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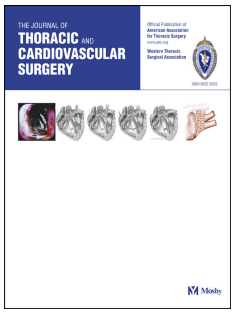

Factor XII promotes the thromboinflammatory response in a rat model of veno-arterial extracorporeal membrane oxygenation.

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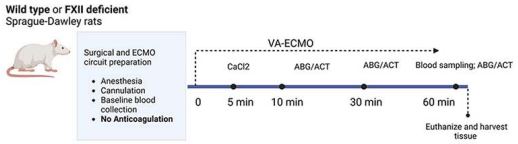
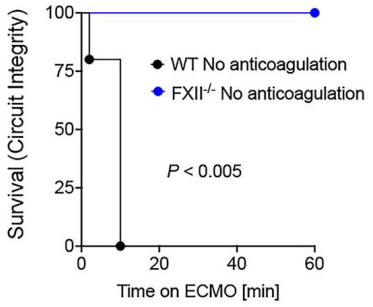
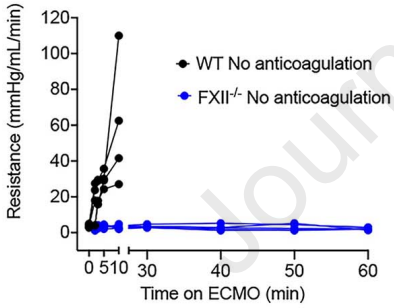
What is the role of factor XII in ECMO associated thromboinflammation?

THROMBOSIS







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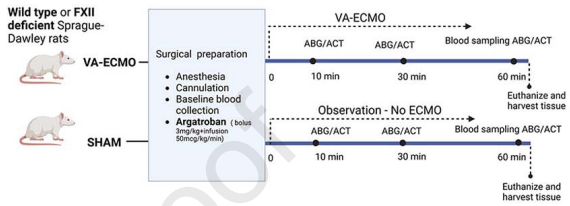

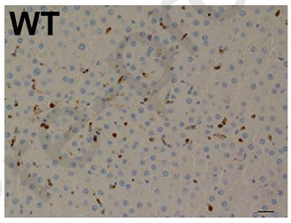

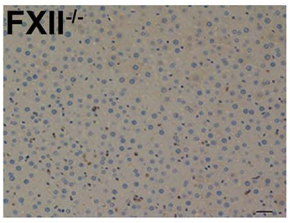

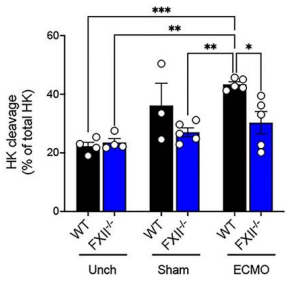

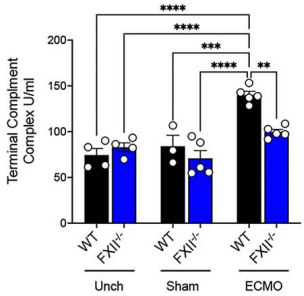

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Abbreviations: VA-ECMO - veno-arterial extracorporeal membrane oxygenation; FXII - factor XII; FXII$^{-/-}$ - factor XII deficient; WT - wild type; ABG - arterial blood gas; $CaCl_2$ - calcium chloride; HK - high molecular weight kininogen

Factor XII promotes the thromboinflammatory response in a rat model of veno-arterial extracorporeal membrane oxygenation.

