MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

ORIGINAL ARTICLE

NEOANGIOGENESIS AND MICROVASCULAR DENSITY IN MYELODYSPLASTIC SYNDROME – A SINGLE CENTER EXPERIENCE

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Abstract: Angiogenesis has a significant part in the pathogenesis of hematological malignancies, such as leukemia and myelodysplastic syndromes (MDS). We evaluated the relationship between morphometric, morphological and clinical features of MDS. Blood vessels of 31 newly diagnosed MDS bone marrow biopsies were immunohistochemically analyzed using CD34 and compared with 8 controls and 13 chronic myelomonocytic leukemias (CMML). MDS were categorized into three risk groups: low-, intermediate- and high-risk MDS.
Microvascular density (MVD) and major and minor axis length were analyzed using digital image analysis. Overall, MDS had significantly higher MVD and lower minor axis values than the control group and CMML. High-risk MDS had significantly higher MVD compared to the controls, while all MDS risk groups had lower minor axis values than the control group. Increased minor and major axis values were prognostic predictors of shorter overall survival in all MDS risk groups and CMML patients. In conclusion, angiogenesis presents one of the essential factors in MDS pathogenesis and progression characterized by descriptive marrow microvascular network transformation. The size-related features are powerful indicators of survival in MDS patients.

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INTRODUCTION

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell diseases characterized by cytopenia, dysplasia in one or more of the major myeloid lineages, ineffective hematopoiesis, recurrent genetic abnormalities and increased risk of developing acute myeloid leukemia (AML).[1-4]
MDS subtypes are based on morphological features, the percentage of blasts in the bone marrow (BM) and peripheral blood, the type and degree of dysplasia, and the percentage of ring sideroblasts, according to the WHO classification of MDS.[4]
Most subtypes are characterized by progressive BM failure or progression to AML that is the natural course in many cases of MDS, but the biological course of some subtypes is prolonged and indolent, with a very low incidence of evolution to AML.[5, 6]
The subtypes of MDS can be generally categorized into three risk groups on the basis of survival time and incidence of evolution to AML. The importance of cytogenetic features as prognostic indicators in MDS was codified by The Revised International Prognostic Scoring System (IPSS-R score) that predicts survival and risk of evolution to AML.[7]
The formation of new blood vessels from pre-existing vessels is essential to growth, invasion and metastasis by solid tumors and occurs in other diseases, including autoimmune disease and diabetes mellitus.[8-11]
Angiogenesis has recently been reported in hematological malignancies, such as leukemia and myelodysplastic syndromes.[12-18] The issue of angiogenesis in MDS has been validated in several studies providing evidence of increased bone marrow MVD in MDS.[13, 17, 18] Impaired homeostasis of inducers and inhibitors of angiogenesis released from tumor cells

and recruited host cells may occur, usually in response to alterations in the microenvironment (e.g. hypoxia).[19] Moreover, intracellular levels of vascular endothelial growth factor (VEGF), a potent angiogenic molecule, have prognostic significance in AML[20] and chronic lymphocytic leukemia.[21]

In some studies, the highest MVD counts were observed in the MDS subgroups, chronic myelomonocytic leukemia (CMML) and refractory anemia with excess of blasts in transformation (RAEB-t),[20] while in other studies they were found in refractory anemia (RA) and RAEB,[23] and one study failed to show significant differences between subtypes.[16] Vascular variables were associated with progression-free survival, and overall survival.[23]

Using von Willebrand factor or CD31[16, 22, 23] as markers for vascular endothelial cells (EC) to estimate vascularization can be insufficient since they are also expressed in megakaryocytes and platelets.[20, 24]

In the present study, we used the CD34 marker for EC to investigate the relationship between MVD and patient survival, common clinicopathological factors, and the IPSS–R.

The study results might ascertain the evolution of the disease and assess the clinical significance of angiogenesis in MDS patients.

PATIENTS AND METHODS

Patients

Paraffin-embedded BM biopsies obtained at diagnosis from 31 consecutive patients with MDS between 2011 to 2016 were studied. A control group of eight subjects with no evidence of BM disease were evaluated: four cases as part of staging procedure for Hodgkin's disease, two cases of non-Hodgkin's lymphomas, one case of solid tumor, and one case of osteoporosis-related pathological fracture.

The study was approved by the University Hospital Merkur Ethical committee, and informed consent was obtained from patients.

MDS diagnosis was established by morphologists, the classification of MDS was confirmed by hematologists, according to the WHO classification.[4]

MDS patients were categorized into three risk groups.[6]

The low-risk group (MDS-LR) includes MDS with single lineage dysplasia, MDS with ring sideroblasts and single lineage dysplasia, and MDS with isolated del (5q).

The intermediate-risk group (MDS-IR) contains MDS with multilineage dysplasia and MDS with ring sideroblasts and multilineage dysplasia.

The high-risk group (MDS-HR) consists of MDS with excess of blast 1 and 2.

The category of MDS, unclassifiable, was not evaluated in this study.

Although chronic myelomonocytic leukemia (CMML) was excluded from MDS, these entities share similar features, and 13 cases of CMML were included in our study to examine the possible differences between CMML and MDS subtypes.

In the MDS group there were 18 males and 13 females with a median age of 74 years (range, 42-90). The frequencies of MDS risk groups were: MDS-LR six cases, MDS-IR nine cases and MDS-HR 16 cases. In the CMML group there were 12 males and one female with a median age of 70 years (range, 44-90). In the control group there were five males and three females with a median age of 68 years (range, 46-8). The results of laboratory tests are shown in Table 1 and 2.

Cytogenetic abnormalities were scored according to the CCSS for MDS (Table 3).

Table 1. Laboratory and clinical features of myelodysplastic syndrome, myelodysplastic syndrome risk groups and chronic myelomonocytic leukemia (median, range).

