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ORIGINAL ARTICLE

Thrombus formation during ECMO: Insights from a detailed histological analysis of thrombus composition

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Abstract

Objectives: Intra-device thrombosis remains one of the most common complications during extracorporeal membrane oxygenation (ECMO). Despite anticoagulation, approximately 35% of patients develop thrombi in the membrane oxygenator, pump heads, or tubing. The aim of this study was to describe the molecular and cellular features of ECMO thrombi and to study the main drivers of thrombus formation at different sites in the ECMO circuits.

Approach and Results: Thrombi ($n = 85$) were collected immediately after veno-arterial-(VA)-ECMO circuit removal from 25 patients: 23 thrombi from the pump, 25 from the oxygenator, and 37 from the tubing. Quantitative histological analysis was performed for the amount of red blood cells (RBCs), platelets, fibrin, von Willebrand factor (VWF), leukocytes, and citrullinated histone H3 (H3Cit). ECMO thrombi consist of a heterogenous composition with fibrin and VWF being the major thrombus components. A clustering analysis of the four major histological parameters identified two typical thrombus types: RBC-rich and RBC-poor/fibrin-rich thrombi with no significant differences in VWF and platelet content. Thrombus composition was not associated with the thrombus location, except for higher amounts of H3Cit that were found in pump and oxygenator thrombi compared to tubing samples. We observed higher blood leukocyte count and lactate dehydrogenase levels in patients with fibrin-rich thrombi.

Conclusion: We found that thrombus composition is heterogenous, independent of their location, consisting of two types: RBC-rich and a fibrin-rich types. We also found

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that NETs play a minor role. These findings are important to improve current anticoagulation strategies in ECMO.

KEYWORDS
ECMO, fibrin, histology, NETs, thrombosis, thrombus composition

1 | INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) provides temporary mechanical support to the heart, lung, or both in patients with cardiovascular and/or respiratory failure in the neonatal, pediatric, and adult population. The use of adult ECMO has considerably expanded during the past 2 decades and has become a valuable lifesaving therapy in critical care medicine. During ECMO support, blood is drained from the vascular system, circulated outside of the body by a mechanical pump, oxygenated, and decarboxylated using an oxygenator before it is reinfused into the patient's circulatory system.[1,2]

Despite increasing experience, systemic anticoagulation and recent advances in ECMO technologies, device thrombosis is the most common source of technical complications during ECMO treatment. Indeed, in a recent ELSO registry analysis, it was shown that 17.2% of ECMO-supported patients experience thrombotic events of which the majority are related to intra-device thrombosis.[3] Intra-device thrombosis can lead to decreased oxygenation and decarboxylation of the patient's blood, increased flow resistance, hemolysis, and embolic complications.[1,4] This often results in equipment malfunctioning and circuit loss, necessitating elective or full system replacements, which are high-risk procedures. In fact, Lubnow et al. found that acute thrombosis of the oxygenator or blood pump is responsible for more than one-third of ECMO system exchanges.[4] Furthermore, ECMO-induced thrombosis greatly affects patient outcomes, further emphasizing the relevance of understanding the underlying drivers of thrombosis in ECMO circuits.[1,5] The exact mechanisms of in-device thrombus formation remain, indeed, poorly understood and involve different critical factors including long-lasting contact of blood with foreign bio-artificial surfaces, low flow zones and turbulences in connectors and high shear forces that prime the activation of platelets, leukocytes and the coagulation system.[6,7]

In patients undergoing ECMO, adequate antithrombotic strategies are key to maintain circuit patency and to prevent thrombotic complications. Current anticoagulation strategy in ECMO mainly focuses on the use of unfractionated heparin (UFH) as a first-line therapy, with or without aspirin or other antiplatelet therapy.[1,8,9] However, despite the release of anticoagulation guidelines by the Extracorporeal Life Support Organization (ELSO) in 2014,[10] large variations still exist among ECMO centers, also including the use of direct thrombin inhibitors or single/dual antiplatelet therapy.[8,11] An additional challenge is the increased risk of bleeding when using systemic anticoagulation during ECMO.[1,2,12]

To date, little is known about the composition and architecture of thrombi formed in the different components of the ECMO circuit.

Essentials

- Intra-device thrombosis is a common complication in extracorporeal membrane oxygenation (ECMO).
- Little is known regarding the composition and main drivers of ECMO thrombus formation.
- ECMO thrombi are heterogenous, independent of their location, consisting of two thrombus types.
- The findings are important to improve anticoagulation and device modifications in ECMO.

NOVELTY AND SIGNIFICANCE

What Is Known?

- Despite anticoagulation, intra-device thrombosis remains one of the most common complications during extracorporeal membrane oxygenation (ECMO)
- Thrombi typically form in the blood pump, the membrane oxygenator and in the circuit's tubing
- This often results in equipment malfunctioning which necessitates hazardous elective or full system exchanges

What New Information Does this Article Contribute?

In contrast to previous studies, this study performed a detailed qualitative and the first quantitative histological description of veno-arterial (VA)-ECMO thrombi to better understand the underlying drivers of thrombus formation during ECMO. The study's main findings are that VA-ECMO thrombi consist of a heterogenous composition with fibrin and von Willebrand factor (VWF) being the main components. Using a novel unbiased clustering analysis for the main thrombus components (red blood cells, fibrin, platelets, and VWF), we identified two distinct thrombus types: (1) RBC-rich, fibrin-poor thrombi and (2) RBC-poor, fibrin-rich thrombi. Intriguingly, these thrombus types are not associated with the respective location of the thrombus within the device, but are associated with the leukocyte count further underscoring a role for immunothrombosis in ECMO. NETs are more frequent in pump and oxygenator thrombi. These results provide novel insights into the architecture and composition of these complication-causing ECMO thrombi, which are important to further improve current anticoagulation strategies (e.g., combination therapy) and device modifications in ECMO.

Clearly, better understanding of ECMO thrombus specimens could help to better understand the mechanisms leading to intra-device thrombosis and thus could be useful to improve current antithrombotic regimens and to develop safer ECMO technologies. The aim of this study therefore was to collect, analyze, and describe the most important molecular and cellular features of ECMO thrombi collected from different sites in ECMO circuits.

