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Characterization of the endotheliopathy, innate-immune activation and hemostatic imbalance underlying CAR-T cell toxicities: laboratory tools for an early and differential diagnosis

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ABSTRACT

Background Chimeric antigen receptor (CAR)-T cell-based immunotherapy constitutes a revolutionary advance for treatment of relapsed/refractory hematological malignancies. Nevertheless, cytokine release and immune effector cell-associated neurotoxicity syndromes are life-threatening toxicities in which the endothelium could be a pathophysiological substrate. Furthermore, differential diagnosis from sepsis, highly incident in these patients, is challenging. Suitable laboratory tools could be determinant for their appropriate management.

Methods Sixty-two patients treated with CAR-T cell immunotherapy for hematological malignancies (n=46 with CD19-positive diseases, n=16 with multiple myeloma) were included. Plasma samples were obtained: before CAR-T cell infusion (baseline); after 24–48 hours; at suspicion of any toxicity onset and 24–48 hours after immunomodulatory treatment. Biomarkers of endothelial dysfunction (soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble TNF receptor 1 (sTNFRI), thrombomodulin (TM), soluble suppression of tumorigenesis-2 factor (ST2), angiopoietin-2 (Ang-2)), innate immunity activation (neutrophil extracellular traps (NETs), soluble C5b-9 (sC5b-9)) and hemostasis/fibrinolysis (von Willebrand Factor antigen (VWF:Ag), ADAMTS-13 (A13), α2-antiplasmin (α2-AP), plasminogen activator inhibitor-1 antigen (PAI-1 Ag)) were measured and compared with those in cohorts of patients with sepsis and healthy donors.

Results Patients who developed CAR-T cell toxicities presented increased levels of sVCAM-1, sTNFRI and ST2 at the clinical onset versus postinfusion values. Twenty-four hours after infusion, ST2 levels were good predictors of any CAR-T cell toxicity, and combination of ST2, Ang-2 and NETs differentiated patients requiring intensive care unit admission from those with milder clinical presentations. Association of Ang-2, NETs, sC5b-9, VWF:Ag and PAI-1 Ag showed excellent discrimination between severe CAR-T cell toxicities and sepsis.

Conclusions This study provides relevant contributions to the current knowledge of the CAR-T cell toxicities pathophysiology. Markers of endotheliopathy, innate immunity activation and hemostatic imbalance appear as potential laboratory tools for their prediction, severity and differential diagnosis.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Emerging evidence points to endothelial and hemostasis dysfunction underlying chimeric antigen receptor (CAR)-T cell toxicities.

WHAT THIS STUDY ADDS

⇒ Biomarkers of endotheliopathy, innate-immune activation and hemostasis imbalance can be used for the prediction of CAR-T cell toxicities, their clinical severity and for their differential diagnosis with sepsis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study lays the grounds for future pre-emptive treatments targeting the endothelium to be applied in selected patients.

INTRODUCTION

Immunotherapy with chimeric antigen receptor (CAR)-T cells has emerged as a feasible option for the treatment of relapsed/refractory (R/R) hematological malignancies. CAR-T cell technology is based on the cytotoxic effect of the patient's T cells (autologous T lymphocytes) modified in vitro to react against antigens present in tumor cells. CAR-T cells against CD19 antigen have proved to be effective for the treatment of patients

with R/R B-cell acute lymphocytic leukemia (B-ALL) and non-Hodgkin's lymphomas, and against B-cell maturation antigen (BCMA) in patients with myeloma, all without further therapeutic options. Despite the encouraging remission rates, toxicities related to the in vivo product expansion can be life-threatening and constitute a true limitation of this therapeutic approach. Several toxicities have been described in patients treated with anti-CD19 CAR-T cells,[1] two of them being especially important due to their incidence and potential severity, often requiring intensive care management and urgent anti-inflammatory treatment: the cytokine release syndrome (CRS) and CAR-T cell-associated neurotoxicity or immune effector cell-associated neurotoxicity syndrome (ICANS).[2] The clinical spectrum of both complications goes from mild symptoms to death, and can be clinically graded following a consensus classification,[3] which is also used globally as a reference for clinical management.

CRS is characterized by fever and, depending on the severity of the case, hypoxemia, hypotension, capillary leak and/or signs of specific-organ toxicity. ICANS comprises a huge range of symptoms and signs, such as headache, delirium, cognitive impairment, motor defects and seizures. Several authors have identified risk factors for the development of CRS and ICANS: conditioning regimens containing fludarabine, high disease burden—especially with bone marrow involvement, high doses of CAR-T cells or high peaks of in vivo proliferation of the CAR-T cells, among others[1 4 5]

The pathophysiology of CRS and ICANS has been extensively explored. A direct relation between elevated levels of some cytokines (interleukin (IL)-6, interferon (IFN)-γ and tumor necrosis factor (TNF)-α) early after CAR-T cell infusion, and the CRS/ICANS severity has been reported.[6 7] Moreover, an increased risk of neurotoxicity was observed in patients with early onset of CRS after CAR-T cell infusion.[7] Coagulopathy is another derived complication of severe CRS and ICANS.[7 8] Furthermore, the blood-brain barrier (BBB) increased permeability was noticed in patients developing ICANS by the detection of CAR-T cells on the cerebrospinal fluid.[7] There is growing evidence pointing to the role of endothelial dysfunction and hemostatic alterations in CAR-T cell-associated toxicities,[7 9–11] similar to other endothelial injury syndromes in the context of cellular therapies, such as sinusoid obstructive syndrome (SOS) (formerly known as veno-occlusive disease),[12] transplant-associated thrombotic microangiopathy,[13] graft-versus-host disease (GVHD)[14–16] and engraftment syndrome.[17] Occasionally, it is difficult to differentiate these toxicities from other entities, mainly infections or sepsis, as they present similar clinical and analytical profiles, but with different therapeutic approaches.

Therefore, we aimed at investigating the interplay between series of well-established biomarkers of endothelial dysfunction, innate immunity activation and hemostatic alterations during CAR-T cell treatment and the presence of its associated toxicities. In addition, a comparative analysis was carried out in patients with sepsis to prove usefulness as differential diagnosis tools.