	MDS -LR (N=6)	MDS-IR (N=9)	MDS-HR (N=16)	MDS (N=31)	CMML (N=13)	Control (N=8)	Summary (N=44)
Age	1949 (1944-1953)	1944 (1941-1947)	1943 (1933-1958)	1945 (1937 - 1953)	1943 (1939-1954)	1951 (1942-1962)	
Hemoglobin (g/L)	101.5 (83-116)	100 (97-114)	102 (94-109.5)	100 (90-113)	107 (101-110)	-	104.5 (95-112)
Neutrophils ($x10^9$/L)	2.17 (1.51-2.46)	1.14 (0.67-1.41)	0.97 (0.64-2.07)	1.34 (0.67 - 2.45)	5.57 (1.91-6.03)	-	1.62 (0.92-4.72)
Platelets ($x10^9$/L)	125.5 (73-277)	88 (80-149)	78.5 (50-118)	87 (52 - 149)	132 (90-362)	-	91.5 (58-153.5)
Blasts (%)	1 (1-1)	2 (1-3)	8 (5.5-12)	5 (1 - 8)	3 (1-6)	-	4 (1-7.5)
CCSS karyotype	1.5 (1-3)	1.5 (1-2.5)	1.5 (1-3)	1.5 (1 - 3)	1 (1-2)	-	1 (1-3)
IPSS-R score	3 (2.5-3)	2.5 (2-4.25)	4.75 (3.5-8)	3.5 (2.75 - 5.25)	2.75 (1.75-3.5)	-	3.5 (2.5-5)
ECOG	0 (0-0)	0 (0-1)	1 (0.5-2)	1 (0 - 2)	0 (0-0)	-	0 (0-1)
Survival (days)	1185.5 (393-1428)	1049 (894-1837)	1403.5 (1046-1652)	1260 (955 - 1683)	1201 (993-1447)	-	1230.5 (958-1585.5)

Legend: CCSS karyotype and IPSS-R score variables were analyzed in MDS-LR (N=6), MDS-IR (N=8), MDS-HR (N=14), CMML (N=8).
MDS - myelodysplastic syndrome, LR - low risk, IR - intermediate risk, HR - high risk, CMML - chronic myelomonocytic leukemia, CCSS karyotype - Comprehensive Cytogenetic Scoring System karyotype,[25] IPSS-R score -The Revised International Prognostic Scoring System,[7] ECOG - Eastern Cooperative Oncology Group Performance Status.

Table 2. The Revised International Prognostic Scoring System values distribution of hemoglobin, absolute neutrophil count and platelets in myelodysplastic syndrome risk groups and chronic myelomonocytic leukemia patients±

	HB	NEU	PTL
MDS-LR			
Normal*	4	6	3
Decreased – milder degree**	2	0	2
Decreased – higher degree***	1	0	1
MDS-IR			
Normal*	6	6	4
Decreased – milder degree**	2	0	3
Decreased – higher degree***	1	3	3
MDS-HR			
Normal*	11	10	6
Decreased – milder degree**	4	0	6
Decreased – higher degree***	1	6	4
CMML			
Normal*	10	13	7
Decreased – milder degree**	2	0	3
Decreased – higher degree***	1	0	2

± IPSS-R score – The Revised International Prognostic Scoring System values for myelodysplastic syndromes.[7]
Legend: MDS - myelodysplastic syndrome, LR - low risk, IR - intermediate risk, HR - high risk, CMML - chronic myelomonocytic leukemia, HB - hemoglobin concentration, NEU - absolute neutrophil count, PTL - platelets.
*Hemoglobin concentration ≥100 g/L; Absolute neutrophil count ≥$8x10^9$/L, Platelets ≥$100x10^9$/L
**Hemoglobin concentration <100 g/L; Platelets 50 to <$100x10^9$/L
***Hemoglobin concentration <50 g/L; Absolute neutrophil count <$8x10^9$/L, Platelets <50 $x10^9$/L

Transfusion was administered in 3/6 (50%) cases of MDS-LR, 8/9 (88.8%) cases of MDS-IR, 8/16 (50%) cases of MDS-HR, and 7/13 (53.8%) cases of CMML. Chemotherapy was administered in 1/9 (11.1%) cases of MDS-IR, 10/16 (62.5%) cases of MDS-HR, and 4/13 (30.7%) cases of CMML. Patients were treated with azacidine in all cases except one where idarubicin was administered.

A median follow-up period for patients without disease progression or lethal outcome was 48.3 (range 31.80-91.46) months. A median follow-up period of patients' disease progression was 8.01 (range 1.43-30.03) months in 14 (45.1%) MDS patients, and six (46%) CMML patients. Overall, 5/14 (35.7%) MDS cases, and 4/6 (66.6%) CMML cases progressed to AML. The rest of MDS cases progressed into higher risk MDS (9/14 cases; 64.2%), while 2/6 (33.3%) cases of CMML progressed to myelofibrosis.

A median follow-up period with lethal outcome was 10.85 (range 1.00-36.56) months, 11/44 (25%) patients had died of which 8/11 (72.2%) died of disease-related causes.

Table 3. Cytogenetic abnormalities in myelodysplastic syndrome risk groups and chronic myelomonocytic leukemia patients

	MDS-LR	MDS-IR	MDS-HR	CMML
CCSS karyotype				
Very good	0	0	0	0
Good	3	4	7	5
Intermediate	1	2	1	2
Poor	2	1	4	1
Very poor	0	1	3	0
NA	0	1	2	5

Legendy: CCSS karyotype - Comprehensive Cytogenetic Scoring System (CCSS) karyotype25, MDS - myelodysplastic syndrome, LR - low risk, IR - intermediate risk, HR - high risk, CMML - chronic myelomonocytic leukemia

Methods

FFPE decalcified (Osteosoft, Merk) BM biopsies and smears of peripheral blood and BM aspirate smears were processed routinely, and evaluated at the time of the diagnosis.