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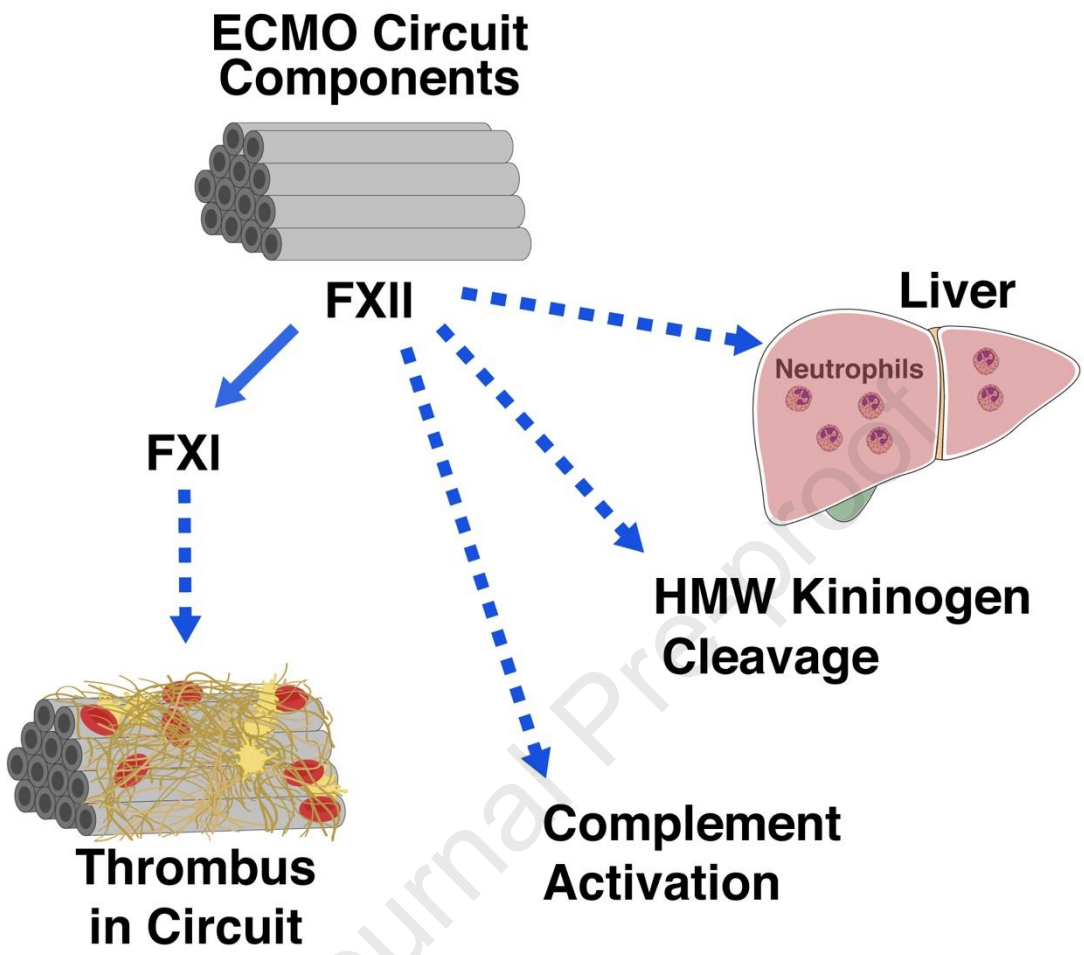
Glossary of Abbreviations:

CPB	Cardiopulmonary bypass
ECMO	Extracorporeal membrane oxygenation
MPO	Myeloperoxidase
uPAR	Urokinase plasminogen activator receptor
cHK	Cleaved high molecular weight kininogen

78 Central Picture



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80

81

82 **Central Message:** The present study suggests that targeting FXII in ECMO could have the dual
83 benefit of limiting thromboembolism, and inflammatory-mediated organ damage.

84

85 **Perspective Statement:** Previous studies have linked factor XII (FXII) to ECMO-induced
86 thrombosis. A model using novel gene-targeted FXII deficient rats on VA-ECMO demonstrated
87 FXII drives thrombosis, supports neutrophil migration into liver, and triggers HMW kininogen

cleavage and complement activation Future studies are needed to better elucidate these mechanisms and reveal additional FXII-dependent pathologies.

