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Phase IIB, Randomized, Double-Blind, Placebo-Controlled Clinical Trial of Intravenous Defibrotide for the Prevention and Treatment of Respiratory Distress and Cytokine Release Syndrome in COVID-19

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ABSTRACT

Introduction: Endothelial dysfunction is key in COVID-19 pathogenesis. This randomized, double-blind phase IIb trial investigated continuous intravenous infusion of defibrotide in patients hospitalized with SARS-CoV-2 infection and respiratory failure.

Methods: One-hundred and fifty patients were randomized (2:1) to defibrotide or placebo, stratified by disease severity (WHO COVID-19 severity scale 4/5 vs. 6). The primary endpoint was clinical improvement time (days from first improvement through Day 30).

Results: Median clinical improvement time was not significantly different with defibrotide versus placebo (15.0 [IQR: 0–24] vs. 20.0 [IQR: 9–25] days; $p = 0.10$). Day-30 (23.0% vs. 22.0%) and Day-60 (26.0% vs. 22.0%) mortality, reduction in mean fraction of inspired oxygen during treatment, and median duration of hospitalization did not differ with defibrotide versus placebo. Defibrotide demonstrated favorable safety, with no differences versus placebo in serious adverse events (34.0% vs. 36.0%), hypotension (16.0% vs. 12.0%), or hemorrhage (13.0% vs. 8.0%). Exploratory pre-specified biomarker analyses showed greater early D-dimer reduction and lymphocyte recovery with defibrotide, although these results require validation.

Conclusion: Continuous intravenous infusion of defibrotide was safe but did not improve clinical outcomes in severe COVID-19. Further analyses will explore mechanistic actions and pharmacokinetics of defibrotide and the pathophysiology of endothelial dysfunction in COVID-19.

Trial Registration: EudraCT identifier: 2020-001409-21. Clinicaltrials.gov identifier: NCT04348383

1 | Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) resulted in >760 million cases of COVID-19 and 6.9 million deaths worldwide [1, 2]. Severe COVID-19 frequently progressed to acute respiratory distress syndrome (ARDS) [3–6]. The pandemic exposed critical vulnerabilities in global healthcare systems, with a scarcity of personal protective equipment (PPE) [7] and no vaccines or targeted therapies for severe disease [8] at the onset in 2020. Clinicians instead relied on repurposed drugs with limited efficacy; for example, dexamethasone reduced mortality by 17% in ventilated patients with COVID-19 [9], remdesivir modestly accelerated recovery [10], and tocilizumab showed inconsistent benefits [11–13]. In Spain, during the first wave (February–May 2020), mortality rates exceeded 40% in mechanically ventilated patients with COVID-19, and healthcare worker infection rates exceeded 15% due to PPE shortages [14]. Thus, the crisis demanded evaluation of therapies addressing both patient outcomes and staff safety through simplified administration protocols.

Early in the pandemic, the vascular endothelium was recognized as a critical therapeutic target in severe COVID-19 [15–26]. SARS-CoV-2 infection triggers endotheliitis through direct viral invasion via angiotensin-converting enzyme 2 receptors and subsequent rupture of alveolo-capillary membranes, cytokine release, and activation of platelets and coagulation. This manifests clinically with vascular leakage and alveolar edema, an increased cytokine storm, generalized inflammation, and immuno-thrombosis, plus elevated inflammatory and coagulation biomarkers that are strongly associated with mortality. Therapies mitigating these effects and providing endothelial protection may potentially be of benefit in COVID-19 management.

Defibrotide, a polydisperse oligonucleotide with endothelial-protective, anti-inflammatory, and anti-thrombotic properties, is approved for treating hepatic sinusoidal obstruction syndrome (SOS) at a dose of 6.25 mg/kg every 6 h (Q6hr) in adults, adolescents, and children aged >1 year [27, 28]. Preclinical studies demonstrated defibrotide could significantly reduce levels of endothelial activation markers intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by 62%–75%, suppress interleukin-6 (IL-6) and tumor necrosis factor-α release ($p < 0.01$), and improve survival rates in a murine model of hyperinflammatory disease such as graft-versus-host disease (87.5% vs. 0% controls) [29]. Because defibrotide directly targets endothelial activation, microvascular thrombosis, and cytokine-driven vascular injury—all hallmarks of severe COVID-19—we hypothesized that defibrotide would accelerate sustained clinical improvement in hypoxemic, hospitalized patients [30–32]. Furthermore, defibrotide is not incompatible with the COVID-19 treatments used during the early stages of the pandemic [33]. Its efficacy in hepatic SOS is also supportive of a potential role in COVID-19, as both conditions share features of severe thrombotic microangiopathy [34, 35].

We therefore conducted a phase IIb, multicenter, randomized, double-blind, placebo-controlled trial to evaluate the efficacy and safety of continuous intravenous (IV) defibrotide in hospitalized patients with COVID-19 and hypoxemic respiratory failure, that is, World Health Organization (WHO) severity scale grades 4–6 [36]. This study aimed to determine whether defibrotide's multimodal mechanisms of action could improve outcomes in severe COVID-19 in terms of clinical improvement time and 30−/60-day mortality, addressing an urgent unmet need. Furthermore, instead of using standard Q6hr dosing, we hypothesized that continuous IV infusion might maintain or improve defibrotide pharmacokinetics, achieving target coverage in the endothelium while avoiding peak concentrations that could be associated with hypotension, an important safety consideration in critically ill COVID-19 patients, or other toxicities such as bleeding. The rationale for this approach was also to maintain sterility via closed systems, reducing nursing interventions by 80% compared to Q6hr dosing, and thereby mitigating PPE use and staff exposure at a time of acute resource shortages in Spain.

2 | Methods

2.1 | Study Design and Patients

This trial (DEFACOVID-HCUVA; EUDRACT: 2020-001409-21; ClinicalTrials.gov NCT04348383) was conducted at eight tertiary hospitals in Spain (Table S1). The trial protocol and amendments were approved by the institutional review board (IRB) of University Clinical Hospital Virgen de la Arrixaca, IRBs at participating centers, and the Spanish Agency of Medicaments and Health Products. The trial was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and was compliant with the European Union's General Data Protection Regulation and Spanish data protection laws. An independent Data Monitoring Committee reviewed safety data every 30 patients (~2-monthly schedule) during enrollment and at the end of the trial.

The trial population comprised high-risk adult patients (aged ≥ 18 years) with a reverse transcription polymerase chain reaction (PCR)-confirmed SARS-CoV-2 infection who were hospitalized and had Grade 4, 5, or 6 clinical status per the WHO 7-category ordinal scale for COVID-19 severity (Table S2) [36, 37]. Other eligibility criteria are listed in Supporting Information Methods. All participants provided written informed consent.

2.2 | Treatment and Randomization

Patients were randomized 2:1 to defibrotide or placebo, plus standard care and conventional COVID-19 therapies per institutional protocols at time of inclusion. Defibrotide 25 mg/kg/day was administered as a continuous IV infusion in 250 mL saline (rate: 10.4 mL/h) for 15 days. Matching placebo (0.9% saline) was administered using identical infusion protocols. Shorter treatment duration (minimum 7 days) was permitted in patients who improved clinically (no oxygen requirement/return to baseline oxygen requirement) and could be discharged home. Randomization was stratified by disease severity (WHO scale 4/5 vs. 6) using computer-generated blocks via a centralized electronic system (Biomédica-IMIB [Biomedical Research Institute of Murcia] platform). Information on blinding is provided in Supporting Information Methods.