2 | MATERIALS AND METHODS

2.1 | Patients

This study is ancillary to the WITECMO-H trial (NCT 03070912) multicenter study conducted in the intensive care units of the Lille University Hospital. The WITECMO-H trial was approved by the French National Ethic Review Committee (CPP No. 2017-A00256-47) and written informed consent was obtained from patients, their person of trust, or their next of kin, whomever was available first at the inclusion. From November 2017 until May 2019, consecutive adult patients aged 18 years and older referred to the intensive care unit for an ECMO were considered for inclusion in the WITECMO-H trial.

To limit the heterogeneity between patients in this ancillary study, we only included patients supported with a veno-arterial ECMO (VA-ECMO). This specific patient selection will exclude the ECMO type as a potential confounding variable (veno-venous vs. VA-ECMO). Patients were prospectively and consecutively considered for inclusion in case of VA-ECMO circuit change or removal during the study period and if thrombi extraction and fixation were feasible in the 48 following hours. The histological substudy started in 2018 after 2 months of feasibility of thrombi collection from November to December 2017. Clinical variables recorded include anthropometric data, comorbidities, and clinical management data. Characteristics of VA-ECMO support and course were also recorded (i.e., etiology of shock, peripheral or central cannulation, the type of circuit and oxygenator, the reason of ECMO weaning or circuit change, the duration of support and outcome).

2.2 | ECMO types and management

During the study period, centrifugal pump-based ECMO systems (Rotaflow [Maquet Gentige group], Revolution [LivaNova Group], Centrimag [Thoratec], or Biomedicus Bio-pump X 80 [Medtronic]) were used in combination with heparin-coated or phosphorylcholine-coated polyvinyl chloride tubing and a hollow fiber membrane oxygenator (Quadrox [Maquet Gentige group], Eos ECMO [LivaNova Group], or A.LONE ECMO [Euroset]) ventilated with an oxygen-air blender (Sechrist Industries). All cannulations were performed in the femoral vein and femoral or subclavian. Weaning modalities were recovery from initial disease, bridge to heart transplantation, temporary/long-term ventricular assist devices, or death. Weaning for recovery was considered for recovery when the aortic velocity time integral >10 to 12 cm/s despite lowering ECMO flow to minimal for 30 min, with no or reduced inotropic support. ECMO bridging to heart transplantation or long-term ventricle assist devices was considered after 5–7 days of support according to patients' conditions. Circuit change was considered by the attending physician in case of clinical suspicion of circuit thrombosis, breakdown of oxygenator performance, or severe hemolysis.

2.3 | Anticoagulation and transfusion management

In all patients, anticoagulation was initiated at the cannulation with a bolus of 100 IU/kg of UFH followed by a continuous infusion, adapted to obtain a target anti-factor Xa activity of 0.2–0.4 IU/ml with a monitoring of UFH performed according to the ELSO guidelines.[10]

2.4 | Thrombotic deposit collection, processing, and histology

Immediately after collection, each circuit was drained of its blood content and filled with 1000 ml of NaCl 0.9% solution. Thereafter, the NaCl 0.9% solution was removed and the whole ECMO circuit was inspected for thrombi. All pump heads, oxygenators, and circuit junctions isolated from the circuit were mechanically opened and were evaluated for the presence of thrombi. Identified thrombi were gently removed, kept in a phosphate buffered saline solution at 4°C for a maximum 48 h before fixation.[13] Importantly, filling of the circuit was performed according to previous studies[14,15] and does not alter the composition of ECMO thrombi as investigated during the feasibility study (November–December 2017). Furthermore, no clear signals were seen as to washing out of red blood cells (RBCs; main concern) from the thrombus. For each thrombus, a macroscopic photograph was taken and thrombus location in the circuit as well as thrombus shape, size, weight, and color were recorded. Thrombus color was scored as dark/red/brown, pink/mixed, or white. To fixate, retrieved thrombi were incubated in 4% paraformaldehyde for 24 h at room temperature, subsequently embedded in paraffin and sectioned into 5-µm sections. One section per thrombus, exposing a large cross-sectional area of the entire thrombus and with a good tissue quality (e.g., limited amount of tissue folds), was used for every staining as this method allows for a representative measurement (as demonstrated in stroke thrombi).[16]

As previously described,[17,18] thrombus sections were stained with hematoxylin and eosin and with Martius Scarlett Blue (fibrin [dark pink/red], RBCs [yellow], and collagen [blue]). In addition, immunohistochemical stainings were performed for platelets (GPIbα, MA5-11642, Invitrogen), von Willebrand factor (VWF; A008202-, Dako) and citrullinated histone H3 (H3Cit, ab5103, Abcam). For immunohistochemical staining procedures, nucleated cells were stained using a Methyl Green solution (H-3402, Vector Laboratories) and negative controls were obtained by omitting the primary antibody or by using isotype-matched primary antibodies (Figure S1). Isotype controls were performed by incubating a section with the respective isotype of

the primary antibody (polyclonal rabbit IgG isotype control antibody [910801, Biolegend] for VWF and H3Cit or a monoclonal mouse IgG1κ isotype control antibody [401402, Biolegend]) for platelets to confirm the absence of aspecific binding of the primary antibodies to the tissue. A more detailed description of all histological methods is provided in the Online Supplementary Methods.

2.5 | Quantification of histological components

Stained thrombus sections were digitized using a slide scanner (Axio Scan Z1, Zeiss or Nanozoomer SQ, Hamamatsu Photonics). Images were exported at an 5× resolution using Zen Blue Edition version 3.1 (Zeiss) or NDP view2 (Hamamatsu). Next, background noise surrounding the thrombus section (e.g., dust particles, air bubbles) was removed using Adobe Photoshop CS5 to prevent noise-derived false-positive signals in the quantification. Histological stainings were examined using color segmentation and planimetric analysis to quantify the areas staining positive for fibrin (dark pink/red), RBCs (yellow), platelets (purple), VWF (purple), and H3Cit (purple) using ImageJ software 1.49v (National Institutes of Health, Bethesda, MD; http://imagej.nig.gov/ij/). For each staining, one section per thrombus was used for quantification.

