

ORIGINAL ARTICLE



Late transplant-associated thrombotic microangiopathy verified in bone marrow biopsy specimens is associated with chronic GVHD and viral infections

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Abstract

Objectives: To describe late transplant-associated thrombotic microangiopathy (TA-TMA) as chronic endothelial complication in bone marrow (BM) after allogeneic hematopoietic stem cell transplantation (HSCT).

Methods: BM specimens along with conventional diagnostic parameters were assessed in 14 single-institutional patients with late TA-TMA (more than 100 days after HSCT), including 11 late with history of early TA-TMA, 10 with early TA-TMA (within 100 days), and 12 non TA-TMA patients. Three non-HSCT patients served as control. The time points of BM biopsy were +1086, +798, +396, and +363 days after HSCT, respectively.

Results: Late TA-TMA patients showed an increase of CD34+ and von Willebrand Factor (VWF)+ microvascular endothelial cells with atypical VWF+ conglomerates forming thickened VWF+ plaque sinus in the BM compared to patients without late TA-TMA and non-HSCT. Severe chronic ($p = .002$), steroid-refractory GVHD ($p = .007$) and reactivation of HHV6 ($p = .002$), EBV ($p = .003$), and adenovirus ($p = .005$) were pronounced in late TA-TMA. Overall and relapse-free survival were shorter in late TA-TMA than in patients without late TA-TMA (5-year OS and RFS: 78.6% vs. 90.2%, 71.4% vs. 86.4%, respectively).

Conclusion: Chronic allo-immune microangiopathy in BM associated with chronic, steroid-refractory GVHD and/or viral infections are key findings of late, high-risk TA-TMA, which deserves clinical attention.

KEYWORDS

alloimmune microangiopathy, atypical VWF+ conglomerates forming thickened VWF+ plaque sinus, bone marrow specimens, late transplant-associated thrombotic microangiopathy, von Willebrand factor (VWF)+ microvascular endothelial cells



Novelty statements

What is the new aspect of your work?

Our work provides evidence of microangiopathic features of late TA-TMA in bone marrow specimens proceeding day +100 post-HSCT.

What is central finding of your work?

Diagnostic delays in patients with late TA-TMA beyond day +100 post-HSCT can be improved by perception of chronic allogeneic reactive-mediated endothelial alterations in bone marrow specimens, and early specific treatment may reduce dismal outcome of these patients. In addition, we found that inadequate treatment of prior early TA-TMA and/or frequently viral reactivation with HHV-6, adenovirus and EBV are in favor of late TA-TMA development.

What is (or could be) the specific clinical relevance of your work?

The findings of chronic allo-immune microangiopathy in the bone marrow of late TA-TMA patients may modify our diagnostic routine, when TA-TMA is clinically suspected. Further validation of these findings should be performed in prospective clinical trials.

1 | INTRODUCTION

Transplant-associated thrombotic microangiopathy (TA-TMA) is a severe complication after allogeneic hematopoietic stem cell transplantation (HSCT) with a 3-year cumulative incidence of 3% and high mortality rates of 60%–90% in untreated patients.^{1,2} Clinical features of TA-TMA overlap with other complications after allogeneic HSCT such as capillary leakage syndrome, engraftment syndrome, acute GVHD, diffuse alveolar hemorrhage, disseminated intravascular coagulation, or veno-occlusive disease (VOD).

The gold standard for the diagnosis of TA-TMA is the detection of microangiopathy in tissue biopsies, which is however underinvestigated.^{3–8} The kidney is the organ most affected in TA-TMA.⁹ Typical pathological criteria of renal TA-TMA include thickened capillary walls, fragmented erythrocytes, occluded vascular lumens, endothelial separation with swelling, fibrinoid deposition and debris, microthrombi in the glomeruli, and characteristic patterns of C4d deposition in the arterioles.^{10–12} Remarkable characteristics of microangiopathy in the gut are crypt loss or degeneration with detachment, interstitial edema with hemorrhage, apoptotic epithelial cells, residual neuroendocrine cells, and platelet thrombi. These findings help to distinguish TA-TMA from GVHD or viral infection like CMV-colitis.¹³ In addition, several reports provide the evidence of TA-TMA in skin, lung, and brain tissue specimens.^{14,15}

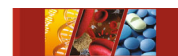
Studies on microangiopathy and endothelial complication in bone marrow biopsy specimens linked to TA-TMA are still not available. Since we observed numerous atypical microvessels in bone marrow biopsy specimens proceeding days +100 after HSCT, we aimed to incorporate our current histopathological findings into the established diagnostic consensus criteria for TA-TMA.⁶ Here, we analyzed pathological features, laboratory parameters, and treatment outcome of 36 single-institutional patients with a median follow-up of 13 years.

2 | PATIENTS, MATERIALS AND METHODS

2.1 | Patients

Between September 2002 and June 2004, we assessed bone marrow biopsies from 36 post-transplantation patients after day +100 post-HSCT at the Bone Marrow Transplantation Unit of the University Hospital of Munich. All patients participated in a prospective, monocentric phase II therapy study on bone loss after HSCT and the use of zoledronic acid with a long-term follow-up after allogeneic transplantation and provided written informed consent for BM biopsy. Inclusion criteria were signed informed consent, allogeneic HSCT within 4 years until inclusion, age ≥ 18 years, osteoporosis (T -score ≤ -2.5 SD) or osteopenia (T -score -1.0 to -2.4), KPS $\geq 70\%$. Exclusion criteria were relapsed underlying malignancy, and serum creatinine >1.5 mg/dL, history of tooth extraction or surgery of the jaw during the last 6 months, prior bisphosphonate treatment, or women with hypogonadism without adequate hormone replacement therapy.¹⁶ The trial was performed in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the institutional ethics committee of the University of Munich. For grading acute and chronic GvHD we used the IBMTR Severity Index published in 1997 by Rowlings and colleagues and the NIH consensus criteria published in 2005 by Filipovich and colleagues. The study was designed by WH in cooperation with all authors. All the authors vouch for the accuracy and completeness of the data. All the authors contributed to drafting the manuscript and no one who is not an author contributed to writing the manuscript.

Late TA-TMA was diagnosed based on modified Jodele criteria, like de novo prolonged or progressive thrombocytopenia <50 G/L or a $>50\%$ decrease in platelet counts in combination with an increased activity of lactate dehydrogenase (LDH), and a decreased hemoglobin concentration, hypertension, soluble C5b-9 exceeding the ULN, and proteinuria (≥ 1 mg/mg random urine protein-to-creatinine ratio).



