ORIGINAL ARTICLE

Modulation of thromboinflammation in hospitalized COVID-19 patients with aprotinin, low molecular weight heparin, and anakinra: The DAWn-Antico study

Matthias M. Engelen MD[1,2] | Quentin Van Thillo MD[3] |
Albrecht Betrains MD[4] | Iwein Gyselinck MD[5,6] | Caroline P. Martens MD[2] |
Valérie Spalart MD[1,2] | Anna Ockerman PhD[1] | Caroline Devooght MSc[1] |
Joost Wauters MD, PhD[7] | Jan Gunst MD, PhD[8] | Carine Wouters MD, PhD[9,10] |
Christophe Vandenbriele MD, PhD[1,2] | Steffen Rex MD, PhD[11,12] |
Laurens Liesenborghs MD, PhD[2] | Alexander Wilmer MD, PhD[7] |
Philippe Meersseman MD[7] | Greet Van den Berghe MD, PhD[8] |
Dieter Dauwe MD, PhD[8] | Ann Belmans PhD[13] | Michiel Thomeer MD, PhD[14,15] |
Tom Fivez MD, PhD[15,16] | Dieter Mesotten MD[15,16] | David Ruttens MD, PhD[14] |
Luc Heytens MD[17] | Ilse Dapper MD[17] | Sebastiaan Tuyls MD[18] |
Brecht De Tavernier MD[19] | Peter Verhamme MD, PhD[1,2] |
Thomas Vanassche MD, PhD[1,2] | DAWn Consortium Members

[1]Department of Cardiovascular Diseases, University Hospitals Leuven, Leuven, Belgium

[2]Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, KU Leuven, Leuven, Belgium

[3]Center for Cancer Biology, VIB, Leuven, Belgium

[4]Department of Microbiology, Immunology and Transplantation, Laboratory for Clinical Infectious and Inflammatory Disorders, KU Leuven, Leuven, Belgium

[5]BREATHE Lab, Department CHROMETA, KU Leuven, Leuven, Belgium

[6]Department of Respiratory Diseases, UZ Leuven, Leuven, Belgium

[7]Department of General Internal Medicine, Medical Intensive Care Unit, University Hospitals Leuven, Leuven, Belgium

[8]Clinical Department and Laboratory of Intensive Care Medicine, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

[9]Pediatric Rheumatology, University Hospitals Leuven, Leuven, Belgium

[10]Laboratory of Adaptive Immunology & Immunobiology, Department of Microbiology and Immunology, KU Leuven, Leuven, Belgium

[11]Department of Cardiovascular Sciences, University Hospitals Leuven, Leuven, Belgium

[12]Department of Anesthesiology, University Hospitals Leuven, Leuven, Belgium

[13]Interuniversity Institute for Biostatistics and statistical Bioinformatics (I-BioStat), KU Leuven, Leuven, Belgium

[14]Department of Respiratory Medicine, Ziekenhuis Oost-Limburg, Genk, Belgium

[15]Department of Medicine and Life Sciences, Hasselt University, Diepenbeek, Belgium

[16]Department of Anaesthesiology, Intensive Care, Emergency Medicine and Pain Therapy, Ziekenhuis Oost-Limburg, Genk, Belgium

[17]Department of Intensive Care, GZA Hospital Group, Antwerp, Belgium

[18]Respiratory Medicine, GZA Hospital Group, Antwerp, Belgium

[19]Department of Emergency Medicine, GZA Hospital Group, Antwerp, Belgium

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Correspondence
Matthias M. Engelen, Herestraat 49 Box 911 – ON1, 3000 Leuven, Belgium.
Email: matthias.engelen@uzleuven.be

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Abstract

Background: Thromboinflammation plays a central role in severe COVID-19. The kallikrein pathway activates both inflammatory pathways and contact-mediated coagulation. We investigated if modulation of the thromboinflammatory response improves outcomes in hospitalized COVID-19 patients.

Methods: In this multicenter open-label randomized clinical trial (EudraCT 2020-001739-28), patients hospitalized with COVID-19 were 1:2 randomized to receive standard of care (SOC) or SOC plus study intervention. The intervention consisted of aprotinin (2,000,000IE IV four times daily) combined with low molecular weight heparin (LMWH; SC 50IU/kg twice daily on the ward, 75IU/kg twice daily in intensive care). Additionally, patients with predefined hyperinflammation received the interleukin-1 receptor antagonist anakinra (100mg IV four times daily). The primary outcome was time to a sustained 2-point improvement on the 7-point World Health Organization ordinal scale for clinical status, or discharge.

Findings: Between 24 June 2020 and 1 February 2021, 105 patients were randomized, and 102 patients were included in the full analysis set (intervention $N = 67$ vs. SOC $N = 35$). Twenty-five patients from the intervention group (37%) received anakinra. The intervention did not affect the primary outcome (HR 0.77 [CI 0.50-1.19], $p = 0.24$) or mortality (intervention $n = 3$ [4.6%] vs. SOC $n = 2$ [5.7%], HR 0.82 [CI 0.14-4.94], $p = 0.83$). There was one treatment-related adverse event in the intervention group (hematuria, 1.49%). There was one thrombotic event in the intervention group (1.49%) and one in the SOC group (2.86%), but no major bleeding.

Conclusions: In hospitalized COVID-19 patients, modulation of thromboinflammation with high-dose aprotinin and LMWH with or without anakinra did not improve outcome in patients with moderate to severe COVID-19.