2.6 | Statistical analysis

Categorical variables are presented as frequencies (percentage). Continuous variables are presented as medians (interquartile range). Normality of distributions was assessed using histograms and the Shapiro–Wilk test. Based on the non-normal distribution of the histological parameters, we assessed the correlations between the histological parameters by calculating Spearman rank correlation. To classify ECMO thrombi in different homogeneous groups, a principal component analysis and a hierarchical clustering analysis were performed. Four histological parameters (RBCs, fibrin, platelets, and VWF) were considered for a cluster analysis. The cluster analysis was based on the k-means method, in which similarity between individuals is measured using the usual Euclidian distance. We performed several analyses with different numbers of clusters, and the optimal number was determined by consensus among three statistical properties: local peaks of the cubic cluster criterion, pseudo F, and R2 (ratio between inter-cluster variation and total variation). The distribution of the four parameters were compared between the two clusters (Mann–Whitney *U* tests), and represented after standardizations (*z*-scores), on a radar graph. A χ^2 test was used to assess whether the two clusters were associated with the respective location in the ECMO device. Comparisons of histological parameters with the respective location in the ECMO device were done using Kruskall–Wallis tests. Finally, the biological parameters assessed at six times (1, 2, 3, 4, 5 and 6 days) were compared between the two clusters using linear mixed models for longitudinal data (an unstructured covariance pattern model to account for the correlation between repeated measures within the same ECMO thrombi) with baseline values, times, clusters, and interaction between times and clusters as fixed effects. The overall effect of clusters (taken into account the heterogeneity in evolution) was assessed using likelihood ratio test. Adjusted means values of biological parameters were obtained from linear mixed model using the least-squares means values. Statistical testing was conducted at the two-tailed α-level of 0.05. Data were analyzed using SAS software package, release 9.4 (SAS Institute, Cary, NC, USA) for all statistical tests except for the hierarchical clustering analysis where R ([version 3.6.2, R Core Team]) was used.

3 | RESULTS

3.1 | Patient population and thrombus collection

During the study period, a total of 29 ECMO circuits from 25 patients were collected at the time of ECMO explantation either for a circuit change or for weaning. A flow chart is given in Figure 1. Of

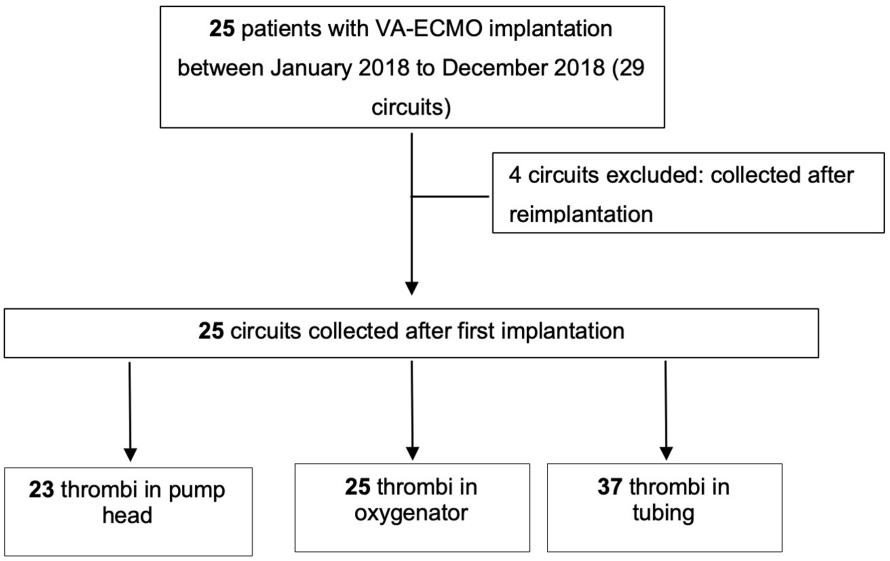

FIGURE 1 Study flow chart

those 29 circuits, four collected after reimplantation were excluded because only the first collected circuit from every patient is kept for analysis. We collected a median of three thrombi (interquartile range, 2–4) per circuit. A total of 85 thrombi were collected from these circuits, of which 23 thrombi originated from the pump head, 25 from the oxygenator, and 37 from the tubing. The demographic, clinical, and ECMO characteristics of the patient cohort are shown in Table 1. Fourteen patients (56%) were male. Hypertension (36%), chronic heart failure (52%), and/or coronary artery disease (40%) were the most frequent risk factors. Postcardiotomy accounted for the largest proportion of reason for ECMO support.

3.2 | Thrombus composition

To better understand the composition and potential mechanisms involved in thrombosis during VA-ECMO, all intra-device thrombi were macroscopically and histologically analyzed. Thrombi were highly heterogeneous in size, weight, and color. They had a predominate red or mixed color (combinations of red, white, and pink) (Table 2). Figure 2 shows representative images of all the histological staining procedures. In general, RBCs and fibrin are interspersed throughout ECMO thrombi (Figure 2), suggesting spatial segregation (most likely via clot contraction) of RBC, fibrin, and platelets into separate areas. Leukocytes (blue nuclei on hematoxylin and eosin staining) are not frequently present in ECMO thrombi (Figure S2A). VWF was found dispersed throughout both the RBC-rich areas and fibrin/platelet-rich areas (Figure S2B,C). Higher magnifications of the H3Cit staining indicate the presence of cellular and extracellular staining patterns (Figure 2D). A characteristic circular morphology was observed in some thrombi formed around the pump shaft and occurred in 9/23 (39.1%) of pump thrombi (Figure 2). Interestingly, higher magnification of this circular thrombi indicates a homogenously stained yellow RBC-rich area in which the morphology of individual RBCs is lost (Figure 2E), whereas individual RBCs can be identified in other ECMO thrombi (Figure 2F). This suggests the presence of hemolyzed RBCs because the RBC morphology was lost but the specific yellow RBC staining was still present. Moreover, histological examination of these circular thrombi again suggests the spatial segregation (most likely mediated via clot contraction) of fibrin, platelets, and VWF to the thrombus periphery.