MATERIALS AND METHODS

Study population and sample collection

We prospectively included adults aged ≥18 years (n=62) with R/R hematological malignancies (either CD19 positive or multiple myeloma (MM) after several lines of treatment), admitted to our center to receive immunotherapy with CAR-T cells with any construct available (varnimcabtagene autoleucel-the former ARI0001-, tisagenlecleucel-Kymriah-, axicabtagen ciloleucel-Yescarta-, lisocabtagene maraleucel-JCAR0017-, (all of them for CD19 malignancies)) or ARI0002h (academic CAR-T against BCMA for MM treatment) at the recruiting period (from 2018 to 2021). All patients received conditioning treatment with fludarabine and cyclophosphamide at the doses recommended by each manufacturer. Nine of the included patients received a reinfusion, which was of the same product as in their first immunotherapy in all cases (n=4 for varnimcabtagene autoleucel, prescribed for relapsed disease; and n=5 for CAR-T ARI0002h, as intensification). Five of these cases were included twice, since the reinfusion was considered a new independent treatment; and in four cases only the second infusion was included. Patients with HIV, hepatitis C virus or hepatitis B virus active infection were excluded. The following clinical variables were collected: age, sex, basal hematological disease, previous treatments (including allogeneic hematopoietic cell transplantation (allo-HCT) or autologous hematopoietic cell transplantation (auto-HCT)), previous HCT-derived complications, the CAR-T-related toxicities presented, their grade and onset, the treatment received, the clinical response to the immunosuppressant treatment and the need for admission to the intensive care unit (ICU).

Blood samples were drawn in 3.2% citrate tubes, at different points during the immunotherapy: before the CAR-T cell infusion (baseline); 24–48 hours after (24h-INF); at the suspicion of the onset of any toxicity (fever, hypotension, hypoxia and/or neurotoxicity) (Tox-onset) and 24–48 hours after immunomodulatory treatment was given (mainly tocilizumab in CRS cases and dexamethasone in ICANS) (post-IMT). Plasma was separated by centrifugation within 4 hours after the extraction, aliquoted and stored at −40°C until used.

Varnimcabtagene autoleucel and ARI0002h were administered in several aliquots (1, 2 or 3 with 10%, 30% and 60% of the total dose, depending on patient tolerance) while axicabtagene ciloleucel and lisocabtagene maraleucel were dispensed as single doses, following the corresponding protocols.

For comparison studies, we used samples from our own collection of healthy donors' plasma. In addition, samples from a previous cohort of patients with sepsis[18] (severe sepsis n=7, and septic shock n=14), collected at their admission in the ICU for any infection, were used.

Soluble levels of endothelial dysfunction biomarkers

Plasma levels of soluble vascular cell adhesion molecule 1 (sVCAM-1) (Sigma-Aldrich, USA), soluble TNF receptor 1 (sTNFRI) (Biomatik, Delaware, USA), soluble suppression of tumorigenesis-2 factor (ST2), thrombomodulin (TM) and angiopoietin-2 (Ang-2) (R&D Systems, Minnesota, USA) were measured by ELISA, following manufacturers' instructions. Absorbance was read by MultiSkan Ascent (Thermo Electron, Finland).

Evaluation of innate immune activation

Neutrophil extracellular traps (NETs) were determined by the quantification of circulating double-stranded DNA (dsDNA), using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher, Massachusetts, USA), according to the manufacturer's instructions, by fluorimetry (Fluoroskan Ascent FL; Thermolab Systems, Massachusetts, USA). The soluble terminal fraction of the complement system membrane attack complex (sC5b-9) was determined by ELISA (Quidel, California, USA).

Hemostasis and fibrinolysis assessment

Plasma levels of circulating von Willebrand Factor antigen (VWF:Ag) were measured, by immunoturbidimetry, in the Atellica 360 COAG coagulometer (Siemens Healthineers, Germany). VWF multimeric analysis was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1.2%) and western blot analysis with a primary antibody against VWF (DAKO, Denmark), followed by horseradish peroxidase (HRP)-conjugated antibody anti-VWF[19] (DAKO). HRP-labeled antibodies were detected by chemiluminescence, in ImageQuant LAS 500 (GE Healthcare Europe, Freiburg, Germany). Plasma ADAMTS-13 activity (A13) was determined by fluorescence resonance energy transfer (FRET), using a synthetic 73 amino acid VWF peptide as a fluorescence-quenching substrate (FRET-VWF73). α2-Antiplasmin activity (α2-AP) was determined by Berichrom α2-Antiplasmin Kit (Siemens Healthineers, Germany), at the coagulometer Atellica COAG 360 (Siemens Healthineers). Plasminogen activator inhibitor-1 antigen (PAI-1 Ag) plasma levels (Imubind, Toronto, Canada) were measured by ELISA, following manufacturers' instructions.

Statistical analysis

Kolmogorov-Simirnov or Shapiro-Wilk normality tests were applied for each continuous variable, depending on the *n*. Results are expressed as mean±SD for normal continuous variables; as median±IQR for non-parametric continuous variables and as absolute count and percentages for qualitative variables. Statistical analysis was performed with parametric or non-parametric tests, as needed: Student's t-test and paired Student's t-test or Mann-Whitney U and Wilcoxon test, respectively. Cross tables and χ^2 tests were applied for the evaluation of frequencies among categorical variables. Analyses with receiver operating characteristic (ROC) curves were applied for establishing the diagnostic or predictive potential of each biomarker individually and their cut-off values. Regression curves were calculated for the assessment of the predictive model with the combination of biomarkers with area under the curve (AUC) >0.7 and p<0.05 in the individual ROC analysis. The outcomes for the predictive models determined were 'presence of any CAR-T cell-related toxicity', 'ICU admission' and diagnosis of 'sepsis', and were established and analyzed retrospectively. Statistical analysis was processed with SPSS statistical software (V.25; SPSS, Chicago, Illinois, USA). Results were considered statistically significant when p<0.05.

