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Antibody inhibition of contact factor XII reduces platelet deposition in a model of extracorporeal membrane oxygenator perfusion in nonhuman primates

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Abstract

Background: The contact factor XII (FXII) activates upon contact with a variety of charged surfaces. Activated FXII (FXIIa) activates factor XI, which activates factor IX, resulting in thrombin generation, platelet activation, and fibrin formation. In both in vitro and in vivo rabbit models, components of medical devices, including extracorporeal oxygenators, are known to incite fibrin formation in a FXII-dependent manner. Since FXII has no known role in hemostasis and its inhibition is therefore likely a safe antithrombotic approach, we investigated whether FXII inhibition also reduces accumulation of platelets in extracorporeal oxygenators.

Objectives: We aimed to determine the effect of FXII inhibition on platelet deposition in perfused extracorporeal membrane oxygenators in nonhuman primates.

Methods: A potent FXII neutralizing monoclonal antibody, 5C12, was administered intravenously to block contact activation in baboons. Extracorporeal membrane oxygenators were temporarily deployed into chronic arteriovenous access shunts. Radiolabeled platelet deposition in oxygenators was quantified in real time using gamma camera imaging. Biochemical assays were performed to characterize the method of action of 5C12.

Results: The anti-FXII monoclonal antibody 5C12 recognized both the alpha and beta forms of human and baboon FXII by binding to the protease-containing domain, and inhibited FXIIa activity. Administration of 5C12 to baboons reduced platelet deposition and fibrin formation in the extracorporeal membrane oxygenators, in both the presence and absence of systemic low-dose unfractionated heparin. The antiplatelet dose of 5C12 did not cause measurable increases in template bleeding times in baboons.

Conclusions: FXII represents a possible therapeutic and safe target for reducing platelet deposition and fibrin formation during medical interventions including extracorporeal membrane oxygenation.

KEYWORDS
blood platelet, extracorporeal membrane oxygenation, factor XII, hemostasis, thrombosis

Essentials

- The blood zymogen factor FXII (FXII) is activated upon contact with charged surfaces.
- We generated an FXII antibody, 5C12, that inhibits activated FXII.
- Intravenous 5C12 administration reduced platelet accumulation in oxygenators in primates.
- Inhibition of FXII may be useful in preventing platelet deposition on vascular devices.

1 | INTRODUCTION

Blood-contacting medical devices, including catheters, stents, grafts, filters, and extracorporeal organ support (ECOS) systems can fail due to thrombus accumulation in the system and may also trigger device-associated thromboembolism.[1] To maintain patency, devices that are perfused for various lengths of time require prophylactic anticoagulation, which can increase the incidence and/or severity of bleeding. Inhibiting the blood coagulation contact activation pathway has been proposed as an alternative approach to safer anticoagulation.[2–4] Extracorporeal membrane oxygenation (ECMO) is an ECOS system that has been increasingly used for short-term management of acute respiratory failure, such as in cases of complicated influenza, or in the temporal alleviation of acute heart failure; however, its benefits are reduced by anticoagulation-associated bleeding.[5–7] ECMO systems have several components that promote the activation of platelets and the blood coagulation contact system, including the hollow fibers and membranes that are exposed to flowing blood.[8]

Activation of the coagulation cascade in vivo leads to thrombin generation, platelet activation, and subsequent fibrin formation to support both hemostasis and pathological vaso-occlusive thrombosis/thromboembolism.[9] Pharmacological thromboprophylaxis can be effectively achieved with existing antithrombotics; however, these currently available treatments also cause bleeding since they inhibit key hemostatic plasma proteins such as thrombin or activated coagulation factor Xa (FXa).[10,11] Accordingly, these drugs cannot be dosed to full efficacy due to dose-limiting antihemostatic toxicity, and thus thrombotic vessel occlusion remains the leading cause of mortality in industrialized countries.[12] To address the issue of antithrombotic safety, we proposed that inhibiting contact system activation would be a safer alternative to current antithrombotic therapy.[2] Steadily increasing efforts are under way in both industry and academia to develop inhibitors to members of the contact activation complex to improve the safety of therapeutic and prophylactic anticoagulation.[13]

The plasma contact activation complex, which includes FXII, prekallikrein (PK), and high-molecular-weight kininogen (HK), has been shown to promote pathological thrombus formation. This complex is activated following exposure of blood to negatively charged surfaces, including a variety of biological molecules and artificial materials.[4,14,15] FXII is a key member of this pathway, with a plasma concentration of 30-40 μg/mL (375-500 nM).[16] Surface-catalyzed cleavage of FXII, an 80-kDa single-chain zymogen, after Arg353 generates the protease α-FXIIa. Cleavage of α-FXIIa after Arg334 generates β-FXIIa, which is comprised of the FXIIa catalytic domain. α-FXIIa activates factor XI (FXI) to FXIa and ultimately leads to thrombin (factor IIa [FIIa]) generation. Concomitantly, α-FXIIa activates the zymogen PK to α-kallikrein, which then converts additional FXII to α-FXIIa. β-FXIIa can activate components of the complement system and as part of the kallikrein-kinin system cleaves the cofactor HK to liberate the potent systemic vasoregulatory and proinflammatory molecule bradykinin.[17] Thus, contact activation initiates both prothrombotic and proinflammatory processes.[18]

Contact activation of plasma drives thrombin formation in the activated partial thromboplastin time (aPTT) assay in vitro. Plasma samples from mammals lacking any of the contact system proteins have prolonged aPTTs, yet aPTT prolongation is not diagnostic of hemostasis impairment.[19] Although hereditary FXI deficiency produces an aPTT prolongation and can cause a mild bleeding disorder (hemophilia C), deficiency of FXII, PK, or HK all result in aPTT prolongation but remain asymptomatic.[20–22] Importantly, while playing a minor to no role in hemostasis, these proteins appear to contribute significantly to thrombosis in experimental animal models.[23–25]

FXIIa inhibition using the anti-FXIIa antibody 3F7 in a rabbit model of ECMO reduced fibrin deposition in the oxygenator without evidence of increased bleeding.[26–28] Since platelets are critical to the formation of occlusive thrombi, here we extended this approach using a potent new anti-FXII monoclonal antibody, and show that FXII inhibition also reduces platelet activation and deposition within membrane oxygenators in both heparinized and nonheparinized nonhuman primates.

2 | MATERIALS AND METHODS

2.1 | Generation of anti-FXII monoclonal antibodies

FXII-deficient mice were immunized with 30 μg of a mixture of recombinant murine and human FXII[29] by intraperitoneal injection in Freund's complete adjuvant on day 0 and 20 μg in Freund's incomplete adjuvant on day 18. A 10-μg booster dose in saline was given on day 46. On day 49, spleens were removed and lymphocytes were fused with P3X63Ag8.653 myeloma cells using a standard

polyethylene glycol–based protocol. Antibodies were tested for capacity to recognize human and/or mouse FXII by ELISA and western blot and to prolong the aPTT of platelet-poor plasma. The clone 5C12 produces an antibody that binds to FXII and FXIIa. 5C12 hybridoma cells were subcloned, expanded in a CL1000 bioreactor (Corning Inc, Corning, NY, USA), and purified by mercaptoethylpyridine and protein-A/G affinity chromatography, characterized in vitro and then used in experiments in vivo. The humanized form of this antibody (h5C12) was produced by complementarity determining region grafting from the murine precursor, expressed in HEK293 cells and purified protein A chromatography.