Quantification was performed to determine the relative contribution of RBCs, fibrin, platelets, VWF, and H3Cit for all thrombi via color segmentation and planimetric analysis. The color of thrombi was significantly associated with its composition, the content of RBC was the highest in dark to red thrombi whether the content of platelets was the highest in white thrombi, as expected (Table S1). As shown in Table 2, the amounts of the different components span wide ranges with fibrin and VWF being the major components, followed by platelets, RBCs, and smaller amounts of citrullinated histones.

To determine whether an association exists between the histological parameters, we performed a correlation analysis (Table 2).

TABLE 1 Clinical characteristics and thrombus load of the patient population

	Histologically analyzed patients ($n = 25$)
Demographics	
Age (years), median (IQR)	51 (47–67)
BMI[a], median (IQR)	25.7 (21.9–30.1)
Male gender, *n* (%)	14 (56.0)
Comorbidities and chronic medical illness, *n* (%)	
Hypertension	9 (36.0)
Diabetes	7 (28.0)
Stroke	3 (12.0)
Chronic obstructive pulmonary disease	5 (20.0)
Atrial fibrillation	7 (28.0)
Chronic heart failure	13 (52.0)
Coronary artery disease	10 (40.0)
Chronic kidney disease	3 (12.0)
Venous thromboembolism	2 (8.0)
Thrombophilia	2 (8.0)
Neoplasia	2 (8.0)
Reason for ECMO support, *n* (%)	
Dilated cardiomyopathy	6 (24.0)
Myocardial infarction	3 (12.0)
Postcardiotomy	11 (44.0)
Pulmonary embolism	1 (4.0)
Cardiac arrest	1 (4.0)
Others	3 (12.0)
ECMO parameters	
ECMO circuit duration, days, median (IQR)	7 (3–9)
P2Y12 before ECMO, *n* (%)	1 (4.0)
P2Y12 during ECMO, *n* (%)	3 (12.0)
AAS, *n* (%)	22 (88.0)
Anticoagulation, *n* (%)	23 (92.0)
Pumps heads, *n* (%)	
Rotaflow (Maquet)	13 (52.0)
Revolution (Livanova)	10 (40.0)
Centrimag (Thoratec)	1 (4.0)
Biomedicus (Medtronic)	1 (4.0)
Oxygenators, *n* (%)	
Quadrox (Maquet)	12 (48.0)
Eos ECMO (Livanova)	10 (40.0)
A.L.ONE ECMO (Euroset)	3 (12.0)
Patients with thrombi per device location, *n* (%)	
Pump	19 (76.0)
Oxygenator	16 (64.0)
Tubing	23 (92.0)

(Continues)

TABLE 1 (Continued)

	Histologically analyzed patients ($n = 25$)
Oxygenator type in patients with oxygenator thrombi, *n* (%)	
Quadrox (Maquet)	11 (68.75)
Eos ECMO (Livanova)	2 (12.5)
A.L.ONE ECMO (Euroset)	3 (18.75)
Pump type in patients with pump thrombi, *n* (%)	
Rotaflow (Maquet)	8 (42.1)
Revolution (Livanova)	10 (52.6)
Centrimag (Thoratec)	0 (0)
Biomedicus (Medtronic)	1 (5.3)
Ablation type, *n* (%)	
Change	7 (28.0)
Definitive ablation	18 (72.0)
Cause of system change, *n* (%)	
Thrombosis	2 (28.6)
Other[b]	5 (71.4)
Cause of definitive ablation, *n* (%)	
Death	1 (5.5)
Recovery	14 (77.8)
Heart transplantation	1 (5.6)
Ventricular assist device (LVAD/BIVAD)	2 (11.1)

Abbreviations: AAS, acetylsalicylic acid; BIVAD, biventricular assist device; BMI, body mass index; ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; LVAD, left ventricular assist device.

[a]5 missing values.

[b]Other: oxygenator failure, hemolysis, severe thrombocytopenia.

TABLE 2 Macroscopic and histological characteristics of ECMO thrombi

Macroscopic characteristics of ECMO thrombi ($n = 85$)	
Thrombus length[a] (mm), median (IQR)	16 (9–30)
Thrombus weight (mg), median (IQR)	40.3 (23.7–113.7)
Thrombus color[b], *n* (%)	
Dark/red/brown	35 (42.2)
Pink/mixed	35 (42.2)
White	13 (15.6)
Histological parameters, median (IQR)	
RBCs (%)	11.8 (4–35.6)
Fibrin (%)	36.7 (18.9–58.6)
Platelets (%)	15.6 (6.8–36.9)
VWF (%)	24.4 (5.3–34.6)
H3Cit (%)	3.2 (1.2–5.4)

Abbreviations: ECMO, extracorporeal membrane oxygenation device; IQR, interquartile range; RBC, red blood cell; VWF, von Willebrand factor.

[a]3 missing values.

[b]2 missing values.

For the amounts of fibrin, a moderately inverse correlation exists with RBCs ($r = -.51$) and a weak positive correlation exists with VWF ($r = .24$) and H3Cit ($r = .24$). Platelets are weakly positively correlated with VWF ($r = .33$) and H3Cit ($r = .26$) and VWF is weakly positively correlated with H3Cit content ($r = .29$).

