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Full Length Article
Biomarkers

Easix Score Correlates With Endothelial Dysfunction Biomarkers and Predicts Risk of Acute Graft-Versus-Host Disease After Allogeneic Transplantation

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A B S T R A C T

Plasma biomarkers of endothelial dysfunction have been postulated for the diagnosis and prognosis of acute graft-versus-host disease (aGVHD). However, their use is not validated in clinical practice yet. The endothelial activation and stress index (EASIX), a simple score based on routine laboratory parameters, is considered to be an indirect marker of endothelial damage. High value of EASIX was correlated with worse non-relapse mortality (NRM) and overall survival (OS) and a high risk of sinusoidal obstructive syndrome and transplant-associated thrombotic microangiopathy (TA-TMA). This study investigates the predictive value of plasma biomarkers and the EASIX score for the prediction of aGVHD. We assessed vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor receptor 1 (TNFR1), and VWF:Ag plasma levels and the EASIX score before allogeneic hematopoietic stem cell transplantation (allo-HSCT) and on days 0, 3, 7, 14, and 21 in an experimental cohort (n = 33). EASIX was transformed to a base-2 logarithm to perform the analysis. For the most relevant biomarkers, we estimate the optimal cutoff values and the discriminatory ability to differentiate patients with high-risk of aGVHD. The conclusions obtained in the experimental cohort were validated in a large cohort of 321 patients at the same institution. Plasma biomarkers and EASIX showed similar post-transplantation dynamics consisting of a progressive increase.

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Multivariate analysis showed an association between high TNFR1 levels and Log-2 EASIX score on day 7 after transplantation with an increased likelihood of developing aGVHD (hazard ratio [HR] = 1, P = .002; HR = 2.31, P = .013, respectively). Patients with TNFR1 ≥1300 ng/mL (HR = 7.19, P = .006) and Log2-EASIX ≥3 (HR = 14.7, P < .001) at day 7 after transplantation were more likely to develop aGVHD with high predictive accuracy (C-index of 74% and 81%, respectively). In the validation cohort, patients with Log2-EASIX ≥3 on day 7 after transplantation presented a significantly higher incidence of grade II-IV aGVHD (HR = 1.94, P = .004) independent of GVHD prophylaxis (HR = 0.38, P = .004), conditioning regimen (HR = 0.59, P = .02) and type of donor (HR = 2.38, P = .014). Differential degree of endothelial damage can be measured using both EASIX score and plasma biomarkers in the early post-transplantation period. Patients at risk of developing aGVHD could be easily identified by a high EASIX score. Both indicators of endothelial activation represent a promising approach to predict aGVHD.

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Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Diagnosis of acute GVHD (aGVHD) is based on clinical features, patient symptoms, laboratory values, and, in most cases, histological confirmation. In recent years, efforts have been made to identify potential noninvasive peripheral blood biomarkers with diagnostic and clinical value in aGVHD, and some promising results have been achieved with markers related to endothelial damage [1–3].

Similar to other early transplant-related complications such as sinusoidal obstruction syndrome, transplant-associated thrombotic microangiopathy (TA-TMA), or engraftment syndrome (ES), endothelial activation and dysfunction have been implicated in the pathophysiology of aGVHD [4–8]. Several soluble biomarkers of endothelial dysfunction have been postulated for the diagnosis and prognosis of aGVHD. These biomarkers include von Willebrand factor (VWF), thrombomodulin (TM), tumor necrosis receptor 1 (TNFR1), plasminogen activator inhibitor type 1, adhesion molecules such as E-selectin, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), suppression of tumorigenicity-2 (ST2), angiopoietin-2 (ANG2), hyaluronic acid (HA), L-Ficolin, and circulating endothelial cells (CECs), among others [9–14]. In general, the majority of studies have shown an increase in the levels of the different markers compared to baseline, especially in patients with transplant-related complications, including aGVHD [2,3,15-17]. We have previously reported that levels of VWF and TNFR1 above an optimal cutoff at day 7 after allo-HSCT could positively predict that approximately 90% of patients will develop aGVHD [9]. Some biomarkers are now being incorporated into clinical trials design to validate and standardize their value in current clinical practice. In addition to validation, technical issues, such as adequate sample processing, availability of the tests in all centers, and rapidity of results, may be one of the main limitations to the widespread implementation of these plasma biomarkers in clinical practice.

The endothelial activation and stress index (EASIX) is a simple laboratory formula (creatinine [mg/dL] × LDH [U/L]/platelets [$\times 10^9$/L]) considered an indirect measurement of endothelial activation. A high EASIX score before and after allo-HSCT has been correlated with worse nonrelapse mortality (NRM) and overall survival (OS) [1,18-22] and a high risk of post-transplantation vascular endothelial complications such as sinusoidal obstruction syndrome and TA-TMA [23,24]. However, its role in predicting aGVHD has not been extensively investigated.

Based on the ability of EASIX to predict early post-transplantation vascular endothelial complications and the lack of easily applicable biomarkers for predicting aGVHD in the allo-HSCT setting, the present study investigates whether the measurement of endothelial activation biomarkers and EASIX during the early post-transplantation period can predict the onset of aGVHD.