KEYWORDS
anakinra, aprotinin, COVID-19, heparin, inflammation, low-molecular-weight, thrombosis

MODULATION OF THROMBOINFLAMMATION IN MODERATE TO SEVERE COVID-19: DAWn-ANTICO

MULTICENTER OPEN-LABEL RANDOMIZED CLINICAL TRIAL

Intention-to-treat
102

INTERVENTION N= **67**
high-dose **aprotinin (IV)**
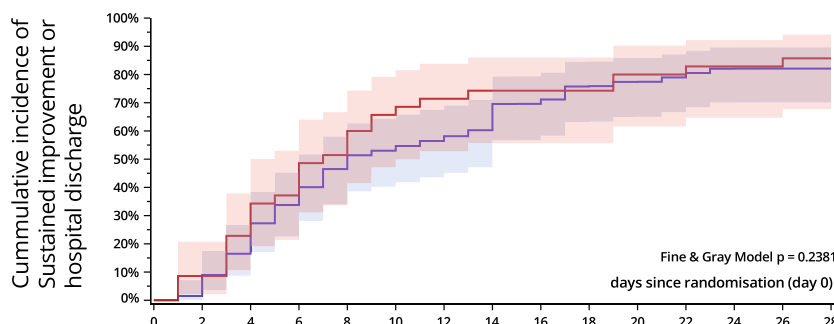intensified **LMWH (SC)**
± **anakinra (IV)**

STANDARD OF CARE N= **35**

time to sustained clinical improvement or hospital discharge (28 days)

Estimated Event Rate (95% CI) by Randomised Treatment

82.1% (70.1%, 89.6%) **85.7% (67.7%, 94.1%)**

Fine & Gray Model p = 0.2381

days since randomisation (day 0)

Number at risk	0	2	4	6	8	10	12	14	16	18	20	22	24	26	28
INTERVENTION	67	66	55	42	34	29	27	25	18	13	12	11	9	9	9
STANDARD OF CARE	35	32	27	22	17	12	10	9	9	8	5	5	4	4	3

Essentials

- Severe COVID-19 is associated with a high incidence of blood clots and inflammation.
- This trial studied a multi-target strategy to prevent blood clots and inflammation.
- This strategy did not improve clinical outcome compared to the standard treatment.
- Overall mortality was low with both the experimental and standard treatment.

1 | BACKGROUND

COVID-19 continues to dominate global health. In patients who develop severe disease, a thromboinflammatory response follows the initial phase of viral replication. Indeed, markers of inflammation and hypercoagulation are associated with disease severity and outcome.[1,2] We therefore designed a multitarget multistep intervention to study whether modulation of this excessive thromboinflammatory response is feasible and improves outcome in patients with severe COVID-19.[3] The study intervention consists of aprotinin to suppress the kallikrein-kinin system and higher dose low molecular weight heparin (LMWH). Additionally, anakinra was added to inhibit the interleukin-1 (IL-1) pathway in patients with signs of hyperinflammation.

The kallikrein-kinin system might drive the pathogenesis of thrombo-inflammation in COVID-19 because it induces inflammation and activation of coagulation through the contact pathway.[4–7] Specific inhibitors of the kallikrein-bradykinin-system are not readily available. However, the nonspecific serine protease-inhibitor aprotinin strongly suppresses kallikrein activity. Being a nonspecific

serine protease inhibitor, aprotinin also could inhibit the protease activity of transmembrane serine protease 2 (TMPRSS2). This human protease cleaves the spike protein between the S1 and S2 subunit, but thereby facilitates SARS-CoV-2 cell entry. Indeed, preliminary research showed that by inhibiting TMPRSS2, aprotinin inhibits SARS-CoV-2 entry and replication *in vitro*.[8–10] LMWH, on the other hand, inhibits coagulation factors Xa and IIa and reduces thrombotic events in patients with severe COVID-19.[11,12] The optimal dose of thromboprophylaxis has been the focus of various dedicated clinical trials. This clinical trial, however, is not designed to determine the optimal dose of thromboprophylaxis but instead investigates the concept of multitargeted thromboinflammatory modulation (aprotinin and LMWH). In patients with hyperinflammation, the recombinant interleukin-1 receptor antagonist anakinra was added to suppress the hyperinflammatory state induced by the overproduction of inflammatory cytokines in COVID-19. Anakinra has been successfully investigated in other pathologies with hyperinflammatory properties and is, therefore, an available compound to suppress the IL-1–IL-6 pathway.

2 | METHODS

2.1 | Study design and oversight

This open-label multicenter randomized clinical trial was performed in three Belgian hospitals. The study's design has been published and the full protocol is in Appendix S2.[3] The Ethics Committee an institutional review board at each participating center approved the protocol. The clinical study was registered as EudraCT 2020-001739-28. The steering committee and Data and Safety Monitoring Board (DSMB) were responsible for the oversight of the trial. A safety and feasibility review was planned after the pilot phase (November 2020). After this prespecified interim analysis of day 6 D-dimer in the first 50 patients, the DSMB recommended to continue the study but ordered a midway safety and futility analysis after randomizing 105 patients (i.e., 50%). After this futility analysis, on 31 March 2021, the DSMB advised terminating the study because of a conditional power of <0.1% to reach the primary endpoint with the planned sample size.

2.2 | Patients

Male or nonpregnant female adult patients aged 18 years or older at the time of enrollment with confirmed diagnosis of SARS-CoV-2 infection were eligible for participation. The main exclusion criteria included known thromboembolic disease, recent myocardial infarction, creatine clearance <20 ml/min or renal replacement therapy, active bleeding or increased bleeding risk, platelet count <30,000/μl, indication for therapeutic anticoagulation, heart failure, and suspicion of latent tuberculosis or severe bacterial infection before randomization

or start of anakinra. Known thromboembolic disease also includes new venous thromboembolism diagnosed before enrollment. Patients were not routinely screened through computed tomography pulmonary angiogram or venous ultrasound to exclude asymptomatic venous thromboembolism. The full list of exclusion criteria is available as an online supplement and in the published study protocol paper.[3] All included patients provided written informed consent.

2.3 | Randomization and masking

Briefly, eligible and consenting patients were randomized to receive standard of care (SOC) or study intervention, according to a 2:1 allocation scheme stratified by study site, using randomly selected block sizes of 6 or 9. Randomization was done using a centralized web-based randomization application. In this open-label study, patients, clinicians, and study personnel were aware of the assigned treatment. The trial statistician was not given access to the full database and was not aware of the allocated treatments until database lock.

