reduced to 1.3-fold when IPSS, age, and sex were included in the model (adjusted hazard ratio, 1.26; 95% CI = 0.65-2.42); only IPSS was closely associated with an increased hazard of death as the adjusted hazard ratio between the highest and lowest IPSS risk category was 78.71 (95% CI = 18.1-342). These results did not substantially change when IPSS was replaced by WPSS (data not shown).

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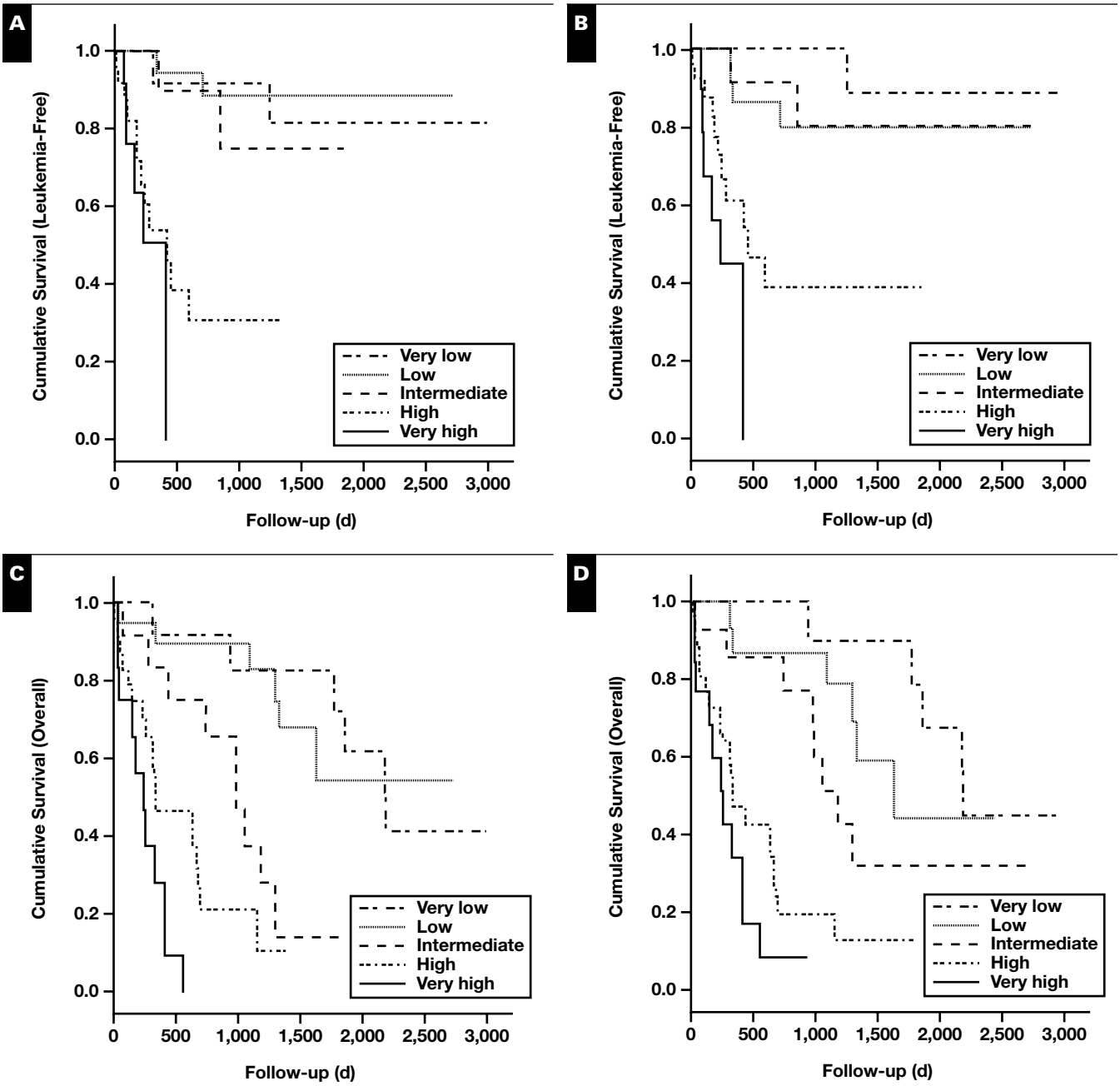

Figure 2 Kaplan-Meier curves showing leukemia-free and overall survival in the 5 categories of the classic WHO classification–based prognostic scoring system (WPSS) (**A** and **C**) and the WPSS modified by adding the vascular endothelial growth factor index to the other parameters (**B** and **D**).

