Loss of endothelial thrombomodulin predicts response to steroid therapy and survival in acute intestinal graft-*versus*-host disease

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*Citation: Andrulis M, Dietrich S, Longerich T, Koschny R, Burian M, Schmitt-Gräf A, Schirmacher P, Ho AD, Dreger P, and Luft T. Loss of endothelial thrombomodulin predicts response to steroid therapy and survival in acute intestinal graft-*versus*-host disease. Haematologica 2012;97(11):1674-1677. doi:10.3324/haematol.2011.061051*

Online Supplementary Results

There was no significant difference in disease- and transplantation-related variables between steroid-sensitive and steroid-refractory patients. Control patients had less frequently mismatched unrelated donors (P=0.06) and less frequently received myeloablative conditioning regimens (P=0.09). Although more refractory patients (12 of 24, 50%) than sensitive patients (5 of 16, 31%) had steroid therapy prior to the biopsy, this difference was not statistically significant (P=0.39). Reasons for prior steroid therapy included engraftment syndrome (n=1), liver and/or skin GvHD (n=5), empiric treatment of intestinal GvHD triggered by clinical symptoms (n=7), initial steroid replacement of calcineurin inhibitors due to side effects (n=2), and delayed endoscopy due to patients' preferences (n=2) (*Online Supplementary Table S1*).

The endoscopy reports and pictures were graded according to the two different staging systems published. None of the staging systems revealed a statistically significant difference between refractory patients and sensitive patients (*Online Supplementary Table S2*). Comparison of steroid-refractory and steroid-sensitive intestinal GvHD patients at disease onset revealed a trend toward higher clinical grade GvHD (P=0.09) and a higher proportion of patients with multi-organ involvement in steroid-refractory disease: skin (P=0.08), liver (bilirubin levels, P=0.04) (*Online Supplementary Table S2*).

Fisher's test analysis showed a significant association between loss of thrombomodulin (TM) expression (TM-score cut off ≥ 2) and clinical course of GvHD. Loss of TM expression was not observed within the group of sensitive patients (*Online Supplementary Table S3*).

The numbers of infiltrating T cell intracellular antigen (TIA)-1 positive cytotoxic T/NK-cells were stained and quantified immunohistochemically. TIA-1 positive cytotoxic cells were found to infiltrate the epithelial cell layer and associate with apoptotic bodies inside the crypts. When comparing steroid-refractory, steroid sensitive and control patients, we observed that patients with histological proven GVHD had higher numbers of cytotoxic T/NK-cells compared to the control group (steroid-refractory: 35/HPF, sensitive: 20/HPF, controls: 10/HPF; refractory vs. control: P=0.02; sensitive vs. control patients: P=0.05). However, there was no significant difference in counts of TIA-1 positive cytotoxic cells between biopsies of steroid-sensitive and steroid-refractory patients (*Online Supplementary Figure S3*).

Microvessel density was increased in colon biopsies of patients with histologically proven GvHD compared to control patients (median: 39.3 *vs.* 16; P=0.002), but no difference was found between steroid-sensitive and steroid-refractory cases (median: 29.6 *vs.* 32.5; P=0.58). Additional morphological features of endothelial damage, such as intimal lymphocytic infiltrates (n=1), or perivascular hemorrhage (n=2), were rare. Furthermore, we did not detect any microthrombi in our series (*Online Supplementary Figure S4*).

Online Supplementary Table S1. Patients characteristics

Parameter	Control (no GVHD) n=11	p[1] (χ^2 -or t-test)	GvHD steroid-sens. n=16	steroid-refract. n=24	p[2] (χ^2 - or t-test)
Median age at HSCT	52	0.56	48	50	0.37
Donor		0.06			0.22
RD	5/11		9/16	10/24	
MUD	6/11		5/16	5/24	
MMUD	0/11		2/11	9/11	
Sex mismatch R/D		0.52			0.31
Female-female or male-male	7/11		9/16	18/24	
Female-male	2/11		4/16	4/24	
Male-female	4/11		3/16	0/24	
Disease stage *		0.26			0.17
0	3/11		2/16	9/24	
1	4/11		4/16	3/24	
2	4/11		10/16	11/24	
n.a.	0/11		0/16	1/24	
ATG	7/11	0.30	6/16	13/24	0.20
Stem cell source		0.25			0.62
PBS	11/11		15/16	20/24	
BM	0/11		1/16	4/24	
Conditioning		0.09			0.82
RIC	11/11		11/16	16/24	
MAC	0/11		5/16	8/24	
Cause of death					
NRM	0/11	0.001	1/16	16/24	0.02
PD	6/11	0.03	5/16	3/24	0.22
Disease		0.33			0.25
AML, MDS	5/11		5/16	11/24	
Lymphoma, CLL	3/11		5/16	6/24	
Myeloma	1/11		5/16	3/24	
Other	2/11		1/16	4/24	
HCT comorbidity index					0.23
0-1	6/11		12/16	20/24	
2-3	3/11		3/16	4/24	
4-5	2/11		1/16	0/24	