2.3 | Endpoints and Assessments

The primary endpoint was "clinical improvement time," defined as the cumulative number of days alive between first achievement of improvement (≥ 1 category) on the WHO scale for COVID-19 severity and Day 30, inclusive. Patients who died before Day 30 were assigned 0 days. This endpoint, adapted from ordinal-scale response endpoints in ACTT-1 and related COVID-19 trials [38, 39], was selected to maximize sensitivity for detecting a treatment effect and integrate both onset and durability of improvement, avoiding informative censoring due to early death or relapse. Secondary safety, efficacy, biomarker, and radiographic endpoints are summarized in Supporting Information Methods. Patients were evaluated at baseline (Day 0), daily during treatment (Days 1–14), with secondary endpoint assessment on Day 7, and during follow-up (Days 15, 30, 60). Assessments and adverse reactions of interest for defibrotide are summarized in Supporting Information Methods.

2.4 | Statistical Analysis

The sample size of 150 patients (100/50 in the defibrotide/placebo arms) provided 80% power ($\beta = 0.20$) to detect an increase in mean clinical improvement time from 12 days with placebo (based on data from ACTT-1 [38, 39]) to 16 days with defibrotide (two-sided $\alpha = 0.05$), assuming a 10% drop-out rate. For the primary efficacy analysis, statistical comparison between arms was done using the Wilcoxon rank-sum test. For other statistical comparisons, p-values < 0.05 were considered nominally significant. Full statistical methodology is provided in Supporting Information Methods.

3 | Results

3.1 | Patients

Between April 14, 2020, and June 29, 2021, 150 patients were enrolled at six participating sites (Table S1) and randomized, 100 to defibrotide and 50 to placebo (Figure S1). Overall, median age was 61.0 years, 78.0% of patients were male, mean body mass index was 29.6 kg/m^2, and WHO COVID-19 severity category was 4, 5, and 6 in 24.0%, 49.3%, and 26.7%, respectively, with no differences between arms (Table 1). The most common comorbidity was hypertension (45.3%), with lower prevalence in the defibrotide versus placebo arm (38.0% vs. 60.0%, $p = 0.01$). Other common comorbidities showed similar prevalences between arms (Table 1). Baseline D-dimer levels were higher in the defibrotide versus placebo arm (Table 1).

3.2 | Clinical Improvement Time

For the primary endpoint, there was no statistically significant difference between the defibrotide and placebo arms (Figure 1A). Median clinical improvement time was 15.0 days (IQR: 0–24) with defibrotide and 20.0 days (IQR: 9–25) with placebo, with no statistical difference between arms (Wilcoxon rank-sum test, $p = 0.10$). Analyses of clinical improvement time by baseline disease severity did not show differences between arms ($p > 0.05$) (Figure 1B–D).

Using a more stringent measure of clinical improvement—sustained reduction to Category 3 (WHO 7-point severity scale)—median clinical improvement time was 0 days (IQR: 0–17) with defibrotide and 0 days (IQR: 0–19) with placebo (Figure 1A), with no significant difference between arms ($p = 0.57$). The wide IQRs in both arms for this endpoint, and the median of 0 days reflecting the proportion of patients who did not achieve this more stringent threshold, indicate greater inter-patient variability in clinical improvement time when applying more stringent improvement criteria compared with the primary endpoint.

TABLE 1 | Clinical characteristics and baseline biomarker levels of patients included in the study, overall and by treatment arm.

Characteristics	All patients ($N=150$)	Defibrotide ($N=100$)	Placebo ($N=50$)	p[a]
Age (years), mean (SD)	60.2 (11.98)	60.1 (11.14)	60.3 (13.62)	0.93
Median (IQR)	61.0 (51.3–69.8)	60.0 (52.8–68.0)	61.5 (49.0–72.0)	
Range	34–89	34–89	36–86	
Sex, *n* (%)				
Male	117 (78.0)	78 (78.0)	39 (78.0)	0.42
Female	33 (22.0)	22 (22.0)	11 (22.0)	
BMI (kg/m^2), mean (SD)	29.6 (4.96)	29.7 (5.13)	29.3 (4.64)	0.66
Median (IQR)	29.0 (26.1–32.1)	29.1 (26.3–32.2)	28.8 (26.1–31.8)	
Range	20.4–45.2	20.4–45.0	21.2–45.2	
Comorbidities, *n* (%)				
Hypertension	68 (45.3)	38 (38.0)	30 (60.0)	**0.01**
Diabetes mellitus	38 (25.3)	26 (26.0)	12 (24.0)	0.80
Cardiac ischemic disease	21 (14.0)	12 (12.0)	9 (18.0)	0.33
History of cancer	12 (8.0)	8 (8.0)	4 (8.0)	0.99
Respiratory disease	10 (6.7)	7 (7.0)	3 (6.0)	0.81
WHO COVID-19 ordinal scale, *n* (%)				
4	36 (24.0)	22 (22.0)	14 (28.0)	0.42
5	74 (49.3)	53 (53.0)	21 (42.0)	0.20
6	40 (26.7)	25 (25.0)	15 (30.0)	0.51
Baseline biomarker levels, median (IQR); [reference range]				b
Interleukin-6, pg/mL [0–7]	110.3 (283.8)	88.5 (316.3)	114.5 (256.2)	0.72
ALC, $\times 10^3/\mu$L [1.0–4.0]	0.8 (0.5)	0.7 (0.6)	0.8 (0.4)	0.39
D-dimer, ng/mL [0–243]	1076.0 (2186.0)	1358.0 (2992.0)	692.5 (796.3)	**<0.01**
C-reactive protein, mg/L [0–5.0]	7.5 (11.7)	8.2 (11.8)	5.9 (12.0)	0.23
Ferritin, ng/mL [30–400]	1193.0 (1230.0)	1247.0 (1513.0)	1167.0 (1261.8)	0.30
LDH, U/L [135–225]	435.0 (209.0)	435.0 (220.0)	435.0 (197.0)	0.56
CPK, U/L [38–174]	64.0 (102.0)	60.0 (119.3)	70.0 (60.0)	0.96

Abbreviations: ALC: absolute lymphocyte count; BMI: body mass index; CPK: creatine phosphokinase: CI: confidence interval; COVID-19: coronavirus disease 2019; IQR: interquartile range; LDH: lactate dehydrogenase; WHO: World Health Organization.
[a]Descriptive p-value determined by Student's t-test for continuous variables and χ^2 test for categorical variables.
[b]Descriptive p-value determined using the Mann–Whitney U test, as the Shapiro–Wilk test for normality of continuous variables indicated nonparametric distributions ($p<0.01$) for all biomarkers. $p<0.05$ (in bold font) indicates nominally significant difference between the defibrotide arm and the placebo arm.

3.3 | Safety

Overall, 131/150 patients (87.3%) had any adverse event (AE), including 91 (91%) and 40 (80%) in the defibrotide and placebo arms, respectively ($p=0.06$) (Table 2). In total, 820 AEs were recorded, 626 and 194 in the defibrotide and placebo arms, respectively, of which 57 were serious AEs (SAEs; 38 and 19 in the defibrotide and placebo arms, respectively, occurring in 34 and 18 patients). Overall, 702 AEs (85.6%) were classified as unrelated to treatment. Percentages of non-serious AEs considered unlikely, possibly, or definitely related to treatment were low (≤7.6%), with no differences between arms (Table 2). Percentages of SAEs considered unlikely, possibly, or definitely related to treatment were ≤5.3%.