Notably, the condition of $\geq 2\%$ schistocytes per high power field in peripheral blood was underrepresented.^{4,6,17}

2.2 | Histopathological diagnosis on bone marrow biopsy specimens

5- μm thick sections of BM biopsies were stained with hematoxylin-eosin, Giemsa and silver reticulin. Vascular endothelial cells were evaluated via positive staining of CD34 and VWF. CD34+ cells were stained by mouse monoclonal antibody (Serotec Cat: MCA547; Oxford, UK) and detected by mouse APAAP complex (DAKO Cat F4648; Hamburg, Germany) and fast red (Sigma Cat F4648; St. Louis, Missouri, USA). VWF+ cells were stained by mouse monoclonal antibody (DAKO Cat: M0616) and detected by streptavidin complex (DAKO Cat: PO397) and 3 Amino-9-Ethylcarbazole (Sigma Cat 5754). CD8 T-cells were stained by mouse monoclonal antibody to human CD8 (DAKO Cat: M7103) and detected as described for CD34. A pathologist (KS) and a hematologist (WH) reviewed the specimens. The numbers of CD34+ and VWF+ microvessels, as well as CD8+ T-cells were quantitatively assessed in 1-mm² bone marrow area using a Zeiss microscope Axioplan II at magnifications of 100 $\times$ and 400 $\times$. Characteristic findings were subsequently described as VWF+ plaque sinus. One plaque sinus was defined as a micro sinus that had a thick VWF+ deposit of at least $15 \times 8 \mu\text{m}$ in the sinus endothelial cells or had a thick vascular wall. Six areas (mean) per bone marrow section were used for the assessment. Control samples of three non-HSCT patients aged 43, 52 and 64, biopsied as staging procedure for lymphoma, without lymphoma infiltration, were used.

2.3 | Statistical analysis

In the univariate analysis, box plots illustrated the differences of continuous clinical parameters between study subgroups. Where hypothesis

testing like relations of laboratory values to patient subgroups performed, a non-parametric Kruskal-Wallis or Mann-Whitney - test was used where appropriate. The Fisher's exact test was applied for testing the association of two binary variables. The statistical testing was conducted using an exploratory approach, the maximum type I error probability associated with all statistical tests in the analyses is 0.05. In multivariate analysis, a stepwise binary logistic regression model was fitted to the data to determine the extent to which parameters can explain late MA. For survival analysis, Kaplan-Meier plots were generated, and Cox regression assessed the influence of covariates. For all statistical analyses SPSS version 26 for Windows was utilized.

3 | RESULTS

3.1 | Patient characteristics

We included a group of 36 post-HSCT patients having bone marrow biopsy (BM) between days +100 and +4624 post-HSCT. Fourteen patients met the diagnostic criteria for late TA-TMA.^{6,15,17,18} Of them, 11 patients had a history of early TA-TMA, while three patients did not. Ten had a history of early TA-TMA and 12 patients never had TA-TMA. Three patients did not receive HSCT (non-HSCT) (Figure 1).

Patient baseline and characteristics are summarized in Tables 1–3. Patients with late TA-TMA were younger compared to non TA-TMA ($p = .001$). Cyclosporine A (CSA) was a standard for prophylactic immunosuppression either in combination with methotrexate (MTX) ($n = 25$) or mycophenolate (MMF) ($n = 11$). Compared to non TA-TMA patients, remarkably higher CSA dose accumulation was observed in patients with late TA-TMA (median, 13.5 g vs. 8.7 g; $p = .048$) and treatment duration of CSA was significantly prolonged in late TA-TMA patients (median, 162 days vs. 97 days; $p = .007$). Longer treatment duration of corticosteroids was found in the late compared to non TA-TMA patients (median, 347 days vs. 145 days, $p = .012$).

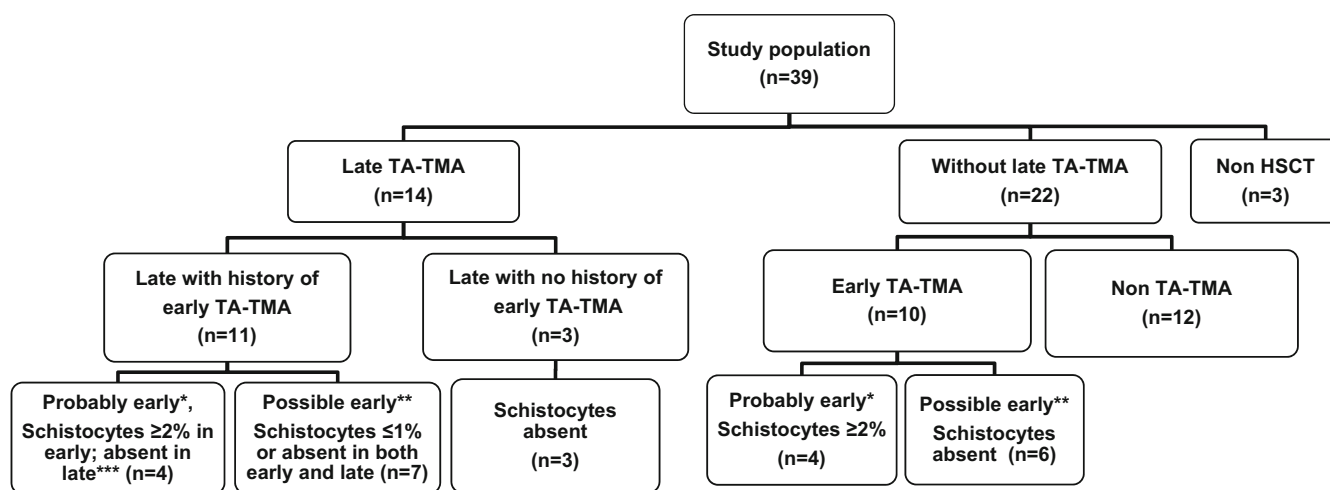


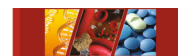
FIGURE 1 Study population: Late ($n = 14$) and without late TA-TMA ($n = 22$) at the time of bone marrow biopsy. In the late group, 11 pts had history of early TA-TMA and three with no history of early TA-TMA. The group without late TA-TMA included 10 pts with early TA-TMA and 12 non TA-TMA. Three patients were non-HSCT. Sub-classification of patients based on presence and/or absence of schistocytes in the peripheral blood (*probable early according to proposed consensus of CHO et al. 2010; **possible early as a newly characterized subtype; *** one patient with 8% schistocytes).