3.3 | Clustering analysis of thrombosis composition

We performed a hierarchal clustering analysis to identify potential subgroups of thrombi that are similar based on their composition (amounts of RBCs, fibrin, platelets, and VWF), without any a priori assumption. H3Cit was not included in this clustering analysis because it is a minor component in the majority of thrombi. Interestingly, the clustering analysis revealed two types of thrombi, as shown in Figure 3. Cluster 1 ($n = 24$) was mainly characterized by lower amounts of fibrin and higher amounts of RBCs, whereas thrombi in cluster 2 ($n = 61$) were fibrin-dominant and contained smaller amounts of RBCs. The histological composition of both clusters is given in Figure 3B and Table S2. Of note, a small overlap (<25%) in fibrin-composition of both clusters can be found (Figure 3B). This overlap, however, does not influence the identification into two clusters because of the use of multiple histological parameters in concertation. Different thrombi from one patient are not necessarily similar in composition as approximately one half of the patients (44%) generated both thrombus types, whereas 4% of patients only had cluster 1-type thrombi and 52% of patients only cluster 2-type thrombi.

Clinical characteristics were well balanced according to the type of thrombus (Table S4). RBC-poor/fibrin-rich cluster 2-type thrombi were associated with higher blood leukocyte count ($p < .0001$) and higher lactate dehydrogenase (LDH) levels ($p < .001$) during the first 6 days of ECMO (Figure 4), but not with other clinical parameters (platelet count, hemoglobin level, aPTT, prothrombin time, fibrinogen, free hemoglobin, and anti-Xa activity).

3.4 | Thrombus composition versus thrombus location

Local differences in fluid dynamics could promote different thrombotic mechanisms, leading to the formation of thrombi that differ in cellular and biochemical makeup. When analyzing thrombus according to the different type of colors observed, we did not observe a significant relation with location (Table S1). Furthermore, we did not find a significant difference between RBC, fibrin, platelet, and VWF content or the two thrombi cluster-types and the respective location within the device (Table 3). H3Cit content was significantly

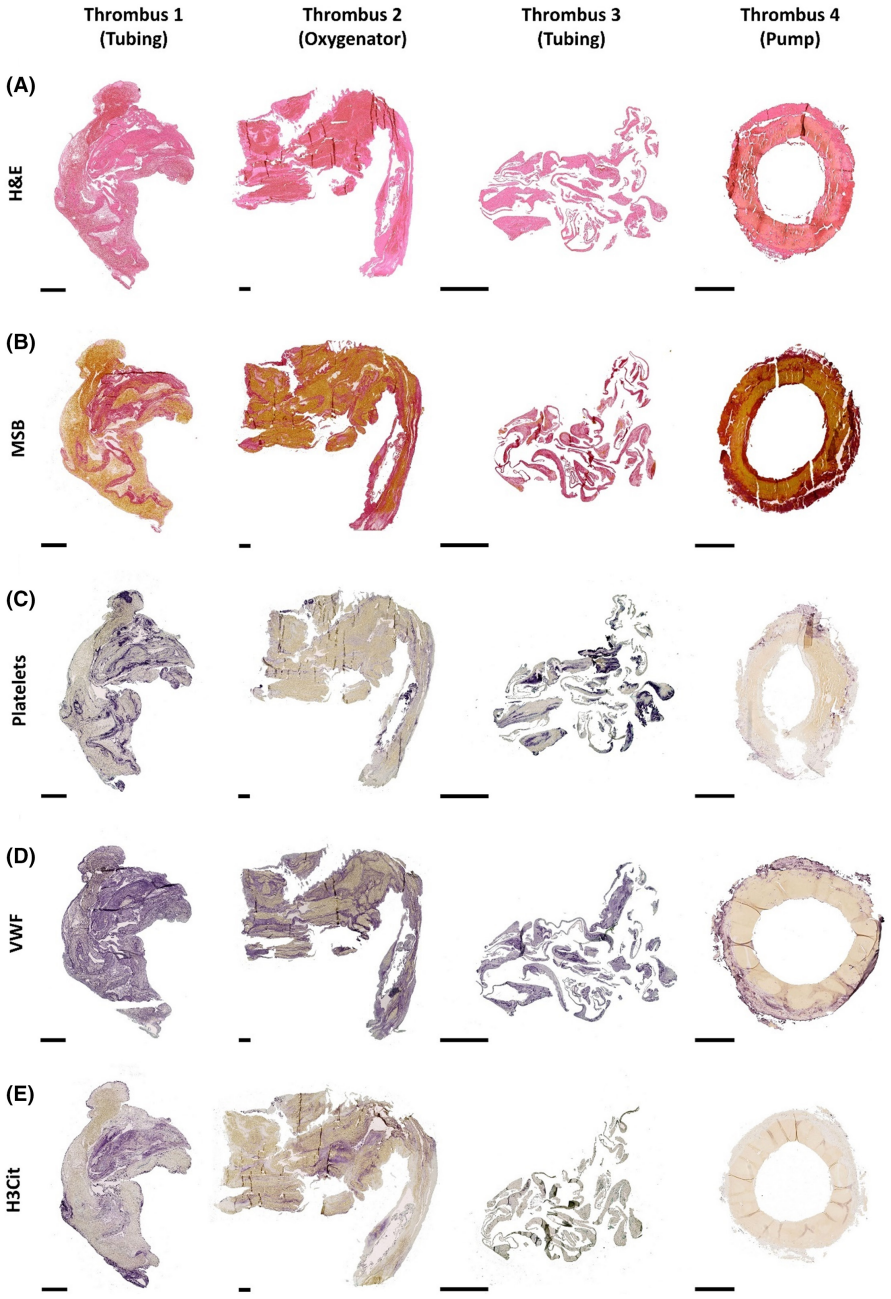

FIGURE 2 Examples of histologically stained ECMO thrombi. Thrombus sections were stained with (A) H&E to visualize overall thrombus composition and organization and (B) MSB to identify RBCs (yellow) and fibrin (dark pink/red). Immunohistochemical stainings were used to identify (C) platelets (CD42b; purple), (D) VWF (purple), and (E) H3Cit (purple). Examples of ECMO thrombi are shown in A-D, illustrating a broad heterogeneity in composition. Thrombus 1 consists of a mixed RBC/fibrin, platelet-poor and VWF-rich content containing a large H3Cit positive area. Thrombus 2 is a RBC/VWF-rich and fibrin/platelet/poor thrombus containing a small H3Cit positive area. Thrombus 3 is a RBC/platelet-poor, fibrin/VWF-rich thrombus containing little to no H3Cit. Thrombus 4 is a circular shaped, RBC-rich, and platelet/fibrin/VWF-poor thrombus containing no H3Cit positive signal. Scale bar: 1 mm (A–E). CD42b, cluster of differentiation 42b; ECMO, extracorporeal membrane oxygenation; H&E, hematoxylin and eosin; H3Cit, Citrullinated histone H3; MSB, Martius Scarlet Blue; RBCs, red blood cells; VWF, von Willebrand factor.

higher in the pump and oxygenator thrombi compared to the tubing thrombi ($p = .01$; Table 3). Taken together, these data indicate that major ECMO thrombus components are not associated with the respective location of the collected thrombus.