Severity information was available for 816/820 AEs: 546 (66.9%) were Grade 1, 135 (16.5%) Grade 2, and 135 (16.5%) Grade ≥3, including 31 (3.8%) Grade 5 (fatal) AEs (Table 2). The distribution of severity grades did not differ between treatment arms, and no association was observed between treatment arm and overall severity distribution ($p=0.945$).

The most frequently reported AEs were elevated liver enzymes (68 events), anemia (53), fever (39), and hypotension (37)

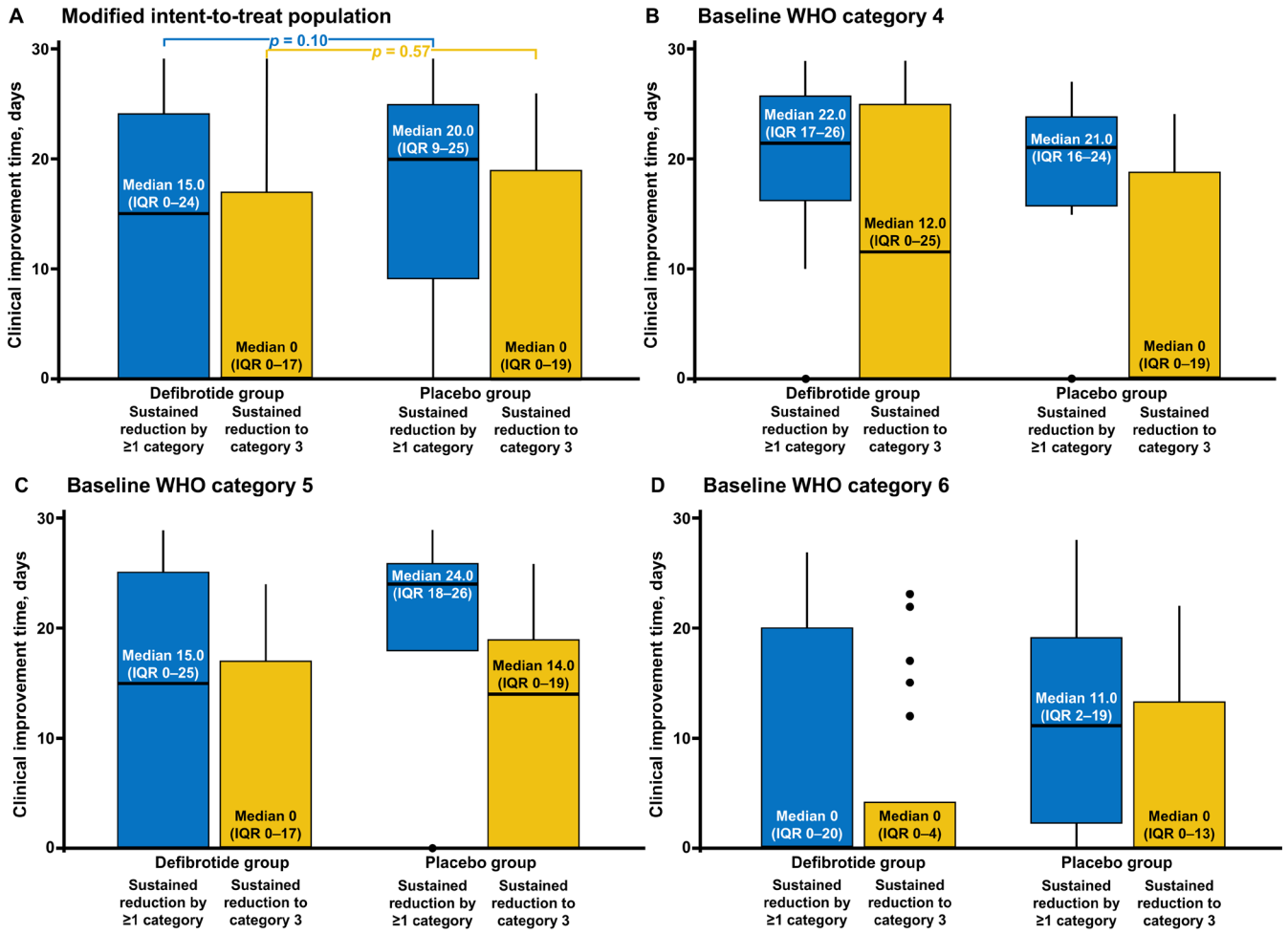


FIGURE 1 | Clinical improvement time through Day 30 in the defibrotide and placebo group. Clinical improvement time (A) in the modified ITT population (all treated patients) and (B–D) according to baseline WHO ordinal scale score in terms of sustained reduction by ≥1 category (primary endpoint; blue boxes) and sustained reduction to Category 3 (hospitalized, not requiring oxygen therapy; yellow boxes) on the WHO 7-point ordinal scale. The Shapiro–Wilk test confirmed non-normal distributions for the primary endpoint in both arms (defibrotide $p < 0.0001$; placebo $p < 0.0001$); thus, the prespecified Wilcoxon rank-sum test was the statistical approach used for comparison between arms. Boxes represent IQR, horizontal lines show median values, and whiskers show 1.5 × IQR; black dots show outliers with values greater than or less than 1.5 × IQR from the median. CI: confidence interval; IQR: interquartile range; ITT: intent-to-treat; WHO: World Health Organization.

(Table 2). Most events of elevated liver enzymes and anemia were grade 1/2 (Table 2), suggesting limited clinical impact. At the patient level, anemia occurred in 50 patients (33.3%), elevated liver enzymes in 44 (29.3%), hypoalbuminemia in 26 (17.3%), fever in 23 (15.3%), hypotension in 22 (14.7%), pain in 19 (12.7%), and hemorrhage in 17 patients (11.3%); anemia, elevated liver enzymes, and hypoalbuminemia were more common in the defibrotide versus placebo arm (Table 2). Although hypotension and hemorrhage were numerically higher with defibrotide, these differences, plus differences in fever and pain, were not significant. The distribution of severity grade for hypotension and hemorrhage did not differ significantly between arms.

3.4 | Mortality

Overall mortality was 22.7% (34/150) at Day 30 and 25.3% (38/150) at Day 60. Day-30 mortality was similar in the defibrotide and placebo arms (23/100, 23%, and 11/50, 22%, respectively). By Day 60, mortality was 27.0% (27/100) with defibrotide and 22.0% (11/50) with placebo, with no significant difference between arms ($p = 0.89$) (Figure S2). The leading causes of death by day 60 ($n = 38$) were multiorgan failure (13, 34.2%; defibrotide 9, placebo 4), respiratory failure (13, 34.2%; defibrotide 9, placebo 4), cardiorespiratory arrest (5, 13.2%; defibrotide 4, placebo 1), and severe thrombotic events (3, 7.9%; defibrotide 1, placebo 2). Four deaths were due to metabolic coma, necrotizing pneumonia, refractory shock with oliguria and metabolic acidosis, and distributive septic shock (each 1, 2.6%); all occurred in the defibrotide arm and were not considered treatment-related.

Mean time from treatment initiation to death was 19.5 days (standard deviation [SD] 12.0; median 16.0 days, IQR: 11.2–23.2) in the defibrotide arm ($n = 26$) and 12.1 days (SD 9.0; median 10.0 days, IQR 6.0–18.5) in the placebo arm ($n = 11$). The longer time to death in the defibrotide arm was not statistically significant compared with placebo ($p = 0.065$), despite the numerical difference and the exploratory biomarker effects described below.

TABLE 2 | AEs during the study according to number of patients experiencing events and number of individual events.