2.2 | Characterization of 5C12

To measure aPTT prolongation, one-tenth volume of 200 μg/mL of purified 5C12 stock (0.13 μM final concentration in plasma) or phosphate buffered saline (PBS) vehicle pH 7.4 was incubated for 3 minutes with human or baboon platelet-poor plasma that was serially diluted with FXII-deficient plasma (Affinity Biologicals, Ancaster, Ontario, Canada) to yield an FXII concentration range in plasma and keeping the antibody concentration constant. In the second set of aPTT experiments, the concentration of 5C12 was varied (0-4 μM) and tested in normal baboon or human plasma and also compared to the FXIIa inhibitor, corn trypsin inhibitor (CTI). 5C12 was also used as the primary antibody in western blots to detect FXII in human and baboon plasma, as well as purified α- and β-FXIIa.

For FXII activation experiments, human FXII (40 nM; Haematologic Technologies, Inc, Essex Junction, VT, USA) was incubated with 0 to 80 nM 5C12 (10 min) followed by 1 μg/mL dextran sulfate (20 min). Spectrozyme FXIIa (0.5 mM; Sekisui Diagnostics GmbH, Lexington, MA, USA) was then added to measure hydrolysis by activated FXII (FXIIa). Next, FXII (100 nM) and 5C12 (0-200 nM) were coincubated (10 min) and then diluted 50/50 with HK (12.5 nM), PK (12.5 nM) (Enzyme Research Laboratories, South Bend, IN, USA), or short- or long-chain polyphosphate (10 μM) (60 min, final concentrations).[30] Amidolytic activity was quantified after addition of polybrene (6 μg/mL) and soybean trypsin inhibitor (50 μg/mL). To test FXIIa inhibition, Spectrozyme FXIIa was added to mixtures of FXIIa (20 nM) and 5C12 (0-40 nM). To test the effect of 5C12 (0-100 nM) on FXII (100 nM) activation by kallikrein, CTI (40 μg/mL), kallikrein (5 nM) and dextran sulfate (10 μg/mL) were co-incubated (0-60 min). Samples were separated by reducing SDS-PAGE and immunoblotted with an anti-FXII polyclonal antibody (Santa Cruz Biotechnology, Inc, Dallas, TX, USA).

For measurement of FXa, FXIa, kallikrein, and thrombin activity, select concentrations of FXa, FXIa, kallikrein, or thrombin (0-2 nM) were incubated in the absence or presence of 5C12 (100 nM) for 10 minutes at 37°C. The activity was determined by adding the chromogenic substrates Chromogenix S-2375 (0.6 mM), Chromogenix S-2366 (0.8 mM), Chromogenix S-2302 (0.6 mM), or Chromogenix S-2238 (0.8 mM), respectively, and the rate of substrate hydrolysis was measured at 405 nm.

2.3 | Baboon model of thrombogenesis in extracorporeal membrane oxygenators and grafts

All animal experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Chronic exteriorized femoral arteriovenous (AV) shunt-bearing baboons (n = 3) were used as described elsewhere.[2,25,31] In brief, baboons received ^{111}In-labeled autologous platelets and ^{125}I-labeled homologous fibrinogen and then their AV shunts were extended to incorporate a saline-primed oxygenator cartridge (Terumo-CAPIOX RX05, coated hollow fiber design; Terumo Cardiovascular Group, Ann Arbor, MI, USA). This model does not contain additional components beyond the membrane oxygenator component that are present in a complete ECMO circuit. Blood perfusion was arterial pressure gradient–driven at a restricted 0.1 L/min flow rate without the use of a pump. The type of oxygenator used has an approximately 0.5 m^2 functional surface area for gas exchange. Since we were interested in adhesion and retention of platelets within the oxygenator as a marker of platelet-dependent thrombogenesis, we recorded platelet radioactivity within the oxygenator in real time using gamma camera imaging (GE-Brivo NM 615 interfaced with Xeleris 3.1 software, GE Healthcare, Chicago, IL, USA). Platelet deposition was calculated as previously described.[2,31–33] For quantification of ^{125}I-fibrin content at the 60-minute end point, the cartridge was removed, rinsed, dried, and stored refrigerated until processing. In brief, the oxygenators were filled with digest buffer (10 mM Tris-H_3PO_4 pH 7.0, 35 mM SDS) for 3 days. The radioactivity of the digest solution was measured using a gamma counter (Wizard-3, PerkinElmer, Waltham, MA, USA), and the amount of trapped fibrin/fibrinogen was calculated as previously described.[2,32] Collagen- and tissue factor–initiated vascular graft thrombosis in the chronic AV shunt model has been described previously.[2,31–33]

2.4 | Anticoagulation of baboons

In an initial dose-finding experiment, 6 consecutive 5C12 doses of 1 mg/kg were injected intravenously at 40-minute intervals, and aPTT prolongation of plasma samples was measured. In AV shunt perfusion experiments, pretreatment with heparin (20 U/kg), 5C12, or their combination were evaluated for their effect on platelet deposition and fibrin formation in the oxygenator cartridge compared with no treatment controls. Experiments were performed on 3 consecutive days in the same animal. Heparin was given 15 minutes prior to oxygenator perfusion. In select experiments, 5C12 was initially dosed at 5 mg/kg, followed by daily 2 mg/kg doses in an effort to maintain FXII inhibition for the 3 consecutive days of experiments (Figure S1). For vascular graft studies, a single dose of 9 mg/kg h5C12 was administered, and experiments were performed at 30-minute and 24-hour time points.

2.5 | Hemostasis assessment

Primary skin hemostasis was evaluated using the adult Surgicutt device (International Technidyne, Edison, NJ, USA). Template bleeding time (BT) was recorded twice during each experiment and averaged.[34,35] A 40-mm Hg pressure cuff was applied to the upper arm of the animal, and a 5-mm-long and 1-mm-deep incision was made on the shaved inside of the lower arm. Blood drops emerging from the wound were collected on a Whatman filter paper every 30 seconds until the bleeding stopped. The volume of blood loss during the BT tests was measured by dissolving the dried blood in Drabkin's reagent (Sigma-Aldrich, St Louis, MO, USA) and measuring the absorption at 540 nm. For quantification, a standard curve of the animal's blood taken on the same day was generated. The skin wounds were observed for rebleeding.