An analysis of FFPE BM biopsies, peripheral blood and BM aspirate smears was performed to establish the morphologic classification of MDS and CMML according to the WHO classification.[4]

Recurrent MDS cytogenetic abnormalities were identified by conventional karyotyping and interphase FISH.

Sequentially sectioned 4-µm-thick slides were used for performing immunohistochemical staining performed by an automated immunostainer (Dako Autostainer Plus, Dako-Cytomation, Glostrup, Denmark) using LSAB HRP and HRP+ kits according to the manufacturer's instructions.

CD34 antibody was used for establishing EC (mouse monoclonal, Dako; QBEnd 10, RTU) according to the manufacturer's instructions. A CD34 expression positive control was the appendix. For the negative control, adjacent sections were stained in the absence of a primary antibody.

Analysis of bone marrow microvasculature

Immunohistochemically stained slides were analyzed using an Olympus 71 digital camera and an Olympus BX51 microscope. Complete BM section was scanned at x100 magnification to assess the area showing the most intense vascularization, the "hot spot".[26]

After determining the "hot spots" at x100 magnification, the same areas were consecutively analyzed at x200 magnification until the highest number of microvessels was included within the x200 field. The "hot spots" at x200 magnification within intertrabecular cellular area were eligible for analysis avoiding connective and fat tissue as well as trabeculae.

"Hot spots" were saved as uncompressed 24-bit RGB TIFF files, analyzed and measured in the software program AnalySIS (Olympus Soft Imaging System GmbH, Munster, Germany) calibrated with the adequate micrometer scale.

The consensus of morphological criteria for microvessels was followed.[26, 27] Any brown-stained EC

or cluster, with or without a lumen, which was clearly separated from adjacent microvessels and other BM cells was considered as a single, countable microvessel. Blood cells or fibrin without any detectable EC were not sufficient for defining a microvessel. Vessels with muscular walls were also not counted; however, there was no restriction regarding the size of the countable vessels.[28]

In addition to EC, the myeloblast is also CD34 positive but can be distinguished from EC by characteristic morphology and granular intracytoplasmic and Golgi-type positivity with alterative membrane positivity. EC show predominantly membranous positivity.

Microvasculature morphometric parameters estimated in this study were major axis length (the distance between the two points along the vessel periphery that are furthest apart), minor axis length (the longest axis perpendicular to the major axis formed by two points along the vessel periphery), and the total count of microvessels per optical field (MVD).[28]

Morphological analysis was performed independently as blind study.

Statistical analysis

Descriptive statistics are presented through frequencies and percentages for nominal variables, and through medians and interquartile ranges for continuous variables. Deviations from normal distributions for each group were assessed using Kolmogorov-Smirnov tests and visual inspection of result distributions. Almost all variables deviated significantly from the normal distribution for at least one group, and visual inspection did not suggest normality. Because of deviations from normality and ordinal nature of some variables, non-parametric Kruskal-Wallis H tests were used to compare groups. Group differences by nominal variables were assessed using chi-square tests of independence with exact p-values to avoid difficulties due to cells with expected frequencies lower than five. Survival analyses were carried out to determine differences in survival functions for different variables. Statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). The alpha value was set to 5%.

RESULTS

Morphometric morphological features were measured for MDS, MDS risk groups CMML, the control group (Table 4, Figure 1).

Kruskal-Wallis H tests

Two sets of intergroup analyses were performed. CCSS karyotype, hemoglobin, platelets and IPSS-R score did not differ significantly across groups; one with separate MDS low-risk, intermediate-risk and high-risk groups, and one with grouped MDS patients.

Separate group differences are examined first (Table 5, Separate MDS). The test found statistically significant omnibus differences in MVD between groups with relatively strong effect size (P=.003, ε^2=.321). Bonferroni-corrected post hoc test found that high-risk MDS patients had higher MDS than the control group (P=.003). Minor axis length also showed strongly pronounced significant differences across groups (P<.001, ε^2=.413), with the control groups having greater minor axis length than low-risk (P=.016), intermediate-risk (P=.006) and high-risk MSD patients (P=.003). Major axis length was significantly different across groups, a relatively strong effect (P=.007, ε^2=.278), with CMML patients having greater major axis length than the control group (P=.008). While neutrophils analysis showed significant differences in the omnibus test (P=.014, ε^2=.247), post hoc tests found no significant differences between groups. It is concluded that there are no significant neutrophil differences across groups. The strongest effect was found in the analysis of the number of blasts (P<.001, ε^2=.623), with high-risk MDS patients having significantly more blasts than low-risk (P<.001) and intermediate-risk MDS patients (P=.001) as well as CMML patients (P=.006). The IPSS-R score differed relatively strongly and significantly across groups (P=.006, ε^2=.356), with high-risk MSD patients having higher scores than CMML patients (P=.045).

Table 4. Morphometric morphological features of myelodysplastic syndrome, myelodysplastic syndrome risk groups and chronic myelomonocytic leukemia (median, range)

	MDS -LR (N=6)	MDS-IR (N=9)	MDS-HR (N=16)	MDS (N=31)	CMML (N=13)	Control (N=8)	Summary (N=52)
MVD	13.5 (11-16)	22 (12-36)	24 (22.5-32)	23 (15 - 32)	21 (16-25)	12.5 (8-16)	21.5 (12.5-25)
MIN	1.67 (1.4-2.5)	1.43 (0.9-2.6)	1.86 (1.0-3.2)	1.43 (0.9 - 2.8)	4.09 (2.5-5.1)	6.98 (5.5-7.5)	2.64 (1.4-5.0)
MAX	17.5 (10.9-54.1)	27.5 (23.8-33.2)	40.44 (17.4-141.9)	27.5 (17.2 - 79.4)	55.69 (30.3-102.1)	13.97 (9.9-21.5)	29.62 (17.0-74.2)

Legend: MDS - myelodysplastic syndrome, LR - low risk, IR - intermediate risk, HR - high risk, CMML – chronic myelomonocytic leukemia, MVD - microvascular density, MIN, MAX - minor, major axis length