**TABLE 1** Baseline pre-transplant characteristics of 14 patients with late TA-TMA and 12 patients with non TA-TMA.

	Late TA-TMA ($\pm$ prior early TA-TMA)		Non TA-TMA		p value
	N = 14	%	N = 12	%	
Median days of bone marrow biopsies after HSCT (IQR)	1086 (439–1513)		363 (309–608)		.007
Median age (years) at HSCT (IQR)	36 (24–45)		53 (48–56)		.001
Female sex (n)	3	21	5	42	.246
Underlying disease (n)					
AML/sAML	4/2	29/14	7/0	58/0	.304
ALL	1	7	0	0	
CML	6	43	4	33	
MDS RAEB-2	1	7	0	0	
NHL	0	0	1	8	
Pretransplantation risk Category (n) ^a					
Low	6	43	4	33	.879
Intermediate	3	21	2	17	
High	5	36	6	50	
Donor/recipient gender (n)					
Male/male	6	43	4	33	.536
Male/female	1	7	3	25	
Female/male	5	36	3	25	
Female/female	2	14	2	17	
Donor HLA-type (n)					
Identical related	7	50	5	42	.940
Mismatch related	2	14	2	17	
Matched unrelated	4	29	3	25	
Mismatch unrelated	1	7	2	17	
No of mismatch loci (n)					
0/1/2/3	11/0/2/1	79/0/14/7	8/2/1/1	67/17/8/8	.562
Donor/recipient CMV status (n)					
Positive–positive	4	29	6	50	.753
Negative–negative	5	36	2	17	
Positive–negative	1	7	1	8	
Negative–negative	4	29	3	25	
ABO/Rh incompatibility					
Absent/major or minor	9/5	64/36	6/6	50/50	.368
Conditioning Cyclophosphamide and TBI/Busulfan (n)	8/6	57/43	9/3	75/25	.296
Reduced intensity no/yes	11/3	79/21	8/4	67/33	.404
Source of stem cells (n)					
BMT/PBSCT/Both ^b	10/2/2	72/14/14	5/5/2	42/42/17	.222
Prophylactic immunosuppression					
CSA-MTX/CSA-MMF	11/3	79/21	8/4	67/33	.404

^aLow risk: CML in chronic phase. AML in first or second remission. High risk: sAML. MDS RAEB-2. Primary refractory AML. Intermediate risk: all others.

^bHaplo HSCT with bone marrow and CD6 depleted mobilized peripheral blood stem cells.

**TABLE 2** Incidences of GvHD and viral infections in 14 patients with late TA-TMA and 12 patients with non TA-TMA.

	Late TA-TMA ($\pm$ prior early TA-TMA)		Non TA-TMA		p value
	N = 14	%	N = 12	%	
Acute GVHD no/yes	3/11	21/79	8/4	67/33	.026
Grade 0/I/II/III/IV	3/1/2/7/1	21/7/14/50/7	8/4/0/0/0	67/33/0/0/0	.002
Grade 0–I/II/III–IV	4/2/8	29/14/57	12/0/0	100/0/0	.001
Steroid no/refractory/sensitive	4/8/2	29/57/14	12/0/0	100/0/0	.001
Organ-specific					
Skin stage 0 plus 1/2–4	4/10	29/71	12/0	100/0	.053
Liver stage 0 plus 1/2–4	7/7	50/50	12/0	100/0	.002
Intestinal stage 0 plus 1/2–4	8/6	57/43	12/0	100/0	.013
Chronic GVHD no/yes (n)	0/14	0/100	8/4	67/33	.001
Score					
0/mild/moderate/severe	0/2/2/10	0/15/15/70	8/0/2/2	67/0/17/17	.001
Category no/classic/overlap	0/8/6	0/57/43	8/2/2	67/17/17	.001
Subtype absent/quiescent/progressive/de novo	0/2/9/3	0/14/64/22	8/2/0/2	67/17/0/17	.001
Steroid no/refractory/sensitive	0/13/1	0/93/7	8/0/4	67/0/33	.001
Skin 0 plus mild/moderate plus severe	5/9	36/64	9/3	75/35	.053
Liver 0 plus mild/moderate plus severe	3/11	21/79	10/2	10/2	.002
Mouth 0 plus mild/moderate plus severe	9/5	64/36	11/1	92/8	.117
Eyes 0 plus mild/moderate plus severe	6/8	43/57	11/1	92/8	.012
Lung 0 plus mild/moderate plus severe	6/8	79/21	11/1	92/8	.012
Early TA-TMA before no/yes	3/11	21/79	12/0	100/0	.001
Probable/possible subtype no/yes	3/4/7	21/29/50	12/0/0	100/0/0	.001
CSA cumulative median dosis (IQR) (g)	13.5 (7.7–23.8)		8.7 (6–9)		.048
CSA duration median days (IQR)	162 (113–275)		97 (62–108)		.007
Corticosteroid cumulative median dosis (IQR) (g)	7.6 (4.7–14.5)		2.4 (0.47–3.6)		.200
Corticosteroid duration median days (IQR)	347 (181–1058)		223 (50–450)		.182
Viral infections (n)					
HHV6 no/yes	3/11	21/79	10/2	83/17	.002
Adeno no/yes	5/9	36/64	11/1	92/8	.005
EBV no/yes	2/12	14/86	9/3	75/25	.003
CMV no/yes	10/4	71/29	10/2	83/17	.404
HHV6, Adeno or EBV no/yes	0/14	0/100	7/5	58/42	.001

Late with history of early TA-TMA were younger compared to early TA-TMA ($p = .027$). BM alone or in combination with peripheral blood stem cells was more frequently used as stem cell source in late with history of early TA-TMA patients compared to early TA-TMA patients ($p = .045$).

Compared to early TA-TMA patients, remarkably higher CSA dose accumulation was observed in late with history of early TA-TMA (median, 18 g vs. 6.4 g; $p = .014$) and treatment duration of CSA was considerably prolonged in late with history of early TA-TMA (median, 182 days vs. 62 days; $p = .014$). Longer treatment duration of corticosteroids was found in the late with history of early TA-TMA compared to early TA-TMA patients (median, 268 days vs. 145 days; $p = .025$).

3.2 | Histopathological characteristics of bone marrow specimens

By applying immunohistochemistry to identify and enumerate CD34+ and VWF+ endothelial cells in BM microvessels and VWF+ deposits in the BM microvessel walls, we found characteristically altered, small, and sometimes dilated VWF+ microvessels with irregular formed plaques in the wall, which we describe as “plaque sinus” hereinafter (Figure 2A–D). Three non-HSCT patients showed normal, non-thickened VWF+ microvessels (Figure 2E,F).

Elaborative analysis of endothelial cell markers revealed a remarkably higher number of VWF+ microvessels, VWF+ plaque sinuses and CD8+ T lymphocytes in patients with late TA-TMA compared to

**TABLE 3** Important parameters of 11 patients with late TA-TMA with history of early TA-TMA and 10 patients with early TA-TMA.