2.4 | Intervention and procedures

Standard of care included LMWH thromboprophylaxis per hospital protocol (Table S1).[13,14] As illustrated in Figure 1, the study intervention consisted of aprotinin (2,000,000 IE four times per day for 4 days) combined with weight-adjusted LMWH (enoxaparin or nadroparin, 50 IU/kg twice daily in the ward, 75 IU/kg twice daily in intensive care unit [ICU]; for 14 days and reduced to once daily when creatinine clearance dropped below 30 ml/min/1.73 m^2). Additionally, patients developing
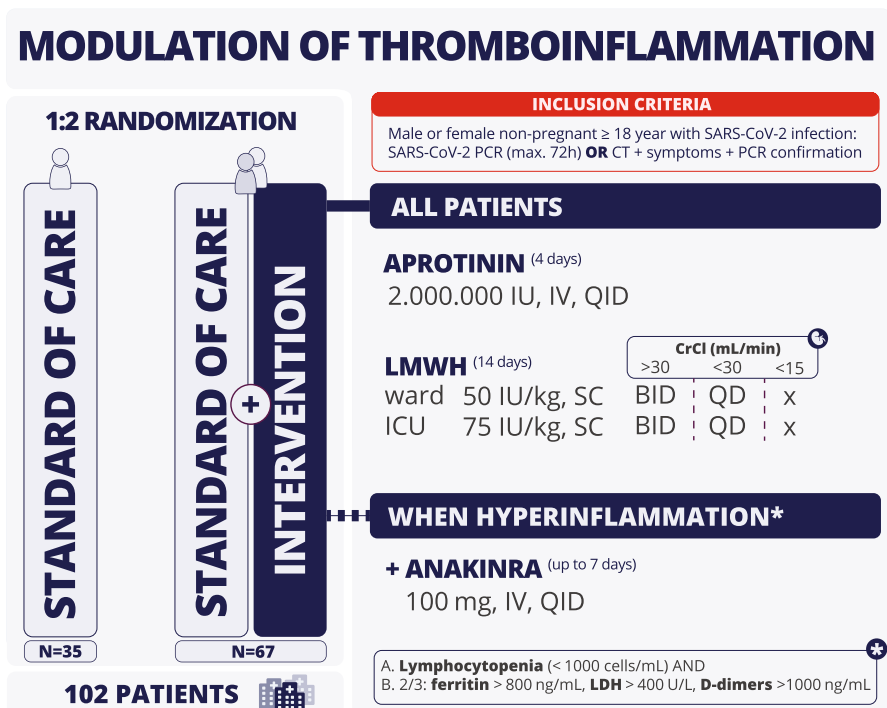

FIGURE 1 Study overview. BID, twice daily; CrCl, creatinine clearance; CT, computed tomography; ICU, intensive care unit; IU, international units; IV, intravenous; LMWH, low molecular weight heparin; PCR, polymerase chain reaction; QD, once daily; QID, four times a day; SC, subcutaneous.
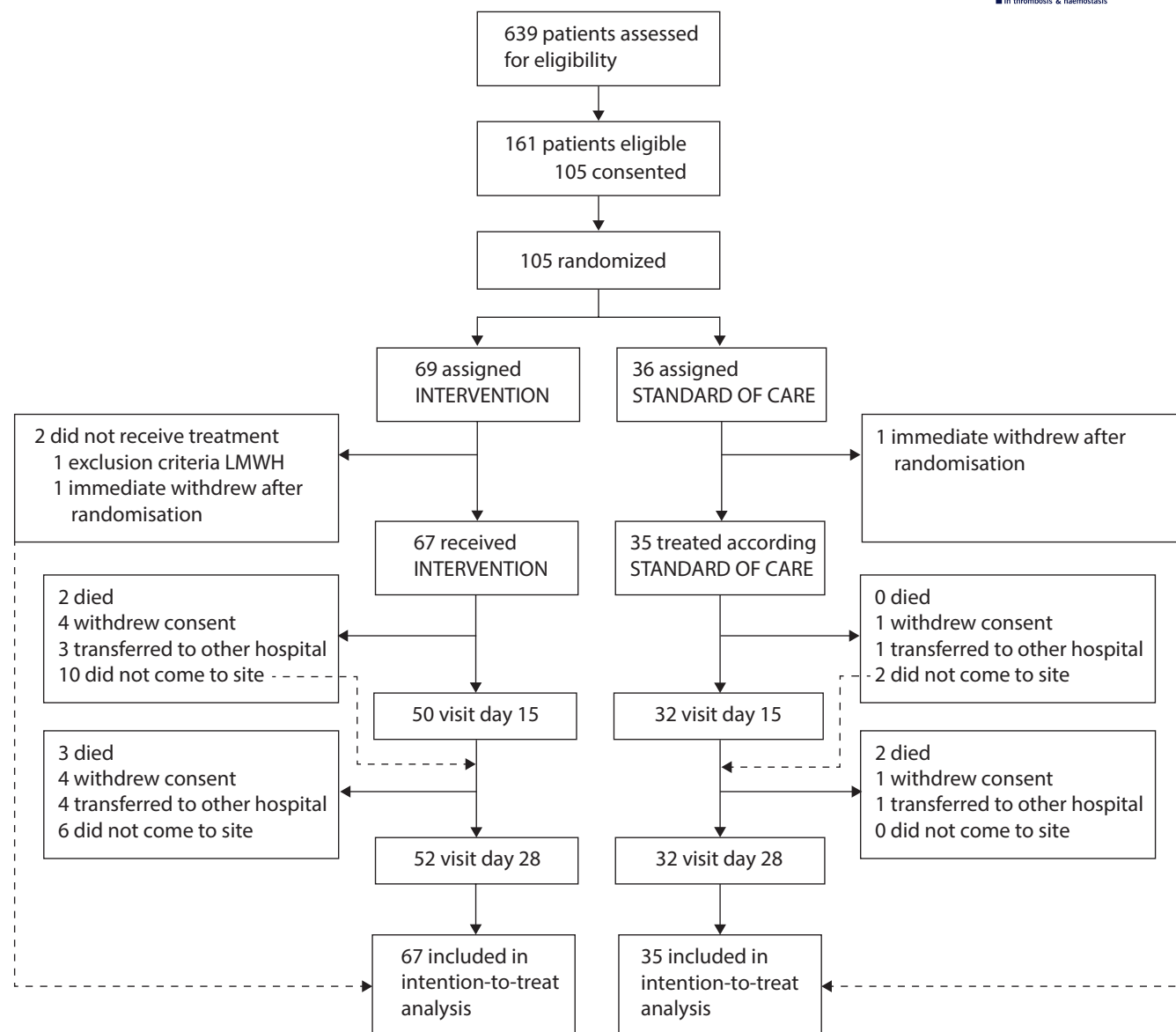
FIGURE 2 Study profile. Low molecular weight heparin, LWMH (CONSORT flowchart also in Appendix S1).