Prothrombin time (PT) of plasma samples was measured using a KC4 Analyzer (TCoag, Ltd, Wicklow, Ireland) coagulometer. PT assay was performed using Dade Innovin (Siemens Healthcare Diagnostics, Flanders, NJ, USA) according to the manufacturer's protocol.

2.6 | Blood sample analyses

Blood samples were collected into citrate and plasma aPTT was measured in duplicates using SynthASil (Instrumentation Laboratory, Bedford, MA, USA) and a KC4 Analyzer. Activated clotting time (ACT) of blood was determined in duplicates using LupoTek KCT (r^2-Diagnostics, South Bend, IN, USA). Plasma thrombin-antithrombin complex (TAT) and platelet factor 4 (PF4) were measured with ELISA kits from Siemens (Flanders, NJ, USA) and R & D Systems (Minneapolis, MN, USA), respectively.

2.7 | Flow chamber analysis

Glass capillary tubes (0.2 × 2×200 mm; Vitrocom, Mountain Lakes, NJ, USA) were coated with 100 µg/mL of fibrillar type I collagen (Chrono-Log Corp, Havertown, PA, USA) in the absence or in combination with 0.1-nM tissue factor (Dade Innovin, Siemens, Flanders, NJ, USA) for 1 hour at room temperature (RT). Capillaries were washed with PBS and then blocked with 5 mg/mL denatured bovine serum albumin for 1 hour at RT before connecting them to a syringe pump and a blood reservoir. Human venous blood was drawn by venipuncture into syringes containing 3.8% (w/v) sodium citrate (one-tenth of blood volume) in the absence or presence of 100 µg/mL 5C12; blood was recalcified prior to perfusion as previously described.[36] After blood perfusion, capillaries were washed with PBS, fixed with 4% paraformaldehyde, and sealed with Fluoromount G. Platelet aggregates and fibrin formation in the capillaries were then imaged for analysis using a 63 × Zeiss Axio Imager M2 microscope as described.[37]

2.8 | Data analyses

Numeric values are shown as mean and range in the text; mean and SEM are shown in the figures. Means were compared by 1-way analysis of variance (ANOVA) for treatment using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Comparisons on platelet deposition over time were performed by repeated measures ANOVA for time and treatment using SigmaPlot 11 (Systat, Inc, Santa Clara, CA, USA). For differences between means, a probability of < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characterization of 5C12

Initial experiments were designed to validate the role of FXII in initiating clotting in an aPTT assay. An increase in aPTT was observed following serial dilution of human or baboon plasma with FXII-depleted plasma (Figure 1 A-B). An increase in aPTT was observed in the presence of the anti-FXII monoclonal antibody 5C12 across the range of serially diluted plasmas (Figure 1 A-B); moreover, an increase in aPTT was observed in complete plasma as a function of concentration (Figure 1 C). Consistent with previous reports, the well-characterized FXIIa inhibitor CTI nearly doubled the aPTT from the baseline of approximarely 30 seconds, while a nearly 10-fold increase in aPTT was observed at an equimolar concentration of 5C12 (Figure 1 D). As analyzed by western blot, 5C12 recognized both human and baboon FXII as well as the protease domain-containing alpha and beta forms of recombinant FXIIa (Figure 1 E).

We next assessed the ability of 5C12 to inhibit FXIIa activity. Addition of the contact activators dextran sulfate or long- or short-chain polyphosphate to purified FXII was used to induce FXII activation and amidolytic activity toward an FXIIa-specific chromogenic substrate (Figure 2 A). We found that 5C12 potently inhibited the amidolytic activity of FXIIa. This result was validated with data showing that 5C12 blocked the amidolytic activity of an equimolar amount of FXIIa, suggesting active site inhibition (Figure 2 B). In contrast, 5C12 did not affect the activation of FXII by kallikrein in the presence of dextran sulfate (Figure 2 C), or the amidolytic activity of the serine proteases FXa, FXIa, kallikrein, or thrombin (Figure 2 D-G).

3.2 | Characterization of the anticoagulant activity of 5C12 in vivo

We next tested the antibody in a single juvenile baboon. 5C12 was administered in a stepwise manner consisting of 6 doses of 1 mg/kg given 40 minutes apart. Our results show that 5C12 caused a nearly 3-fold increase in the plasma aPTT from baseline after the second dose; a 4-fold increase in aPTT above baseline was recorded after the fifth dose (Figure 3A). A corresponding increase in ACT was recorded