	Late TA-TMA with history of early TA-TMA		Early TA-TMA		p value
	N = 11	%	N = 10	%	
Median days of bone marrow biopsies after HSCT (IQR)	798 (438–1261)		396 (258–809)		.126
Median age (years) at HSCT (IQR)	34 (23.8–44.8)		45 (35–56)		.027
Female sex (n)	3	27	3	30	.633
Underlying disease (n)					
AML/sAML	3/1	27/9	3/0	30/0	.436
ALL	1	9	4	40	
CML	5	45	2	20	
MDS RAEB-2	1	9	1	10	
NHL	0	0	0	0	
Source of stem cells (n)					
BMT/PBSCT/Both ^a	7/2/2	64/18/18	3/7/0	30/70/0	.045
Acute GVHD no/yes	1/10	9/91	0/10	0/100	.524
Grade 0/I/II/III/IV	1/0/2/7/1	9/0/18/64/9	0/0/7/2/1	0/0/70/20/10	.035
Grade 0–I/II/III–IV	½/8	9/18/73	0/7/3	0/70/30	.047
Organ-specific					
Liver stage 0 plus 1/2–4	4/7	36/64	10/0	100/0	.003
Chronic GVHD no/yes (n)	0/11	0/100	2/8	20/80	.214
Score					
0/mild/moderate/severe	0/2/1/8	0/18/9/73	2/4/2/2	20/40/20/20	.013
Steroid no/refractory/sensitive	0/11/0	0/100/0	2/5/3	20/50/30	.012
Liver 0 plus mild/moderate plus severe	2/9	18/82	8/2	80/20	.007
Mouth 0 plus mild/moderate plus severe	6/5	55/45	10/0	100/0	.023
Eyes 0 plus mild/moderate plus severe	5/6	45/55	10/0	100/0	.009
CSA cumulative median dosis (IQR) (g)	18.0 (1–25)		6.4 (1–9)		.014
CSA duration median days (IQR)	182 (124–305)		62 (21–119)		.014
Corticosteroid cumulative median dosis (IQR) (g)	8.9 (6.4–20.0)		6.0 (5.0–9.3)		.075
Corticosteroid duration median days (IQR)	268 (153–1015)		145 (105–366)		.025
Viral infections (n)					
HHV6 no/yes	3/8	27/73	6/4	60/40	.142
Adeno no/yes	3/8	27/73	9/1	90/10	.006
EBV no/yes	2/9	18/82	6/4	60/40	.063
HHV6, Adeno or EBV no/yes	0/11	0/100	4/6	40/60	.035

^aHaplo HSCT with bone marrow and CD6 depleted mobilized peripheral blood stem cells.

that of non TA-TMA and non-HSCT, but comparable with early TA-TMA patients (Figure S1a,b,d). Notably, the increase of CD34+ microvessels was in correlation with history of prior early TA-TMA in late but not in non-late patients ($p = .014$, Figure S1C). We did not observe intravascular thrombin. The number of VWF+ megakaryocytes was in upper normal interval ($6.1/\text{mm}^2$ vs. $5.1/\text{mm}^2$) (not shown).

Especially in view of an association of cGVHD with TA-TMA, we further assessed vascular endothelial markers in all patients with

cGVHD. Compared to patients without cGVHD, patients who suffered from mild and moderate to severe cGVHD grades, exhibited a highly increase of VWF+ microvessels (19.7 vs. $7.3/\text{mm}^2$; $p < .001$), VWF+ plaque sinus (2.9 vs. $0.5/\text{mm}^2$; $p < .001$), CD34+ endothelial cells (19.7 vs. $8.5/\text{mm}^2$; $p < .001$), and CD8+ T-cells (43.8 vs. $21.2/\text{mm}^2$; $p < .001$) (Figure S2a–d). This also held true for patients with quiescent, progressive and *de-novo* subtype of cGVHD (not shown).

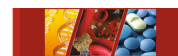
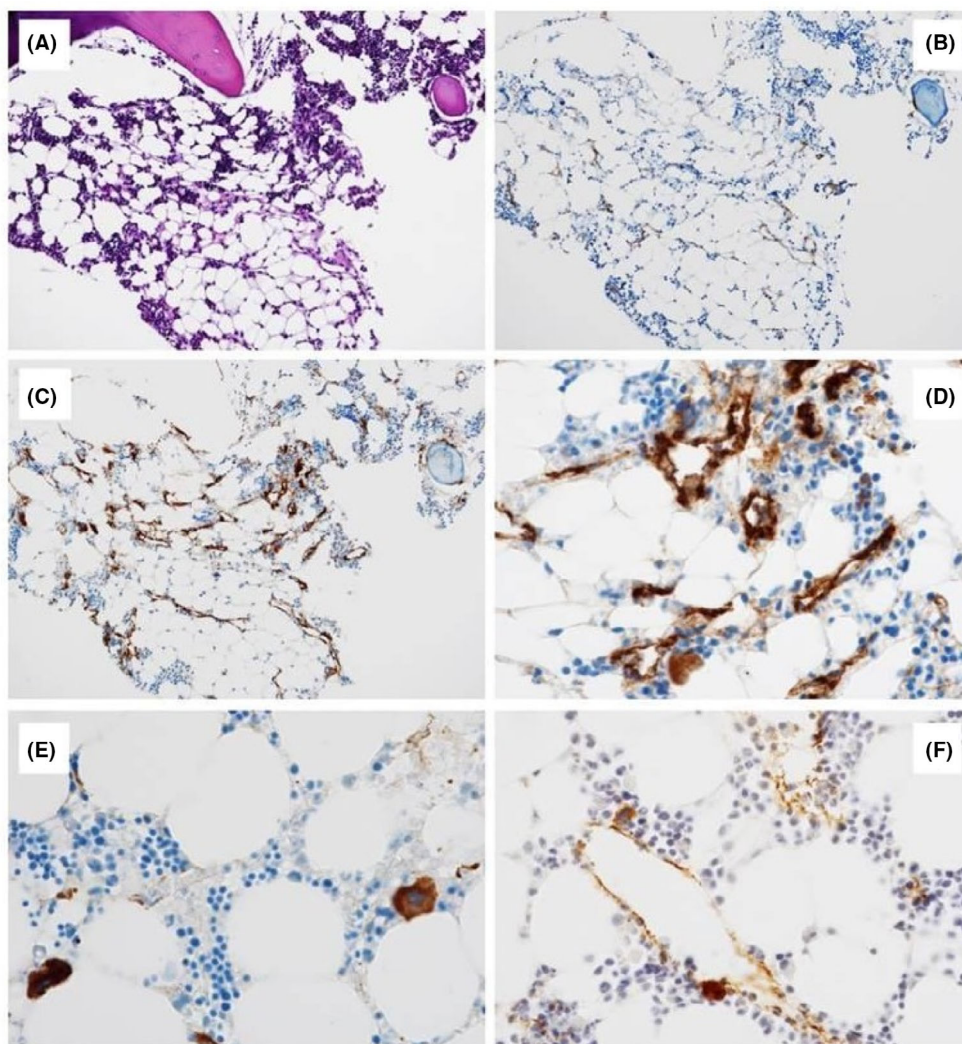


FIGURE 2 Bone marrow immunohistopathology identifies endothelial cells and plaque sinus in a patient with severe chronic GVHD (A–D) and two other non-HSCT patients (E, F).