hyperinflammation during the first 14 days after randomization were treated with anakinra unless contraindicated (100 mg IV four times per day for up to 7 days). Hyperinflammation was defined as lymphocytopenia (<1000 cells/ml) with two of the following: (1) ferritin >800 ng/ml, (2) LDH >400 U/L, or (3) D-dimer >1000 ng/ml. During the first 14 days after randomization, there was daily follow-up until discharge. Additional follow-up was planned on days 15 and 28. A follow-up visit was provided 5 to 7 weeks after discharge.

2.5 | Trial outcomes

The primary clinical outcome was time to discharge or sustained 2-point improvement in the World Health Organization 7-point ordinal scale for clinical status or hospital discharge. A 2-point improvement was defined as an improvement of >2 points compared with the highest value recorded on day 0 or 1 and sustained for at least 3 days. Clinical status was recorded daily until discharge and on days 15 and 28 and follow-up visit 5 to 7 weeks after discharge. Secondary outcomes included all-cause mortality on days 15 and 28, incidence and duration of supplemental oxygen and mechanical ventilation up to day 28, duration of hospital and ICU stay, and thromboinflammatory parameters at predefined timepoints. Details on laboratory assays for outcome parameter D-dimer (cutoff 500 μg/L fibrinogen equivalent units; Werfen and Stago) and C-reactive protein (Roche and Abbott) are summarized in Table S2. Safety outcomes included adverse events, thrombotic events (venous thromboembolism and others) and major bleeding as defined by the International Society on Thrombosis and Haemostasis.[15] A detailed description of secondary and safety end points is available as an online supplement or in the published study protocol.[3]

2.6 | Statistical analysis

sLearning from early randomized trials, we assumed a 40% improvement rate on day 15 in the control group for our power calculations. Based on the log-rank test, with a two-sided significance level of 5% and 80% statistical power and using a 2:1 randomization ratio in favor of the intervention, we estimated that a total sample size of 196 patients would suffice to detect an absolute improvement of 20% (60% in the intervention group). We proposed a pragmatic sample size of 210 patients considering early dropouts. A detailed description of the analysis is provided in the Statistical Analysis Plan, which was finalized and filed before database lock (Appendix S3). A summary is provided here. Analysis sets were finalized prior to database lock when the investigators were unaware of the study results. The Full Analysis Set (FAS) included all randomized patients, except one patient with chronic alcoholism and three patients who withdrew consent to use any data immediately after randomization and before treatment administration. The Per Protocol Set included all FAS patients, except for patients randomized to intervention who had fewer than 3 days of aprotinin dosing, who had hyperinflammation but received no anakinra, or who received anakinra but had no hyperinflammation. The primary analysis set of interest was the FAS, but all efficacy analyses were repeated on the Per Protocol Set as a sensitivity analysis.

Missing clinical status data were accounted for by means of multiple imputation, using a total of 100 imputations. Treatment effects for all end points were estimated by an appropriate measure and presented with 95% confidence intervals and were adjusted for study site and period (before and after start of second peak of COVID hospitalizations [7 September 2021]). The primary end point was compared using competing-risk methodology, using cumulative incidence functions to estimate event rates and a Fine & Gray regression model to obtain cause-specific hazard ratios. Daily clinical status was analyzed using a proportional odds logistic regression to estimate the common odds ratio. All-cause mortality and survival without mechanical ventilation up to 30 days were assessed using a Cox regression to obtain hazard ratios. Incidence rates were estimated using Kaplan–Meier methodology. Time to hospital discharge, incidence and duration of supplemental oxygen, mechanical ventilation, and ICU stay were analyzed using the same methodology as for the primary end point. Cumulative clinical status scores were analyzed using a general linear model on the log-transformed scores to obtain a treatment ratio of geometric means between the treatment groups.

Prespecified subgroup analyzes were performed for the primary end point, considering the following subgroups: age (according to observed median), study period, admission to ICU at hospital admission, presence of hyperinflammation at baseline, clinical status on day 0 (≥5 vs. <5).

All tests were two-sided and assessed at a significance level of 5%. No correction was made for multiple secondary endpoints. All analyses were performed using SAS software version 9.4 for Windows 10.

2.7 | Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

3 | RESULTS

3.1 | Baseline demographics

Between 24 June 2020, and 1 February 2021, 105 patients were randomized at three hospitals in Belgium. Thirty-six patients were randomized to SOC and 69 to intervention, of whom 25 patients (37%) were additionally treated with anakinra. After excluding one patient in the SOC group and two patients in the intervention group, 102 patients were analyzed in the FAS (Figures 1 and 2). The mean age was 58 years (SD 13) and 75% of patients were male. There was no significant difference in ethnicity between both groups; 86% of patients was Caucasian (details in Table S3). Except for chronic systemic corticosteroid therapy, there were no baseline differences in demographics, medical history, vital signs at admission, and medical treatment or mechanical support started on admission between SOC and intervention (Table 1). Patients had elevated D-dimer (median 665 μg/L, IQR 460–1090 μg/L) and C-reactive protein (median 65 mg/L, IQR 41–112 mg/L) at admission, confirming baseline activation of coagulation and inflammatory pathways. At admission, criteria of hyperinflammation were met in 29% of cases and D-dimer were elevated in 29% of cases.