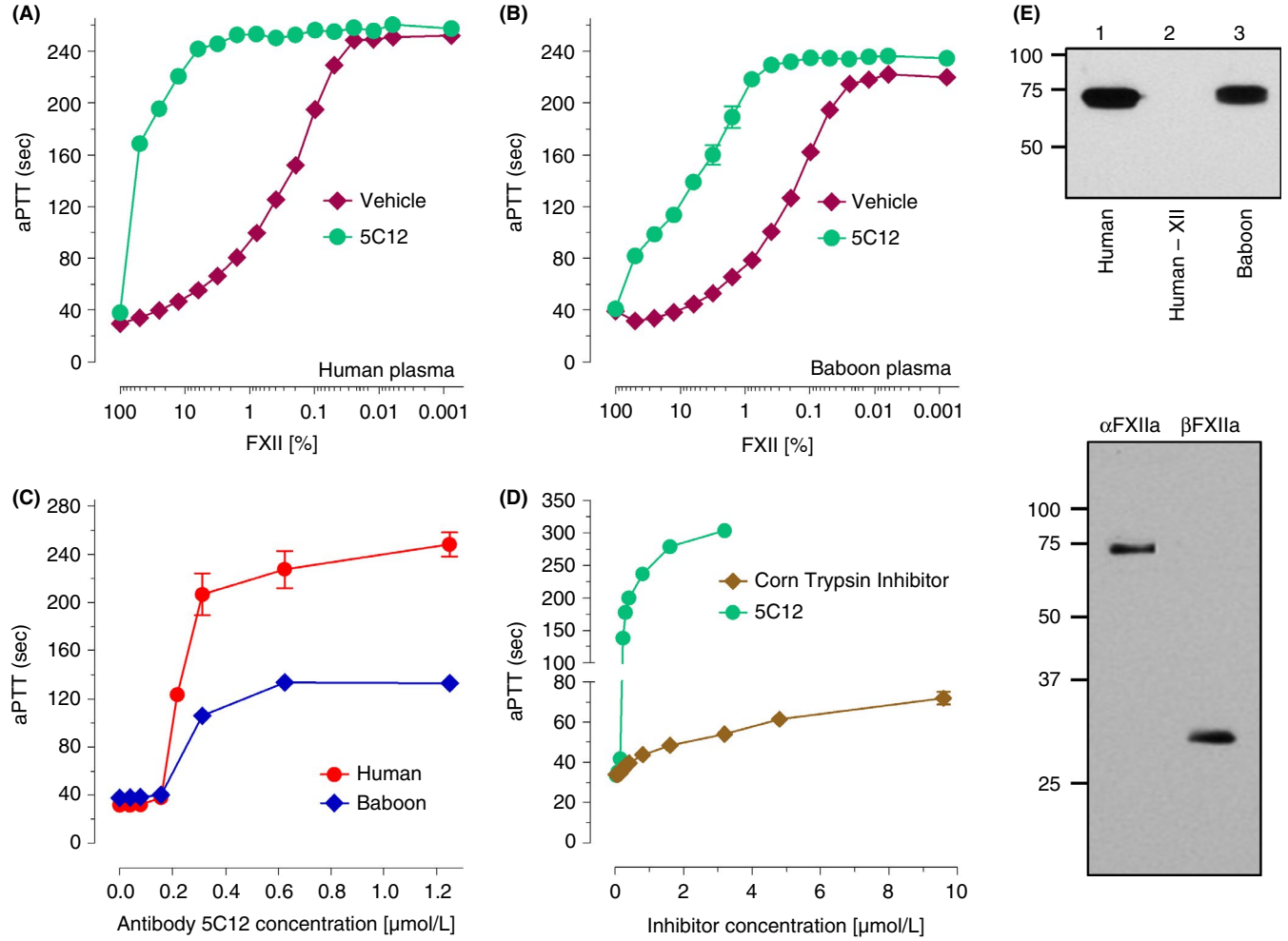

FIGURE 1 In vitro characterization of anti-FXII(a) monoclonal antibody 5C12. Platelet-poor plasma from (A) humans or (B) baboons was serially diluted with FXII-deficient human plasma to achieve a range of FXII levels decreasing from 100% to 0%. Clotting times were measured following addition of an aPTT reagent; in select experiments, plasma was pretreated with 5C12 (20 μg/mL; 133.3 nM) for 5 min at 37°C. Measurements were performed in duplicate. (C) Increasing concentrations of 5C12 prolonged aPTT in human or baboon platelet-poor plasma. (D) Comparison of the effect of equimolar concentrations of 5C12 and CTI on aPTT. (E) Top: 5C12 recognized both human and baboon FXII zymogen in platelet-poor plasma but showed no reactivity in human FXII-deficient plasma; Bottom: 5C12 recognized both the alpha and beta forms of purified activated FXII. Data represent mean ± SEM from n = 2-5. aPTT, activated partial thromboplastin time; CTI, corn trypsin inhibitor; FXII, factor XII; FXIIa, factor XIIa

(Figure 3B). As expected, 5C12 did not affect prothrombin time or baseline circulating TAT levels or blood cell counts (Figure 3C-H).

3.3 | Effect of 5C12 on thrombus formation under flow

Experiments were designed to determine the effect of inhibiting FXIIa activity with 5C12 on platelet deposition and fibrin formation on graft surfaces coated with collagen or tissue factor and inserted into the external loop of an AV shunt in the baboon. Under vehicle conditions, a robust degree of platelet deposition and fibrin formation was observed on either surface after 60 minutes of blood flow at a shear rate of 265 /sec. We found that 5C12 significantly reduced platelet deposition and fibrin formation on surfaces of collagen but not tissue factor (Figure 4A-D). This result was validated in vitro, whereby 5C12 was shown to inhibit thrombus formation on collagen alone but not in the presence of tissue factor (Figure 4E-H). These data are in line with the concept that contact activation of FXII promotes thrombus formation on extracellular matrix proteins such as collagen, yet is not essential for extrinsic pathway–mediated thrombus formation or hemostatic plug formation.

3.4 | Effect of 5C12 on platelet deposition in an extracorporeal membrane oxygenator

We next quantified autologous radiolabeled platelet deposition in an extracorporeal membrane oxygenator that had been placed in the loop of a baboon AV shunt. In the absence of anticoagulation, platelets readily deposited in the oxygenator by 5 minutes and achieved a platelet retention rate of 1.64 ± 0.13 billion/min