RESULTS

Patients' characteristics

The descriptive characteristics of the patients included are summarized in table 1. Thirty-four patients (55%) developed CRS and 10 (16%) ICANS. All patients who developed ICANS had received the complete dose of the construct in a single aliquot (p=0.001) and in nine cases (90%) it was associated with the infusion of axicabtagene ciloleucel. Seventeen patients (27.4%) needed ICU admission. The different hematological diseases that were treated, their frequencies and the treating construct are presented in online supplemental table 1. The clinical phenotype of the toxicities and the treatments applied are shown in online supplemental table 2.

The number of cases with documented septic complications without toxicities in the CAR-T cell patients included was extremely low (n=1).

Endotheliopathy biomarkers in CAR-T cells-treated patients versus healthy controls

The first objective of the present study was to demonstrate endothelial dysfunction in CAR-T cell patients, especially underlying the related toxicities. For these purposes, the levels of all the biomarkers analyzed at points baseline, 24h-INF, Tox-onset and post-IMT were compared with those in healthy controls. We observed that, globally, the levels of sVCAM-1, sTNFRI, TM, sC5b-9, VWF:Ag and α2-AP were significantly higher in CAR-T cell patients at all the time points, even at their baseline sample, than in controls, whereas A13 activity was decreased in CAR-T cells-treated patients (table 2). Of note, points baseline and 24h-INF samples were collected from all patients, and points Tox-onset and post-IMT were only collected in 19 patients out of the 35 that developed toxicities.

Basal endotheliopathy assessment depending on previous clinical conditions

Baseline levels of biomarkers were analyzed regarding previous treatments and basal hematologic malignancy. We observed that VWF:Ag levels were significantly increased in patients who previously underwent an allo-HCT than in patients who did not (225%±194% vs 153%±88%, respectively; p=0.011) (online supplemental figure 1, panel A). However, patients who received an auto-HCT

Table 1 Patients' clinical characteristics

Variables	N (n=62)
Age (years); median (range)	51 (19–72)
Gender (female); n (%)	30 (48.4)
Hematological disease; n (%)	
CD19+	
Acute lymphoblastic leukemia	22 (35)
Diffuse large B cell lymphoma (and aggressive transformations from indolent lymphomas)	18 (30)
Mantle cell lymphoma	2 (3)
Indolent B cell lymphoproliferative disorders	3 (4)
Hodgkin's lymphoma transformed to diffuse large B cell lymphoma	1 (2)
Multiple myeloma/Plasma cell dyscrasias	16 (26)
Construct received; n (%)	
Varnimcabtagene autoleucel	29 (47)
ARI0002h	16 (26)
Axicabtagene ciloleucel	14 (22)
Lisocabtagene maraleucel	3 (5)
Reinfusion (yes); n (%)	9 (14)
Aliquoted infusion (yes); n (%)	45 (73)
Disease status before auto-HCT; n (%)	
CR	10 (16)
PR	1 (2)
VGPR	6 (10)
TF	41 (66)
≥2 TL; n (%)	59 (95)
Previous auto-HCT (yes); n (%)	23 (37.1)
Previous allo-HCT (yes); n (%)	22 (35.5)
Previous treatment with inotuzumab-ozogamicin (yes, n; %)	15 (24.2)
CRS; n (%)	34 (55)
Grade ≥2 or persistent CRS grade 1; n (%)	12 (35)
Onset of CRS (days after infusion) (median±IQR)	4±6
ICANS; n (%)	10 (16)
Grade ≥2; n (%)	8 (80)
Onset of ICANS (days after infusion) (median±IQR)	6±5
Both (CRS and ICANS); n (%)	9 (14.5)
ICU admission; n (%)	17 (27.4)

allo-HCT, allogeneic hematopoietic cell transplantation; auto-HCT, autologous hematopoietic cell transplantation; CR, complete response; CRS, cytokine release syndrome; ICANS, immune effector cell-associated neurotoxicity syndrome; ICU, intensive care unit; PR, partial response; TF, therapeutic failure; TL, therapeutic lines; VGPR, very good partial response.

before the CAR-T therapy presented significantly lower levels of sVCAM-1 than patients who did not (97 ng/mL±261 ng/mL vs 224 ng/mL±421 ng/mL, respectively, p=0.003) (online supplemental figure 1, panel B). Patients with lymphoid neoplasms had increased levels of NETs and sVCAM-1 at their basal sample versus those with plasma cell dyscrasias (NETs of 8±6 vs 5±2, p<0.001, respectively; and sVCAM-1 of 226±395 vs 81±112, p<0.001, respectively) (online supplemental figure 1, panel C). An opposite tendency was observed for TM levels, which were lower in patients with lymphoid neoplasms (TM of 3148±1590 vs 3980±1252, p=0.043, respectively). There were no significant differences neither regarding sex or age when a cut-off value of ≥60 years was defined.

One patient had presented SOS 2 months before the CAR-T cell therapy. The levels of all the biomarkers in this patient were higher than in the rest of the patients, being significantly higher for sVCAM-1 (937 ng/mL vs 171 ng/mL±268 in non-SOS patients, p<0.001).

Increase of levels of endothelial activation markers after construct's infusion

The in vivo effects of CAR-T cell infusion on endothelial function were also explored. The levels of endothelial biomarkers were analyzed in individual postinfusional plasma samples (24h-INF point) and compared with those obtained in the basal sample (baseline). Globally, a significant increase of sTNFRI and Ang-2 was observed in the first 24–48 hours after the construct's infusion (sTNFRI of 2646 pg/mL±1939 pg/mL at baseline vs 3146 pg/mL±2111 pg/mL at 24h-INF point, p=0.029; and Ang-2 of 1434 pg/mL±985 pg/mL at baseline vs 2034 pg/mL±2014 pg/mL at 24h-INF point, p<0.001) (online supplemental figure 2). When the analysis was performed by the different constructs administered, only significant changes were found in the group of patients that received varnimcabtagene autoleucel, with an increase of Ang-2 and NETs and a decrease of A13 levels after infusion (online supplemental table 3).

Of note, in the five patients who underwent a reinfusion and both administrations were included, no significant differences in the levels of none of the biomarkers assessed were observed when comparing samples from baseline or from 24h-INF point among them.