4 | DISCUSSION

Intra-device thrombosis remains an important challenge during ECMO. In this study, we performed a detailed histological examination of 85 thrombi collected from VA-ECMO circuits and provide a detailed qualitative and the first quantitative description of VA-ECMO thrombus composition with regard to the amount RBCs, fibrin, platelets, VWF, and H3Cit. Our main findings are that VA-ECMO thrombi have a heterogenous composition, with fibrin and VWF as the major thrombus components. Second, we identified two histological thrombus types: (1) fibrin-poor, RBC-rich thrombi and (2) fibrin-rich, RBC-poor thrombi. Finally, the overall thrombus composition including thrombus types was not associated with the respective location, although we found higher amounts of H3Cit in the pump and the oxygenator compared to the tubing.

The macroscopic evaluation of ECMO circuits in this study reveal a considerable thrombus load at various locations within the device. These results are to some extent in line with a report by Hastings et al., illustrating that ECMO thrombi are predominantly located at

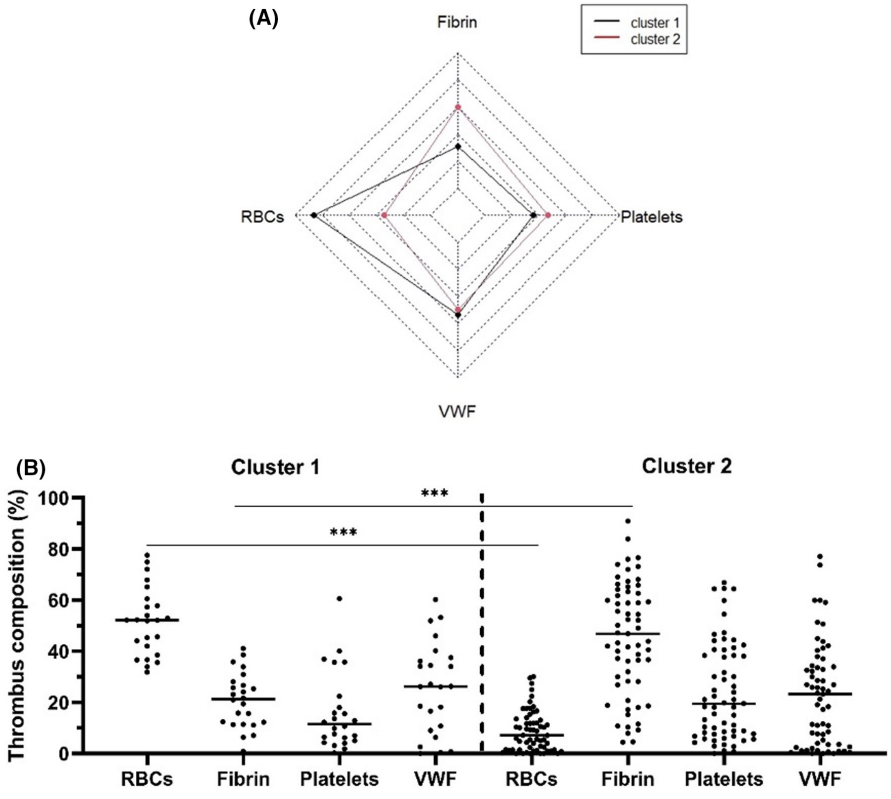

FIGURE 3 Hierarchal clustering identifies two histological cluster types. Hierarchal clustering analysis was performed using the quantitative histological RBC, fibrin, platelets, and VWF data. (A) Two histological cluster types were identified. (B) Cluster 1 ($n = 24$) was mainly characterized by significantly lower amounts of fibrin and higher amounts of RBCs compared with cluster 2, whereas thrombi in cluster 2 ($n = 61$) were fibrin-dominant and contained smaller amounts of RBCs compared with cluster 1. VWF and platelet content does not significantly differ between the two cluster types. RBCs, red blood cells; VWF, von Willebrand factor.