METHODS

Study Design

We hypothesized that the dynamics of the EASIX score in the early post-transplantation period would be similar to the dynamics of endothelial damage plasma biomarkers; therefore it could be used for prediction of clinically relevant aGVHD. The present study was conducted in 2 phases. In the first part, the dynamics plasma

biomarkers of endothelial damage and EASIX score were measured at different time points before and after allo-HSCT in an experimental cohort of 33 allo-HSCT recipients using peripheral blood stem cell grafts and reduced-intensity conditioning (RIC) regimens. Then the potential association between the plasma biomarkers levels and EASIX score with the risk of developing grade II-IV aGVHD was evaluated.

In the second part of the study, we investigated whether EASIX score results in predicting aGVHD were reproducible in a larger validation cohort of 321 patients undergoing allo-HSCT at the same institution. Because endothelial dysfunction after allo-HSCT can be induced by RIC regimens, but more commonly by myeloablative conditioning (MAC) regimens, the validation cohort included patients who received RIC and MAC regimens. This was done to investigate whether the results observed in the analysis were independent of the intensity of the conditioning regimen administered as part of the transplant platform [25]. The local ethics committee approved the study (HCB/2021/0948), which was conducted in accordance with the Declaration of Helsinki.

Patients and Allo-HSCT Procedure

Patients suffering from malignant hematologic diseases who consecutively underwent allo-HSCT at the Hospital Clínic of Barcelona between January 2014 and October 2020 were included in the study. To form the experimental cohort, a group of 33 patients with plasma samples available for biomarker analysis at all predefined timepoints of the study before and after transplantation was selected.

The conditioning regimens used were based on the type of hematological malignancy and patient characteristics. Patients aged >50 years or who had previously undergone autologous HSCT received an RIC regimen; otherwise, MAC regimens were administered. A summary of the different types of conditioning regimens used is shown in Table 1.

Our institutional protocol for GVHD prophylaxis historically consisted of conventional calcineurin inhibitor-based GVHD prophylaxis with cyclosporine (5 mg/kg from days −1 to 3 and then 3 mg/kg to day 270 in the absence of GVHD) plus methotrexate (15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11) or mycophenolate mofetil (10 mg/kg every 8 hours, maximum 3 g daily, from day 0 to day 60). Since 2016, it has been progressively introduced PTCY-based schemes (50 mg/kg/day on days 3 and 4) for the prevention of GVHD, initially in transplants from haploidentical and mismatched unrelated donor and more recently in transplants from matched unrelated donor and matched related donor. Antithymocyte globulin or alemtuzumab were not administered.

Donor selection and supportive care have been described in detail previously [26]. All patients included in the study received T-cell–replete peripheral blood stem cell grafts. None of the patients received letermovir for cytomegalovirus prophylaxis. Acute GVHD was clinically and histologically diagnosed and graded using standard criteria [27,28]. Patients with aGVHD were treated homogeneously throughout the study period.

Plasma Biomarker Assessment

The sample size of the experimental cohort was selected in accordance with the previous studies of our group [9,15,29]. Only patients with plasma samples available at all study time points were chosen for the study.

Based on our previously published work, we chose to analyze plasma levels of VCAM-1, TNFR1, and VWF:Ag as biomarkers of endothelial damage [9,15,29]. Individual blood samples were collected prospectively at different time points during allo-HSCT: before conditioning (pre-transplantation), immediately before transplantation (day 0), and on days 3, 7, 14, and 21 after allo-HSCT. Plasma was obtained by centrifugation of anticoagulated blood samples collected in 3.8% sodium citrate tubes, within 4 hours of collection. The samples were aliquoted in cryovials without additives and stored at −80°C until use. The biomarkers VCAM-1 (R&D Systems, Minneapolis, MN), TNFR1 (BosterBio, Pleasanton, CA), and VWF:Ag (Grifols, Barcelona, Spain) were assessed by enzyme-linked immunosorbent assay.

EASIX Score Assessment

The EASIX score was calculated retrospectively in both cohorts of patients according to the following formula: creatinine (mg/dL) × LDH (U/L) / platelets ($\times 10^9$/L). The analytical parameters used to calculate the score were obtained from routine patient blood samples before conditioning (pre-transplantation), immediately before transplantation (day 0), and on days 3, 7, 14, and 21 after allo-HSCT.

Statistical Methods

First, using the experimental cohort data, our study examined the dynamics of VCAM-1, TNFR1, VWF:Ag plasma levels and EASIX score during the

Table 1
Patient and Transplantation Characteristics of Experimental and Validation Cohorts