CAR-T-related toxicity and endothelial activation

To explore whether the onset of the toxicity (Tox-onset) was associated with changes in the biomarkers with respect to the postinfusional values (24h-INF), levels at both points were compared. This analysis was performed in samples from 19 (out of 35) patients who developed either CRS or ICANS with any construct. Levels of sVCAM-1, sTNFRI and ST2 presented a significant increase at the clinical onset of the toxicity versus values at the postinfusional point (sVCAM-1 of 359 ng/mL±455 ng/mL at Tox-onset point vs 223 ng/mL±499 ng/mL at 24h-INF point, p=0.028; sTNFRI of 4252 pg/mL±4733 pg/mL at Tox-onset point vs 3559 pg/mL±2259 pg/mL at 24h-INF point, p=0.023 and ST2 of 191 ng/mL±130 ng/mL at Tox-onset point vs 124 ng/mL±130 ng/mL at 24h-INF point, p=0.031).

The VWF multimeric analysis was performed only in six patients who presented severe CRS and severe ICANS, requiring ICU admission. The analysis showed a normal VWF structure which correlated with the normality in

Table 2 Biomarkers' levels of CAR-T patients at all time points versus controls

Biomarker Median±IQR	Controls	CAR-T cell patients (baseline)	P value (CAR-T at baseline vs control)	CAR-T cell patients (24h-INF)	P value (CAR-T at 24h-INF vs control)	CAR-T cell patients (Tox-onset)	P value (CAR-T at Tox-onset vs control)	CAR-T cell patients (post-IMT)	P value (CAR-T at post-IMT vs control)
	N=49	N=62		N=62		N=19		N=19	
sVCAM-1 (ng/mL)	77±27	176±283	<0.001*	203±362	<0.001*	359±455	<0.001*	498±1078	<0.001*
sTNFRI (pg/mL)	291±106	2467±2076	<0.001*	2678±2350	<0.001*	4252±4733	<0.001*	3052±4096	0.001*
TM (ng/mL)	14±13	3164±1645	<0.001*	3222±1312	<0.001*	3765±2938	<0.001*	3032±2042	<0.001*
ST2 (ng/mL)	11±8	42±42	<0.001*	59±170	<0.001*	210±241	<0.001*	187±515	<0.001*
Ang-2 (pg/mL)	1188±660	1439±999	0.098	2058±2566	<0.001*	2826±3660	<0.001*	1959±1640	0.002*
NETs (µg/mL)	1±9	8±5	0.954	7±3	0.219	8±5	0.021*	8±4	0.045*
sC5b-9 (ng/mL)	199±66	516±403	<0.001*	472±424	<0.001*	689±795	<0.001*	685±284	0.001*
VWF:Ag (%)	107±33	162±137	<0.001*	185±136	<0.001*	226±111	<0.001*	211±98	0.001*
A13 (%)	100±12	90±20	0.009*	75±20	<0.001*	74±24	<0.001*	65±29	<0.001*
α2-AP (%)	88±45	112±31	<0.001*	108±29	<0.001*	92±27	<0.001*	100±15	0.001*
PAI-1 Ag (ng/mL)	27±16	29±20	0.180	32±31	0.211	39±32	<0.001*	41±40	0.281

Biomarker's values from controls (in a single time point) are used for the comparison with the CAR-T cell group in each of the time points analyzed. Statistical significance (p) for the comparisons between Tox-onset versus post-IMT: sVCAM-1: 0.063; sTNFRI: 0.642; TM: 0.836; ST2: 0.501; Ang-2: 0.234; NETs: 0.196; sC5b-9: 0.098; VWF:Ag: 0.820; A13: 0.959; α2-AP: 0.234; PAI-1 Ag: 0.134.

*P<0.05.

A13, ADAMTS-13 activity; Ang-2, angiopoietin-2; Baseline, sample collected before the CAR-T cell infusion; 24h-INF, sample collected 24–48 hours after infusion; NETs, neutrophil extracellular traps; PAI-1 Ag, inhibitor of the activator of plasminogen antigen; post-IMT, sample collected 24–48 hours after immunomodulatory treatment; sC5b-9, soluble C5b9; ST2, soluble suppression of tumorigenesis-2; sTNFRI, soluble TNF receptor 1; sVCAM-1, soluble vascular cell adhesion molecule 1; TM, thrombomodulin; Tox-onset, sample collected at the suspicion of the onset of any toxicity (cytokine release syndrome or immune effector cell-associated neurotoxicity syndrome); VWF:Ag, von Willebrand factor antigen; α2-AP, α2-antiplasmin.

the functional tests (ratio of VWF:GPIbM/VWF:Ag >0.7), at all the time points, and the absence of hemorrhagic diathesis. There was an increased density of the protein at Tox-onset point, compared with the control (online supplemental figure 3).

The type of neoplasm had also an impact on the levels of biomarkers at the onset of the toxicity (Tox-onset), A13 levels being significantly lower in patients with lymphoid neoplasms with respect to those with plasma cell dyscrasias (A13 of 72±27 vs 89±20, respectively, p=0.016). Treatment with either an allo-HCT or an auto-HCT, or previous development of SOS, acute GvHD or chronic GvHD did not have any significant impact on the biomarker's levels at Tox-onset point.

In the five patients included in their first treatment and in their reinfusion, the incidence of toxicity was very variable between the two admissions. Thus, we did not have samples from all time points to make proper comparisons and to assess whether the biomarkers had similar kinetics between the two admissions.

Potential role of endothelial and innate-immune activation biomarkers for the prediction of the CAR-T cell toxicity and its severity

We looked for predictors of CAR-T cell toxicity among the assessed biomarkers. Levels of ST2 >29 ng/mL at 24h-INF point had a sensitivity and a specificity of 70% and 60%, respectively, for the prediction of any CAR-T cell-related toxicity (AUC 0.7; 95% CI 0.54 to 0.81, p=0.020) (figure 1).