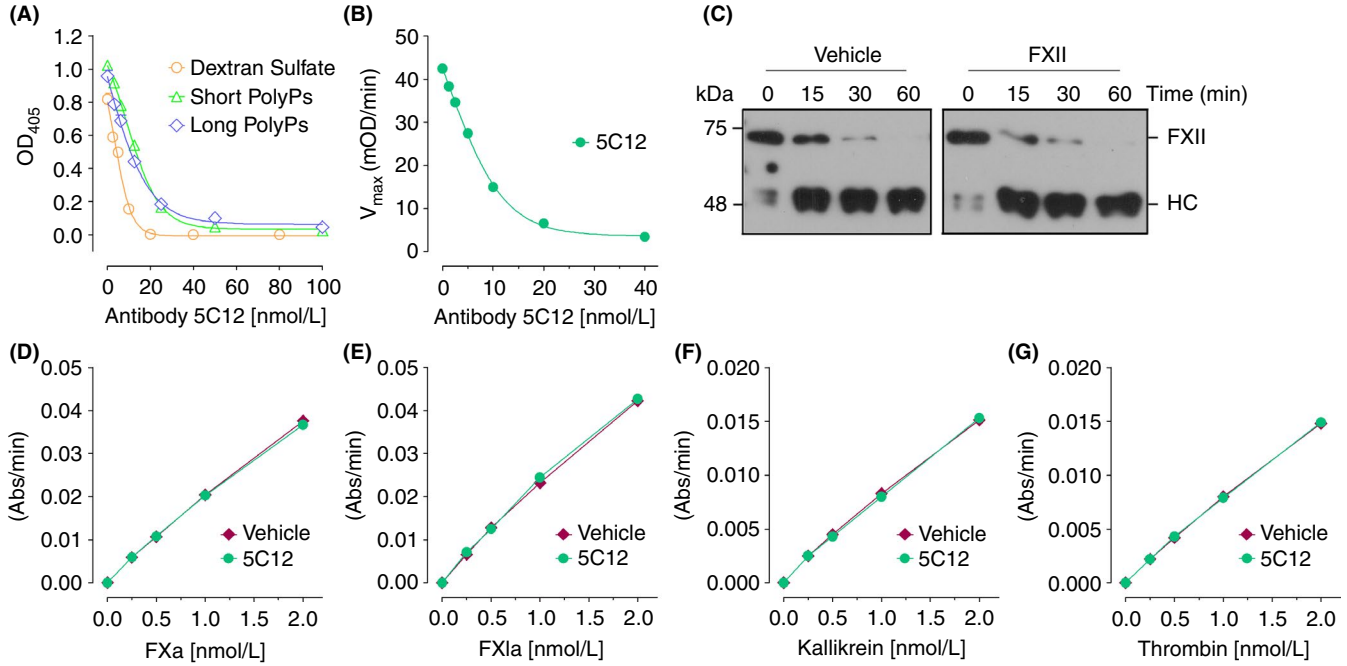

FIGURE 2 5C12 inhibits FXII(a) activity. (A) Increasing concentrations of 5C12 were added to a solution of purified FXII. FXII was activated by either 1 μg/mL dextran sulfate (circle) or 10 μM short-chain or long-chain polyphosphate (polyP). Amidolytic activity was quantified as hydrolysis of the chromogenic substrate Spectrozyme FXIIa. (B) Activated FXII (FXIIa; 20 nM) was incubated with increasing concentrations of 5C12 for 5 minutes before addition of Spectrozyme FXIIa. The velocity of hydrolysis was measured for 20 minutes and V_{max} was calculated for each reaction. Each data point was measured in duplicate. (C) FXII (100 nM) was incubated with kallikrein (5 nM) and dextran sulfate (10 μg/mL) in the presence of vehicle or 5C12 (100 nM). FXIIa activity was blocked by 40 μg/mL CTI. 5C12 did not block the activation of FXII by kallikrein. (D-G) 5C12 (100 nM) had no effect on the amidolytic activity of FXa, FXIa, kallikrein, or thrombin. Amidolytic activity was quantified as hydrolysis of a chromogenic substrate. Data represent mean ± SEM from n = 2. CTI, corn trypsin inhibitor; FXa, factor Xa; FXIa, factor XIa; FXII, factor XII; FXIIa, factor XIIa; HC, heavy chain

between 30 and 60 minutes from the start of perfusion (Figure 5A-B). A robust degree of fibrin formation was observed at the 60-minute end point under vehicle conditions (Figure 5C). Pretreatment of the baboon with a low dose of heparin caused a significant reduction in both the rate and final extent of platelet deposition and degree of fibrin formation (Figure 5A-D). The combination of 5C12 with heparin further reduced the rate and extent of platelet deposition as compared to low-dose heparin alone, lending credence to the concept that combining a contact activation pathway inhibitor with low-dose heparin may provide protection against vascular device–associated thrombotic complications. Moreover, anticoagulation with 5C12 alone significantly reduced the rate and extent of platelet deposition rate and fibrin formation in the oxygenator. A change in template bleeding time for all treatment groups, including heparin, was not observed for any of the groups tested (Figure 5E).

3.5 | Effect of 5C12 on systemic markers of coagulation

Blood samples taken at the beginning and end of each oxygenator perfusion experiment were analyzed for markers of thrombin generation (TAT levels) and platelet activation (PF4 levels) (Figure 6), while levels of anticoagulation were assessed by measuring the aPTT, ACT, and PT at the 0- and 60-minute time points. A robust increase in systemic levels of TAT and PF4 were observed at the end of the 60 minutes of oxygenator perfusion in the absence of any anticoagulant. Pretreatment of the baboons with 5C12 either alone or in combination with low-dose heparin resulted in a significant reduction in both TAT and PF4 levels (Figure 6A-B). A corresponding increase in aPTT and ACT was observed for both cohorts, while the PT was unaffected by 5C12 alone (Table 1). The study was underpowered to draw a conclusion regarding the effect of low-dose heparin alone on TAT and PF4 levels compared to no treatment; we did confirm that low-dose heparin caused an increase in aPTT, ACT, and PT as compared to baseline (Table 1).

4 | DISCUSSION

In this study, we demonstrate that intravenous administration of the newly described anti-FXII monoclonal antibody, 5C12, significantly reduces platelet accumulation in extracorporeal membrane oxygenators in a nonhuman primate model. 5C12 binds to the FXII beta chain, where it is in position to block the protease active site upon FXII zymogen conversion to FXIIa by kallikrein. The 5C12 antibody inhibits clotting in contact activation-initiated coagulation