(A) Hematoxylin–eosin stained hypocellular bone marrow. (B) CD34 staining highlights the number and distribution of microvessels. (C, D) von Willebrand factor (VWF)+ microvessels in the same area showed an intensive cytoplasmatic and membranous VWF expression. Characteristic changes of VWF+ microvessels with irregular formed plaques in the wall of small sometimes dilated microvessels, so-called plaque sinuses. Megakaryocytes serve as internal controls. (E, F) Non-thickened VWF+ microvessels found in non-HSCT. Images at magnifications of 100 $\times$ and 400 $\times$.



3.3 | Laboratory and clinical characteristics of late TA-TMA patients

At diagnosis of late TA-TMA, 14 patients showed *de-novo* prolonged or progressive thrombocytopenia with platelet count of 51 G/L (IQR 17–58), maximal LDH of 354 U/L (IQR 310–383), hemoglobin 9.5 g/dL (IQR 8.1–11.4) and reticulocytes of 51 % (IQR 37–62) (Table S1).

When comparing late patients with history of early TA-TMA to early TA-TMA, we found a tendency for an increase of hemolytic parameters (maximal LDH: median, 476 U/L vs. 299 U/L; $p = .087$ and reticulocyte: mean, 70 % vs. 47 %; $p = .095$), but no difference of the consumption parameters like minimal thrombocytes. A difference of hemoglobin concentration between late and early patients might suggest frequent RBC transfusions for the treatment of acute anemia (Table S1). Thus, we noted that worsened thrombocytopenia presents as the earliest marker of late TA-TMA diagnosis at median days +176 (IQR 120–532) before any other signs of hemolysis. LDH increase becomes apparently at median days +214 (IQR 123–543). In contrast, early TA-TMA is first diagnosed by elevated LDH at median days +30 (IQR 20–51), followed by thrombocytes decrease of at

median days +55 (IQR 40–70) (Table S2). Time interval to laboratory abnormalities for the diagnosis of late TA-TMA was broader compared to that for early diagnosis (Tables S2 and S3).

The disease courses of 11 late patients with history of early TA-TMA varied widely (Figure 3). At the time of best response to early TA-TMA therapy, eight patients showed improvement to normal labor ranges, that is, median LDH of 210 U/L (IQR 203–230) between days +17 and +624 (IQR 41–113) and median platelets of 124 G/L (IQR 39–164) between days +26 and +606 post-HSCT (IQR 56–96). Subsequently, six patients with normalized hematological values deteriorated at the diagnosis of late TA-TMA, without reaching the pathological values of TA-TMA.

Changes of disease course in 14 patients from diagnosis of late TA-TMA are presented in Figure S4. Eleven patients achieved best response to therapy of late TA-TMA with improvement of LDH and platelets to normal levels, i.e. median LDH of 231 U/L (IQR 192–245) between days +123 to +644 (IQR 145–562) and median platelets of 158 G/L (IQR 94–180) between days +147 and 916. (IQR 178–600). Six patients sustained normalized platelets and LDH until last follow-up ($p = .016$ and $.016$, respectively).

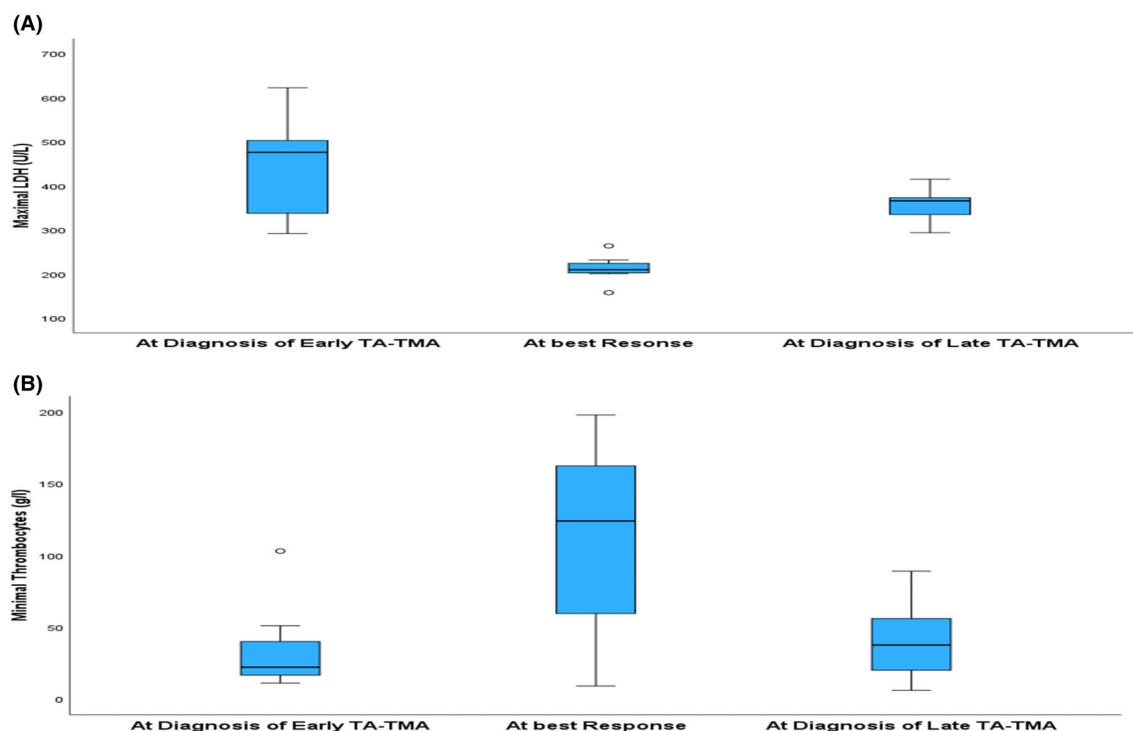


FIGURE 3 Disease course of 11 late TA-TMA patients with history of early TA-TMA from diagnosis of early TA-TMA to time of best response to treatment of early TA-TMA and to diagnosis of late TA-TMA. (A) Maximal LDH elevation: At diagnosis of early TA-TMA on day +30 (IQR 20–51): 476 (IQR 330–515) U/L. At time of best therapy response on day +57 (IQR 41–113): 210 (IQR 203–230) U/L. At diagnosis of late TA-TMA on day +205 (IQR 115–588) 366 (IQR 329–374) U/L. Comparison of early vs. late TA-TMA: $p = .062$. (B) Minimal thrombocyte counts: At diagnosis of early TA-TMA on day +55 (IQR 40–71): 22 (IQR 16–48) G/L. At time of best therapy response on day +80 (IQR 56–96) 124 (IQR 39–164) G/L. At diagnosis of late TA-TMA on day +189 (IQR 129–586): 38 (IQR 19–57) G/L. Comparison of early vs. late TA-TMA: $p = .097$.