[1]P value comparing no GVHD vs. GVHD; [2]P value comparing sensitive vs. refractory GVHD, RD: related donor; MUD: matched unrelated donor; MMUD: mismatched unrelated donor; R/D: recipient/donor; BM: bone marrow; PBSC: peripheral blood stem cells; RIC: reduced intensity conditioning; MAC: myeloablative conditioning; NRM: non-relapse mortality; PD: progressive disease; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; MPS: myeloproliferative syndrome; ALL: acute lymphoid leukemia; CLL: chronic lymphocytic leukemia; Myeloma: multiple myeloma; HCT comorbidity index: hematopoietic cell transplantation-specific comorbidity index. Disease stage as defined in the "EBMT risk score for stem cell transplants".

Online Supplementary Table S2. GVHD associated characteristics at biopsy.

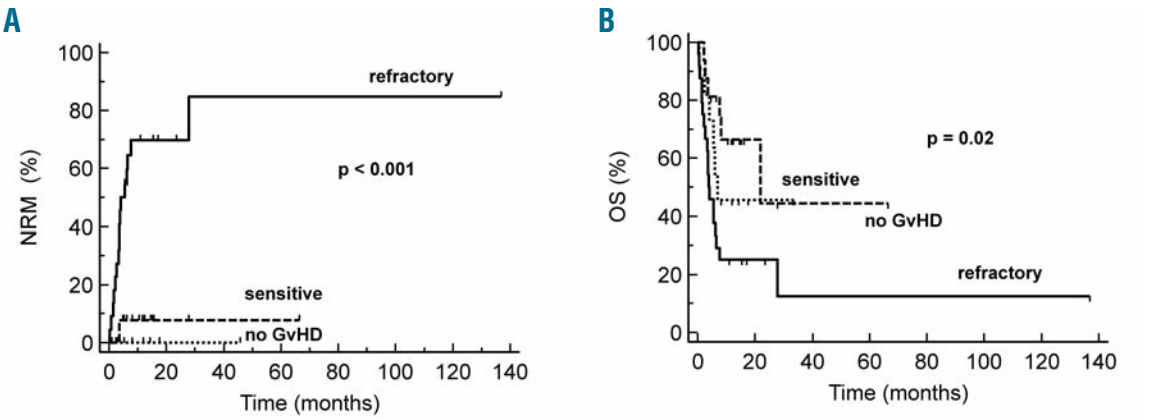
Parameter	GvHD steroid-sens. n=16	GvHD steroid-refract. n=24	*P* (χ^2- or Mann Withney-test)
Gut GvHD at biopsy			0.70
1-2	13/16	17/24	
3-4	3/16	7/24	
Skin GvHD at biopsy			0.08
1-2	10/16	7/24	
3-4	6/16	17/24	
Clinical GvHD Grade at biopsy			0.09
1-2	9/16	6/24	
3-4	7/16	18/24	
Histological Grade at 1st biopsy			0.73
1-2	11/16	14/24	
3-4	5/16	10/24	
Bilirubin (mg/dL, median+range)	0.6 (0.2-4)	1.3 (0.3-5.3)	0.04
Liver function Test (GPT U/l, median+range)	34 (8-83)	25 (12-209)	0.73
Albumin (g/L, median+range)	30 (26-48)	28 (21-41)	0.35
Endoscopic scoring (Cheung *et al.*)			0.30
0	5/16	4/24	
1-2	5/16	8/24	
3-4	6/16	8/24	
n.a.	0/16	4/24	
Endoscopic scoring (Martinez *et al.*)			0.29
0-1	11/16	11/24	
2-3	5/16	9/24	
n.a.	0/16	4/24	
Months after SCT	2.5	3.7	0.37
Steroids prior to biopsy			0.39
yes	11/16	12/24	
no	5/16	12/24	

Comparison of steroid-refractory and steroid-sensitive intestinal GVHD patients at biopsy (disease onset) reveals a trend toward higher clinical grade GVHD and multiorgan involvement (skin, liver) in steroid-refractory patients. The endoscopy reports and pictures were graded according to the two different staging systems published None of the staging systems revealed a statistically significant difference between refractory patients and sensitive patients (Table 2). Comparison of steroid-refractory and steroid-sensitive intestinal GVHD patients at disease onset revealed a trend toward higher clinical grade GVHD (p=0.09) and a higher proportion of patients with multiorgan involvement in steroid-refractory disease (skin (p=0.08), liver (bilirubin levels, P=0.04)) (Table 2).