	Experimental Cohort (N = 33) (%)	Validation Cohort (N = 321) (%)
Patient information		
Age (yr), median (range)	60 (41-69)	52 (18 -70)
Male sex	16 (48)	170 (53)
Baseline diagnosis		
AML	11 (33)	128 (40)
ALL	2 (6)	44 (14)
MDS/CMML	10 (30)	55 (17)
MPN	3 (9)	30 (9)
NHL/CLL	4 (13)	46 (15)
Others	3 (9)	18 (5)
High-very high Disease Risk Index	16 (48)	54 (17)
60%-80% Karnofsky Performance Status	12 (36)	80 (25)
HCT-CI score >3	10 (30)	74 (23)
Transplant information		
Age donor median (y), (range)	41 (19-68)	38 (15-73)
Donor sex		
Male	20 (61)	208 (65)
Female to Male (Donor/recipient)	13 (39)	50 (16)
Donor selection		
HLA MSD	10 (30)	87 (27)
10/10 HLA MUD	13 (40)	125 (39)
7/8 HLA MMUD	10 (30)	68 (21)
Haploidentical	–	41 (13)
Conditioning regimen		
Myeloablative (MAC)		
Cy/Bu 4 days	–	19 (6)
Cy/TBI 12Gy	–	13 (4)
FLUBU 4 days	–	58 (18)
FLU/TBI 12Gy	–	38 (12)
RIC		
FLUBU 2–3 days	26 (79)	116 (36)
FLUTBI 2-8Gy	4 (12)	38 (12)
FLUMEL	–	13 (4)
IDA/FLAG/MEL (sequential)	2 (6)	13 (4)
FLU/CFM/TBI2Gy (Baltimore)	1 (3)	13 (4)
GVHD Prophylaxis		
CNI/MTX	-	48 (15)
CNI/MMF	11 (33)	68 (21)
CNI/RAPA	1 (3)	2 (1)
PTCY/TK	18 (54)	148 (46)
PTCY/TK/MMF	3 (10)	55 (17)
Main post-transplantation outcomes		
Cumulative Incidence of aGVHD (95% CI)		
Grade II-IV acute GVHD (+100)	18.8 (7.5-34)	26.2 (21.5-31.1)
Grade III-IV acute GVHD (+100)	9.1 (2.3-21.9)	7.5 (4.9-10.7)
2-year non-relapse mortality [% (95% CI)]	25.2 (11.6-41.4)	14.9 (11.2-19.1)
2-year Cumulative incidence of relapse, % (95% CI)	31.3 (16.1-47.8)	27.8 (23.0-32.9)
2-year Overall survival, % (95% CI)	49.5 (31.2-65.4)	67.8 (62.3-72.8)
2-year Relapse free survival, % (95% CI)	43.5 (26.0-59.7)	57.3 (51.5-62.6)
Follow-up (mo), median (IQR)	21 (4.1-45.1)	28.8 (9.3-53.8)

HCT-CI indicates hematopoietic cell transplantation comorbidity index; MSD, matched sibling donor; MUD, matched unrelated donor; MMUD, mismatched unrelated donor.

early post-transplant period and the ability of EASIX and the selected endothelial damage biomarkers to predict grade II-IV aGVHD. Plasma biomarkers and EASIX score results are presented as medians and ranges. To graphically represent the statistical trend in which plasma biomarkers and EASIX score move over time after transplantation with respect to the pretransplantation time point, we used the Stacked Line Chart provided by the Microsoft Excel program. Second, the ability of EASIX to predict grade II-IV aGVHD was evaluated in the 321 adults included in the validation cohort.

EASIX was transformed into a base-2 logarithm (Log2-EASIX) for statistical analysis. Descriptive information was calculated using counts and percentages and compared using the chi-square test, Fisher's exact test, and Mann-Whitney U test. The cumulative incidence function of grade II-IV aGVHD was estimated using cumulative incidence regression analyses and accounting for death and relapse as competing events. The variations of Log^2-EASIX, VCAM-1, TNFR1 and VWF:Ag values during the early post-transplantation period, and their correlation with grade II-IV aGVHD was explored using landmark and regression analyses. Those patients who died or those who were diagnosed with the study time-dependent adverse event before each landmark point were excluded from the next time point.

The association between higher endothelial activation and damage (measured using endothelial damage biomarkers and EASIX score) and grade II-IV aGVHD risk occurring during the first 100 days after allo-HSCT was analyzed using Fine-Gray proportional hazard regression for competing events. This association was investigated treating the explanatory variables as continuous and discrete after estimating the optimal cutoff values for discriminating high-risk patients. Because of the sample size, in the experimental cohort, the variables included in the univariate analysis as predictive of aGVHD were the research variables (plasma biomarkers of endothelial damage and log2-EASIX). A multivariate analysis was performed to investigate whether the predictive ability of a particular biomarker was superior to that of the others. In the validation cohort, in addition to the experimental variable log2-EASIX, all clinical variables classically associated with the risk of developing aGVHD were included in the univariate and multivariate analysis. Last, the accuracy of Log2-EASIX, VCAM-1, TNFR1 and VWF:Ag for predicting grade II-IV aGVHD was explored using the concordance index (C-index). This index confirms the predictive power of the evaluated score for values superior to 0.5 with perfect discrimination of 1. All P values were 2-sided, and $P < .05$ indicated a statistically significant result. SPSS and R software were used to perform statistical analysis.

RESULTS

Patient, Transplant Characteristics, and Outcomes

The baseline characteristics of the patients and the transplant procedure are summarized in Table 1. The experimental and validation cohorts included 33 and 321 patients, respectively. Acute leukemia and myelodysplastic syndrome were the most common pretransplantation diagnoses in both cohorts. All patients in the experimental cohort and 60% of the validation cohort received RIC regimens. Most patients in both groups received allo-HSCT from HLA-identical related and unrelated donors. PCTy-based prophylaxis was used in 64% of the patients included in the experimental cohort and in 63% of the adults included in the validation cohort.