At early TA-TMA diagnosis, schistocyte counts of greater or equal to 2% were evident in 4/11 late patients with history of early TA-TMA and in 4/10 patients of early TA-TMA. Subsequently, at the time of late TA-TMA diagnosis, three of the former (except one patient with 8% schistocytes) and three of the later did not show schistocytosis. In contrast, 7/11 late patients with history of early TA-TMA and 6/10 patients of early TA-TMA showed less or equal to 1% schistocytes or absence of schistocytes at the time of both early and/or late diagnosis (Figure 1, Table S2). Here we noted two variants of possible und probable early TA-TMA subtypes based on schistocyte levels (Figure 1). Subsequent analysis of the two subtypes merged with routine hematological parameters of TA-TMA failed to show a diagnostic impact. All patients had normal blood coagulation. Level of creatinine was normal. Relative increase of serum VWF levels were found in three patients with late and in two early patients. Normal level of ADAMTS13 was in one patient of the late group (Table S1).

Fourteen patients diagnosed with late TA-TMA had multiple clinical complications. Specifically, four patients with arterial hypertension (AHT) CTC grade 3 to 4 and 11 patients with AHT history of CTC grade 1 to 2, all required between one and three antihypertensive medications. Intermediate renal impairment by CSA or antiviral therapy was evident in one patient with late TA-TMA, and in seven patients with early TA-TMA.

3.4 | Late TA-TMA and chronic GVHD

Twenty-six of 36 patients developed chronic GVHD (cGVHD), with major involvement of the liver, eyes and mouth. cGVHD with mild, moderate and severe scores occurred more frequently in late TA-TMA compared to non TA-TMA ($p = .001$; Table 2). Notably, moderate and severe cGVHD was more commonly in late patients with history of early TA-TMA compared to early ($p = .013$; Table 3). Time interval from HSCT to onset of cGVHD (median days +275, IQR 138–445) was comparable to the interval from HSCT to onset of late TA-TMA (minimal thrombocytes: median days +176, IQR 120–532; maximal LDH: median days +214, IQR 123–543). Remarkably, 12 of 14 late TA-TMA patients with cGVHD were on CSA and/or MMF or on treatment with corticosteroids and additionally immunosuppressive therapy more than 100 days prior biopsy. Compared to non TA-TMA, late TA-TMA patients suffered more frequently from steroid-refractory cGVHD ($p = .001$), and had rarely steroid-sensitive cGVHD ($p = .007$; Tables S4 and S1). At the time of bone marrow biopsy, active cGVHD was present in 7/14 late TA-TMA, 2/10 early TA-TMA and 3/12 non TA-TMA patients (Tables S3 and S4), who received instantly corticosteroid treatment, showing that late TA-TMA is present in patients with chronic steroid refractory GVHD. Response to the treatment of late TA-TMA was assessed in 14 prior



early to late TA-TMA patients, which showed 11 patients in complete response and three patients with no response (Table S3).

3.5 | History of early TA-TMA and acute GVHD

Overall, 20 patients with history of early TA-TMA developed acute GVHD (aGVHD), mainly involving the liver. Specifically, occurrence of aGVHD grade II-IV was more frequently in early TA-TMA than in late patients with history of early TA-TMA ($p = .035$; Table 1). Notably, aGVHD developed neither in three late patients without history of early TA-TMA or in all 12 non TA-TMA patients.

Early TA-TMA coincided with aGVHD in all 20 patients. The interval from HSCT to onset of aGVHD (median days +22, IQR 17–26) was notably shorter than to onset of early TA-TMA (maximal LDH: days +44, IQR 22–52, $p < .001$; minimal thrombocytes: days +56, IQR 35–74, $p < .001$). During the course of early TA-TMA, every patient required treatment of early TA-TMA, that is, discontinuation of CSA and transfusion of fresh-frozen plasma. Eight patients received antithrombotic therapy with defibrotide. Eventually, 20 patients received corticosteroid (CS) therapy for aGVHD of whom 15 developed steroid-refractory aGVHD and 5 steroid-sensitive aGVHD, which was however equally distributed among the late and non-late groups (Tables S3, S4 and Table 1).

Over time, four patients of the non TA-TMA received corticosteroid (CS) therapy for cGVHD and had steroid-sensitive cGVHD disease. However, none of the non TA-TMA patients had steroid-refractory aGVHD or cGVHD disease (Tables S3 and S4). Response assessment of early TA-TMA treatment in 11 patients with history of early TA-TMA showed six patients in complete response, two patients in

partial response and three patients with no response (Table S3). In contrast, all 10 non-late patients with history of early TA-TMA responded to the therapy of early TA-TMA, including nine patients in complete response and one patients in partial response (Table S4).

3.6 | Viral infections associated with TA-TMA

All 14 patients with late TA-TMA suffered from clinical manifestation of one or more viral infections, which involved predominantly the gastrointestinal tract with diarrhea in nine patients, lower respiratory tract in eight patients including two with pneumonia, three with bronchiolitis obliterans and the urogenital tract in nine patients with hemorrhagic cystitis amongst some other manifestations. Virus reinfections occurred more frequently in patients with late TA-TMA compared to non TA-TMA, i.e. Humane Herpes Virus 6 ($p = .002$), Epstein-Barr Virus (EBV) ($p = .003$), Adenovirus ($p = .005$). Notably, HHV6 targeted multiple organs of late TA-TMA patients. Reactivation of other viruses, like cytomegalovirus (CMV) was less common. The onset of virus infections was between days +22 and +39 post-HSCT (not shown), along with onset of early TA-TMA (Tables 1 and 4, Table S2).

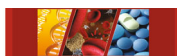
3.7 | Potent risks associated with late development of TA-TMA

Variables with $p < .05$ evaluated in univariate analysis for late TA-TMA vs. non TA-TMA patients were further tested using a binary logistic regression model, including patient ages, aGVHD, cGVHD, source

TABLE 4 Frequency of viral infections documented in various organs in late and without TA-TMA.