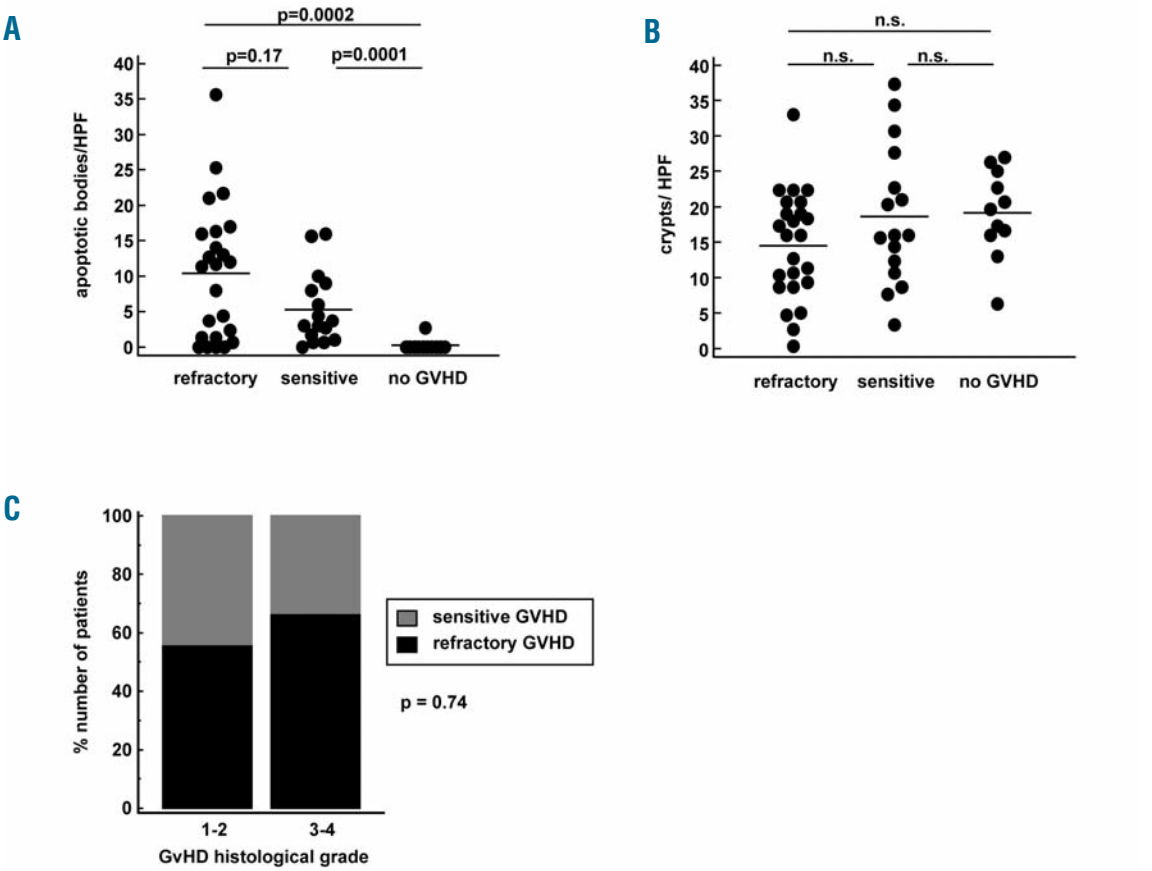
Online Supplementary Table S3. Loss of TM expression and steroid-response.

	Refractory GVHD	Sensitive GVHD	No GVHD	Total
TM negative	14	0	2	16 (31%)
TM positive	10	16	9	35 (69%)
	24	16	11	
	(47.1%)	(31.4%)	(21.6%)	51

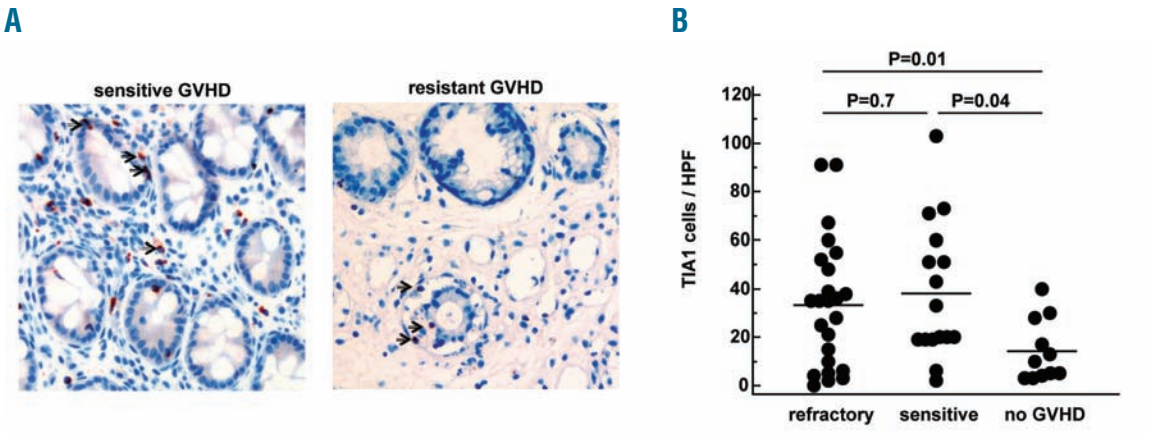
P<0.001 was considered significant.