Overall, 32% of patients developed grade aGVHD at a median of 40 days (interquartile range [IQR] = 26-64) after transplantation [40 days for grade II-IV (IQR = 26-59) and 42 days for grade III-IV (IQR = 27-67)]. The cumulative incidences of grade II-IV and grade III-IV aGVHD at day 100 were 18.8% and 9.1% for the experimental cohort, and 26.2% and 7.5% for the validation cohort. The median follow-up of survivors was 21 (range 4.1-45.1) and 28.8 (range 9.3-53.8) months, respectively. For all patients, the 2-year overall survival (OS) and progression-free survival rates were 67.8% (95% confidence interval [CI], 62.3-72.8) and 57.3 (95% CI, 51.5-62.6), respectively.

Association Between Plasma Biomarkers, the EASIX Score, and the Development of Acute GVHD in the Experimental Cohort

Plasma levels of VCAM-1, TNFR1 and VWF:Ag showed an increasing dynamic in the post-transplantation period in all patients from before transplantation until day 21. A graphical representation of the statistical trend in which plasma biomarkers and Log2-EASIX data move over time after transplantation with respect to pretransplantation values is shown in Figure 1. Log^2-EASIX score increased rapidly after transplantation and peaked at day 7 (median Log^2-EASIX 1.64, IQR = 0.53-3.16), followed by a slight decline at days 14 (median Log2-EASIX 1.45, IQR 0.22-

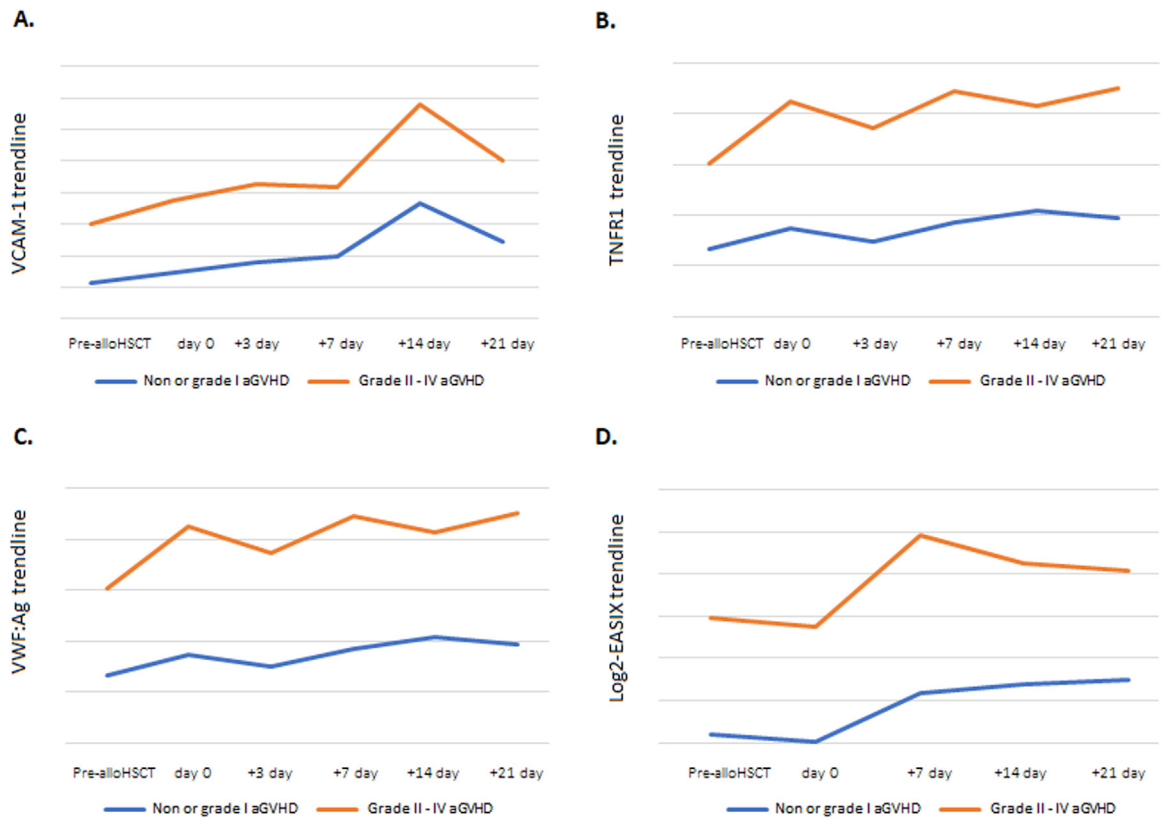

Figure 1. Graphical representation of the statistical trend in which plasma biomarkers and Log2-EASIX data move over time after transplant with respect to pre-transplant values at the experimental cohort: (A) VCAM-1, (B) TNFR1, (C) VWF:Ag, and (D) Log2-EASIX.

2.58) and +21 (median Log^2-EASIX 1.07, IQR = 0.57-1.47), remaining above the pretransplantation baseline (Table 2, Figure 1D).