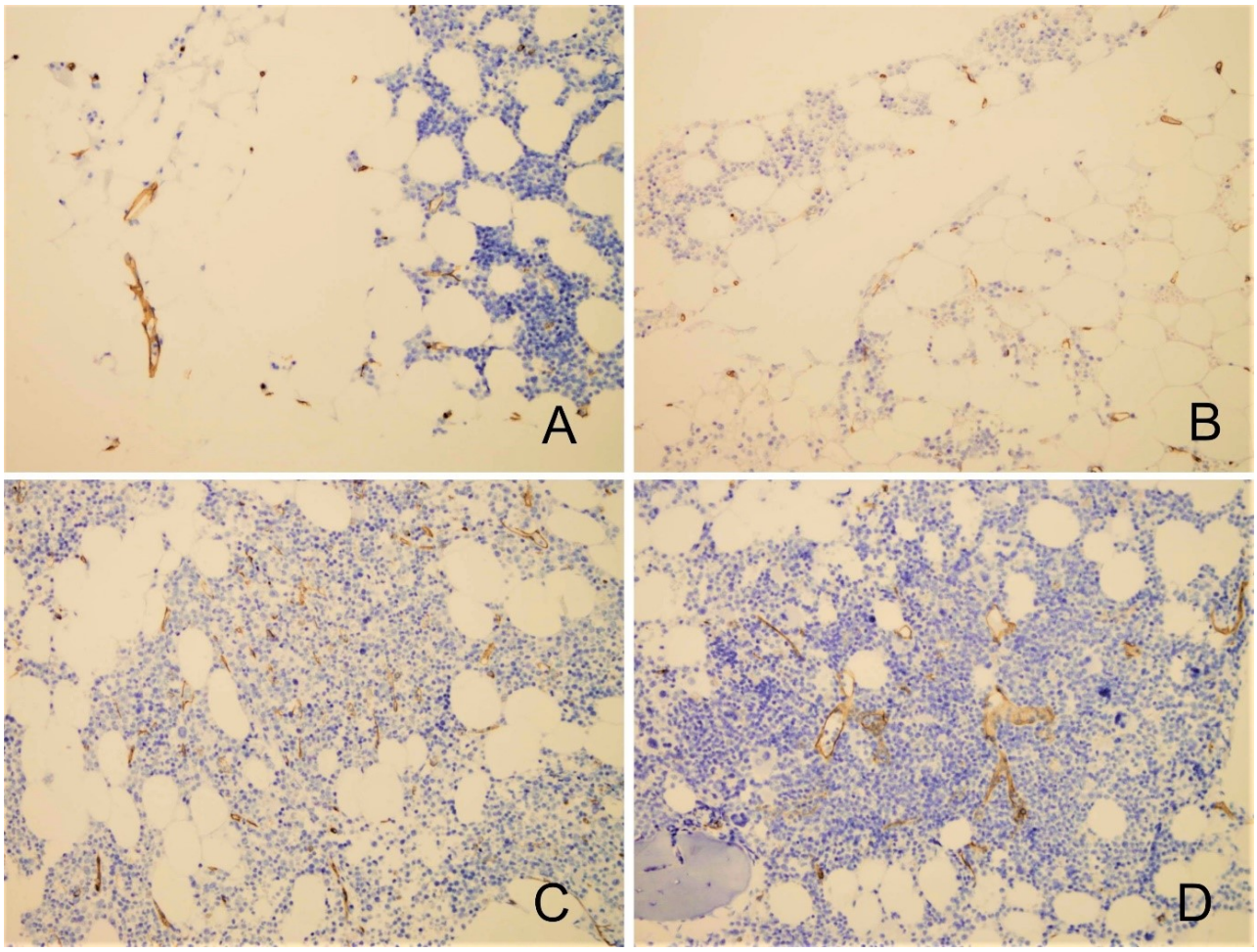

Figure 1. Immunohistochemical staining CD34 of bone marrow microvasculature (magnification x100); A. Control group (PHD#2492-16), B. Myelodysplastic syndrome - low risk (PHD#2164-15), C. Myelodysplastic syndrome - high risk (PHD#9555-14), D. Chronic myelomonocytic leukemia (PHD#2965-16)

ECOG results were overall significantly different with a relatively strongly pronounced effect (P=.021, ε^2=.226), and with high-risk MSD patients having higher results than CMML patients (P=.043). CCSS karyotype, hemoglobin, platelets and IPSS-R score did not differ significantly across groups.

Analyses of grouped MDS patients produced the following results (Table 5, Grouped MDS). MVD differed across groups relatively strongly and significantly (P=.006, ε^2=.356). Post hoc test found significantly higher results for MDS patients than the control group (P=.008). Minor axis length differed strongly and significantly across groups as well (P<.001, ε^2=.402), with MDS patients achieving lower results than CMML patients (P=.008) and the control group (P<.001). Relatively strong significant differences were found in major axis length (P=.004, ε^2=.222), with CMML patients having greater lengths than the control group (P=.002). The rest of the analyses did not include a control group, leaving only MDS and CMML patients, so no post hoc tests were performed. CMML patients had significantly more neutrophils than MDS patients, with a relatively strongly pronounced effect size (P=.004, ε^2=.195). CMML patients also achieved a significantly higher performance status than MDL patients, with a moderately pronounced effect site (P=.044, ε^2=.094). CCSS karyotype, hemoglobin, platelets, blasts and IPSS-R score did not differ significantly across groups.

Chi-square tests of independence

Chi-square tests of independence found statistically significant differences between groups based on type of therapy applied, with a strongly pronounced effect size (χ2 (6)=18.54, P=.004, V=.649) (Table 6). To examine which specific cells differed from their expected frequencies significantly, adjusted standardized residuals were calculated. Intermediate-risk MDS patients received transfusions more frequently than expected by chance (z=2.52, p=.012). High-risk MDS patients received chemotherapy significantly more frequently (z=3.01, p=.003). Disease outcome, transfusion and progression of disease did not differ significantly among groups.