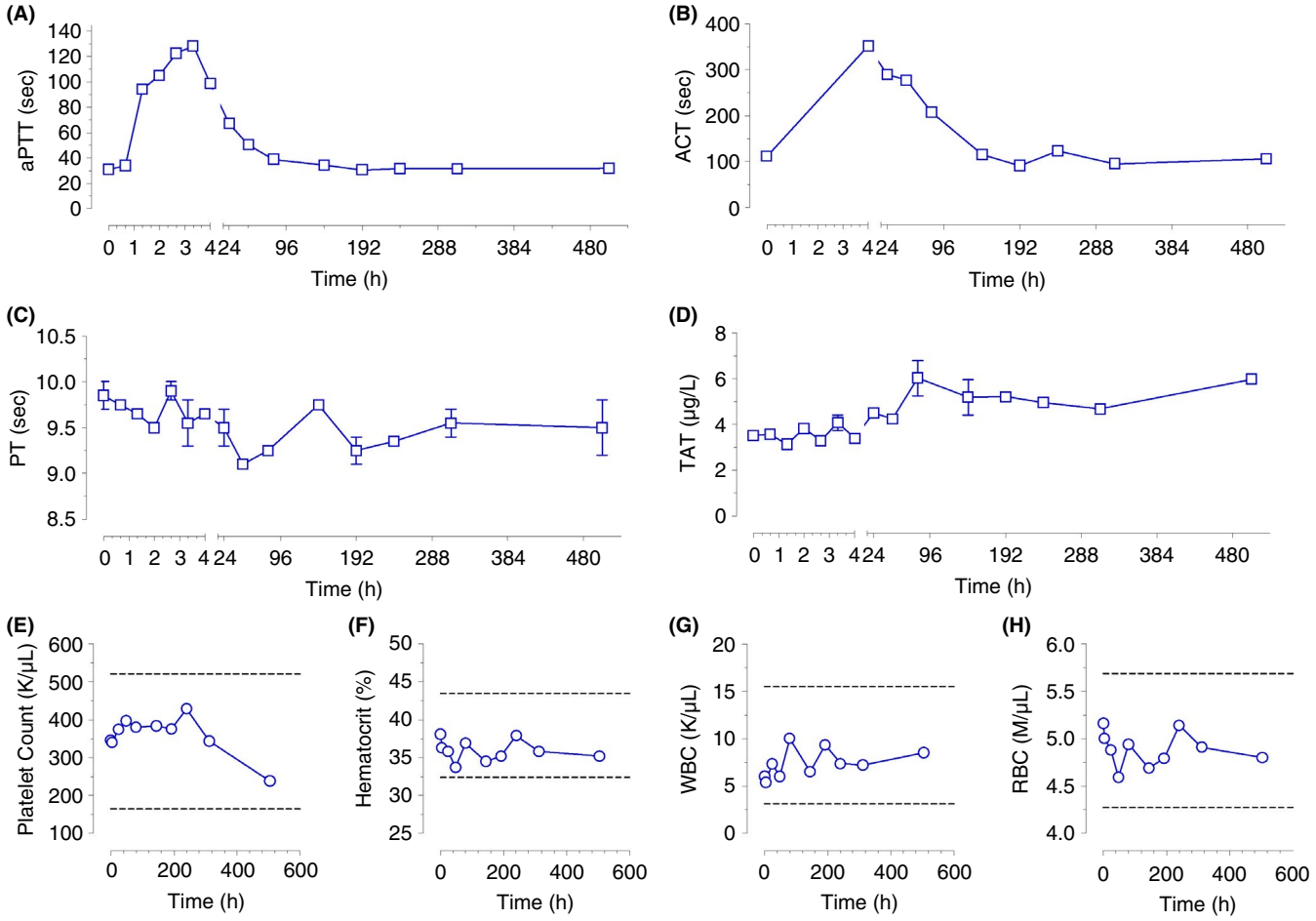

FIGURE 3 Time course of coagulation parameters after 5C12 administration in a nonhuman primate. A baboon was administered subsequent iv doses of 1 mg/kg 5C12 every 40 min over a 4-hour period, equaling a total of six doses. Blood samples were taken at select times for up to three weeks post administration. (A) aPTT, (B) ACT, (C) PT, and (D) TAT levels were measured. Complete blood count (CBC) was measured and compared to the upper and lower limits of reference values as indicated by dashed lines: (E) platelet counts, (F) hematocrit, (G) WBC, and (H) RBC. ACT, activated clotting time; aPTT, activated partial thromboplastin time; PT, prothrombin time; RBC, red blood cell count; TAT, thrombin-antithrombin complex; WBC, white blood cell count

tests such as aPTT and ACT, primarily (if not exclusively) by limiting thrombin generation, but does not affect the PT. Our results link FXIIa activity with platelet activation and deposition in an extracorporeal membrane oxygenator, which likely contributes to occlusive device failure. The exact mechanism of the observed antiplatelet activity of 5C12 was not determined in this study, but the possibility of direct effects of FXIIa activity on platelet behavior under flow conditions in vivo cannot be completely excluded. Dampened thrombin generation in 5C12-treated animals suggests that the observed apparent antiplatelet effect may be a result of diminished cleavage of platelet protease-activated receptors by serine proteases including thrombin or by reducing the degree of platelets that expose phosphatidylserine as a potential catalyst of FXII-mediated thrombus formation.[38] Reduced fibrin formation may also lead to destabilization of large platelet-rich aggregates, increasing detachment and limiting accumulation in the graft or oxygenator.[39]

In this model, we used lower levels of heparin anticoagulation than one would use under clinical conditions. We minimally heparinized the baboons and determined whether 5C12 had an effect on platelet retention in the oxygenator. The low dose of heparin produced a modest but clearly antithrombotic effect in the animals, and reduced but did not completely prevent quantifiable thrombus formation in the membrane oxygenator, allowing for the observation of change in platelet deposition in either direction. This low but anticoagulant dose of heparin prevented occlusion of the cartridge while still allowing for the antithrombotic effect of 5C12 to be studied. In the clinic, the patients undergoing ECMO procedures typically receive an initial bolus of 50 to 100 U/kg of unfractionated heparin followed by adjusted anticoagulation using anti-FXa activity or coagulation tests. Profound anticoagulation is generally sustained and maintained for as long as several weeks during ECMO.[40] These desperately ill patients often bleed as a result of anticoagulation, and this bleeding is a significant contributor to the observed high mortality rate of ECMO patients. In search of a safer alternative, we now propose that a lower dose of heparin could potentially be used in combination with a potent FXII inhibitor, such as 5C12, and that this combination or targeting FXII alone could offer a safer approach to