	All		Defibrotide		Placebo		p[a]	
AEs	**Patients ($N=150$)**	**Events**	**Patients ($N=100$)**	**Events**	**Patients ($N=50$)**	**Events**	**Patients**	**Events**
Any AE, *n* (%)/*n*	131 (87.3)	820	91 (91.0)	626	40 (80.0)	194	0.06	—
Any nonserious AE, *n* (%)	125 (83.3)	763 (93.0)	91 (91.0)	588 (93.9)	34 (68.0)	175 (90.2)		
Causality		$N=763$		$N=588$		$N=175$		
Unrelated	117	651 (85.3)	84 (84.0)	500 (85.0)	33 (66.0)	151 (86.3)	0.45	0.72
Unlikely related	16	44 (5.8)	12 (12.0)	37 (6.3)	4 (8.0)	7 (4.0)	1.00	0.36
Possibly related	43	58 (7.6)	36 (36.0)	45 (7.7)	7 (14.0)	13 (7.4)	0.06	1.00
Definitely related	10	10 (1.3)	6 (6.0)	6 (1.0)	4 (8.0)	4 (2.3)	0.46	0.25
Any serious AE, *n* (%)	52 (34.7)	57 (7.0)	34 (34.0)	38 (6.1)	18 (36.0)	19 (9.8)	0.44	0.08
Causality		$N=57$		$N=38$		$N=19$		
Unrelated	48 (32.0)	51 (89.5)	31 (31.0)	34 (89.5)	17 (34.0)	17 (89.5)	—	—
Unlikely related	3 (2.0)	3 (5.3)	2 (2.0)	2 (5.3)	1 (2.0)	1 (5.3)	—	—
Possibly related	2 (1.3)	2 (3.5)	1 (1.0)	1 (2.6)	1 (2.0)	1 (5.3)	—	—
Definitely related	1 (0.7)	1 (1.8)	1 (1.0)	1 (1.6)	0	0	—	—
AE by max grade,[b] *n* (%)		$N=816$		$N=625$		$N=191$		
Grade 1	119 (79.3)	546 (66.9)	88 (88.0)	421 (67.4)	31 (62.0)	125 (65.4)	1.00	0.66
Grade 2	48 (32.0)	135 (16.5)	34 (34.0)	104 (16.6)	14 (28.0)	31 (16.2)	1.00	1.00
Grade 3	40 (26.7)	66 (8.1)	29 (29.0)	48 (7.7)	11 (22.0)	18 (9.4)	0.60	0.50
Grade 4	25 (16.7)	38 (4.7)	18 (18.0)	29 (4.6)	7 (14.0)	9 (4.7)	1.00	1.00
Grade 5	30 (20.0)	31 (3.8)	22 (22.0)	23 (3.7)	8 (16.0)	8 (4.2)	0.70	0.83
Common AEs, *n* (%)/*n*								
Elevated liver enzymes[c]	44 (29.3)	68	35 (35.0)	54	9 (18.0)	14	**0.03**	0.66
Anemia[d]	50 (33.3)	53	41 (41.0)	44	9 (18.0)	9	**<0.01**	0.32
Fever	23 (15.3)	39	16 (16.0)	30	7 (14.0)	9	0.81	1.00
Hypotension[e]	22 (14.7)	37	16 (16.0)	28	6 (12.0)	9	0.63	1.00
Pain	19 (12.7)	31	12 (12.0)	18	7 (14.0)	13	0.80	**0.03**
Hypoalbuminemia	26 (17.3)	27	24 (24.0)	25	2 (4.0)	2	**<0.01**	0.06
Hemorrhage[f]	17 (11.3)	17	13 (13.0)	13	4 (8.0)	4	0.425	1.00
Leukocytosis	16 (10.7)	16	13 (13.0)	13	3 (6.0)	3	—	—
Low-grade fever	12 (8.0)	16	7 (7.0)	10	5 (10.0)	6	—	—
Constipation	15 (10.0)	15	11 (11.0)	11	4 (8.0)	4	—	—
Diarrhea	13 (8.7)	15	10 (10.0)	11	3 (6.0)	4	—	—
Headache	11 (7.3)	15	9 (9.0)	12	2 (4.0)	3	—	—
Urinary tract infection	12 (8.0)	13	8 (8.0)	8	4 (8.0)	5	—	—

(Continues)

TABLE 2 | (Continued)

AEs	All		Defibrotide		Placebo		p^a	
	Patients ($N=150$)	Events	Patients ($N=100$)	Events	Patients ($N=50$)	Events	Patients	Events
Orotracheal intubation	13 (8.7)	13	9 (9.0)	9	4 (8.0)	4	—	—
Bacteremia	14 (9.3)	14	12 (12.0)	12	2 (4.0)	2	—	—

Abbreviations: AE: adverse event; BMI: body mass index; CI: confidence interval; COVID-19: coronavirus disease 2019; max: maximum; NCT CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events; WHO: World Health Organization.
[a]Descriptive *p*-value determined by Fisher's exact test. $p<0.05$ (in bold font) indicates a nominally significant difference between the defibrotide arm and the placebo arm. Dashes indicate data that were not statistically compared between arms.
[b]Maximum toxicity grade determined according to NCI CTCAE v5.0.
[c]Elevated liver enzymes including aspartate aminotransferase, alanine aminotransferase, and/or gamma-glutamyl transferase, graded per NCI CTCAE v5.0. Of 68 events, 49, 9, 7, and 3 were grade 1, 2, 3, and 4, respectively.
[d]Anemia defined as hemoglobin below the sex-adjusted lower limit of normal. Of 53 events, 37, 10, and 6 were grade 1, 2, and 3, respectively.
[e]Hypotension severity grade distribution (grade 1/2/3 13.0/3.0/2.0% vs. 12.0/0/0%) did not differ significantly between arms.
[f]Hemorrhage severity grade distribution (grade 1/2/3 7.0/3.0/3.0% vs. 4.0/0/0%) did not differ significantly between arms.

Evaluation of concomitant COVID-19 medications as a potential confounding factor in the mortality analysis showed no differences between arms (Table 3).

3.5 | Changes in FiO_2, and Mechanical Ventilation and Hospital/Intensive Care Unit (ICU) Requirements

Fraction of inspired oxygen (FiO_2) was assessed over time to compare respiratory improvement between arms (Table 3). The mean reduction in mean FiO_2 from Day 1 to end of treatment (Day 15) was numerically greater with defibrotide versus placebo (28.6 vs. 26.6), but this difference was not statistically significant. The ICU admission rate was 72.0% in both arms. Extracorporeal membrane oxygenation (ECMO) was required more often in the defibrotide versus placebo arm (12.0% vs. 4.0%), but this difference was not statistically significant. Dialysis requirement was similar between arms. In surviving patients, mean duration of hospitalization was 25.8 versus 21.7 days in the defibrotide versus placebo arm (median 16.5 vs. 14.0 days; $p=0.13$) (Table 3).

3.6 | Biomarkers

Biomarker analyses were exploratory and hypothesis-generating. Seven biomarkers were assessed at baseline and on Days 7, 15, 30, and 60: IL-6 (inflammation), absolute lymphocyte count (ALC; immune recovery), D-dimer (coagulation, fibrinolysis), C-reactive protein (CRP; inflammation), ferritin (inflammation), lactate dehydrogenase (LDH; tissue injury, inflammation), and creatine phosphokinase (CPK; muscle injury, ischemia). Baseline values were generally similar between arms, except for a higher D-dimer level in the defibrotide versus placebo arm ($p<0.01$) (Table 1).