3.2 | Efficacy outcomes

There was no effect of the intervention on the primary outcome of time to sustained clinical improvement or hospital discharge (Figure 3A). Additionally, there were no significant differences between intervention and SOC in secondary clinical outcomes (Table 2A and Figure 3B). During hospitalization, one of three patients were admitted to the ICU (29% SOC vs. 42% intervention; HR 1.56 [CI 0.79-3.06], p = 0.20) and 20% of patients needed invasive ventilation (11% SOC vs. 24% intervention; HR 2.35 [CI 0.79-6.96], p = 0.12). Table 2B shows the evolution of secondary biochemical outcomes. Of note, after a significant reduction of D-dimer levels at day 3 (estimated treatment ratio 0.43 [CI 0.30-0.61], p < 0.01), D-dimer levels rose again at days 6 and 15 without a significant difference between the two groups. There was no sustained effect on the biochemical outcomes D-dimer and C-reactive protein (Table 2B).

3.3 | Safety outcomes

There was one thrombotic event in the SOC group (not further specified) and one in the intervention group (pulmonary embolism). There was no major bleeding in either study groups. There was no difference in study-related adverse events (Table 3).

TABLE 1 Patient characteristics

		Randomized treatment		
	Statistic	Intervention	Standard of care	*p* value
Baseline characteristics	N	67	35	
Age, y	[*n*] Mean (SD)	[67] 58 (13)	[35] 59 (14)	0.75
Male	n/N (%)	51/67 (76.1%)	25/35 (71.4%)	0.61
Caucasian	n/N (%)	56/66 (84.85%)	31/35 (88.57%)	0.91
Body weight, kg	[*n*] Mean (SD)	[65] 92 (22)	[34] 91 (19)	0.79
Medical history				
Diabetes mellitus	n/N (%)	13/67 (19.4%)	10/35 (28.6%)	0.46
Arterial hypertension	n/N (%)	34/67 (50.8%)	16/35 (45.7%)	0.66
Smoking status				
Active	n/N (%)	3/67 (4.5%)	2/35 (5.7%)	0.16
Former	n/N (%)	9/67 (13.4%)	10/35 (28.6%)	
Never	n/N (%)	55/67 (82.1%)	23/35 (65.7%)	
COPD	n/N (%)	3/67 (4.5%)	2/35 (5.7%)	0.57
Asthma	n/N (%)	11/67 (16.4%)	4/35 (11.4%)	0.45
Heart failure	n/N (%)	2/67 (3.0%)	0/35 (0%)	0.25
Ischemic heart disease	n/N (%)	5/67 (7.5%)	1/35 (2.9%)	0.37
Moderate or severe liver disease	n/N (%)	0/67 (0%)	0/35 (0%)	0.30
Chronic kidney disease	n/N (%)	4/67 (6.0%)	0/35 (0%)	0.19
Active cancer	n/N (%)	2/67 (3.0%)	0/35 (0%)	0.30
Previous medications				
Antiplatelet agent	n/N (%)	7/67 (10.5%)	4/35 (11.4%)	0.88
Anticoagulation	n/N (%)	5/67 (7.5%)	1/35 (2.9%)	0.59
Statins	n/N (%)	14/67 (20.9%)	6/35 (17.1%)	0.82
Chronic systemic corticosteroid therapy	n/N (%)	0/67 (0%)	6/35 (17.1%)	0.001
Other immune-suppressing therapy	n/N (%)	1/67 (1.5%)	2/35 (5.7%)	0.38
Antibiotics	n/N (%)	7/67 (10.5%)	4/35 (11.4%)	0.88
Laboratory data at admission				
Hemoglobin, g/dl	[*n*] Mean (SD)	[65] 14 (2)	[34] 14 (1)	0.32
WBC, 10^9/L	[*n*] Mean (SD)	[65] 7 (3)	[34] 7 (2)	0.56
Platelet count, 10^9/L	[*n*] Mean (SD)	[65] 231 (96)	[33] 231 (102)	1.00
CRP, mg/L	[*n*] Median (Q1; Q3)	[66] 64 (43; 104)	[35] 73 (31; 114)	0.79
D-dimer, µg/L	[*n*] Median (Q1; Q3)	[60] 735 (465; 1230)	[31] 570 (454; 877)	0.16
Serum creatinine [mg/dl]	[*n*] Mean (SD)	[65] 1 (0)	[34] 1 (0)	0.64
High-sensitivity troponin T, µg/ml	[*n*] Median (Q1; Q3)	[28] 0 (0; 0)	[14] 0 (0; 0)	0.77
NT-proBNP, ng/L	[*n*] Median (Q1; Q3)	[14] 144 (74; 766)	[7] 298 (99; 717)	0.65
Hyperinflammation				
No	n/N (%)	37/57 (64.9%)	27/33 (81.8%)	0.09
Yes	n/N (%)	20/57 (35.1%)	6/33 (18.2%)	
D-dimer >1000 µg/L at baseline				
No	n/N (%)	40/60 (66.7%)	25/31 (80.7%)	0.22
Yes	n/N (%)	20/60 (33.3%)	6/31 (19.4%)	
In-hospital support	N	67	35	
Oxygen	n/N (%)	64/67 (95.5%)	33/35 (94.3%)	1.00
High-flow oxygen	n/N (%)	33/63 (52.4%)	13/33 (39.4%)	0.28

(Continues)

TABLE 1 (Continued)

| | Statistic | Randomized treatment | | |
		Intervention	Standard of care	p value
Noninvasive ventilation	n/N (%)	13/63 (20.6%)	7/33 (21.2%)	1.00
Invasive ventilation	n/N (%)	16/63 (25.4%)	4/33 (12.1%)	0.19
Prone ventilation	n/N (%)	14/63 (22.2%)	4/33 (12.1%)	0.28
ECMO	n/N (%)	1/67 (1.5%)	2/35 (5.7%)	0.27
Inhaled nitric oxide	n/N (%)	3/67 (4.5%)	2/35 (5.7%)	1.00
Dialysis	n/N (%)	1/67 (1.5%)	0/35 (0.0%)	1.00
In-hospital medical treatment	N	67	35	
Hydroxychloroquine	n/N (%)	0/67 (0.0%)	0/35 (0.0%)	
Favipiravir	n/N (%)	0/67 (0.0%)	0/35 (0.0%)	
Remdesivir	n/N (%)	5/67 (7.5%)	7/35 (20.0%)	0.10
Lopinavir/ritonavir	n/N (%)	0/67 (0.0%)	0/35 (0.0%)	
Other antivirals	n/N (%)	1/67 (1.5%)	1/35 (2.9%)	1.00
Tocilizumab	n/N (%)	1/67 (1.5%)	0/35 (0.0%)	1.00
Antibiotics	n/N (%)	36/67 (53.7%)	22/35 (62.9%)	0.41
Antifungal treatment	n/N (%)	5/67 (7.5%)	4/35 (11.4%)	0.49
Systemic corticosteroids	n/N (%)	54/67 (80.6%)	26/35 (74.3%)	0.46

Abbreviations: COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; ECMO, extracorporeal membrane oxygenation; WBC, white blood cell.