	Patients No n/total	Bone marrow	Peripheral blood	Colon/stomach biopsies	Stool	BAL	Throat rinse water	Urine
HHV6								
Late TA-TMA	11/14	2/14	5/14	6/14	1/14	2/14	2/14	1/14
Early TA-TMA	4/10	-	3/10	1/10	-	-	-	-
Non-TA-TMA	1/12	2/12	1/12	-	-	-	1/12	1/12
EBV								
Late TA-TMA	12/14	5/14	11/14	3/14	-	3/14	2/14	2/14
Early TA-TMA	4/10	3/10	3/10	-	-	-	1/10	-
Non-TA-TMA	3/12	1/12	1/12	-	-	-	1/10	-
Adeno								
Late TA-TMA	9/14	2/14	-	-	7/14	-	4/14	3/14
Early TA-TMA	1/10	1/10	-	-	-	-	1/10	1/10
Non TA-TMA	1/12	-	-	-	-	-	1/10	-
CMV								
Late TA-TMA	4/14	-	2/14	-	1/14	1/14	2/14	1/14
Early TA-TMA	2/10	1/10	-	-	-	-	1/10	1/10
Non TA-TMA	2/12	-	-	-	1/12	-	1/10	1/12

Note: Evidence Range: HHV6 18–1900 Geq/mL; EBV 15–80 Mio Geq; Adeno Antigen pos; CMV 140–520 Geq/mL or/20 000 cells or Early Antigen.



of stem cells, viral infections, CSA intake, and the number of VWF+ microvessels and CD8+ T lymphocytes. As a result, we found the presence of cGVHD and age less than 44 years at HSCT as potent risks of development of late TA-TMA ($p = .011$ and $p = .024$, respectively).

3.8 | Outcome of late TA-TMA

Overall survival (OS) was considerably shorter in patients with late TA-TMA, compared to patients without late TA-TMA (OS probability at 5 years: 78.6% vs. 90.2%; $p = .069$; Figure S3). Relapse-free survival (RFS) was considerably shorter in patients with late TA-TMA compared to patients without late TA-TMA (RFS probability at 5 years: 71.4% vs. 86.4; $p = .065$) (data not shown). Two patients without late TA-TMA had relapse of AML resulting in a 5-year relapse rate of 5.6% and two patients with late TA-TMA had a 5-year relapse rate of 8.3%. Five patients with late TA-TMA died of cGVHD and/or additional causes, such as pneumonia with sepsis, cerebral bleeding, cachexia (in two patients) or bronchiolitis obliterans. Two patients without late TA-TMA died of relapse and bronchiolitis obliterans.

4 | DISCUSSION

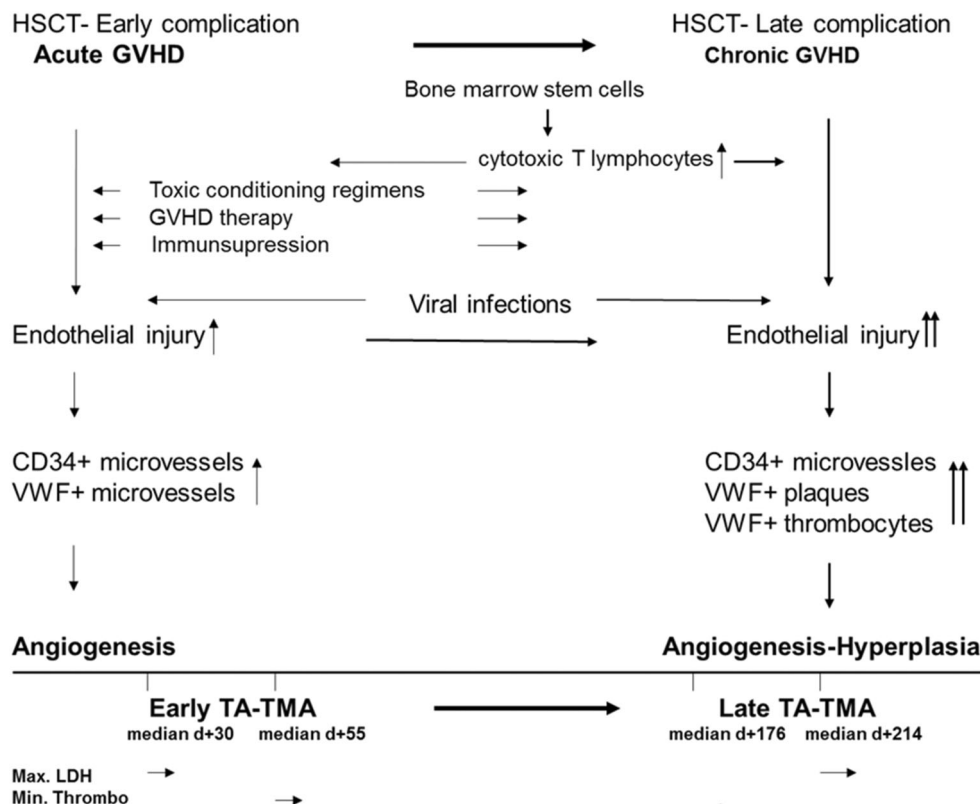
In the current study, we reported the histopathological findings of late TA-TMA in bone marrow specimens with the characteristics of chronic allo-immune microangiopathy proceeding days +100 post-HSCT. Though, there was no evidence of systemic microthrombus formation as published by Ruutu et al.,¹⁹ we described for the first time alterations of vascular sinusoids, so-called plaque sinus, consisting of atypical VWF+ conglomerates forming thickened VWF+ plaques in the endothelial walls of the bone marrow in patients with late TA-TMA. In addition, late TA-TMA manifests in patients at young ages at time of HSCT, likely with history of early TA-TMA under inadequate treatment and/or frequently virus infections, with the consequence of dismal clinical outcome. Previous studies concentrated on early TA-TMA commonly occurring within the first 100 days of HSCT, but there are also cases emerging beyond days +100 in the course of TA-TMA.¹⁸ Here we reported patients diagnosed with late TA-TMA between days +100 and +639, (IQR123-543) mostly with a history of early TA-TMA. Late TA-TMA shared the key laboratory features of classic TA-TMA. In our study, late TA-TMA represented with lower platelet counts and higher LDH compared to early TA-TMA. Previous reports of TA-TMA episodes up to 2 years after transplantation revealed patients with or without evidence of schistocytes.²⁰⁻²² Studies dealing with schistocytosis as evidence for TA-TMA diagnosis are controversial,⁹⁻¹² since in patients with TA-TMA schistocytes may not become apparent until the manifestation of other clinical symptoms or may be absent altogether.²³ Several validations of the proposed criteria have been published.^{4-6,17,24} Additional proposals, like the subtype of “probable early” TA-TMA including patients with schistocytes more or equal to 2% without nephropathy and neurologic