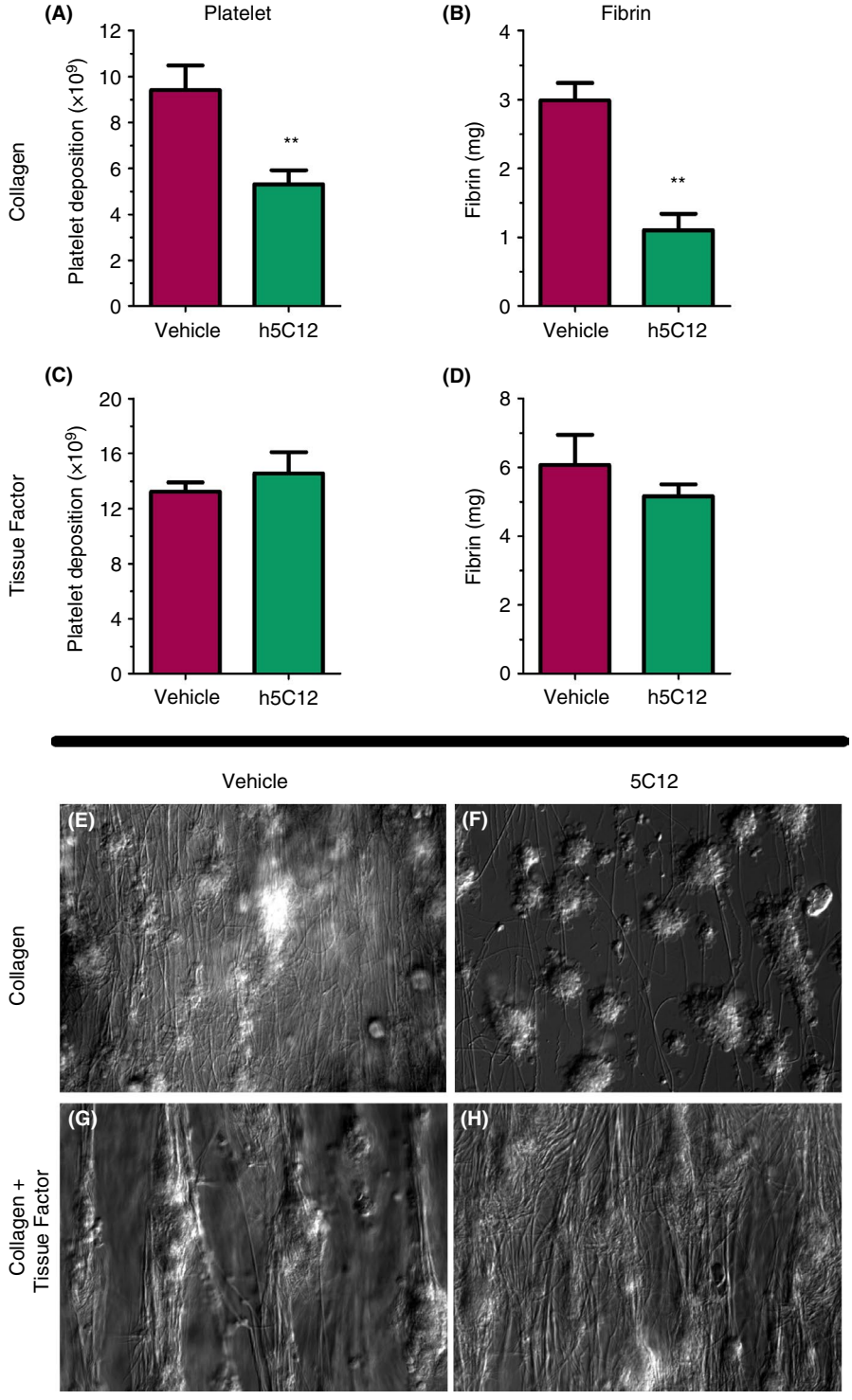

FIGURE 4 Effect of 5C12 on fibrin and thrombus formation on surfaces coated with collagen and/or tissue factor in vivo (A-D) or in vitro (E-H). Expanded polytetrafluoroethylene grafts (4 mm i.d., 20 mm long) were coated with collagen (A, B) or tissue factor (C, D) and inserted into the external loop of a chronic AV shunt of a baboon. Platelet deposition (A, C) and fibrin content (B, D) was measured (n = 3 for vehicle, n = 6 for h5C12). Administration of 9 mg/kg h5C12 given 1-24 hours before significantly reduced both platelet and fibrin accumulation on collagen but not tissue factor. Microfluidic glass capillaries coated with 100 µg/mL collagen alone (E, F) or in the presence of 0.1 nM tissue factor (G, H). 100 µg/mL 5C12 blocked fibrin formation on collagen alone but did not affect thrombus formation on collagen and tissue factor. Representative images from n > 3 experiments; data represents mean ± SEM from n = 3-6; $^{**}P < 0.01$ vs vehicle

anticoagulation during ECMO, as FXII has no known significant role in the maintenance of hemostasis.

The caveat with pharmacologically inhibiting FXII is that the physiological functions have not been established, and therefore it is not known what adverse effects, if any, reducing or eliminating FXIIa activity could cause. The protein, which is produced primarily in the liver and is a close homolog of the hepatocyte growth factor activator enzyme, is found at relatively high plasma concentrations in most terrestrial vertebrates but is absent in birds and is not expressed in cetaceans.[41] Of the terrestrial vertebrates, mammals alone synthesize FXI, which acts as a bridge between FXII and downstream thrombin generation in the in vitro coagulation tests. However, thrombin generation via FXI activation is only one possible activity of FXIIa, since FXIIa can shorten the contact-initiated clotting time even of FXI-deficient plasma in vitro.[30] FXIIa could also cleave several macromolecular substrates, including some inhibitors of coagulation, such as antithrombin. Moreover, it is also possible that the reagents used in the aPTT assay induce enzymatic reactions that are contact initiated but not entirely

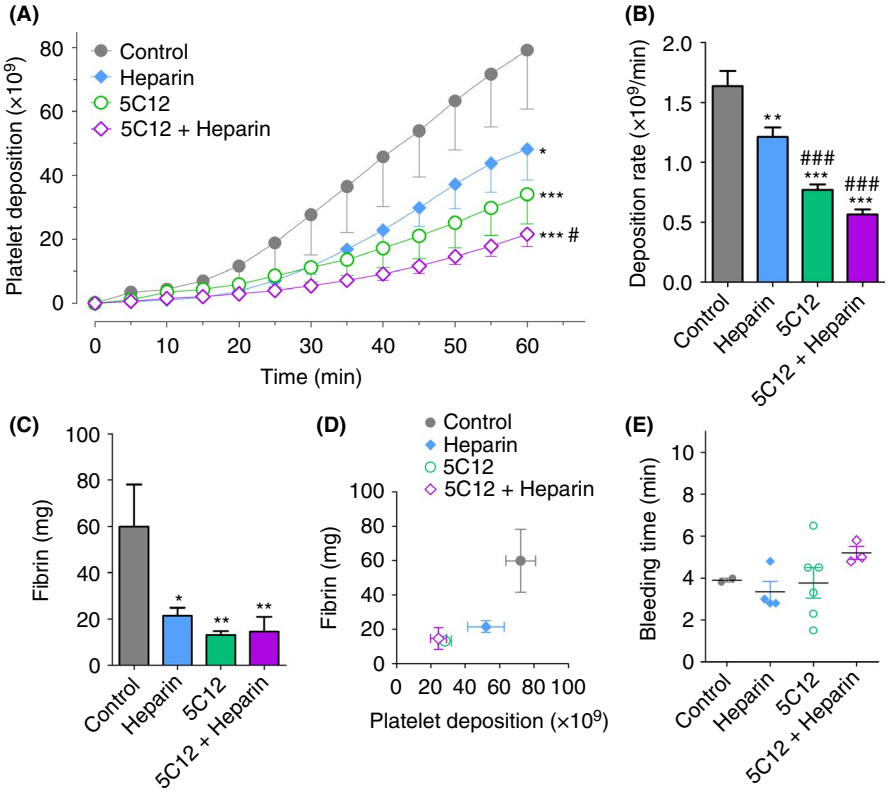

FIGURE 5 Effect of the anti-FXII monoclonal antibody 5C12 on platelet deposition and fibrin formation in extracorporeal membrane oxygenators. (A) Real-time platelet deposition was monitored in an extracorporeal membrane oxygenator inserted in the extended loop of a chronic AV shunt. Study arms included vehicle control (n = 2), heparin alone (20 U/kg 15 min before perfusion, n = 4), 5C12 (5 mg/kg initial day + 2 mg/kg on the following days 30 min before perfusion, n = 6), or the combination of both heparin and 5C12 (as above; n = 3). (B) Average platelet deposition rate calculated from 30 to 60 min. Measurement of terminal (C) fibrin content in the oxygenator and (D) correlation of fibrin and platelet content. (E) Two Surgicutt bleeding time measurements were performed during each experiment and each data point represents the average of the two measurements ($P = 0.36$, 1-way analysis of variance comparison of treatment groups). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$ $^{\#\#\#}P < 0.001$ vs. heparin. AV, arteriovenous; FXII, factor XII

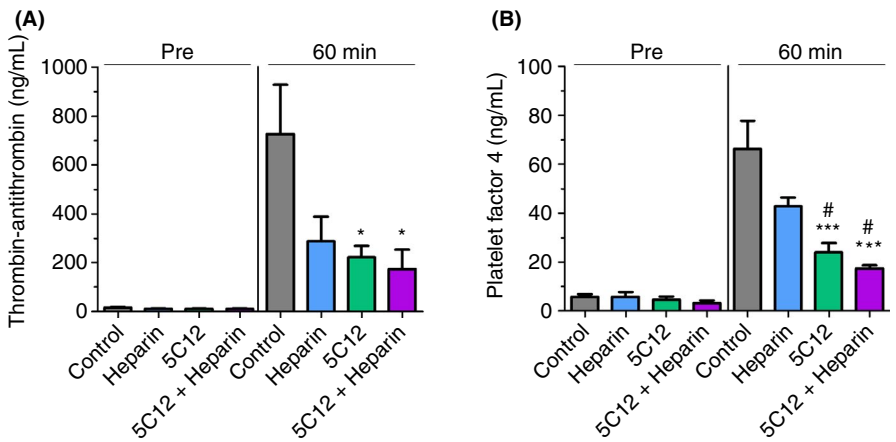

FIGURE 6 Effect of the anti-FXII monoclonal antibody 5C12 on thrombin generation and platelet activation during membrane oxygenator perfusion. (A) Thrombin-antithrombin formation was measured in systemic blood samples before administration of 5C12 (Pre) and at the end of each perfusion experiment (60 min). (B) Platelet activation was measured by quantitative measurement of platelet factor 4 in plasma. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$ vs. heparin. FXII, factor XII

dependent on FXII, since FXII-deficient and -depleted plasmas also clot reasonably fast, in <300 seconds in the aPTT assay. Nevertheless, the mechanism of thrombin generation in recalcified FXI- and/or FXII-blocked platelet-poor plasma in the absence of added tissue factor has not yet been fully explained.