Table 5. Kruskal-Wallis H test results and Bonferroni-corrected Dunn post hoc group comparisons of myelodysplastic syndrome, myelodysplastic syndrome risk groups, chronic myelomonocytic leukemia and the control group

Separate MDS	P	ε^2	Significant group differences		
MVD	.003	.321	MDS(hr) - X_0		
Minor axis length	<.001	.413	X_0 - MDS(lr)	X_0 - MDS(ir)	X_0 - MDS(hr)
Major axis length	.007	.278	CMML - X_0		
CCSS karyotype	.750	.035	-		
Hemoglobin	.860	.018	-		
Neutrophils	.014	.247	-		
Platelets	.273	.091	-		
Blasts	<.001	.623	MDS(hr) - (lr)	MDS(hr) - (ir)	MDS(hr)-CMML
IPSS-R score	.006	.356	MDS(hr)-CMML		
ECOG	.021	.226	MDS(hr)-CMML		
Grouped MDS	**P**	ε^2	**Significant group differences**		
MVD	.011	.177	MDS - X_0		
Minor axis length	<.001	.402	CMML - MDS	X_0 - MDS	
Major axis length	.004	.222	CMML - X_0		
CCSS karyotype	.319	.028	-		
Hemoglobin	.455	.013	-		
Neutrophils	.004	.195	CMML - MDS		
Platelets	.114	.058	-		
Blasts	.215	.036	-		
IPSS-R score	.093	.081	-		
ECOG	.044	.094	MDS - CMML		

Legend: P - significance of Kruskal-Wallis H test, ε^2 - epsilon squared effect size, in group difference comparisons the group to the left of the hyphen has a significantly higher score
MDS(lr) - myelodysplastic syndrome-low risk, MDS(ir) - myelodysplastic syndrome-intermediate risk, MDS(hr) - myelodysplastic syndrome-high risk, CMML - chronic myelomonocytic leukemia, X_0 - control group, MVD - microvascular density, CCSS karyotype - Comprehensive Cytogenetic Scoring System karyotype, IPSS-R score - The Revised International Prognostic Scoring System, ECOG - Eastern Cooperative Oncology Group Performance Status

Kaplan-Meier survival analyses

Log rank tests were calculated as part of Kaplan-Meier survival analysis to find differences in survival functions for different variables. MVD, minor axis length and major axis length values were categorized based on the median value (Table 7). Log rank test found a significant difference in survival functions for minor axis lengths ($\chi2$ (1)=6.418, P=.011) (Figure 2) and major axis lengths ($\chi2$ (1)=8.658, P=.003) (Figure 3). For both variables, patients above the median had lower time until death than those below the median. Differences between survival functions did not differ significantly for examined groups (Figure 4), MVD (Figure 5), CCSS karyotype, progression of disease, therapy treatment and IPSS-R score.

Table 6. Crosstabs and chi-square tests of differences between myelodysplastic syndrome risk groups and chronic myelomonocytic leukemia by outcome, transfusion administration, progression of disease and type of therapy

		MDS-LR	MDS-IR	MDS-HR	CMML	Total	χ^2 (df), P, V
Survived	f (%)	4 (66.67)	6 (66.67)	13 (81.25)	10 (76.92)	33 (75.00)	χ^2 (3) = 0.915
Died	f (%)	2 (33.33)	3 (33.33)	3 (18.75)	3 (23.08)	11 (25.00)	P = .829 V = .144
Transfusion	f (%)	3 (50.00)	8 (88.89)	8 (50.00)	7 (53.85)	26 (59.09)	χ^2 (3) = 4.206
No transfusion	f (%)	3 (50.00)	1 (11.11)	8 (50.00)	6 (46.15)	18 (40.91)	P = .259 V = .309
Progression	f (%)	3 (50.00)	4 (44.44)	7 (43.75)	6 (46.15)	20 (45.45)	χ^2 (3) = 0.075
No progression	f (%)	3 (50.00)	5 (55.56)	9 (56.25)	7 (53.85)	24 (54.55)	P = 1 V = .041
	f (%)	3(50.00)	7(77.78)	1 (6.25)	7(53.85)	18(40.91)	
Transfusion therapy	z	0.49	2.52	-3.53	1.13	-	
	P	.626	.012	<.001	.258	-	
	f (%)	0 (0.00)	1 (11.11)	10 (62.50)	4 (30.77)	15 (34.09)	χ^2 (6) = 18.54
Chemo-therapy	z	-1.90	-1.63	3.01	-0.30	-	P = .004
	P	.058	.103	.003	.763	-	V = .649
	f (%)	3 (50.00)	1 (11.11)	5 (31.25)	2 (15.38)	11 (25.00)	
No therapy	z	1.52	-1.08	0.72	-0.95	-	
	P	.128	.281	.469	.340	-	

Legend: f - frequency, % - percentage of answers within a group, z - adjusted standardized residuals, χ^2 - chi-square test result, P - exact statistical significance of the chi-square test, V - Cramer's V effect size; MDS - myelodysplastic syndrome, LR - low risk, IR - intermediate risk, HR - high risk, CMML - chronic myelomonocytic leukemia

Table 7. Log rank test estimating overall survival in myelodysplastic syndrome and chronic myelomonocytic leukemia

Variable	χ^2	df	P
MDS groups and CMML	3.542	3	.315
MVD*	0.113	1	.737
Minor axis length*	6.418	1	.011
Major axis length*	8.658	1	.003
CCSS karyotype	2.107	3	.551
Progression	1.108	1	.293
Therapy	0.008	2	.996
IPSS-R score	3.906	4	.419