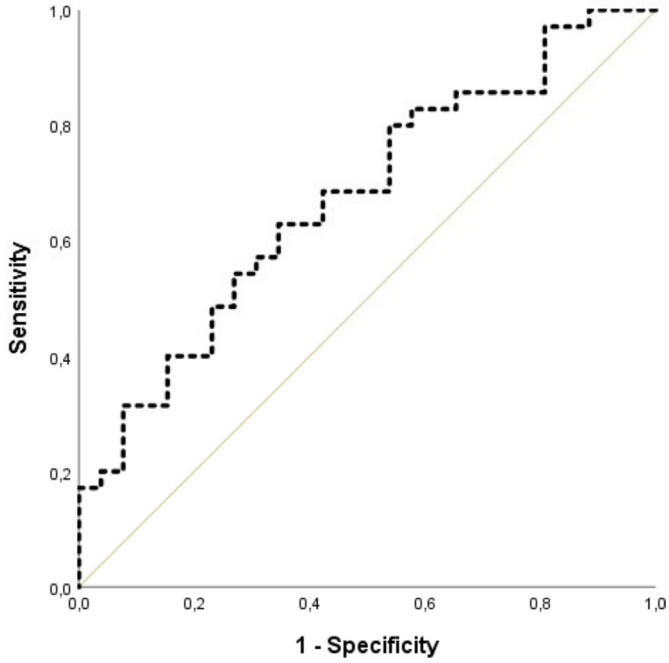

Figure 1 Prediction of toxicity. Predictive model of soluble suppression of tumorigenesis-2 factor (ST2) at sample collected 24–48 hours after infusion (24-INF) point for the outcome 'presence of any chimeric antigen receptor (CAR)-T cell-related toxicity' meant as clinical detection of cytokine release syndrome (CRS) and/or immune effector cell-associated neurotoxicity (ICANS) of any severity grade. Area under the curve (AUC) 0.7 (95% CI 0.54 to 0.81, p=0.020).

Regarding severity prediction, patients requiring admission to ICU for complications derived from the immunotherapy (n=17) had higher levels of Ang-2, ST2, NETs and sC5b-9 in their postinfusional sample (24h-INF) than the rest of the patients (Ang-2 of 2841 ng/mL±2959 pg/mL vs 1729 ng/mL ± 1354 pg/mL, p=0.043; ST2 of 76 ng/mL±176 ng/mL vs 28±29, p<0.001; NETs of 9 µg/mL±6 µg/mL vs 6 µg/mL±2 µg/mL, p=0.008 and sC5b-9 of 763 ng/mL±659 ng/mL vs 440 ng/mL±351 ng/mL, p=0.05, respectively). The regression model created with Ang-2, ST2 and NETs (parameters that showed a discerning potential in the individual ROC analysis) for the prediction of the event 'ICU admission' showed an AUC of 0.8 and p<0.001 (figure 2).

Differentiation between CAR-T cell-related toxicities and septic syndromes

The other objective of the present study was to assess whether the biomarkers analyzed could be used as laboratory tools for the differential diagnosis between the inflammatory syndrome that characterizes the CAR-T-toxicities and septic syndromes. For this purpose, biomarkers' levels at the onset of the toxicity (Tox-onset point in 19 patients who presented CRS and/or ICANS) were compared with those in a cohort of patients with severe septic syndromes (n=21). Levels of TM, Ang-2, NETs, sC5b-9, VWF:Ag and PAI-1 Ag at Tox-onset point were significantly higher in patients with septic shock than in CAR-T cell toxicity patients (table 3). The reliability of these parameters as diagnostic tools was evaluated through a ROC analysis and was confirmed for Ang-2, NETs, sC5b-9, PAI-1 Ag and VWF:Ag, having all of them AUC of 0.8–0.9 and p<0.001 for the outcome 'sepsis'. The cut-off values for sensitivity >70% for the diagnosis of sepsis are shown in figure 3, panel A. A logistic regression model for the classification between sepsis and CAR-T toxicity was applied considering Ang-2, NETs, sC5b-9, VWF:Ag and PAI-1 Ag levels for each patient. The new variable created had an AUC of 0.992 (p<0.001) for the outcome of sepsis (figure 3, panel B). Since 17 out of the 19 CAR-T patients with toxicities and all patients with sepsis included needed ICU admission, the clinical severity is balanced between the two groups. There were no significant differences between the APACHE (Acute Physiology and Chronic Health Evaluation)-II score observed at ICU admission in patients with CAR-T-related toxicities versus patients with sepsis (median±IQR of 21±6 vs 19±11, respectively, p=0.171).

Impact of the immunosuppressant treatment on endotheliopathy biomarkers

The paired analysis performed for the evaluation of the biomarkers' levels at Tox-onset point versus at post-IMT point showed no significant differences for any of them (footnote of table 2).