4 | DISCUSSION

In hospitalized patients with moderate to severe COVID-19, a multi-target strategy to modulate thromboinflammation with high-dose IV aprotinin, intensified weight- and severity-dosed LMWH with or without anakinra, did not show signs of safety issues, but did not improve outcome. Overall mortality was low.

Some aspects of our trial require comment. First, after analysis of 102 randomized patients, early termination was recommended by the DSMB because futility, as stated in the Methods. Second, despite the disease severity of the study population with considerable baseline activation of coagulation and inflammation, overall mortality was low in both SOC and intervention. Sustained clinical improvement by 2 points on the 7-point World Health Organization ordinal scale was obtained in 74% of control patients at day 15, which is more than the anticipated 40% used in the power calculation; this may reflect improved care for COVID-19 patients. It therefore becomes increasingly difficult for clinical trials to demonstrate significant improvement of care with new treatment strategies. Third, despite randomization, there are some differences in baseline demographics. Note that patients in the intervention group had numerically but not significantly elevated D-dimer and hyperinflammation, potentially reflecting a more severely ill intervention group or the higher portion of chronic steroid use at baseline. Despite these trends, clinical outcome did not differ. Fourth, in the intervention group, more intravenous fluids were administrated, which might result in cardiopulmonary deterioration in certain precarious patients. This could explain the (nonsignificant) trend of the intervention curves starting around day 4, when looking at daily clinical status and respiratory support. Fifth, after introducing an intensified COVID-19 thromboprophylaxis,

incidence of venous thromboembolism was low.[14] This antithrombotic, but possibly also anti-inflammatory, effect of LMWH might have helped suppress thromboinflammation. Nevertheless, we observed a significant decrease of D-dimer at day 3, probably reflecting an aprotinin effect.

4.1 | Aprotinin

Other studies have targeted the thromboinflammatory response. Regarding aprotinin, a noncomparative clinical trial investigated the therapeutic *in vivo* antiviral effects of aprotinin in COVID-19.[16] In addition to SOC and thromboprophylaxis with 40 mg enoxaparin, patients without (noninvasive) mechanical ventilation were included in three cohorts with (1) low-dose IV aprotinin (10^6 KIU) plus hydroxychloroquine, (2) nasal aprotinin (625 KIU four times a day) plus hydroxychloroquine, or (3) low-dose IV aprotinin (10^6 KIU) plus avifavir, with the latter being the most effective in the combined primary outcome of normalization of polymerase chain reaction, D-dimer and C-reactive protein. The difference in primary outcome could reflect an avifavir effect compared with hydroxychloroquine in the other cohorts. In a phase III randomized trial with 60 patients with mild COVID-19, nebulized aprotinin seemed to decrease admission time compared with placebo.[17]

4.2 | Low molecular weight heparin

COVID-19 is associated with a high incidence of subclinical and symptomatic venous thromboembolism in hospitalized patients despite prophylactic doses of LMWH.[18] Thrombosis and coagulation markers
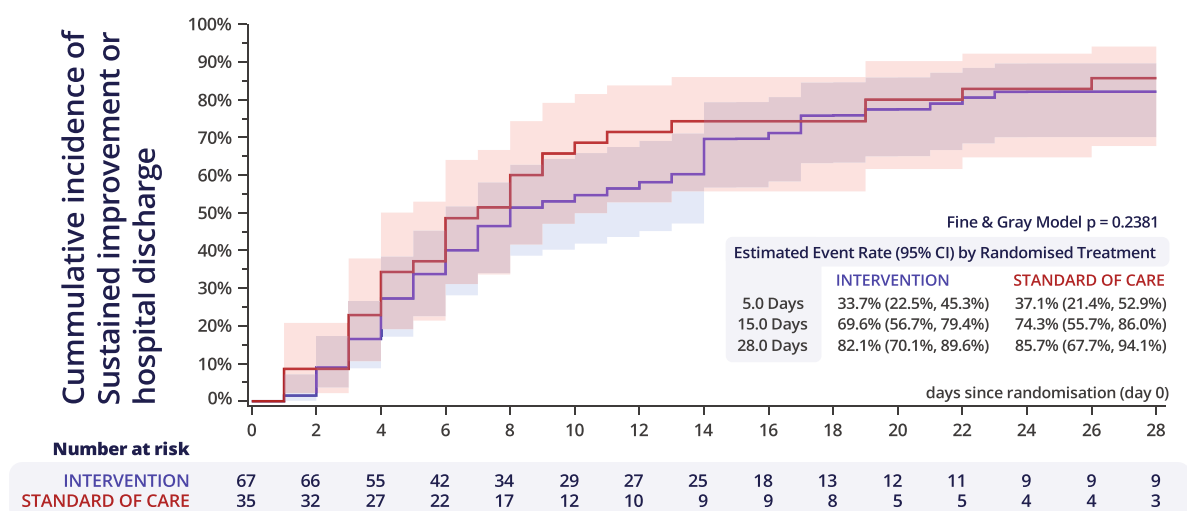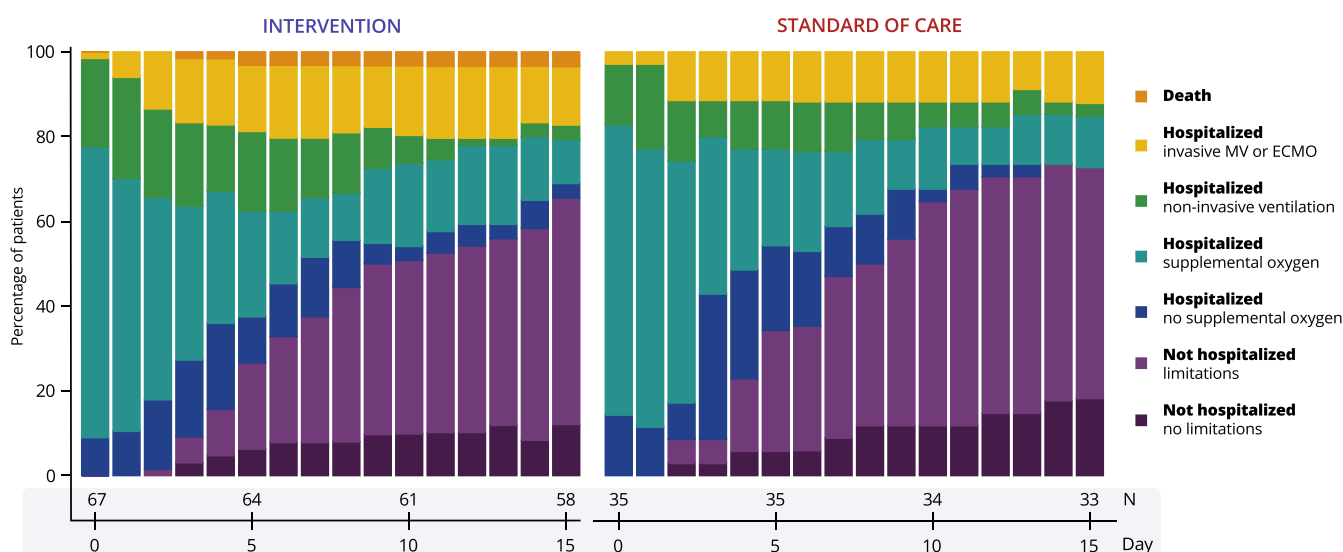
(A) Primary outcome: sustained clinical improvement