Changes from baseline were analyzed as the proportions of evaluable patients achieving either a ≥ 50% reduction or (for ALC) increase or normalization of levels (per established reference ranges, Table 1) (Figure 2). For IL-6, the proportion of patients with a ≥ 50% reduction increased from 28.0% (Day 7) to 42.5% (Day 60) in the defibrotide arm but remained relatively stable in the placebo arm (38.0%–42.0%). IL-6 normalization rates were lower with defibrotide on Days 7 and 15 but increased and were similar between arms on Days 30 and 60 (Figure 2A).

ALC recovery (≥ 50% increase from baseline) and normalization rates were consistently higher or similar with defibrotide compared with placebo (Figure 2B). The rate of ≥ 50% increase in ALC on Day 7 was significantly higher with defibrotide (67.0% vs. 50.0%, $p=0.05$).

Consistently higher rates of ≥ 50% reductions in D-dimer levels were seen with defibrotide versus placebo across time-points, notably on Day 7 (23.0% vs. 8.0%, $p=0.03$) and Day 15 (31.0% vs. 12.0%, $p=0.02$) (Figure 2C) that might reflect a trend toward improving hemostatic recovery, although this requires validation. Conversely, D-dimer normalization rates were numerically higher in the placebo group across time-points, potentially associated with higher baseline D-dimer levels in the defibrotide arm offering greater potential for achieving ≥ 50% reductions but making normalization more challenging.

Rates of ≥ 50% reductions in CRP levels were high and comparable between arms at all time-points (Figure 2D). CRP normalization rates remained low in both arms. Lower rates of ≥ 50% reductions and normalization of ferritin levels were seen with defibrotide versus placebo across time-points, notably on Day 7 (1.0% vs. 18.0%, $p<0.01$, and 5.0% vs. 18.0%, $p=0.02$, respectively), although rates in both arms increased during follow-up (Figure 2E).

There were relatively low rates of ≥ 50% reductions and normalization of LDH in both arms across time-points, with gradual increases over time and no consistent difference between arms (Figure 2F). For CPK, ≥ 50% reductions were similar between arms at Days 7 and 15 but higher with defibrotide at days 30 (34.0% vs. 18.0%, $p=0.04$) and 60 (36.0% vs. 22.0%) (Figure 2G). CPK normalization rates were consistently high and similar in both arms.

3.7 | Pulmonary Infiltrates

Severity of pulmonary inflammatory disease on serial chest radiographs was determined using an 8-point severity score

TABLE 3 | Supportive care by treatment arm—concomitant COVID-19 medications, changes in FiO_2, ICU admission rate, ECMO and dialysis requirements, and length of hospital stay.

Characteristics	Defibrotide ($N=100$)	Placebo ($N=50$)	p[a]
Concomitant COVID-19 medications, *n* (%)	$N=98$	$N=44$	
Dexamethasone	90 (91.8)	40 (90.9)	1.00
Azithromycin	40 (40.8)	23 (52.3)	0.28
Remdesivir	36 (36.7)	14 (31.8)	0.71
Tocilizumab	36 (36.7)	13 (29.5)	0.50
Baricitinib	10 (10.2)	6 (13.6)	0.76
Hydroxychloroquine	2 (2.0)	2 (4.5)	0.78
Lopinavir/ritonavir	1 (1.0)	2 (4.5)	0.48
FiO_2,[b] mean (SD)			
On Day 1	64.1 (23.8)	60.4 (24.7)	0.38
At end of treatment (Day 15)	35.5 (27.0)	33.8 (24.0)	0.72
Δ, Day 1 – end of treatment (Day 15)[c]	28.6 (31.9)	26.6 (30.1)	0.70
At Day 30	26.8 (15.4)	25.1 (9.7)	0.80
Δ, Day 1 – Day 30[c]	26.7 (25.0)	33.2 (23.2)	0.42
At day 60	23.3 (10.3)	21.3 (1.9)	0.29
Δ, Day 1 – Day 60[c]	39.2 (23.0)	38.4 (22.5)	0.85
ICU admission, *n* (%)	72 (72.0)	36 (72.0)	1.00
Life-support requirements, *n* (%)			
ECMO	12 (12.0)	2 (4.0)	0.11
Dialysis	8 (8.0)	3 (6.0)	0.63
Hospital length of stay in surviving patients	$N=74$	$N=39$	
Mean (SD)	25.8 (21.2)	21.7 (18.9)	0.13
Median (IQR)	16.5 (12.2–36.8)	14.0 (10.5–20.5)	

Abbreviations: ECMO: extracorporeal membrane oxygenation; FiO_2: fraction of inspired oxygen; ICU: intensive care unit; IQR: interquartile range; SD: standard deviation.

[a]Descriptive *p*-value determined by Mann–Whitney *U* test (non-parametric comparison) for FiO_2 parameters and hospital length of stay and by χ^2 test for categorical variables. $p<0.05$ indicates nominally significant difference between the defibrotide arm and the placebo arm.

[b]FiO_2 was determined in a mixed population of patients. For mechanically ventilated patients (WHO category 6 – $n=25$ and $n=15$ in defibrotide and placebo arms, respectively), FiO_2 was recorded directly from the ventilator. For non-intubated patients, estimated FiO_2 was calculated using standard clinical formulae: for nasal cannula, $FiO_2 \approx 21 + (3 \times$ flow rate in L/min) (e.g., 2 L/min → 27, 4 L/min → 33, 6 L/min → 39); for simple face masks, $FiO_2 \approx 35$–55 depending on flow and seal; for non-rebreather masks, $FiO_2 \approx 60$–80; for high-flow nasal oxygen, FiO_2 was set directly on the device. It is acknowledged that these estimates are imprecise, particularly at higher flow rates. FiO_2 in non-intubated patients is physiologically distinct from mechanically ventilated patients (in whom positive end-expiratory pressure [PEEP] also influences oxygenation).

[c]Δ values show the mean (SD) difference between FiO_2 at Days 1 and 15 at the end of treatment (respiratory improvement during therapy) and Days 30 and 60 (only patients with both Days 1 and 30 [defibrotide, $n=64$; placebo, $n=34$] or Days 1 and 60 data [defibrotide, $n=60$; placebo, $n=35$] were included).

[40], with continuous quantitative analysis of score over time plus categorical evaluation (Supporting Information Methods). Continuous quantitative analysis using multiple linear regression models showed that baseline severity score was a predictor of ongoing pulmonary involvement at all analysis time points (Days 7, 15, 30, and 60), with the strongest association on Days 7 and 15 ($p<0.01$) and attenuation of effect size by Day 60 ($p<0.05$) (Figure S3).

Defibrotide treatment was not a predictor for pulmonary involvement on Days 7, 15, and 30 but showed a marginal association on Day 60, potentially suggesting slightly less improvement in the defibrotide versus placebo arm at this time-point. The explanatory power of the regression models was strongest on Day 7 and declined over subsequent time-points. The constant term, representing expected severity scores for patients in the placebo arm with baseline scores of 0, was a predictor of severity on Day 7 ($p<0.01$) and had a marginal effect on Day 15 ($p<0.1$), indicating measurable pulmonary involvement even in ostensibly "normal" patients at baseline, potentially due to contributions from unmodeled clinical factors.

Figure S3 shows regression lines for the relationship between baseline severity score and scores on Days 7, 15, 30, and 60.