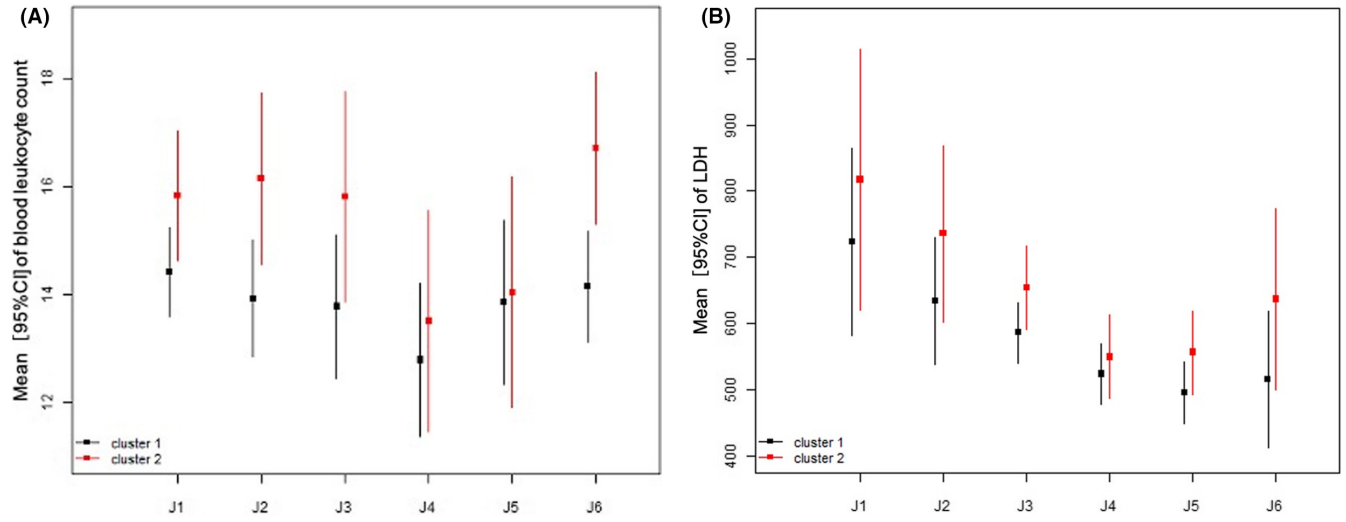

FIGURE 4 Blood leukocyte counts and LDH levels according to the two cluster-type thrombi. Using a linear mixed model including cluster and cluster*time interaction (to account the possibility of different evolution), in comparison to RBC-rich cluster 1-type thrombi (black), RBC-poor/fibrin-rich cluster 2-type thrombi (red) were associated with (A) significantly higher blood leukocyte counts ($p < .001$) and (B) significantly higher LDH levels ($p < .001$) during the first 6 days of ECMO support by comparison to RBC-rich cluster 1-type thrombi (black). LDH, lactate dehydrogenase; RBC, red blood cell.

tubing junction connectors and the pre-membrane side of oxygenators.[14] In addition, our study also reveals the presence of pump thrombotic deposits in the majority of patients.

The exact mechanisms of in-device ECMO thrombosis remain poorly understood. Yet, only few studies investigated the composition of ECMO thrombi.[14,15,19] These reports mainly focused on the descriptive analysis of ECMO thrombi in a pediatric population, in particular RBC, fibrin, platelets, and VWF content, and will be compared with our findings throughout the discussion.[14,15,19] Various elements are thought to contribute to ECMO thrombus formation, and good understanding of this multifactorial thrombogenicity can help design better therapeutic prevention strategies and circuit technologies. The continuous perfusion of blood over a large nonbiological surface, together with sites of high shear stress, particularly in the pump, remains a risk for thrombotic complications in ECMO. These conditions provoke activation of the platelets,

TABLE 3 Analysis of thrombus composition/cluster and thrombus location

Thrombus components (%), Median (IQR)	Thrombus location			
	Pump ($n = 23$)	Oxygenator ($n = 25$)	Tubing ($n = 37$)	Significance (p value)
RBCs	7.2 (1.2–42.0)	18.4 (10.3–52.2)	10.4 (2.5–22.4)	.070[a]
Fibrin	36.6 (20.8–63.3)	30.0 (10.8–58.6)	40.0 (24.2–54.5)	.26[a]
Platelets	26.3 (9.0–40.6)	12.8 (4.0–24.8)	15.9 (7.1–30.1)	.27[a]
VWF	30.0 (10.8–42.2)	18.6 (3.7–34.3)	23.3 (2.6–34.0)	.24[a]
H3Cit	**4.2 (1.1–7.4)**	**4.1 (2.2–13.6)**	**1.8 (0.8–4.0)**	**.011**[a]
Thrombus cluster, n (%)	**Pump (n = 23)**	**Oxygenator (n = 25)**	**Tubing (n = 37)**	**Significance (p-value)**
Cluster 1 ($n = 24$)	7 (30.4)	9 (36.0)	8 (21.6)	.37[b]
Cluster 2 ($n = 61$)	16 (69.6)	16 (64.0)	29 (78.4)	

Note: Significance level is $p < .05$. Bold values highlight the results that are statistically significant.

Abbreviations: RBCs, Red blood cells; VWF, von Willebrand factor; H3Cit, Citrullinated Histone H3; IQR, Interquartile range.

[a]Kruskall-Wallis test.

[b]χ^2 test.

leukocytes, and coagulation factors, leading to fibrinogen deposition and surface-adherent clot formation.

We found that the major components of ECMO thrombi are fibrin and VWF. One of the possible reasons that fibrin is an important constituent is most likely related to the initiation of thrombus formation within the device. Exposure of blood to a nonbiological surface leads to immediate absorption of plasma proteins such as fibrinogen to the surface of these biomaterials, leading to the activation of the contact pathway.[6,20] Moreover, also tissue factor-expressing monocytes potentially contribute to fibrin formation by receptor-mediated binding to the absorbed fibrinogen, resulting in tissue-factor pathway activation.[21,22] This is further corroborated by the consumption of fibrinogen in these ECMO patients.[14,23,24] Interestingly, in our data, fibrin deposition seems to occurs despite anticoagulation therapy with UFH, raising an interesting perspective regarding the effectiveness of heparin in an ECMO setting.

VWF-mediated thrombus formation could provide another explanation as to why heparin is unable to prevent in device thrombotic complications. VWF mediates platelet adhesion to surface-absorbed fibrin(ogen) under conditions of high shear stress.[25,26] In addition, with 18% of ECMO-treated patients having hemolytic complications, free hemoglobin has also been shown to enhance VWF-mediated platelet adhesion, followed by subsequent platelet activation and aggregation.[12,27] Although VWF is abundantly present, it should also be noted that VWF might mainly act as a scaffold for platelets, RBCs, and fibrin. On the other hand, VWF has also been shown to contribute to the complex duality between bleeding and thrombosis in ECMO patients. Indeed, as a result of high shear stresses, the partial or complete loss of high molecular weight VWF leads to acquired von Willebrand syndrome in ECMO patients.[28–30] Our results also show that platelets are an important constituent of almost all thrombi, independent of their location, suggesting that platelets, at least in part, might have contributed to thrombus formation in the ECMO circuit, which is in line with previous reports that show the presence of platelets in ECMO thrombi.[15,19]

Using an unbiased clustering analysis approach, we were able to identify two specific thrombus clusters: an RBC-rich and an RBC-poor/fibrin-rich thrombus type. These results suggest different thrombus formation mechanisms in the ECMO circuit. The predominant nature of the RBC-poor/fibrin-rich thrombus type in ECMO circuits further underscores the important role of fibrin deposition on the artificial surfaces, despite systemic anticoagulation with heparin. Moreover, the presence of a RBC-rich thrombus type hints toward low shear-mediated thrombus formation in the ECMO device.