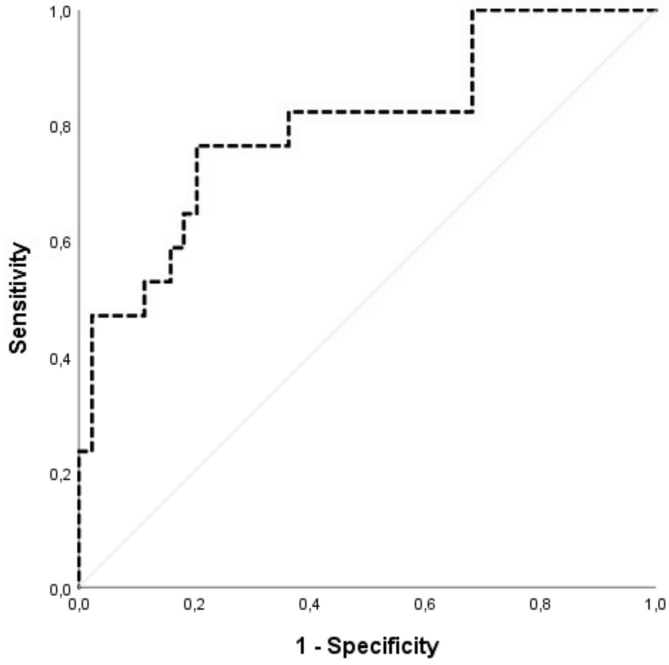

Figure 2 Prediction of severity. Receiver operating characteristic (ROC) curve after the application of a regression model for the predictive value of the composed variable created from soluble suppression of tumorigenesis-2 factor (ST2), angiopoietin-2 (Ang-2) and neutrophil extracellular traps (NETs) levels at sample collected 24–48 hours after infusion (24-INF) point for the outcome 'intensive care unit (ICU) admission'. Area under the curve (AUC) 0.8 (95% CI 0.67 to 0.93, p<0.001). The values of the AUC and 95% CI, and the proposed cutoffs for each biomarker are shown below (graph not shown): Ang-2: AUC 0.7 (95% CI 0.501 to 0.835, p=0.043). Levels of Ang-2 >1877 pg/mL have a sensitivity of 70% and a specificity of 57% for the prediction of 'ICU admission'. ST2: AUC 0.8 (95% CI 0.626 to 0.93, p=0.001). Levels of ST2 >38.7 ng/mL have a sensitivity of 82% and a specificity of 70% for the prediction of 'ICU admission'. NETs: AUC 0.7 (95% CI 0.559 to 0876, p=0.009). Levels of NETs >7.5 µg/mL have a sensitivity of 70% and a specificity of 88% for the prediction of 'ICU admission'.

Table 3 Biomarkers in CAR-T cell-related toxicities versus sepsis

Biomarker	CAR-T cell toxicity (n=19)	Sepsis/Septic shock (n=21)	P value
sVCAM-1 (ng/mL)	240±455	285±823	0.884
sTNFRI (pg/mL)	4243±3929	3520±4347	0.302
TM (ng/mL)	3496±2144	75±44	<0.001*
ST2 (ng/mL)	208±236	210±73	0.860
Ang-2 (pg/mL)	2841±3498	7696±12 043	<0.001*
NETs (µg/mL)	6±4	24±36	<0.001*
sC5b9 (ng/mL)	567±815	1219±912	0.001*
VWF:Ag (%)	222±132	461±258	<0.001*
A13 (%)	74±25	15±24	<0.001*
α2-AP (%)	94±27	84±40	0.042*
PAI-1 Ag (ng/mL)	38±38	119±93	<0.001*

Comparison between levels of biomarkers in patients with CAR-T cell-related toxicities at the onset of CRS/ICANS, before any specific treatment is administered (Tox-onset point) versus patient with sepsis/septic shock at their ICU admission. Values are expressed as median±IQR or percentage (as indicated).
*P<0.05.
A13, ADAMTS-13 activity; Ang-2, angiopoietin-2; CAR, chimeric antigen receptor; CRS, cytokine release syndrome; ICANS, immune effector cell-associated neurotoxicity syndrome; NETs, neutrophil extracellular traps; PAI-1 Ag, inhibitor of the activator of plasminogen antigen; sC5b-9, soluble C5b-9; ST2, soluble suppression of tumorigenesis-2; sTNFRI, soluble TNF receptor 1; sVCAM-1, soluble vascular cell adhesion molecule 1; TM, thrombomodulin; VWF:Ag, von Willebrand factor antigen; α2-AP, α2-antiplasmin.

DISCUSSION

In the present study, circulating biomarkers of endothelial dysfunction, innate-immunity activation, hemostasis alterations and fibrinolytic imbalance were analyzed in patients with R/R hematological malignancies who received immunotherapy with CAR-T cells. The levels of these biomarkers were also compared with those in healthy donors and patients with sepsis. Our results demonstrate that CAR-T cell-related toxicities are associated with the development of an endotheliopathy, with alterations of the linked pathways explored. Furthermore, a panel including sVCAM-1, sTNFRI and ST2 may be helpful for their laboratory confirmation. In addition, ST2, Ang-2 and NETs arise as feasible tools for the early prediction of the CAR-T cell-related toxicities appearance and severity. Also, a panel consisting of Ang-2, NETs, sC5b-9, VWF:Ag and PAI-1 Ag could facilitate the differential diagnosis between CAR-T cell treatment toxicity and severe septic complications. The potential clinical uses of the biomarkers studied are summarized in table 4.

To date, the European Medicine Agency (EMA) has approved three CAR-T cell products against CD19 neoplasms: tisagenlecleucel (Novartis), axicabtagene ciloleucel (Gilead), Tecartus (Gilead) and lisocabtagene maraleucel (Bristol Myers Squibb). Varnimcabtagene autoleucel (the former ARI0001) is a non-commercial construct recently approved by the Spanish Medicines Agency[20–22] for the treatment of adult patients (>25 years of age) with R/R B-ALL, and also a compassionate use program for patients with B-cell malignancies who are not eligible for commercial products. Regarding CAR-T cells targeting B-cell maturation antigen (BCMA) in patients with MM, the EMA recently gave a conditional authorization to ciltacabtagene autoleucel (Janssen) and idecabtagene vicleucel (Bristol Myers Squibb).

The main acute complications of patients receiving CAR-T cell therapy are CRS and ICANS, which are immune-mediated and quite specific to this therapy, and sepsis, more associated with the immunosuppressed phenotype of these patients.

Regarding the pathophysiology of the toxicities in CAR-T cell therapies, the elevation of some pro-inflammatory cytokines, such as IL-6, IFN-γ and TNF-α, early after the