abnormalities have shown clinical significance regarding GVHD-specific survival and overall survival.^{25,26} Here, we found that the subtype of “probable early” TA-TMA was evident in the early but absent in the late TA-TMA. In addition, we reported a “possible” subtype of early TA-TMA with less or equal to 1% or no evidence of schistocytes in late and patients without late TA-TMA both with history of early TA-TMA. Consistently, both subtypes did not have diagnostic impact of TA-TMA. Histopathological assessment of the bone marrow revealed VWF+ enriched microvessels, thickened VWF+ plaque sinuses, as well as platelet aggregation and consumption, but without the consumption of coagulation factors as seen in DIC. We observed a slight obstruction of vascular lumen caused by these conglomerates, which is however only with a consequence of minimal hemolysis and the occasional evidence of schistocytes by destruction of red blood cells.

Late TA-TMA patients are younger than patients without late TA-TMA, irrespective of history of early TA-TMA. As commonly observed by classic TA-TMA,²⁰ early infections with HHV-6, adenovirus and EBV increase the incidence of TA-TMA. It has been shown that expression levels of vascular endothelial cell growth factor (VEGF) were low in patients with TA-TMA, especially in those having severe infections, e.g. most commonly adenovirus, HHV-6 and BK virus.²⁷ In addition, prolonged administration of CSA can also increase risk of TA-TMA.^{5,21,28} In this regard, the high incidence of acute liver GVHD and suspiciously hepatic veno-occlusive disease (VOD) observed in our study may suggest an altered hepatic metabolism of CSA in the course of hepatic GVHD²⁸ and by a high frequency of VOD associated with TA-TMA.²⁹ Notably, aGVHD proceeds early TA-TMA, while late TA-TMA coincides cGVHD. Both events significantly manifest the clinical features of steroid-refractory GVHD. The recent study of Zeisbrich³⁰ showed that early complications of TA-TMA are associated with steroid-refractory aGVHD. Our study provides an additional fatal variant of late TA-TMA associated with steroid refractoriness of cGVHD. In comparison to early TA-TMA, patients with late TA-TMA had milder clinical symptoms but considerably reduced overall survival and much worse clinical outcome. TA-TMA is thought to be a disease of microvascular endothelial complications. Still, late TA-TMA may not be seen as an own entity existing without alloreactive processes of GVHD, as perceived as for classic TA-TMA.^{18,30,31} The increase of bone marrow VWF+ microvessels along with CD8+ cytotoxic T-cells observed from early post-HSCT events may point immunological processes associated with GVHD and act eventually as comparable mechanism to severe chronic GVHD. Here we postulated that late TA-TMA seems to be the print-out of chronic endothelial GVHD. Patients, who suffered from late TA-TMA and progressive cGVHD simultaneously, did not respond to treatment of late TA-TMA. In addition, endothelial injury can be the direct consequence of cytotoxic donor T cells,³¹ causing upregulation of adhesion molecules, VWF and other triggers by endothelial activation.³² A participation of circulating endothelial cells in human vascular repair and potential involvement of CD34+ stem cells in processes of BM-derived re-endothelialization have previously been reported.³³⁻³⁵ As that, pathologic mechanisms underlying the hyperplasia of VWF+ and CD34+ microvascular endothelial cells in patients with cGVHD are postulated.



FIGURE 4 Proposed pathogenesis of post-HSCT complication of TA-TMA and GVHD. The concurrence of GVHD and TA-TMA over the course of post-HSCT complications triggers a sustained activation of alloreactive donor cells accompanying vascular endothelial markers in the bone marrow, and as a consequence of such effects—a progressive allo-reactive process of chronic GVHD along with alloimmune microangiopathy. Chronic endothelial complication may end up with irreversibly angiogenesis-hyperplasia. The very first laboratory abnormality of early TA-TMA involved the increase of LDH (day +30), whereas late TA-TMA was that of thrombocytopenia (day +176).



In contrast to our findings in the bone marrow, Biedermann et al. found a cytotoxic T lymphocyte-mediated loss of microvessels in the skin of patients with cGVHD, simultaneously followed by fibrosis with high serum level of VWF.¹⁴ However, VWF extravasation and signs of vascular proliferation were highly frequent in cutaneous aGVHD compared to control HSCT recipients without GVHD.³⁶ Thus, we attempt to explain this contradiction by different mechanisms of vascular injury in both organs, under the assumption of endothelial cell death of the skin in contrast to endothelial stimulation and angiogenesis in the bone marrow. Here we propose a hypothesis of the pathogenesis underlying TA-TMA and GVHD complications post-HSCT (Figure 4). Yet, we are aware of the relatively small, single-center cohort of patients. However, the selective patients with accessible bone marrow biopsies and mature long-term follow-up allowed us to depict the natural course of TA-TMA development. In our study, a proportion of 58% of patients with early TA-TMA did not exceed the highest proportion of 76% of microangiopathy as previously published.³ In all respects, this is also in agreement with study of Holler et al.²⁸ The portions of patients with aGVHD and cGVHD are in line with previous publications.^{31,37} In conclusion, our newly described characteristics of chronic allo-immune microangiopathy in BM specimens of late TA-TMA patients with unfavorable clinical outcome may help to diagnose late TA-TMA in patients without schistocytes but declining platelets and raising LDH. Alterations of vascular sinusoids, so-called plaque sinus, found in subsequent BM biopsy may confirm the diagnosis of late TA-TMA. Our findings have to be further validated in incorporated clinical trials, that is, which are assessing pharmacological endothelium protection.

AUTHOR CONTRIBUTIONS

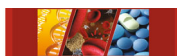
WH planned and designed the study, analyzed and interpreted the data, supervised laboratory and histological testing, performed the histological examination, and wrote the manuscript. KL supervised histological testing and performed the histological examination. AH supervised clinical management. HJK supervised clinical management. MS supervised laboratory and histological testing. JU performed bone marrow biopsies. JT designed and planned the study. TTP analyzed and interpreted the data and was a major contributor in writing the manuscript. MH supervised clinical management, analyzed and interpreted the data and was a major contributor in writing the manuscript. AR designed the study and supervised clinical management of the project and gave final approval. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflicts of interest.



DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are not publicly available due to data privacy restrictions but are available as a complete de-identified patient data set from W.H. (wolfgang.hill@med.uni-muenchen.de) on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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