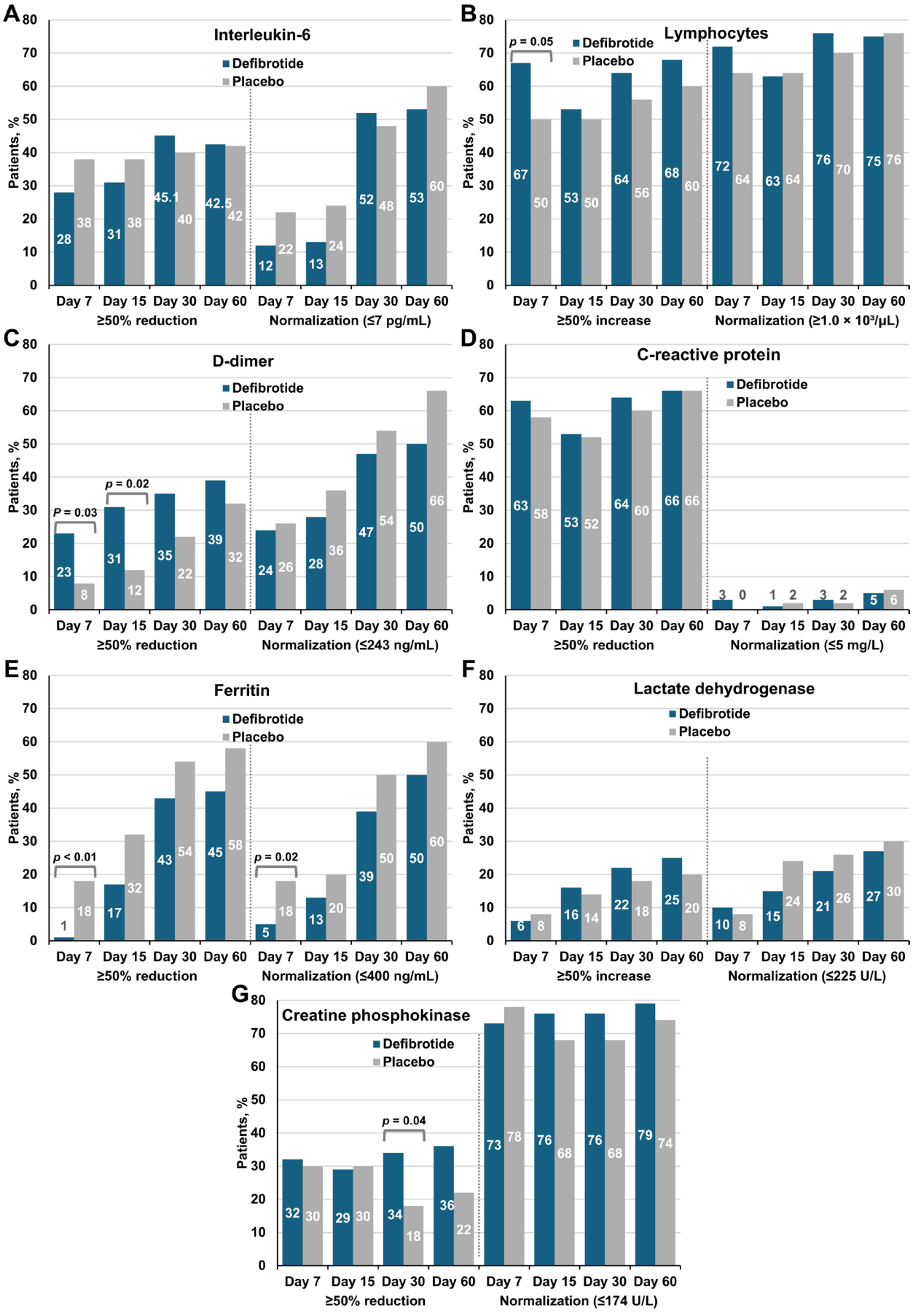


FIGURE 2 | Legend on next page.

FIGURE 2 | Changes from baseline in biomarker levels by treatment group. Figures show the proportions of patients with a ≥ 50% reduction or increase from baseline or normalization of biomarker levels at four analysis time-points (Days 7, 15, 30, 60) for (A) interleukin-6, (B) lymphocytes, (C) D-dimer, (D) C-reactive protein, (E) ferritin, (F) lactate dehydrogenase, and (G) creatine phosphokinase. Between-group comparisons were performed using chi-square tests of independence. All variables were dichotomized as "yes/no" based on whether patients met these endpoints. $p < 0.05$ indicates nominally significant difference between the defibrotide arm and the placebo arm (no adjustments for multiplicity of testing), as indicated in the figure panels.

The steeper slopes of the defibrotide versus placebo regression lines on Days 30 and 60 suggest that severity of pulmonary involvement at these time-points was more dependent on baseline severity in the defibrotide arm. These findings reflect more variable improvement patterns, by which defibrotide-treated patients with higher baseline scores tended to maintain relatively worse severity scores at later time points.

Figure 3 shows categorical changes from baseline in severity of pulmonary involvement, with patients classified as remaining stable, improving, or worsening by at least one severity category compared to baseline. With both defibrotide (Figure 3A) and placebo (Figure 3B), percentages of patients with improvement from baseline increased from Days 7 to 60, with no differences between arms ($p \geq 0.31$).

4 | Discussion

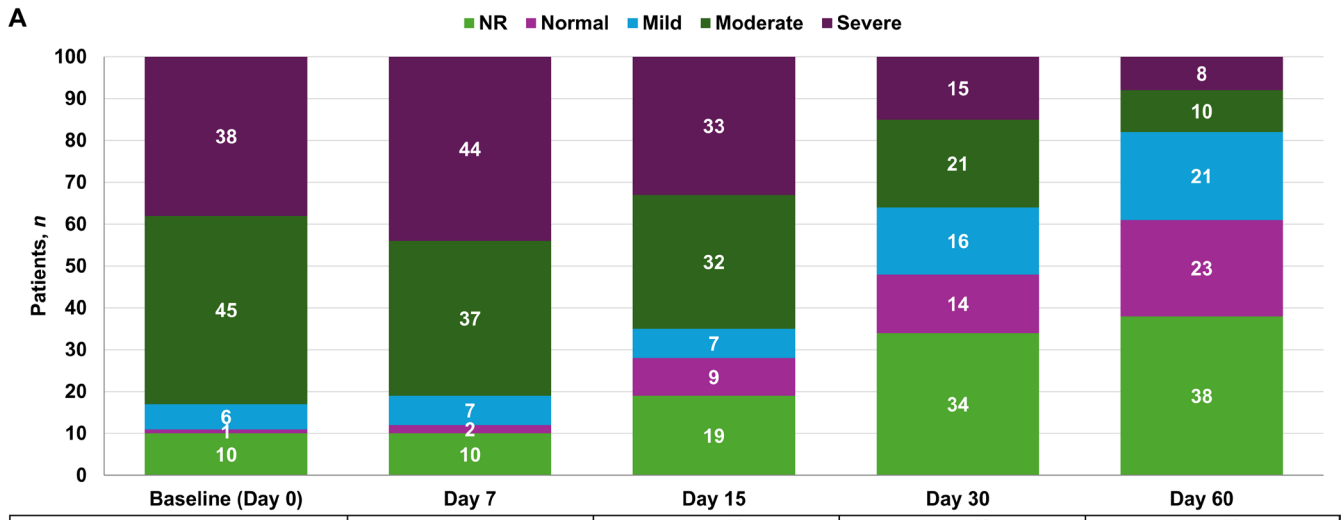
To our knowledge, this was the first randomized controlled trial of defibrotide as an endothelial-protective agent in severe COVID-19. Although continuous IV infusion of defibrotide did not improve clinical outcomes versus placebo, the study confirmed its safety in critically ill, hypoxemic patients, demonstrated pharmacodynamic signals in coagulation/inflammation biomarkers, and identified a likely subtherapeutic exposure profile with continuous IV infusion versus the approved Q6hr dosing. Therefore, these data redefine how defibrotide should be studied in syndromes driven by endothelial injury and suggest an important biological effect in this setting.