Patients who developed grade II-IV aGVHD had higher levels of plasma biomarkers and the Log^2-EASIX score than those without aGVHD or grade I aGVHD (Table 2). In the univariate analysis, VCAM-1 levels measured on day 7 and TNFR1 levels measured at all-time points after transplantation were significantly associated with an increased risk of grade II-IV aGVHD (Table 3). Multivariate analysis confirmed the association between high TNFR1 levels on day 7 after transplantation with an increased likelihood of developing grade II-IV aGVHD (hazard ratio [HR] = 1, P = .002). Similarly, the Log^2-EASIX score measured on days 0, 7, 14, and 21 was predictive of grade II-IV aGVHD, with the highest hazard ratio (HR) for day + (HR - 2.31, P = .013) (Table 3).

Considering these results, optimal cutoff values for TNFR1 and Log2-EASIX score on day 7 were estimated to discriminate patients at high risk of developing aGVHD. We found that patients with TNFR1 $\geq$ 1300 ng/mL (HR = 7.19, P = .006) and Log2-EASIX $\geq$ 3 (HR = 14.7, $P < .001$) at day 7 after transplantation were more likely to develop grade II-IV aGVHD with high predictive accuracy (C-index of 74% and 81%, respectively) (Figure 2).

EASIX Dynamics and Log2-EASIX Cutoff Applicability in the Validation Cohort

Similar to the experimental cohort, the Log2-EASIX score increased from pretransplantation levels to a peak on day 7, followed by a slight decrease on days 14 and 21 after transplantation, which remained above baseline (Figure 3A). Patients who developed aGVHD had higher Log^2-EASIX scores throughout the analysis period, with a peak on day 7 after transplantation (Figure 3A). Patients with Log2-EASIX values $\geq$3 on day 7 after transplantation had a significantly higher cumulative incidence of grade II-IV aGVHD than those with low values (HR = 1.82, P = .004) (Table 4, Figure 3B). Moreover, the univariate analysis show that the use of post-transplant high-dose cyclophosphamide–based GVHD prophylaxis (HR = 0.58, P = .009) and RIC regimen (HR = 0.64, P = .037) were associated with a low risk of aGVHD (Table 4). Multivariate analysis confirmed that Log2-EASIX $\geq$3 had a predictive value for aGVHD (HR = 1.98, P = .003) independently of the age (HR = 0.99, P = .54), the type of GVHD

Table 2
VCAM-1, TNFR1, VWF:Ag, and Log2-EASIX Median Values According to Presence of Grade II–IV aGVHD

	Pre-transplantation	Day 0	Day 3	Day 7	Day 14	Day 21
Experimental cohort						
VCAM-1 (ng/mL)						
None or grade I aGVHD (IQR)	58.1 (35.2-120.7)	73.5 (36.3-114)	93 (75-152)	97.9 (45-225.3)	181(50.2-277)	147.5 (55-247.3)
Grade II-IV aGVHD (IQR)	84 (54.8-241.4)	92.9 (61.8-175.3)	117.1 (68.7-149)	104 (40.7-237.7)	169 (51.5-238.5)	123.9 (51.5-198.8)
TNFR1 (ng/mL)						
None or grade I aGVHD (IQR)	658.7 (521.9-745.3)	868.2 (567.4-1019.8)	745.1 (685-862.2)	791.3 (628.4-1212.6)	981 (748-1278.2)	942.7 (794.4-1429.5)
Grade II-IV aGVHD	849.6 (578.2-980.4)	1115.7 (759.6-1488.8)	965.8 (646.3-1254.9)	932.3 (716.2-1481.4)	1140.6 (993.8-1700.5)	1108.3 (937.3-1421.6)
VWF:Ag (ng/mL)						
None or grade I aGVHD (IQR)	114.3 (98.7-167.3)	166.4 (154.7-208.6)	185 (136.2-242.9)	173.1 (112.7-322.5)	237.8 (119.5-402.6)	284.7 (216.1-404.1)
Grade II-IV aGVHD (IQR)	137.1 (111.4-167.1	182.9 (136.1-253.8)	198 (159.4-353.6)	187.4 (146.6-344.2)	194.3 (155.4-350.5)	250.5 (169.9-373)
Log2-EASIX						
None or grade I aGVHD (IQR)	0.33 (−0.48 to 0.56)	0.72 (−0.69 to 0.96)	—	1.49 (0.78-2.58)	1.12 (0.68-2.11)	0.54 (−1.07 to 1.23)
Grade II-IV aGVHD (IQR)	0.37 (-1.09-3.02)	1.18 (0.68-2.72)	—	2.72 (0.23-4.26)	2.41 (0.55-3.33)	1.34 (0.32-2.05)
Validation cohort						
Log2-EASIX						
None or grade I aGVHD (IQR)	0.4 (−0.37 to 1.38)	0.54 (−0.16 to 1.36)	—	2.4 (1.49-3.30)	1.95 (0.81-3.12)	1.95 (0.51-3.22)
Grade II-IV aGVHD (IQR)	0.88 (−0.02 to 1.97)	0.95 (0.14-2.03)	—	2.91 (1.45-3.63)	2.26 (1.02-3.36)	2.01 (0.97-3.52)