Abstract

Objective: Factor XII (FXII) is a multifunctional protease capable of activating thrombotic and inflammatory pathways. FXII has been linked to thrombosis in extracorporeal membrane oxygenation (ECMO), but the role of FXII in ECMO-induced inflammatory complications has not been studied. We used novel gene-targeted FXII deficient rats to evaluate the role of FXII in ECMO-induced thromboinflammation.

Methods: FXII deficient ($FXII^{-/-}$) Sprague-Dawley rats were generated using CRISPR/Cas9. We used a minimally invasive veno-arterial (VA) ECMO model to compare wild-type (WT) and $FXII^{-/-}$ rats in 2 separate experimental cohorts – rats placed on ECMO without pharmacological anticoagulation, and rats anticoagulated with argatroban. Rats were maintained on ECMO until circuit failure or 1 hour. Comparisons were made with unchallenged rats and rats that underwent a sham surgical procedure without ECMO.

Results: $FXII^{-/-}$ rats were maintained on ECMO without pharmacological anticoagulation with low resistance throughout a 1-hour experiment. In contrast, WT rats placed on ECMO without anticoagulation developed thrombotic circuit failure within 10 minutes. Argatroban provided a means to maintain WT and $FXII^{-/-}$ rats on ECMO for the 1-hour timeframe without thrombotic complications. Analyses of these rats demonstrated that ECMO resulted in an increase in neutrophil migration into the liver that was significantly blunted by FXII deficiency. ECMO also resulted in increases in high molecular weight kininogen cleavage and complement activation that were abrogated by genetic deletion of FXII.

Conclusion: FXII initiates hemostatic system activation and key inflammatory sequelae in ECMO, suggesting that therapies targeting FXII could limit both thromboembolism and inopportune inflammatory complications in this setting.

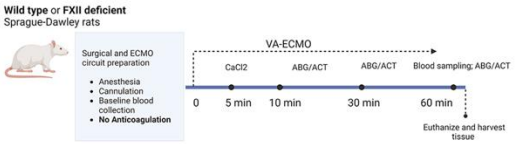
Word count: 250

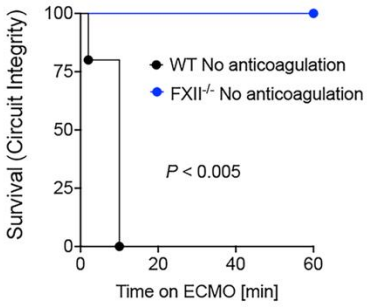
Keywords: Extracorporeal circulation, Factor XII, Neutrophil, Complement, High molecular weight cleavage, inflammation.

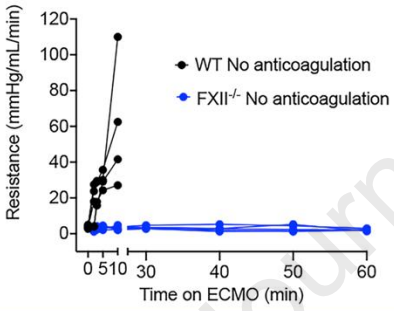
Graphical Abstract

What is the role of factor XII in ECMO associated thromboinflammation?

THROMBOSIS







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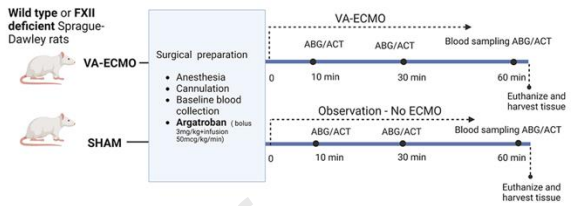