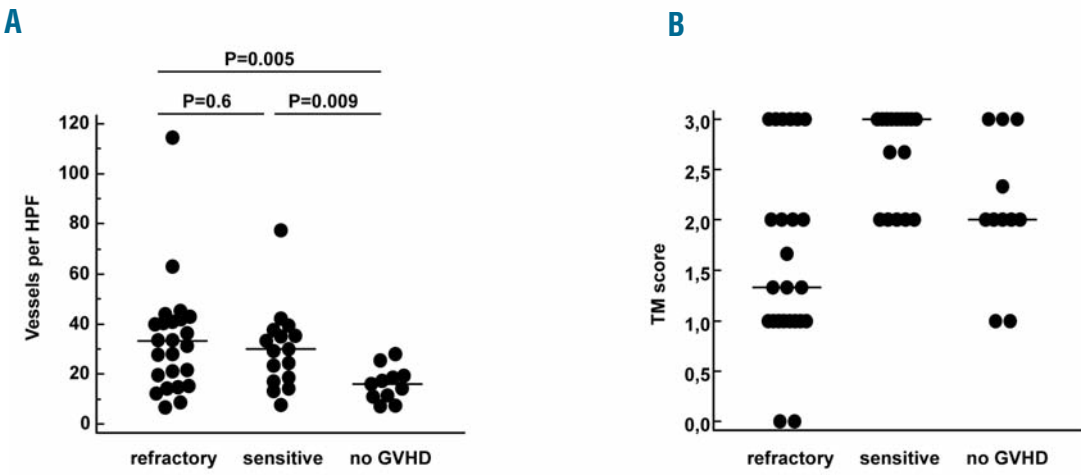
Online Supplementary Figure S1. Survival after allogeneic SCT. (A) Non-relapse mortality from allogeneic SCT of patients without GvHD, with steroid-refractory and therapy-sensitive GvHD. (B) Overall survival after allogeneic SCT of patients without GvHD, with steroid-refractory and therapy-sensitive GvHD.



Online Supplementary Figure S2. Histological grading of intestinal biopsies. (A) Number of apoptotic bodies per crypt (lines: medians+/-95%CI, n=50) and (B) number of crypts per high power field (HPF) (lines: medians+/-95%CI, n=51) in intestinal biopsies of patients with clinical suspicion of GvHD. Patients with histologically proven GvHD had higher numbers of apoptoses and steroid-refractory patients showed a trend toward lower crypt numbers (non-parametric Mann-Whitney test). (C) No significant differences between steroid-refractory and sensitive patients were found in either parameter or in the histological grading of GvHD (Fisher's test).



Online Supplementary Figure S3. TIA-1 positive cytotoxic T/NK-cells in intestinal biopsies. (A) TIA-1 positive T/NK-cells invade the epithelial layer and associate with apoptotic bodies (arrow) in intestinal biopsies of patients with GvHD. Representative examples of a patient with steroid-refractory (right) and a patient with steroid-sensitive (left) GvHD (400x original magnification). (B) Numbers of TIA-1 positive T/NK-cells per high power field (HPF) are significantly increased in patients with GvHD, but no difference was observed between steroid-refractory and sensitive patients (lines: medians±95%CI, n=51, non-parametric Mann-Whitney test).



Online Supplementary Figure S4. Vessel density and TM score in colon mucosa of GvHD-patients. (A) Numbers of $CD34^+$ vessels per high power field (HPF) are significantly increased in patients with GvHD, but no difference was found between steroid-refractory and sensitive patients (lines: medians±95%CI, n=51, non-parametric Mann-Whitney test). (B) Endothelial thrombomodulin expression (TM score) of biopsies of 51 patients with clinical suspicion of GVHD evaluated before start of immunosuppressive treatment for GVHD. Low TM expression (score<2) was found in 14/24 patients with steroid-refractory and 0/16 patients with sensitive disease (lines: medians±95%CI, n=51, non-parametric Mann-Whitney test).