Legend: $\chi 2$ - chi-square value of log rank test, df - degrees of freedom, P - statistical significance. * median split variables.
MDS - myelodysplastic syndrome, CMML - chronic myelomonocytic leukemia, MVD - microvascular density, CCSS karyotype - Comprehensive Cytogenetic Scoring System karyotype, IPSS-R score - The Revised International Prognostic Scoring System

DISCUSSION

Although it is established that angiogenesis is implicated in the progression of hematological malignancies and in particular in MDS, the controversial results of a limited number of studies require further investigation of microvasculature in MDS subtypes and risk-stratified subgroups.
Three studies showed higher MVD in MDS than in controls, but lower than in AML.[16, 17, 20]
MVD differs between morphological subgroups MVD, with higher MVD in CMML and RAEB-t in previous studies.[20, 22] In contrast, Korkolopoulou P et al. showed higher MVD in RA and RAEB. One study found significant differences between subgroups.[16] Vascular variables were associated with progression-free and overall survival.[23]
Also, increased BM angiogenesis and circulating angiogenic cytokines in patients with myeloproliferative diseases highlight the application of anti-angiogenic therapies as alternative or auxiliary treatments in MDS.[29-32]
We confirmed previous observations that patients with MDS exhibit higher levels of
MVD in comparison to the control group. We also obtained higher levels of the major axis in CMML patients in comparison to the control group.[16, 20]
We found significantly higher major axis values in CMML than in the control group.
Minor axis values in MDS were significantly lower in comparison to CMML and the control group.
We extended the investigation further by analyzing microvasculature according to WHO classification MDS subtyping and risk-stratifying groups of MDS.[4, 6]
MDS-HR has significantly higher MVD values than the control group, confirming the data reported by recent studies.[20, 22, 23]
Minor axis values in MDS-LR, MDS-IR, MDS-HR were significantly lower in comparison to the control group.

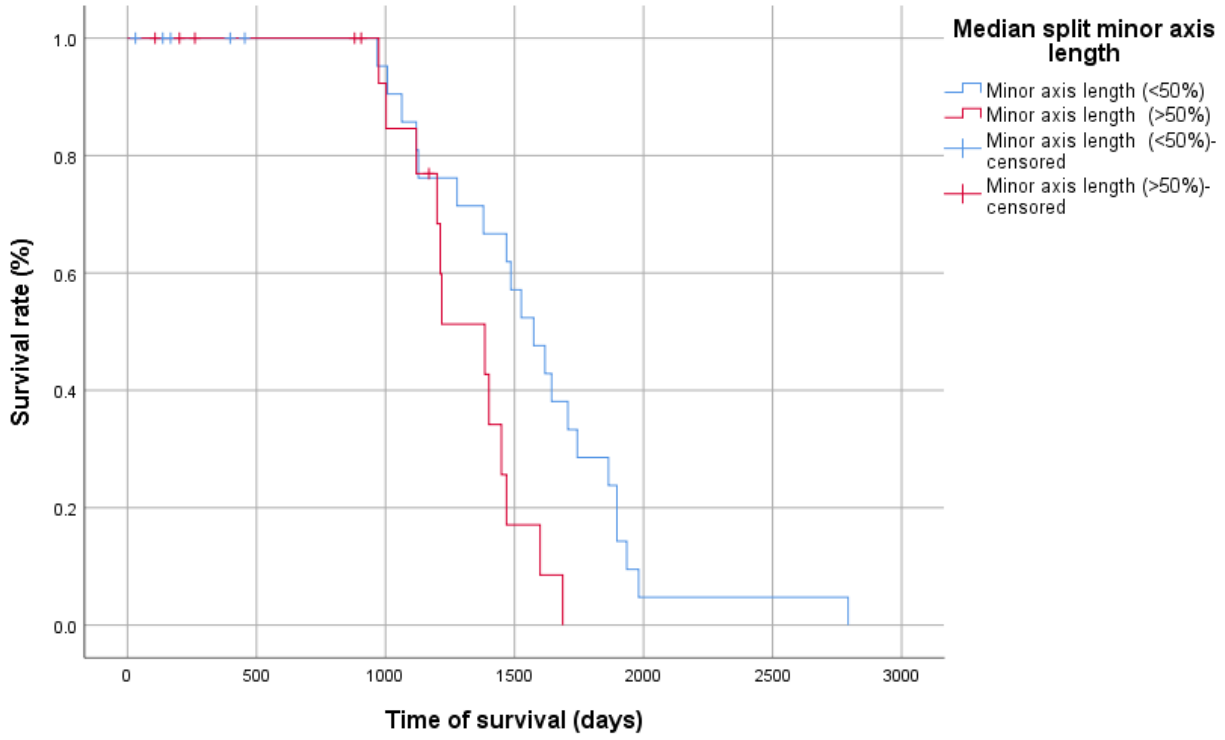

Figure 2. Survival rates through time in relation to median split minor axis length