FXII facilitates embryo implantation [42,43], promotes tissue repair, angiogenesis, and mitogenesis[44]; enhances vasodilation and inflammation through kallikrein activation and bradykinin generation[45]; may contribute to autoimmune disease pathogenesis through dendritic cell interactions[46]; and FXII produced by neutrophils appears to play a role in normal neutrophil function.[47] Despite all of these data, pathology has not been associated with FXII deficiency, suggesting it could be a safe drug target. Since the existence and roles of potential adaptive mechanisms have not been described in

TABLE 1 Changes in coagulation parameters during anticoagulation and extracorporeal membrane oxygenator perfusion

	aPTT (sec)		ACT (sec)		PT (sec)	
Baseline (n = 6)	31.1 ± 0.6		209 ± 10		8.4 ± 0.2	
ECMO study	**Start (0 min)**	**End (60 min)**	**Start (0 min)**	**End (60 min)**	**Start (0 min)**	**End (60 min)**
Vehicle (n = 2)	30.2 ± 0.6	33.4 ± 0.2	201 ± 19	177 ± 20	8.2 ± 0.2	8.3 ± 0.6
Heparin (20 U/kg) (n = 4)	50.8 ± 3.3	34.4 ± 1.0	275 ± 15	191 ± 8	8.7 ± 0.2	8.7 ± 0.2
5C12 (5 mg/kg) (n = 6)	72.3 ± 7.2	78.3 ± 5.9	372 ± 12	395 ± 10	8.5 ± 0.1	8.6 ± 0.1
5C12 (5 mg/kg) +Heparin (20 U/kg) (n = 3)	124.5 ± 13.7	77.7 ± 3.4	473 ± 25	380 ± 14	9.0 ± 0.1	8.7 ± 0.2

aPTT, ACT, and PT values were determined in 3 baboons at baseline prior to treatment with heparin or 5C12 alone or in combination. Measurements were repeated immediately before (0 min) and following extracorporeal membrane oxygenator perfusion experiments (60 min). Data represents mean ± SEM.

Abbreviations: ACT, activated clotting time; aPTT, activated partial thromboplastin time; ECMO, extracorporeal membrane oxygenation; PT, prothrombin time.

FXII-deficient individuals, it is still not certain that pharmacological targeting of FXII would be entirely safe. Nevertheless, based on our and others' data and observations, it remains possible that pharmacological FXII inhibition is reasonably safe and well suited for antithrombotic and anti-inflammatory intervention (eg, in hereditary angioedema), even potentially in actively bleeding patients who need antithrombotic therapy.[13,48] Our data support the premise that FXII inhibition could be used in limited-term medical procedures where both platelet-dependent thrombus formation and bleeding are immediate risks. This may include ECOS systems, including hemodialysis, ECMO, left ventricular assist devices, or cardiopulmonary bypass.[49]

Based on epidemiological observations showing an association between low-normal FXII levels and cardiovascular disease, some investigators extrapolated from these data to suggest that severely reduced FXII activity (ie, FXII deficiency) could be associated with an increased risk of thrombosis.[50,51] However, the incidence, prognosis, or prevalence of cardiovascular disease in FXII deficiency is not established, and observational studies found no association between FXII levels and predisposition to thrombosis.[52,53] Deficiency of FXI, an FXIIa substrate, may cause a mild to moderate bleeding diathesis, while also reducing the risk of thrombosis.[54–56] FXI inhibitors are in clinical development,[13,57] with an FXI antisense oligonucleotide demonstrating improved outcomes in a phase 2 trial for venous thromboembolism prevention following knee replacement surgery.[58] Inhibition of either FXI or FXII with antisense oligonucleotides showed similar antithrombotic efficacy and safety in a rabbit model of catheter-induced thrombosis.[3] The development of FXII inhibitors is at an early stage. Some monoclonal anti-FXII/FXIIa antibodies have already been described, but none have reached human efficacy trials. Several decades ago, the monoclonal anti-FXII antibody C6B7 was found to reduce hypotension in a baboon model of sepsis but did not diminish signs of disseminated intravascular coagulation; however, this antibody only inhibited FXII activity approximately 60% in baboon plasma.[59] More recently, the anti-FXIIa monoclonal antibody 3F7 reduced fibrin clot accumulation in a rabbit ECMO model and is also being evaluated as a potential treatment for hereditary angioedema, as pathological FXIIa activity or reduced inhibition of FXIIa and kallikrein may be responsible for excessive kininogen cleavage and bradykinin generation.[27,28,60] Other approaches to target FXII include a synthetic peptide based on CTI,[61] anti-FXII nanobodies,[62] and nuclease-resistant FXII inhibitory RNA aptamers.[63] FXII antisense oligonucleotides have also been tested in animal studies.[3] Since antisense oligonucleotides require weeks to reach efficacy and produce sustained FXII deficiency,[58] they could well be useful for long-term thromboprophylaxis or prevention of other FXIIa-driven disease conditions.

The results of this study suggest that 5C12 forms an inhibitory complex with circulating FXII in vivo, providing effective reduction of platelet-dependent thrombus formation in an extracorporeal oxygenator device, without appreciable changes in the prothrombin or template BTs. Importantly, we propose that the combination of 5C12 with heparin would improve the antithrombotic efficacy of anticoagulation with heparin alone. The hemostatic safety of this drug combination remains to be further evaluated, as only low doses of heparin that do not cause BT prolongation were used in these experiments. Additional, longer-term perfusion studies that include pumps and other components of the ECMO circuit are needed to determine whether 5C12 administration could represent a useful stand-alone or adjuvant anticoagulant strategy for safely preventing thrombo-occlusive failure of cardiovascular devices, such as those used during ECMO.

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AUTHOR CONTRIBUTIONS

MW, AG, and EIT were responsible for the project concept and design. MW, JJ, and MTH executed and led the primate studies. CUL, MRC, CP, HHSL, and DG contributed in vitro data acquisition and analysis. MW performed data analysis and statistics for in vivo primate data. MW, OJTM, and AG drafted the manuscript. CUL, DG, and EIT critically reviewed and revised the manuscript.

RELATIONSHIP DISCLOSURE

MW, CUL, MRC, EIT, and AG are employees of Aronora, Inc., and they as well as OHSU may have a financial interest in the results of this study. The other authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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