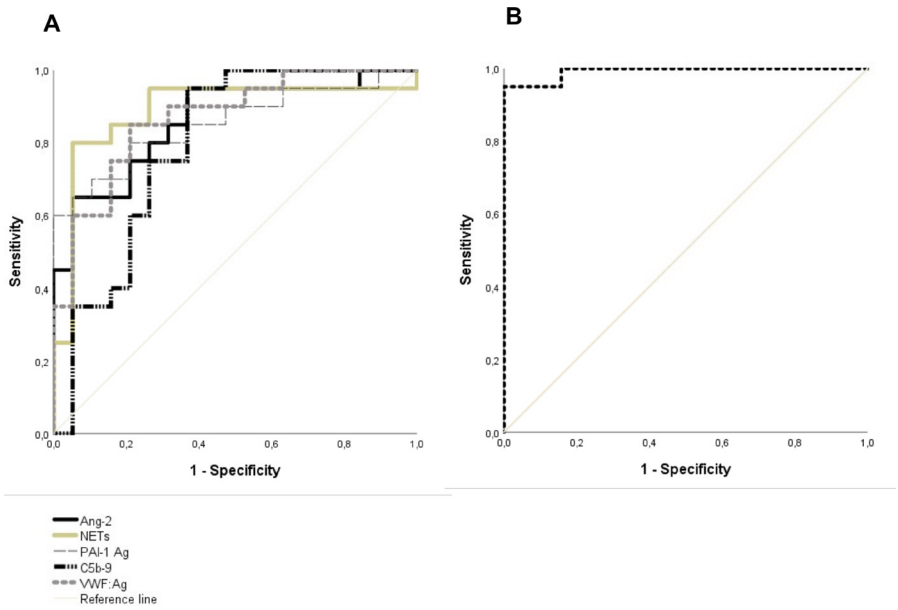

Figure 3 Differential diagnosis: toxicity versus sepsis. (A) Receiver operating characteristic (ROC) curve for the diagnostic value of angiopoietin-2 (Ang-2), neutrophil extracellular traps (NETs), soluble C5b9 (sC5b-9), von Willebrand Factor antigen (VWF:Ag) and plasminogen activator inhibitor-1 (PAI-1) at Tox-onset point, for the outcome 'sepsis'. The values of the area under the curve (AUC) and 95% CI, and the proposed cutoffs for each variable are shown below: Ang-2: AUC 0.861 (95% CI 0.744 to 0.977). Levels of Ang-2 >4823 pg/mL have a sensitivity of 80% and a specificity of 74% for the diagnosis of sepsis over chimeric antigen receptor (CAR)-T toxicity. NETs: AUC 0.887 (95% CI 0.76 to 1); p<0.001. Levels of NETs >16.5 µg/mL have a sensitivity of 80% and specificity of 84% for the diagnosis of sepsis over CAR-T toxicity. sC5b-9: AUC 0.795 (95% CI 0.64 to 0.943); p=0.002. Levels of sC5b-9 >1109 ng/mL have a sensitivity of 75% and specificity of 73% for the diagnosis of sepsis over CAR-T toxicity. VWF:Ag: AUC 0.868 (95% CI 0.757 to 0.898); p<0.001. Levels of VWF:Ag >345% have a sensitivity of 75% and specificity of 84% for the diagnosis of sepsis over CAR-T toxicity. PAI-1 Ag: AUC 0.853 (95% CI 0.73 to 0.975); p<0.001. Cut-off value of PAI-1 Ag >73.6 ng/mL have a sensitivity 70% and specificity of 90% for the diagnosis of sepsis over CAR-T toxicity. (B) Predictive model for the outcome 'sepsis' with the composed variable created from Ang-2, NETs, sC5b-9, VWF:Ag and PAI-1 Ag at the onset of the toxicity in CAR-T cell-patients (Tox-onset point) or at the onset of sepsis. AUC 0.992 (95% CI 0.934 to 1); p<0.001.

construct's infusion has been reported associated with CRS and ICANS severity.[6 7 23] Endothelial damage, which is also a consequence of the cytokine's storm,[24] has been recently postulated as involved pathological substrate in the CAR-T cell-related toxicities, in direct relation with their intensity.[11 25] EASIX index, although based on indirect biomarkers of endotheliopathy, has proven to be useful for the prediction of severe CRS and/or ICANS.[25] In addition, other biological functions altered in CRS and ICANS, such as ongoing coagulopathy[8] and innate immunity activation,[26] are both in tight connection with the endothelium. Specifically, levels of Ang-2 and VWF were found to be higher in patients with grade ≥4 neurotoxicities than in patients with lower severity grades.[27] In addition, a lesser proportion of VWF high molecular weight multimers with lower A13 activity were observed in patients with grade 4 ICANS.[7] Circulating NETs and sC5b-9 proved to be increased proportionally to the severity of other diseases where the endothelium is importantly affected, as in septic syndromes and COVID-19.[28 29]

In our study, some biomarkers of endotheliopathy were found to be increased in patients with CAR-T cell-related toxicities at their debut, being potentially valid for their laboratory confirmation. By contrast, biomarkers of innate-immune activation and hemostasis/fibrinolysis could be useful for discerning between toxicities and sepsis. Therefore, a combination of biomarkers of endotheliopathy and innate-immune activation emerges as a potential tool for the prediction of CAR-T cell toxicities, their severity and differential diagnosis with sepsis (table 4).

By analyzing levels of biomarkers at different time points, we were able to assess the timeline from baseline to CAR-T cell infusion and toxicity appearance. Although some biomarkers of endotheliopathy have proven to be potentially useful for the laboratory confirmation of CAR-T toxicities, quantitative or qualitative changes in other proteins involved in hemostasis, such as A13 and VWF as described by other authors,[7] failed to show significant differences in the toxicity onset in our cohort.

Regarding the assessment of response to treatment, no significant differences were observed when considering biomarker's levels at the onset of the toxicity and 24–48 hours after the immunosuppressant treatment. This time was selected because it is when clinical improvement usually starts. However, indirect evidence points to longer half-life times of clearance of some of the biomarkers analyzed.[30 31] Therefore, the timing of collection of

Table 4 Proposed clinical use of the biomarkers analyzed from our data

Biomarkers	Toxicity lab confirmation	Early predictor of toxicity	Early predictor of ICU admission	Differential diagnosis: CAR-T cell toxicity versus sepsis
Endothelial dysfunction biomarkers				
sVCAM-1	*			
sTNFRI	*			
TM				*
ST2	*	*†	*†	
Ang-2			*†	*†
Innate immune activation biomarkers				
NETs			*†	*†
sC5b-9			*	*†
Hemostasis and fibrinolysis biomarkers				
VWF:Ag				*†
A13				*
α2-AP				*
PAI-1				*†