Table 3
Predictive Value of VCAM-1, TNFR1, VWF:Ag, and Log2-EASIX for Grade II-IV Acute GVHD in the Experimental Cohort

	Pretransplantation		Day 0		Day 3		Day 7		Day 14		Day 21	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Analysis of the predictive value of VCAM-1, TNFR1, VWF:Ag for grade II-IV aGVHD												
	Univariate Analysis											
VCAM-1 ng/mL	1 (0.99-1)	.90	1 (0.99-1)	.92	1 (0.99-1)	.54	1 (1-1)	<**.001**	0.99 (0.99-1)	.27	0.99 (0.99-1)	.44
TNFR1 ng/mL	0.99 (0.99-1)	.49	1 (0.99-1)	.42	1 (1-1)	**.001**	1 (1-1)	<**.001**	1 (1-1)	<**.001**	1 (1-1)	**.002**
VWF:Ag ng/mL	1 (0.99-1.01)	.62	1 (0.99-1)	.89	1 (0.99-1)	.31	1 (0.99-1.01)	.15	0.99 (0.99-1)	.75	1 (0.99-1)	.99
Multivariate regression analysis of the three plasma biomarkers at day 7												
VCAM-1 ng/mL	—		—		—		0.7 (0.99-1.01)	.97	—		—	
TNFR1 ng/mL	—		—		—		1.01(1.00-1.01)	**.002**	—		—	
VWF:Ag ng/mL	—		—		—		1.01 (0.99-1.01)	.99	—		—	
Analysis of the predictive value of log2-EASIX for grade II-IV aGVHD												
	Univariate Analysis											
Log2-EASIX	1.68 (0.96-2.98)	.06	2.03 (1.14-3.61)	**.01**	—		2.31 (1.19-4.48)	**.013**	1.89 (1.16-3.07)	**.009**	1.64 (1.14-2.37)	**.007**

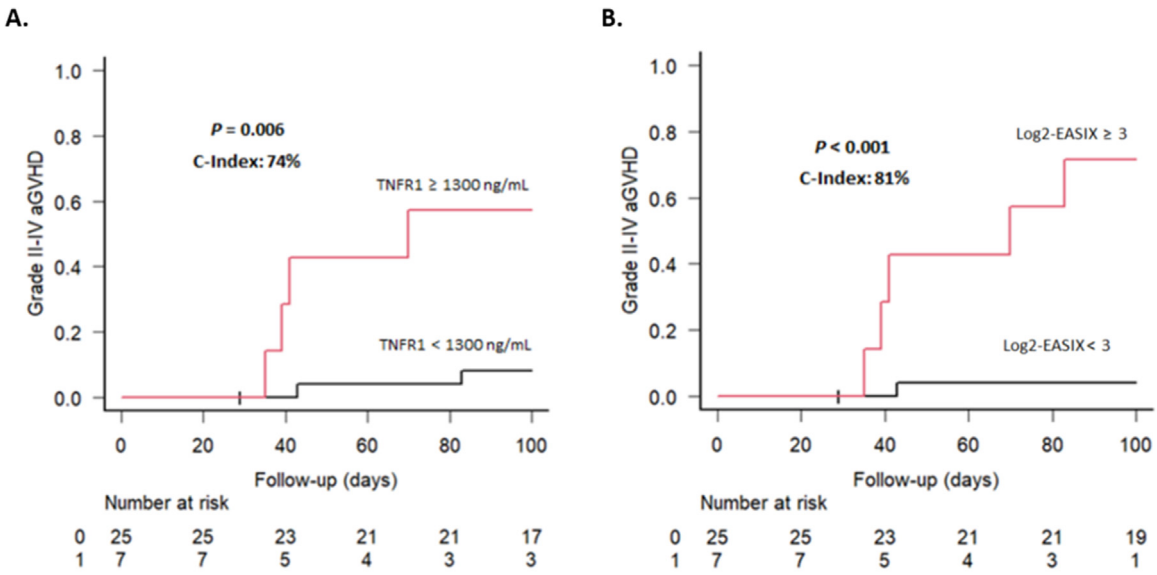

Figure 2. Cutoff value at day 7 to evaluate II-IV aGVHD in the experimental cohort for (A) TNFR1 and (B) Log2-EASIX.

prophylaxis (HR = 0.38, P = .005), intensity of conditioning regimen (HR = 0.65, P = .10), and the type of donor (HR = 2.29, P = .021) (Table 4). Log^2-EASIX values ≥3 on day 7 after transplantation were also significantly associated with higher NRM (HR = 1.18; 95% CI, 1.0-1.3; P = .03), and lower OS (HR = 1.15; 95% CI, 1.0-1.2; P = .01).