The COVID-19 pandemic highlighted the urgent need for effective treatments, particularly, in severe cases progressing to ARDS and cytokine storm. Given limited therapeutic options beyond remdesivir and dexamethasone, and emerging evidence of endothelial dysfunction in COVID-19 pathogenesis, we investigated defibrotide as a potential therapy. To our knowledge, there had been no preclinical studies evaluating defibrotide in animal models of SARS-CoV-2 infection. However, in a murine model of graft-versus-host disease, defibrotide significantly reduced levels of adhesion molecules and cytokines that are critically elevated in the cytokine release syndrome associated with COVID-19 and, importantly, improved animal survival [29]. In addition, the mechanistic rationale for defibrotide was supported by the endothelial dysfunction demonstrated in COVID-19 [15–26] and defibrotide's demonstrated safety and endothelial-protective properties [35, 41, 42].

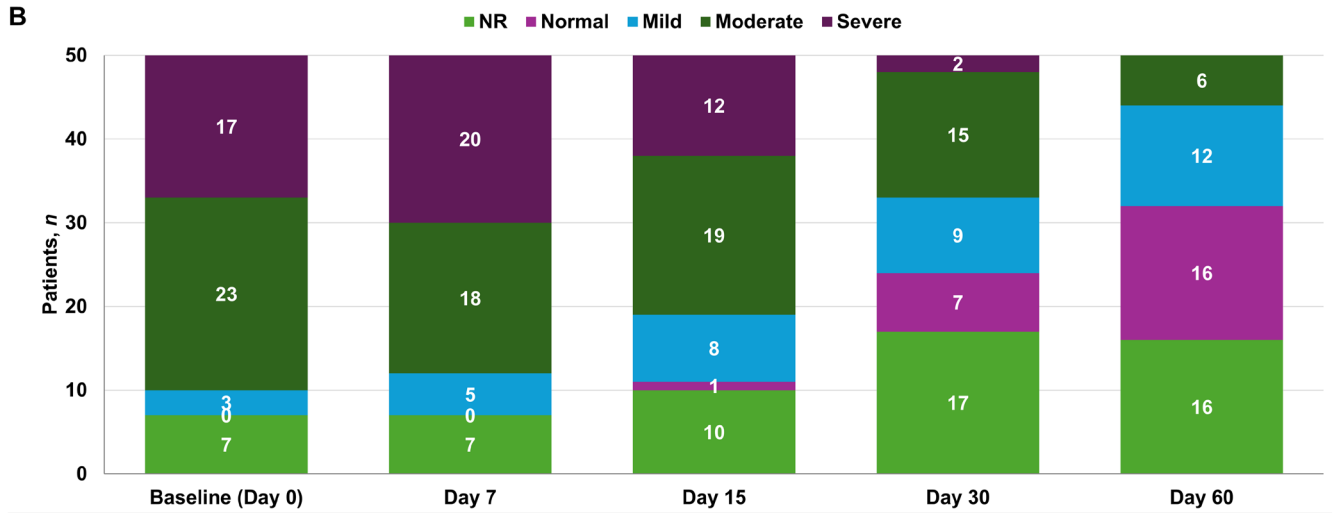
This multicenter trial enrolled a population that was balanced between arms and reflected both the geographic spread of participating institutions and the variable impact of COVID-19 caseloads during different pandemic phases in Spain, enhancing the generalizability of findings while accounting for regional differences in patient populations and hospital protocols. Moreover, causes of patient mortality in this trial reflect the severity of respiratory and vascular involvement in critically ill COVID-19 patients and align with complications described in the literature, particularly in the context of endothelial dysfunction and proinflammatory states [4, 5, 15–17].

Conducting this trial during the early waves of the pandemic, with limited PPE resources and no available vaccine, was challenging but nevertheless justified by the unmet medical need and substantial clinical and mechanistic rationale. The circumstances presented multiple complexities, requiring consideration of numerous factors including logistics of treatment delivery, safety of healthcare professionals, and the evolving understanding of COVID-19. Thus, although the trial did not demonstrate the benefit of defibrotide in clinical improvement time, it provided valuable lessons and key findings informing further investigation of defibrotide in this and related settings.

A key hypothesis emerging from this trial, in the context of other evaluations of defibrotide during the COVID-19 pandemic [30–32], was the potential importance of intermittent Q6hr dosing rather than continuous IV infusion. At the time of trial design, given the challenges associated with clinical administration of therapy during lockdown, the decision to use continuous IV infusion instead of the approved Q6hr schedule was operationally justified, as it reduced exposure of healthcare staff to patients with severe COVID-19 and provided a simplified workflow for the medical and nursing teams. However, this strategy may have limited the ability to detect a clinically significant benefit. In contrast, other studies using conventional Q6hr administration of defibrotide have demonstrated promising efficacy in COVID-19 ARDS [30–32], including the Italian DEFI-VID19 study, which showed a significant increase in post-recovery days and a trend toward improved respiratory failure-free survival with defibrotide versus a contemporaneous, real-world control cohort receiving standard-of-care therapy only [31]. These differing results may reflect exposure differences between continuous IV infusion of 25 mg/kg/day and Q6hr dosing of 6.25 mg/kg by 2-h infusion. Continuous IV infusion (25 mg/kg over 24 h; ~1.04 mg/kg/h) likely produced a substantially lower peak plasma concentration (C_{max}), potentially reducing endothelial protective activity, compared to Q6hr dosing (6.25 mg/kg over 2 h; ~3.125 mg/kg/h), for which the reported pharmacokinetic profile shows a C_{max} of 17.3 ± 3.83 μg/mL [41]. Although clinical data at subtherapeutic exposure are lacking, this hypothesis is supported by dose–exposure pharmacodynamic relationships: lower doses are associated with markedly reduced tissue factor pathway inhibitor release, a key mechanism of action of defibrotide, in preclinical and clinical studies [43, 44].



		Day 7	Day 15	Day 30	Day 60
Severity change from baseline in evaluable patients	Improvement	17.8% Severe to Moderate: *n* = 10 Moderate to Mild/Normal: *n* = 4/1 Mild to Normal: *n* = 1	32.1% Severe to Moderate/ Mild/Normal: *n* = 12/3/1 Moderate to Mild/Normal: *n* = 2/3 Mild to Normal: *n* = 5	60.6% Severe to Moderate/ Mild/Normal: *n* = 12/5/4 Moderate to Mild/Normal: *n* = 9/6 Mild to Normal: *n* = 4	75.8% Severe to Moderate/ Mild/Normal: *n* = 4/8/10 Moderate to Mild/Normal: *n* = 12/8 Mild to Normal: *n* = 5
	Stable	61.1%	51.9%	31.8%	22.6%
	Worsening	21.1% Moderate to Severe: *n* = 16 Mild to Moderate: *n* = 2 Normal to Moderate: *n* = 1	16.0% Moderate to Severe: *n* = 11 Mild to Moderate: *n* = 1 Normal to Mild: *n* = 1	7.6% Moderate to Severe: *n* = 4 Normal to Moderate: *n* = 1	1.6% Moderate to Severe: *n* = 1