Toxicity lab confirmation: potential use of the selected biomarkers for supporting the diagnosis of any CAR-T cell-related toxicity (CRS or ICANS), which diagnostic criteria are, currently, purely clinical.
Early predictor of toxicity: potential use of the selected biomarkers as early predictors of any CAR-T cell-related toxicity, when analyzed within the 24–48 hours after the construct's infusion.
Early predictor of ICU admission: potential use of the biomarkers as severity predictors, specifically of ICU admission, when analyzed within the 24–48 hours after the construct's infusion.
Differential diagnosis: CAR-T cell toxicity versus sepsis: potential use of the selected biomarkers marked to discern between CAR-T cell toxicities (CRS or ICANS) or sepsis, once patients present a clinical picture consisting in fever, hypoxia, dyspnea and/or hypotension, which are manifestations presented in both complications.
*Biomarkers with significant differences in the hypothesis contrast tests.
†Biomarkers considered laboratory discerning tools or included in a logistic regression model with predictive value (ROC curves above AUC >0.7 and p<0.05).
A13, ADAMTS-13 activity; Ang-2, angiopoietin-2; AUC, area under the curve; CAR, chimeric antigen receptor; ICANS, immune effector cell-associated neurotoxicity syndrome; ICU, intensive care unit; NETs, neutrophil extracellular traps; PAI-1 Ag, inhibitor of the activator of plasminogen antigen; ROC, receiver operating characteristic; sC5b-9, soluble C5b-9; ST2, soluble suppression of tumorigenesis-2; sTNFRI, soluble TNF receptor 1; sVCAM-1, soluble vascular cell adhesion molecule 1; TM, thrombomodulin; VWF:Ag, von Willebrand factor antigen; α2-AP, α2-antiplasmin.

samples after IMT was given could have been too early to evaluate an improvement of endotheliopathy through the surrogated biomarkers.

Patients' background influence endothelial function previous to CAR-T cell infusion. In the setting of auto-HCT, a myeloablative treatment with a well-known relation with endothelial damage,[32] decreased levels of the biomarkers of endothelial damage at their basal point were observed in the present study, in comparison with patients not autotransplanted. We hypothesize that this could be explained by a likely 'exhaustion effect' of the endothelium after a previous severe noxa.[33 34] Moreover, patients with lymphoid neoplasms had higher levels of the endotheliopathy biomarkers sVCAM-1 and NETs, whereas patients with plasma cell dyscrasias had increased levels of TM. These results could reflect the more intense previous therapies received in cases with aggressive B-cell lymphomas or leukemia, whereas in patients with myeloma the increase of thrombomodulin (a natural anticoagulant) could be the compensatory response to the use of prothrombotic drugs, such as thalidomide or lenalidomide. These differences can account for different responses in endothelial function after CAR-T cell treatment.

It is interesting to mention that the infusion of the CAR-T cell construct itself causes endothelial activation, as demonstrated here. When subanalyzing depending on the construct, we only could confirm this activation in the varnimcabtagene autoleucel group, the one with greater casuistic. The lack of significant changes in the other groups could be attributed to different causes: differences in the constructs, the baseline hematological disease or previous treatment as well as more reduced sample size, among others.

The beneficial effect of dividing the construct's infusion into several aliquots has been previously reported.[35] However, we could not conclude that the single-dosing rather than a concrete type of construct was responsible for higher rates of toxicity.

Discerning between CAR-T cell toxicities and sepsis with biomarkers of endotheliopathy and hemostatic imbalance is challenging, since these pathways are also involved in the development of the clinical manifestations of sepsis.[18 36] Samples from a previous cohort of ICU patients with sepsis had to be used for the comparative studies, as the casuistic of severe and documented septic complications, without co-existence with toxicities, in the CAR-T cell patients included was very low. Thus, the patients with sepsis included were, mostly, non-hematological patients. However, in absence of previous chemotherapy treatments, this group showed more elevated levels of biomarkers of endotheliopathy and innate-immunity biomarkers, indicating that the sepsis, itself, constitutes an extreme noxa, stronger than the additive effect of treatments and toxicities in CAR-T cell patients.

The present study has some limitations. We could not collect all the samples corresponding to all time points of all the patients presenting toxicities. Another drawback is that we included all patients treated with CAR-T therapy regardless of their baseline disease or CAR-T construct. Thus, the number of patients in each group of CAR-T constructs is variable and our analysis could not be applied to detect differences among groups.

Nevertheless, our study offers valuable data applicable in the clinical setting. To our knowledge, this is the first study in which biomarkers of endotheliopathy have demonstrated their critical role for the laboratory confirmation of the toxicities, and for the differential diagnosis with septic syndromes. The potential utilities are summarized in table 4. Thus, if confirmed and validated, they could be implemented in the clinical routine and applied to guide the treatment and even to advise for closer monitoring in patients with increased biomarkers' levels after the CAR-T cell infusion. Having confirmed the early endotheliopathy in the CAR-T cell toxicities, the protection of the endothelium appears also as an attractive option for their prevention management. Statins[37 38] or defibrotide[39 40] are drugs with a low-toxicity profile that have been demonstrated to improve endothelial function by decreasing pro-inflammatory cytokines and leukocyte-adhesion molecules, or by increasing nitric oxide bioavailability and reducing oxidative stress, respectively. Increasing ANG-1 levels (an endothelium stabilizer molecule as opposed to ANG-2[41–43]) is also under assessment.[7 44]

CONCLUSIONS

This study provides relevant contributions unveiling the pathophysiology of CAR-T cell toxicities, where endotheliopathy, innate-immunity activation and hemostatic imbalance are major cornerstones and potential targets for their treatment. The biomarkers analyzed may have a potential role in the laboratory confirmation of these complications and in the prediction of their clinical severity. These molecules provide a distinctive profile that may be helpful for the differential diagnosis between CAR-T cell toxicities and sepsis. Further prospective studies should be proposed to validate these results in larger cohorts of patients.

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Contributors ABM-C, SF, PC and MD-R designed the study. SF and ABM-C contributed to sample collection. ABM-C, MP, JM-S and AR performed the laboratory tests and analysis. ABM-C and HV analyzed the clinical data and ABM-C performed the statistical analysis. SF, HV, GE, EC, FF-A, OP, VO-M, JD, CFdL, AU-I, GE, EC, FF-A, OP, JMN, AT and PC contributed to the interpretation of data and hypotheses for clinical applications. ABM-C and MD-R wrote the manuscript, and all authors contributed to the editing of the final text. ABM-C, PC and MD-R act as guarantors of the content of the article.

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