DISCUSSION

In the present study we demonstrated that plasma biomarkers of endothelial dysfunction, mainly TNFR1, and the EASIX score showed a parallel post-transplantation dynamic, consisting of a progressive increase through the period of observation after transplantation. Both parameters, TNFR1 levels and EASIX score, were significantly higher in patients who developed grade II-IV aGVHD. We were able to identify a cutoff value of these parameters in an experimental cohort of patients that was significantly associated with an increased risk of developing aGVHD. The predictive value of the EASIX score as a surrogated of endothelial dysfunction in predicting aGVHD was confirmed in a large cohort. Our results suggest that measuring endothelial damage using EASIX score or plasma biomarkers is a promising

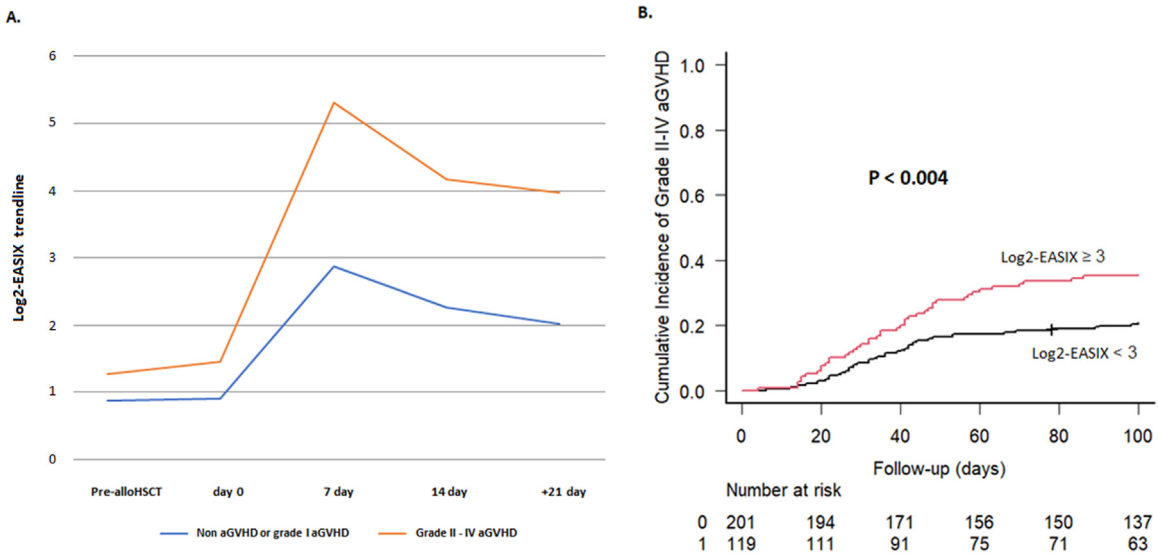

Figure 3. Graphical representation of the statistical trend in which (A) Log2-EASIX data move over time after transplant with respect to pretransplantation values at the validation cohort, and (B) Log2-EASIX cutoff value at day 7 to evaluate II-IV aGVHD.

Table 4
Predictive value of Log2-EASIX for grade II-IV aGVHD in the validation cohort.

	HR (95% CI)	*P* value
Univariate Analysis		
Log2-EASIX ≥3	1.82 (1.21-2.74)	.004
Age (continuous variable)	0.98 (0.97-1.01)	.12
PTCy-based GVHD prophylaxis (versus other)	0.58 (0.38-0.87)	.009
Mismatched and Haplo donor (MSD and 10/10 MUD)	1.12 (0.73-1.71)	.60
RIC (versus MAC)	0.64 (0.43-0.97)	.037
Donor cells from female to male (versus others)	0.89 (0.49-1.61)	.72
Multivariate Regression Analysis: Predictors for grade II-IV aGVHD		
Log2-EASIX ≥3 at day 7	1.98 (1.26-3.13)	.003
Age (continuous variable)	0.99 (0.97-1.01)	.540
PTCy-based GVHD prophylaxis (vs. other)	0.38 (0.19-0.75)	.005
Mismatched and Haplo donor (MSD and 10/10 MUD)	2.29 (1.13-4.64)	.021
RIC (versus MAC)	0.65 (0.39-1.09)	.100

PTCy indicates post-transplantation high-dose cyclophosphamide; MSD, HLA-matched sibling donors.

approach to predict aGVHD development. In comparison to plasma biomarkers, the main advantage of the EASIX score could be its simplicity, low cost, and rapid results.

The early allogeneic post-transplantation period is characterized by endothelial activation and dysfunction, which can be detected by quantification of various biomarkers of endothelial injury. The origin of this endothelial damage is multifactorial and includes the conditioning regimen, cytokines produced by the injured tissues, bacterial endotoxins translocated through the damaged gastrointestinal tract, use of the granulocyte colony-stimulating factor and calcineurin inhibitors, the process of engraftment, and allogeneic reactions with donor-derived immune cells, among others [29,30]. There is increasing evidence that endothelial damage is the pathophysiological substrate for the development of aGVHD and a key factor in the development of steroid refractory forms [8].

Several studies have shown that inflammatory angiogenesis and neovascularization are central events in endothelial damage during the development of aGVHD and refractoriness to steroid treatment [31–33]. Other findings indicative of endothelial dysfunction include increased numbers of circulating endothelial cells and elevated levels of the endothelial stress biomarkers such as ST2, VWF, angiopoietin 2, and thrombomodulin [34–36]. Some of these factors have also been related to an increase in mortality in patients with steroid-refractory aGVHD [10,37]. However, there is less information on their value as predictors of aGVHD risk.