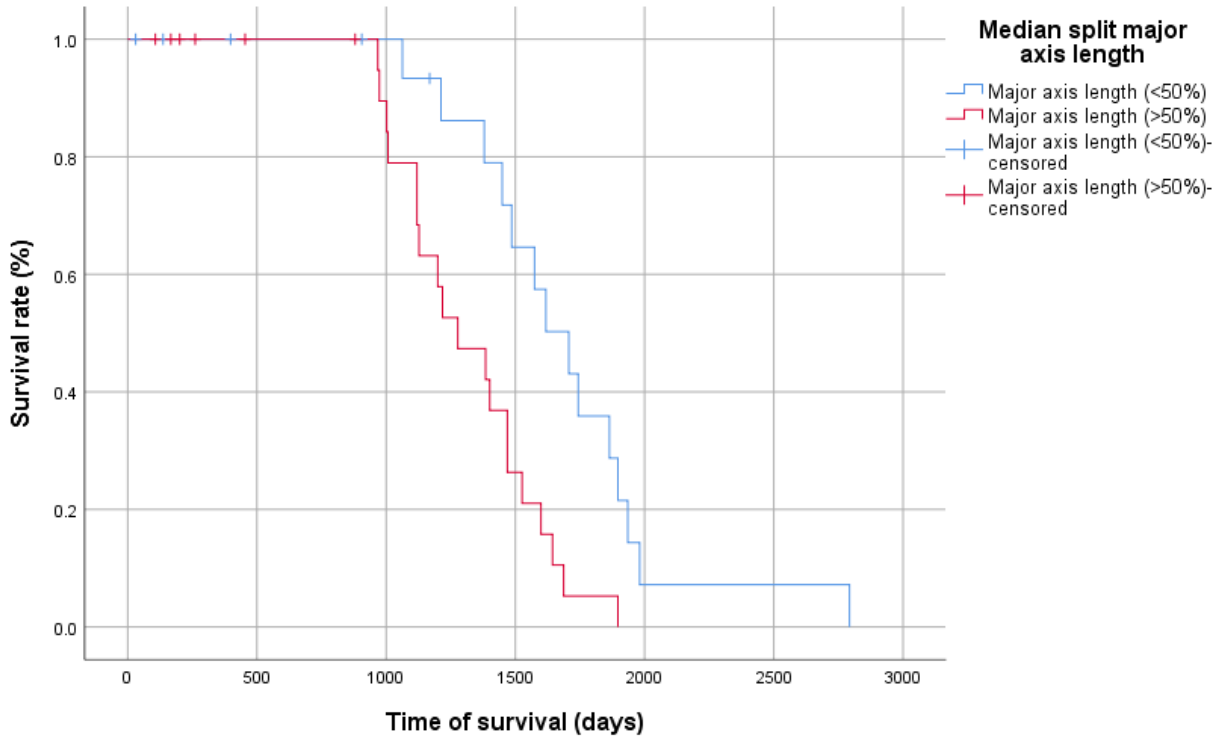

Figure 3. Survival rates through time in relation to median split major axis length