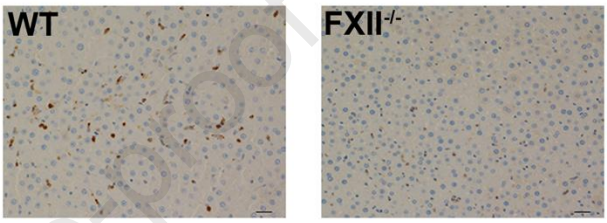

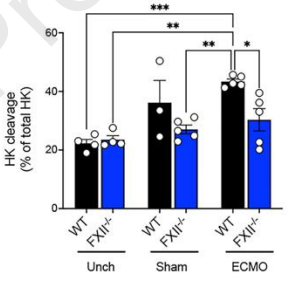

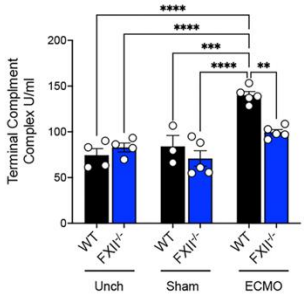

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INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) represents a life-saving treatment for patients with severe refractory cardiopulmonary failure. Thromboembolic complications, including thrombi in the circuit itself and embolic strokes, as well as bleeding complications are common in pediatric and adult ECMO.[1] ECMO is also complicated by a complex, rapid inflammatory response characterized by increased levels of circulating inflammatory cytokines, complement activation and leukocyte activation within hours of ECMO initiation.[2] However, the mechanisms driving the pathological immune responses in ECMO remain poorly defined. Although classically viewed separately, the hemostatic and inflammatory systems are highly integrated with extensive cross talk.[3] The coagulation contact protease factor XII (FXII) is particularly exciting in this context as it lies at the interface of a key nexus between the hemostatic and inflammatory systems. FXII-mediated activation of FXI ultimately results in thrombin generation. FXII proteolytic functions as well as FXII zymogen have been linked to a variety of immune functions, including neutrophil activation, complement activation, and regulation of vascular leak.[4, 5] Moreover, FXII plays no role in surgical hemostasis,[6] making it an attractive therapeutic target. Previous animal studies have linked FXII to ECMO-associated thrombosis,[7-9] but the role of FXII in ECMO-induced pro-inflammatory mechanisms has not been evaluated. In this study, we used novel gene-targeted FXII deficient rats ($FXII^{-/-}$) and a novel minimally invasive model of veno-arterial extracorporeal membrane oxygenation (VA-ECMO) to investigate the role of FXII in ECMO-induced thrombo-inflammation.

METHODS

Generation of gene-targeted FXII deficient rats.

The CRISPR/Cas9 system was used to target the FXII gene (*f12*) in Sprague Dawley rats. Founder animals were interbred with wildtype Sprague Dawley rats purchased from Charles River to generate rats heterozygous for the FXII mutation ($FXII^{+/-}$). $FXII^{+/-}$ rats were interbred generating $FXII^{+/+}$ (WT), $FXII^{+/-}$ and $FXII^{-/-}$ animals. See Supplement for details.

Rat ECMO model.

Adult (ages 8 – 10 weeks) male WT and $FXII^{-/-}$ Sprague-Dawley rats weighing ~300-600 g were used as ECMO recipients. A rat of the same genotype was used as a blood donor to prime the circuit. All animal protocols were approved by Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee.

Briefly, rats were anaesthetized with isoflurane, intubated orotracheally and connected to a ventilator. The right external jugular vein and left common carotid artery were cannulated and connected to a peristaltic pump (Harvard Peristaltic Pump P-230, Harvard Apparatus Inc.) and an oxygenator with a priming volume of 1.6 ml (MICRO-1 oxygenator-Dongguan Kewei Medical Instrument Co) (Fig. 2A). The left femoral artery was cannulated for invasive blood pressure monitoring. The oxygenator inlet and outlet pressures were monitored via pressure transducers and used to calculate resistance by dividing the inlet outlet pressure difference by the flow rate. Circuit dysfunction was considered when blood flow resistance reached three times it's baseline resistance with impairment of gas exchange.