We have previously shown that exposure of endothelial cells in culture to sera from patients who have received allo-HSCT causes endothelial activation and damage, with a more pronounced proinflammatory and prothrombotic phenotype associated with aGVHD [9]. In this study, plasma levels of VWF:Ag and TNFR1 above an optimal cutoff at day 7 after transplantation were able to positively predict that approximately 90% of patients would develop aGVHD. Consistent with these findings, in the present study we observed a progressive increase in the plasma levels of VCAM-1, TNFR1 and VWF:Ag after conditioning, as early as day 0, and before infusion of hematopoietic progenitors. Patients who developed aGVHD had higher levels of all biomarkers than those without aGVHD. Among all the plasma biomarkers, multivariate analysis identified TNFR1 measured on days 7 as having the greatest impact on developing aGVHD. All of these data support the concept that aGVHD is associated with severe endothelial injury, and that this endothelial injury occurs several days before the clinical diagnosis of aGVHD.

The quantification of plasma biomarkers of endothelial dysfunction as predictors of aGVHD risk has, however, technical limitations in clinical practice. In that sense, the EASIX, a simple score based on three laboratory parameters used worldwide, was developed as a surrogate value of endothelial dysfunction. Luft et al. [1] showed that EASIX measured at the time of aGVHD diagnosis was a powerful predictor of NRM and OS. In a second study from the same authors, EASIX score measured before conditioning regimen (EASIX-pre) correlated with biomarkers of endothelial

homeostasis such as CXCL8, interleukin-18, and insulin-like growth-factor-1 serum levels [22]. They also found that higher values of EASIX-pre predicted higher NRM, lower OS, and tended to be associated with a higher risk of grade III-IV aGVHD after allo-HSCT. In agreement with these results, other studies have shown that higher EASIX scores measured at different time points before and after transplantation are associated with poorer overall outcomes [18–20]. In the experimental cohort of our study, endothelial dysfunction biomarkers and the EASIX score were quantified from the pretransplantation period to day 21 after transplantation. We observed a parallel dynamic consisting of a progressive increase in both parameters that was significantly higher in patients who developed aGVHD. Patients with TNFR1 $\geq$ 1300 ng/mL (HR = 7.19, P = .006) and Log2-EASIX $\geq$ 3 (HR = 14.7, $P < .001$) at day 7 after transplantation were more likely to develop grade II-IV aGVHD with high predictive accuracy (C-index of 74% and 81%, respectively). We confirmed the predictive value of EASIX at day 7 in a large validation cohort that was independent of the type of conditioning regimen, GVHD prophylaxis protocol, and type of donor. In agreement with previous published studies, we also found an association between EASIX score and post-transplantation outcomes [18-20,38]. Thus patients with Log^2-EASIX $\geq$ 3 at day 7 had a significant higher risk of NRM and lower OS.

In the experimental cohort, only patients who received RIC allo-HSCT were included, whereas the validation cohort was composed of patients who received both RIC and MAC transplants. This could be considered a limitation of the study. Previous studies from our group have shown that endothelial dysfunction after allo-HSCT can be induced by any type of conditioning regimen [39]. MAC regimens seem to be associated with a higher risk of endothelial damage-related clinical manifestations than RIC [25]. In our study, the analysis conducted in the experimental cohort showed that plasma biomarkers and log^2-EASIX had similar post-transplant dynamics. Patients with TNFR1 $\geq$1300 ng/mL and Log^2-EASIX $\geq$3 at day 7 after transplantation were more likely to develop aGVHD with high predictive accuracy. Therefore patients at risk of developing aGVHD could be easily identified by a high EASIX score. Based on these results, we aimed to confirm the predictive value of EASIX in a large and heterogeneous cohort including both types of conditioning regimens, RIC and MAC. Findings in the validation cohort regarding log^2-EASIX were similar to those observed in the experimental cohort. As expected, RIC was associated with a lower risk of aGVHD. Multivariate analysis confirmed that Log^2-EASIX $\geq$3 had a predictive value for aGVHD independently of the type of GVHD prophylaxis, intensity of conditioning regimen and the type of donor. In contrast to plasma biomarkers, EASIX can be easily used in clinical practice worldwide to predict individual risk of developing aGVHD.

In conclusion, we showed, using 2 different approaches, a differential degree of endothelial damage in the early post-transplantation period between patients who developed aGVHD and those who did not. This endothelial damage appears some days before the clinical diagnosis of aGVHD onset. Patients at risk of developing aGVHD could be easily identified by a high EASIX score. Our results, together with those published previously, may open a new window for preventive strategies in patients at high risk of aGVHD aimed at reducing endothelial dysfunction and damage, such as defibrotide, statins or sildenafil.

DISCLOSURE OF CONFLICTS OF INTEREST

All authors declare no competing financial interests.

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AUTHORSHIP CONTRIBUTIONS

M.Q.S, A.P, C.M and M.P conceived and designed the study; A.P and S.E.S collected and assembled data; M.Q.S, A.P and C.M, analyzed and interpreted the data; and A.P and C.M wrote the manuscript. All coauthors are physicians at our center, who performed the transplants, took care of the patients, made significant contributions to the discussion of the results, and participated substantively in the writing of the manuscript.

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