(B) Secondary outcome: daily clinical status

FIGURE 3 Outcomes. (A) primary outcome of time to sustained 2-point improvement on 7-point World Health Organization scale or hospital discharge. (B) Secondary outcome of daily clinical status. ECMO, extracorporeal membrane oxygenation; MV, mechanical ventilation.

are associated with worse clinical outcomes and intermediate or therapeutic doses of LMWH have been suggested early in the pandemic to improve outcome.[1,2,11,12] Although many (randomized) studies have focused on identifying the optimal dose of LMWH, in-hospital thromboprophylaxis is still highly debatable. The LMWH dose used to treat the patients in the SOC group was based on the guidance published by the Belgian Society on Thrombosis and Haemostasis.[13] Ultimately, the dose of LMWH in the intervention group is higher, although not therapeutic, compared with the dosages in the guidance document to counteract the antifibrinolytic effect of aprotinin. This study was, therefore, not designed to determine the optimal dose of LMWH in COVID-19 thromboprophylaxis. However, even in our SOC group (with prophylactic to intermediate-dosed anticoagulation), the overall mortality is lower compared with the intervention group in both the critically and noncritically ill cohorts of the REMAP-CAP trials (with therapeutic-dosed

anticoagulation) for example, debating the need of therapeutic dosed thromboprophylaxis in COVID-19.[19,20]

4.3 | Anakinra

Anakinra showed promising results in specific subpopulations,[21–28] but in our study, anakinra had no significant effect on clinical or biochemical outcomes in a multitarget approach. However, our trial was designed to evaluate a strategy of modulation of thromboinflammation as a whole and thus lacks the power to evaluate the effect of individual components of the intervention. Based on the studies discussed previously, we believe that anakinra is indeed best investigated early in subgroups with hyperinflammation and biomarkers for clinical progression in COVID-19.

TABLE 2 Secondary outcomes

Secondary outcome up to day 28	Statistic	Estimate (95% CI)		Treatment effect	Estimate (95% CI)
		Intervention (N = 67)	SOC (N = 35)		
A. Clinical secondary outcome					
All-cause mortality	[n] KM [%]	[3] 4.6 (1.5; 13.7)	[2] 5.7 (1.5; 21.0)	Hazard ratio	0.82 (0.14-4.94)
Mechanical ventilation	[n] CIF [%]	[16] 24.0 (14.5; 34.9)	[4] 11.4 (3.5; 24.4)	Subdist. HR	2.35 (0.79-6.96)
ICU admission	[n] CIF [%]	[28] 42.2 (30.1; 53.8)	[10] 28.6 (14.7; 44.1)	Subdist. HR	1.56 (0.79-3.06)
Supplemental oxygen	[n] CIF [%]	[61] 91.0 (80.9; 95.9)	[31] 88.6 (72.0; 95.6)	Subdist. HR	1.02 (0.88-1.18)
Hospital discharge	[n] CIF [%]	[52] 80.9 (68.5; 88.8)	[31] 88.6 (70.2; 95.9)	Subdist. HR	0.72 (0.47-1.09)
Limitations on daily activities on day 28	n/N (%)	34/47 (72)	19/30 (63)		
B. Biochemical secondary outcome					
C-reactive protein, mg/L					
Admission	Geometric mean	63.6 (51.3; 78.7)	66.79 (45.4; 98.2)	Treatment ratio	
Day 3	Geometric mean	27.8 (20.3; 38.1)	27.45 (16.0; 47.0)	Treatment ratio	1.08 (0.61-1.92)
Day 6	Geometric mean	18.2 (12.3; 26.8)	14.7 (7.9; 27.4)	Treatment ratio	1.26 (0.63-2.52)
Day 15	Geometric mean	7.9 (3.8; 16.3)	7.1 (3.4; 15.0)	Treatment ratio	1.13 (0.40-3.25)
D-dimer, µg/L					
Admission	Geometric mean	832.7 (701.7; 988.0)	686.0 (537.8; 875.0)	Treatment ratio	
Day 3	Geometric mean	432.3 (366.0; 510.6)	909.9 (652.7; 1268.5)	Treatment ratio	0.43 (0.30-0.61)
Day 6	Geometric mean	792.3 (641.4; 978.8)	1015.6 (699.5; 1474.4)	Treatment ratio	0.70 (0.48-1.01)
Day 15	Geometric mean	980.6 (727.3; 1322.1)	1478.3 (884.9; 2469.7)	Treatment ratio	0.60 (0.32-1.09)

Note: Hazard ratios were obtained using a Cox regression including factors for randomized treatment, study period, and site. Subdistribution hazard ratios were obtained using a Fine & Gray regression model (accounting for competing risk) including factors for randomized treatment, study period and site. Ratios of geometric means between treatments were obtained using a general linear model including the baseline value as a covariate and factors for randomized treatment, study period and site, after log-transformation of the data; estimated means and treatment differences obtained using the model were back-transformed using the exponential function.