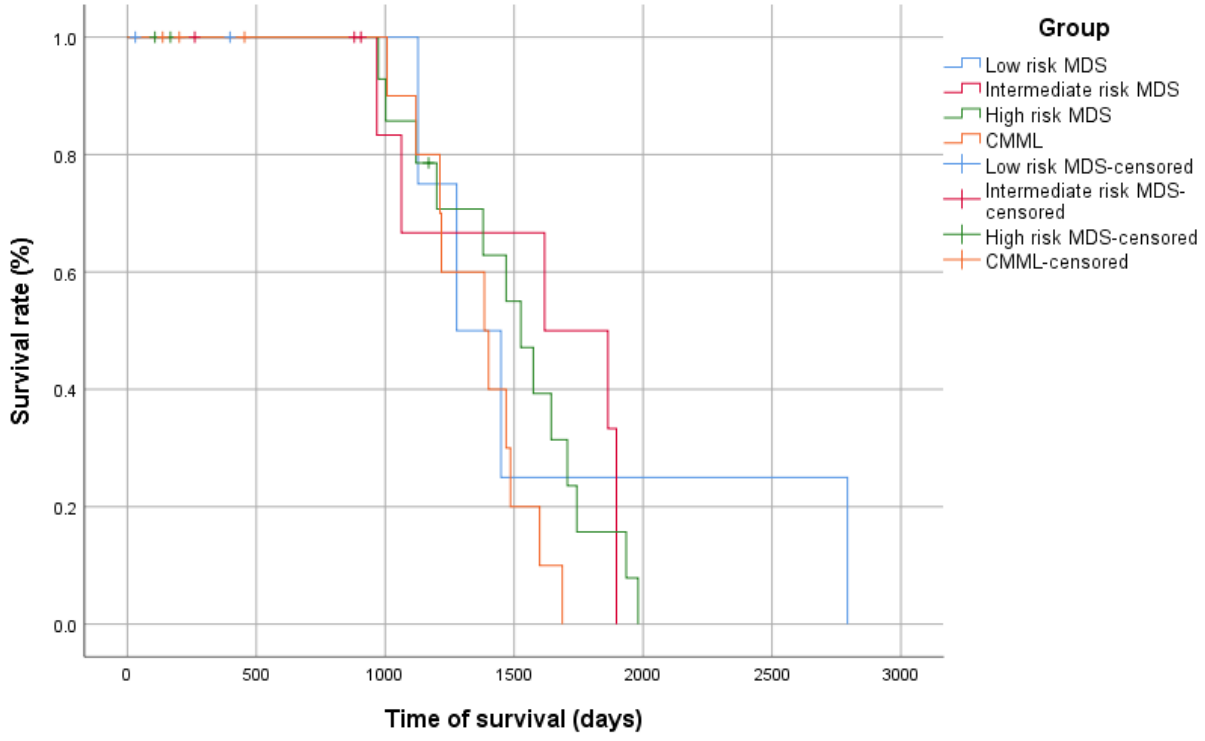

Figure 4. Survival rates through time in relation to explored groups

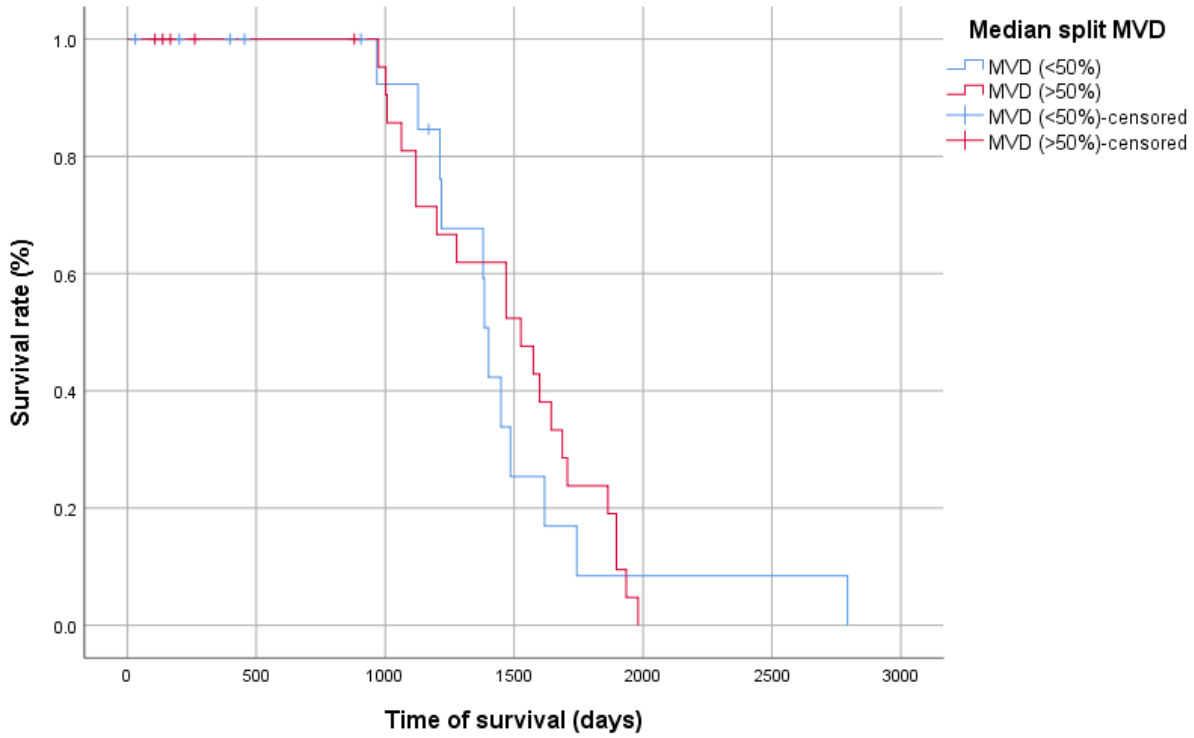

Figure 5. Survival rates through time in relation to median split MVD

Therefore, the lower minor axis values in all three MDS risk groups can point at the different angiogenic processes which occur in BM. Two basic types of angiogenesis, sprouting and intussusceptive angiogenesis, occur in utero and in adults, with the latter one discovered just 20 years ago.[33, 34] In contrast to sprouting angiogenesis characterized by sprouts of EC stimulated with VEGF-A, intussusceptive angiogenesis involves the formation of blood vessels by a splitting process in which elements of interstitial tissues invade existing vessels, forming transvascular tissue pillars that eventually expand. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis because, initially, it only requires the reorganization of existing EC and does not rely on immediate endothelial proliferation or migration.[35-37] The control of intussusceptive angiogenesis is poorly understood compared with sprouting angiogenesis. Since capillary network already exists in BM, we can speculate that intussusceptive angiogenesis is the first process activated by changes in metabolic activity with oxygen as a pivotal player in this regulation since anemia is the leading MDS feature. The splitting of existent vessels can make new vessels with lower minor axis values, and with time blood vessels enlarge, and sprouting angiogenesis can take its part by accelerating angiogenesis resulting in higher MVD.

In contrast, higher minor axis in CMML than MDS can be the result of an even more complex dynamic process in CMML that involves cell-cell and cell-extracellular matrix interactions directed spatially and temporally by growth factors and morphogens.[38-41] This process includes the differentiation of mesodermal stem cells into fibroblasts activated by megakaryocytes producing fibrosis, changing the hemodynamic environment so small tortuous vessels and dilated sinusoids can be identified.[41, 42]

We found no significant difference according to disease outcome, transfusion administration and progression of disease. However, MDS-IR patients received transfusions more frequently, while MDS-HR patients received chemotherapy significantly more frequently. The administration of transfusion in MDS-IR group confirms similar frequency like in the clinical trials.[43-45] Although the risk of progression and shorter overall survival is related to MDS-HR, in our study we found no difference related to it. Either more frequent intermediate and poor cytogenetic profiles in MDS-LR can influence shorter overall survival, or MDS-HR patients had longer survival and lower frequency of progression due to adequate chemotherapy.[4]

More importance is attached to targeted therapy aimed at angiogenesis in hematologic malignancies. DNA methylation (DNMT) inhibitors 5-azacytidine and 5-AZA-20-deoxycytidine were both approved for the treatment of higher-risk myelodysplastic syndromes. DNMT inhibitors appeared to have a role in angiogenesis inhibition, not only indirectly in the angiogenesis inhibition through the re-activation of tumor suppressor genes in cancer cells, but also having direct inhibitory effects through the epigenetic regulation in EC themselves. Reversal of epigenetic modifications can be achieved by DNMT inhibitors mediated by the re-activation of angiogenesis-

suppressing genes that have been silenced in tumor-conditioned EC.[46] Therefore, apart from the 'standard' modulators of angiogenesis, such as VEGF(R) or endothelial nitric oxide synthase, DNMT inhibitors may block or reverse the expression of certain EC genes and may be promising therapeutic targets.

That can be supported by our results, taking into account that we found no difference in overall survival between MDS risk groups and MVD. Patients with minor and major axis median values of more than 50% had shorter overall survival (Figure 2, 3), indicating these are morphological features of prolonged angiogenesis which result in disease progression and drug resistance.

In conclusion, enhanced MDS therapy presents an essential need for the majority of patients in all three MDS risk groups. Targeting tumor angiogenesis from early to advanced stages and relevant regulatory factors angiogenesis, inhibitors are mandatory to restrict tumor growth and metastasis as a new approach and effective oncotherapy. In recent years, combination therapy with multiple targets has provided a brand-new research direction for anti-angiogenesis. Although in this study disease progression within MDS is rather of qualitative than quantitative nature as regards tumor angiogenesis, a larger patient series is needed for clinical validation of MVD in MDS.

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