One of the possible reasons for the variable composition and the different thrombus types in ECMO circuits is probably related to the different device components. It seems reasonable to speculate that low shear conditions in the circuit (e.g., oxygenator) favor coagulation driven-thrombosis, whereas high shear environments (e.g., pump) are prone to form platelet-driven thrombi. Our histological data, however, indicates that both the individual thrombus components (RBCs, fibrin, platelets, and VWF content) and the unbiased thrombus cluster-types are not associated with the respective location in the device. Similar as to our quantitative findings, a recent descriptive report, investigating 53 pediatric ECMO circuits, has shown that thrombi originating from different device locations consist of a mixture of fibrin, platelets, red blood cells, VWF, and leukocytes.[19] In contrast, two descriptive reports by Hastings et al. have shown that oxygenator and tubing thrombi mainly consist of RBCs and fibrin, whereas pump thrombi are more platelet dominant.[14,15]

In recent years, increasing knowledge indicates that also immunothrombotic components play a role in ECMO thrombogenesis. For instance, adhesion and activation of neutrophils, monocytes, and complement factors on the ECMO circuit's surface have been shown to activate both coagulation and platelets.[7,31–33] Interestingly we observed a higher blood leukocyte count during ECMO support

in patients with fibrin-rich thrombi, supporting the involvement of leukocytes that trigger thrombus formation. However, although not specifically stained, our histological data indicate that only few leukocytes are present in VA ECMO thrombi, which could potentially be explained by the fact that the majority of VA-ECMO cases is related to noninfectious etiologies. In contrast to VA-ECMO, we have previously shown the abundant presence of leukocytes in a small cohort of COVID-19 patients using veno-venous ECMO support.[34] Apart from leukocytes, also H3Cit content of ECMO thrombi was measured to identify the presence of NETs. Our histological data indicate the presence of some intra- and extracellular H3Cit signal, suggesting the presence of neutrophils undergoing NETosis and NETs, respectively. In general, few NETs (H3Cit-postive signal) were observed. However, the (rather low) H3Cit levels were particularly increased in both the pump and the oxygenator thrombi compared with tubing thrombi which suggests at least in minor part a NET-related mechanism in both device locations as already hypothesized.[35] This is in line with recent *in vitro* studies which have shown platelet-dependent NET formation in ECMO circuits and high shear rate-induced NET formation in a microfluidic assay.[36,37]

Our study shows the contribution of various factors, including fibrin, platelets, and VWF, which underscores the complexity of ECMO-induced thrombus formation. This indicates that other therapies besides the currently used heparin are worth investigating. These could include the use of direct thrombin inhibitors, FXII and/or FXI blocking and inhibition of the platelet-VWF interaction (e.g. blocking the GPIb-A1 interaction).[38-42] Indeed, data from *in vivo* ECMO animal models suggest that FXII, alone or in combination with FXI inhibition, leads to reduced fibrin and platelet deposition and is associated with fewer bleeding complications, but remains to evaluated in a clinical setting.[43,44] Besides optimization of anticoagulation regimens, modification of ECMO materials, flow conditions, and device technology are also needed to prevent the deposition of prothrombotic factors to further minimize thrombosis. Future studies, however, are required to investigate the safety and efficacy of such potential strategies.

This study has several limitations that are worth considering. First, because different types of pumps or oxygenators are often composed of different biomaterials and surface coatings, it is plausible that the type of pump/oxygenator could influence the composition of thrombi.[45] However, we were unable to investigate this aspect because of the low sample sizes of thrombi formed in different oxygenators or pumps. Second, we cannot exclude that the oxygenator acted as a sieve for thrombi that were dislodged from other locations and might therefore not accurately reflect the composition of thrombi formed at the premembrane oxygenator side. Third, the weight of variation in flow shear rate according to location and during support periods should also be accounted in the interpretation of these results and was not captured in our study. Finally, even though most of our thrombi were kept in phosphate buffered saline solution for less than 24 hours, we cannot rule out bacterial growth and (even limited) protein degradation. Nevertheless, previous reports indicated very limited issues when thrombi are kept less than 48 hours in such condition.[13] Furthermore, although our histological data also suggest that clot contraction may occur within ECMO thrombi, we are currently not able to investigate the presence of contracted RBCs (polyhedrocytes) via scanning electron microscopy because all samples were processed via routine paraffin-embedding procedures. Overall, despite the high number of thrombi analyzed in comparison to previous reports, our results and their interpretation are exploratory and need further confirmation.

In conclusion, our histological study provides insight into the possible underlying drivers of in-device VA-ECMO thrombus formation. We found that thrombus composition is heterogenous, consisting of two distinct thrombus types: an RBC-rich and a fibrin-rich thrombus type. These findings are important to further improve current anticoagulation strategies in ECMO, such as combination strategies. Future studies are needed to investigate the safety and efficacy of novel antithrombotic therapies in ECMO and to evaluate device-related modifications to minimize in-device thrombosis.

AUTHOR CONTRIBUTIONS

S.Su., S.St., M.M., S.F.D.M., and A.R. contributed to the conception and design of the work. S.St., M.M., P.M., D.C., E.B., and K.V. contributed to the acquisition and analysis of data. A.P., J.L., A.D., B.P., and L.D. contributed to the biostatistics ans data management. S.St., D.C., M.M., and S.F.D.M. contributed to the interpretation of data. S.St. drafted the manuscript. A.R., E.V.B., O.M., F.V., and S.S. critically revised the manuscript. All authors have approved the final version of the manuscript. They all agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST

Authors have no relevant relationships to disclose with regard to the contents of this paper.

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SUPPORTING INFORMATION

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

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