Abbreviations: CI, confidence interval; 95% CI calculated using log(–log)-transformation; CIF, incidence estimated using Cumulative Incidence Function accounting for competing risk; HR, hazard ratio; ICU, intensive care unit; KM, incidence estimated using Kaplan–Meier methodology; SOC, standard of care; Subdist. HR, subdistribution hazard ratio.

TABLE 3 Thrombosis, major bleeding, and treatment related serious adverse event

	Statistic	Actual treatment		
		Intervention	Standard of care	Total
Total number of subjects	N	67	35	102
Thrombosis	n/N (%)	1/67 (1.5%)	1/35 (2.9%)	2/102 (2.0%)
Pulmonary embolism	n/N (%)	1/67 (1.5%)	0/35 (0%)	1/102 (1.0%)
Deep vein thrombosis	n/N (%)	0/67 (0%)	0/35 (0%)	0/102 (0%)
Other thrombotic events	n/N (%)	0/67 (0%)	1/35 (2.9%)	1/102 (1.0%)
Major bleeding	n/N (%)	0/67 (0%)	0/35 (0%)	0/102 (0%)
Treatment-related serious adverse event				
Hematuria	n/N (%)	1 (1.5%)	0 (0.0%)	1 (1.0%)

Note: Major bleeds are any in-hospital bleeds that require a blood transfusion.

4.4 | Limitations

Our multicenter randomized clinical trial provides insights into a unique multistep multitarget approach in targeting thromboinflammation in COVID-19. Some limitations are worth mentioning. Because of the state of emergency during the first waves of the pandemic, we opted for an open-label study design to allow for new treatment options as SOC in the rapidly evolving landscape. Rapidly evolving COVID-19 care was also noticeable in the primary outcome that was obtained in 74% of control patients at day 15, which is more than the anticipated 40% used in the power calculation. Because of the better-than-expected outcome, the power calculation was overly optimistic. Additionally, the study faced early termination because of futility. However, neither the primary nor

any relevant clinical or biochemical secondary end point showed any trend toward a benefit of the intervention, making a type II error unlikely. In COVID-19 treatment trials with improvement and time to improvement as outcome, both cause-specific and subdistribution hazards are possible approaches to analyze these outcomes.[29] After peer review, we also performed a Cox regression with deaths censored at the time of death. The resulting cause-specific hazard ratio was 0.782 (CI 0.496-1.234), $p = 0.29$. As expected, because of the very low number of deaths and the equal death rates between treatment groups, the cause-specific hazard ratio is very close to the subdistribution hazard ratio and does not affect our conclusion.

5 | CONCLUSION

In hospitalized patients with moderate to severe COVID-19, a strategy to modulate thromboinflammation with high-dose aprotinin and LMWH with or without anakinra was associated with an overall low mortality, but did not improve clinical or biochemical outcome compared with standard of care.

AUTHOR CONTRIBUTIONS

T.V., P.V., L.L., J.W., J.G., C.W., C.V., and S.R. conceptualized the study and methodology. T.V. and P.V. acquired funding. T.V., P.V., M.M.E., M.T., T.F., D.M., D.R., and L.H. supervised research activity. M.M.E., Q.V.T., A.B., I.G., A.O., C.D., C.P.M., V.S., J.W., J.G., C.W., C.V., S.R., A.W., P.M., G.V.d.B., D.D., M.T., T.F., D.M., D.R., L.H., I.D., S.T., and B.D.T. all contributed to resources. A.O., C.D., M.M.E., T.V., and P.V. contributed to project administration. A.Be. performed statistical analysis. M.M.E., T.V., and A.Be. visualized the data. M.M.E., T.V., and P.V. drafted the manuscript, and all other authors reviewed the drafted manuscript.

DATA AVAILABILITY STATEMENT

Patient level data will not be made available. The study protocol is published and available online (https://doi.org/10.1186/s13063-020-04878-y). Together with the statistical analysis plan, the study protocol will also be attached to the Appendix S2.

ETHICAL STATEMENT

Based on the Declaration of Helsinki, the local Ethical Committee (S63979) approved the study protocol and all the participants provided informed consent.

ORCID

Matthias M. Engelen https://orcid.org/0000-0002-5364-7751
Quentin Van Thillo https://orcid.org/0000-0003-3260-280X
Caroline P. Martens https://orcid.org/0000-0002-2890-7072
Jan Gunst https://orcid.org/0000-0003-2470-6393
Laurens Liesenborghs https://orcid.org/0000-0001-5616-8548
Michiel Thomeer https://orcid.org/0000-0001-5627-0323
Peter Verhamme https://orcid.org/0000-0001-8698-2858
Thomas Vanassche https://orcid.org/0000-0002-7404-8918

TWITTER

Matthias M. Engelen @matthiasengelen
Quentin Van Thillo @QThillo
Albrecht Betrains @Will_I_Am_Osler
Valérie Spalart @ValerieSpalart
Christophe Vandenbriele @Cvandenbriele
Laurens Liesenborghs @LaurensLiesenb1
Dieter Dauwe @DieterDauwe
Michiel Thomeer @MThomeer
Ilse Dapper @IlseDapper1
Sebastiaan Tuyls @SebastiaanTuyls
Brecht De Tavernier @BrechtDeTav
Peter Verhamme @VerhammePeter

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SUPPORTING INFORMATION

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