		Day 7	Day 15	Day 30	Day 60
Severity change from baseline in evaluable patients	Improvement	16.3% Severe to Moderate: *n* = 4 Moderate to Mild: *n* = 3	40.0% Severe to Moderate/Mild: *n* = 9/2 Moderate to Mild: *n* = 4 Mild to Normal: *n* = 1	66.7% Severe to Moderate/ Mild/Normal: *n* = 6/5/1 Moderate to Mild/Normal: *n* = 4/5 Mild to Normal: *n* = 1	88.2% Severe to Moderate/ Mild/Normal: *n* = 3/5/6 Moderate to Mild/Normal: *n* = 6/9 Mild to Normal: *n* = 1
	Stable	65.1%	45.0%	30.3%	11.8%
	Worsening	18.6% Moderate to Severe: *n* = 7 Mild to Moderate: *n* = 1	15.0% Moderate to Severe: *n* = 6	3.0% Mild to Moderate: *n* = 1	0

FIGURE 3 | Change in severity of pulmonary involvement on radiological assessment from baseline. Changes in severity score from baseline to the time-points of day 7, day 15, day 30, and day 60 in evaluable patients at each time-point in the (A) defibrotide and (B) control arms. The differences between arms in the proportions of patients with improvement at each time-point were not nominally significant (Chi-square test of independence *p*-values 0.90, 0.67, 0.64, and 0.31, respectively). Classification per an 8-point severity score (score of 0–4 for each lung): Scores of 0, 1–2, 3–6, and > 6 classed as 'normal', 'mild', 'moderate', and 'severe' involvement, respectively. NR: Not reported, patients not evaluable.

Additionally, DEFI-VID19 enrolled its defibrotide-treated patients over an 8-month period [31], which was shorter than the 14-month enrollment window of our trial that spanned four distinct COVID-19 waves in Spain (Table S3). It is therefore possible that a higher proportion of patients in our trial were infected with the original SARS-CoV-2 strain, whereas the Italian cohort may have been dominated by infections during the Alpha-variant wave [31]. Differences in circulating variants and in vaccination coverage between the two study periods could have influenced the observed differences in defibrotide efficacy.

The rapidly evolving context of the COVID-19 pandemic also posed major challenges to trial conduct. For instance, the WHO 7-point ordinal COVID-19 severity scale [36] used at the time of

study design was subsequently superseded by 8-point [38] and later 10-point [45] versions, the latter incorporating oxygen saturation levels. Moreover, evolving clinical practice and logistical constraints made it unfeasible to assess several of the originally planned secondary endpoints during the trial.

From a safety standpoint, this trial is important: continuous IV infusion of defibrotide 25 mg/kg/day did not increase serious bleeding, hemodynamic instability, or treatment-related SAEs versus placebo, even in patients requiring high levels of ventilatory support, ECMO, or renal replacement therapy. This supports the feasibility of administering defibrotide in severely ill COVID-19/ARDS populations and, more broadly, in syndromes of systemic endotheliitis.

Continued evaluation of defibrotide in patients with severe COVID-19 was planned based on the present study's findings and the dosing hypothesis described above. Protocol amendments were approved by local IRBs and national regulatory authorities to continue the trial using Q6hr dosing of defibrotide. However, due to very slow accrual associated with the changing clinical course of COVID-19 following widespread vaccination, the pharmaceutical sponsor recommended closure of the trial, and the study leadership complied. Evaluation of biomarkers of defibrotide activity remains an important ongoing objective. Despite baseline clinical characteristics being generally representative of COVID-19 cases, imbalances were identified that may have influenced comparative efficacy. Baseline D-dimer levels were significantly higher and CRP levels trended higher in the defibrotide versus placebo arm, suggesting higher endothelial stress at enrollment. Nevertheless, overall biomarker patterns might reflect a trend toward favorable effects on thrombotic pathway modulation, although these data require validation, and continuous IV infusion may have limited the endothelial protective effects of defibrotide.

In conclusion, continuous IV defibrotide was safe but did not improve clinical outcomes in severe COVID-19. Further studies exploring intermittent (Q6hr) dosing and/or the potential role of defibrotide in mitigating post-COVID-19 vascular sequelae are ongoing. Specifically, additional insights are anticipated from an ongoing phase II study of Q6hr defibrotide for patients with severe COVID-19 (NCT04652115). The DEFACOVID study group is continuing analysis of biologic samples from this trial to further elucidate the mechanistic actions of defibrotide and the pathophysiology of endothelial dysfunction in COVID-19, findings that may have broader relevance for other conditions characterized by endotheliitis and ARDS, for example, complications of immunotherapy in hematologic malignancies, including multiple myeloma [34, 46].

Author Contributions

Conceptualization and study design: R.J.R., A.J.M.-M., D.G.-B., F.I., P.G.R., J.M.M. Treated patients and collected data: R.J.R., A.J.M.-M., B.G.-P., P.C., M.D.-R., A.C.-A., A.L.-B., E.B.M., A.R., S.M., F.-J.R.-L., E.S.-M., D.M.-B., C.L.A.M., E.A.S., A.T.S., S.F.M., J.A.N.V., I.C.L., A.P., A.E.-M., M.B., A.S.-S., C.H.G., M.M., A.M.P., A.M.-D., M.M.G., J.M.M. Analyzed the data: R.J.R., A.J.M.-M., J.K.-C., J.G.C., A.M.T.-C., M.S., M.I., C.C.-S., L.-J.W., P.G.R., J.M.M. Performed statistical analyses: J.K.-C., J.G.C., A.M.T.-C., L.-W. Interpreted the data: R.J.R., A.J.M.-M., M.S., M.I., C.C.-S., L.-J.W., P.G.R., J.M.M. Wrote the paper: R.J.R., A.J.M.-M., P.G.R., J.M.M. Reviewed and edited the paper: All authors. Approved the final version of the paper for submission: All authors.

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Ethics Statement

The trial protocol and amendments were approved by the institutional review board (IRB) of University Clinical Hospital Virgen de la Arrixaca, IRBs at participating centers, and the Spanish Agency of Medicaments and Health Products. The trial was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines and was compliant with the European Union's General Data Protection Regulation and Spanish data protection laws.

Consent

All participants provided written informed consent.

Conflicts of Interest

J.K.-C. has received an honorarium for a lecture on herpes zoster vaccine from GSK. P.C. has received honoraria for scientific collaborations from Pfizer, MSD, Gilead, AbbVie, and Mundipharma and support for attending meetings and/or travel from Menarini and Pfizer, and has participated in advisory boards for Alexion, Janssen, Sanofi, and Gilead. M.D.-R. has received honoraria for lectures from Jazz Pharmaceuticals and Zacros and equipment, materials, drugs, medical writing, gifts or other services from Zacros. S.F.-M. has received honoraria for lectures from Gilead and support for attending meetings from Pfizer. P.G.R. has received grants from Oncopeptides and Karyopharm, and consulting fees from Celgene/BMS, GSK, Karyopharm, Oncopeptides, Regeneron, and Sanofi. J.M.M. has received honoraria for lectures from Roche and payments for Data Safety Monitoring Board participation from Rocket Pharmaceuticals Inc. The other authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Tertiary hospitals in Spain that participated in the trial, plus site enrollment. **Table S2:** The World Health Organization 7-category ordinal scale for COVID-19 severity [5]. **Table S3:** Summary of COVID-19 waves in Spain, 2020–2021. **Figure S1:** CONSORT flow diagram. **Figure S2:** Kaplan–Meier analysis of survival/mortality through to Day 60. **Figure S3:** Severity of pulmonary involvement on chest radiography at baseline and over time.