Comparison of the Classic and Modified IPSS and WPSS

Table 3 shows the hazard ratios for progression to AML and death obtained with the Cox proportional hazard model, including the classic or modified WPSS prognostic scores. The modified WPSS not only maintained the significant prognostic role of the standard WPSS in predicting leukemia-free and overall survival, but also seemed capable of better discriminating among the different prognostic classes, especially the low risk classes **Figure 2**.

Similar albeit less striking results were obtained with the standard and modified IPSS (data not shown).

Discussion

In this study we evaluated the immunohistochemical expression of VEGF protein in BMB specimens of patients with MDS with the aim of investigating its relationship with clinical outcomes (transfusion dependence and leukemia-free

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and overall survival). We used a VEGFi to evaluate VEGF expression to avoid the bias caused by the bone marrow cell variability that frequently characterizes MDS.[13,24] The VEGFi was higher in the patients with MDS than in the normal controls, thus confirming the findings of other studies that used different methods to quantify VEGF expression.[14-17] VEGFi levels differed significantly in the different WPSS classes: those in the very high risk group had the highest VEGFi values, and those in the very low risk group had the lowest values.

More importantly, we found a significant nonlinear relationship between the VEGFi and the risk of transfusion dependence: the patients with higher VEGFi required more blood transfusions in the year after diagnosis, regardless of other known risk factors such as their IPSS category or degree of anemia. Transfusion dependence is one of the main clinical challenges in MDS. It is an independent prognostic factor in patients with MDS, and can be considered a reliable indicator of disease severity.[19,25] The VEGFi of BMB specimens may therefore predict the severity of transfusion requirements at the time of diagnosis.

Hemoglobin levels at diagnosis also predicted the development of transfusion dependence. Malcovati et al[25] showed that the degree of anemia at diagnosis influences overall survival in patients with MDS, and suggested that it could replace the transfusion dependence criterion in the WPSS. Our results are in line with these findings and further substantiate the possibility of introducing the degree of anemia at diagnosis as a WPSS parameter.

Overall and leukemia-free survival rates were lower in the patients with MDS with VEGFi more than the 75th percentile at diagnosis than in those with lower values. There are no published data on the prognostic value of the VEGF protein expression evaluated on immunohistochemistry. Aguayo et al[16] assessed the relationship between plasma VEGF levels and overall survival, and did not find any correlation. Using Western blotting and radioimmunoassays, Verstovstek et al[17] quantified VEGF protein levels in bone marrow samples of patients with MDS, and found that higher VEGF levels were associated with decreased survival. Both studies used methods that are substantially different from ours: the immunohistochemical approach is characterized by its ability to identify the expressing cells specifically, thus avoiding the bias due to their dilution in different cell populations.

We also found that a modified version of WPSS (to which we added VEGFi) maintained its significant prognostic value in terms of leukemia-free and overall survival but allowed a better prognostic stratification of patients in the less severe categories. This is of great interest given the already strong predictive value of WPSS risk classes per se, and confirms the physiopathological role of VEGF in MDS.

In conclusion, although further studies on larger series of patients are required to validate our results, our findings support and considerably extend previous data showing increased VEGF protein expression in the bone marrow of patients with MDS, and show that high VEGF levels are a possible independent risk factor of transfusion dependence. The use of VEGF in addition to other prognostic factors in a modified WPSS might help to improve the stratification of patients in the lower risk groups, thus improving their prognostic value.

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