Rats were enrolled onto ECMO in two separate experimental cohorts. The first consisted of WT and FXII$^{-/-}$ rats (n = 5 per genotype) placed on ECMO with no pharmacological anticoagulation. The second group of WT and FXII$^{-/-}$ rats (n = 5 per genotype) was placed on ECMO with argatroban administration. We chose argatroban (Exela Pharma Sciences, LCC, USA) as this short-acting thrombin inhibitor has no effect on FXII activity. Argatroban dosing was determined by pilot studies performed in WT rats. We choose an argatroban dosing schema that resulted in an activated clotting time of > 350 seconds prior to ECMO initiation and provided sufficient anticoagulation to maintain WT rats on ECMO for the predetermined 1 hour timeframe without complications. A continuous infusion of argatroban (1mg/mL) was initiated through a femoral vein catheter at 50 mcg/kg/min starting 30 minutes prior to ECMO initiation. This was followed by administration of bolus of argatroban (3 mg/kg) just before ECMO initiation.

Once cannulated, the ECMO recipient received either no anticoagulation or argatroban anticoagulation as described above. The circuit was primed with 7 ml of whole blood from a rat of the same genotype containing sodium citrate 3.2% (blood:citrate ratio 9:1). ECMO was initiated and flow rate increased to 60-80 mL/kg/min. Once the circuit was running for ~5 minutes, calcium chloride was administered to normalize the calcium, reversing citrate anticoagulation. Animals were euthanized at the end and harvested immediately. The sham surgery consisted of intubation, surgical cannulation, and argatroban administration for the same timeframes as the rats undergoing ECMO, with the exclusion of ECMO. See Supplement for details.

Statistics

Statistical analyses were performed using GraphPad Prism software v.9.

Refer to the Supplement for details regarding the following assays: thrombin generation assay, activated partial thromboplastin time, quantitation of FXII mRNA and protein, FXII activity, plasma fibrinogen, histological analyses, measurement of cleaved HMW kininogen, and analyses of complement activation.

RESULTS

Genetic deletion of factor XII limits thrombus formation and blood flow resistance in ECMO.

To define the role of FXII in ECMO-associated pathologies, we generated FXII-deficient Sprague Dawley rats using the CRISPR/Cas9 system that carry a premature stop codon in exon 4 the of *F12* gene (Fig. 1A). Paralleling FXII-deficient mice,[10, 11] rats homozygous for this mutation ($FXII^{-/-}$) are viable, appear healthy, and exhibit normal fecundity (data not shown). Liver-derived FXII mRNA was below the detection level of the assay in $FXII^{-/-}$ rats (Fig. 1B). ELISA-based quantitation of plasma FXII protein in $FXII^{-/-}$ rats was ~1% of that in FXII sufficient rats ($FXII^{+/+}$) (Fig. 1C). Whether this small amount of protein represents a truncated amino acid fragment of FXII or rare readthrough events of the premature stop codon remains to be determined. $FXII^{-/-}$ rats exhibited significantly prolonged aPTTs (Fig. 1D), and plasma FXII activity that was below the lower limit of detection based on a 1-stage clotting assay (Fig. 1E). Consistent with previous studies showing that FXII plays no role in tissue factor (TF) initiated hemostasis, analyses of TF-initiated thrombin generation in plasma from $FXII^{-/-}$ rats was indistinguishable from that in plasma from $FXII^{+/+}$ rats (Fig. 1F). CBCs and plasma fibrinogen levels were also similar in $FXII^{-/-}$ rats and controls (Supplemental Fig. 1)

To define the role of FXII in ECMO-related thrombosis, we placed 5 $FXII^{-/-}$ and 5 $FXII^{+/+}$ (hereafter referred to as WT) rats on VA-ECMO without any anticoagulation for a predetermined 1-hour timeframe. See Figure 2A&B for details of the ECMO circuit and protocol. All 5 WT rats experienced circuit failure with 10 minutes of ECMO initiation (Fig. 3A), as defined by an exponential increase in the blood flow resistance across the oxygenator (Fig. 3B). Consistent with this there was substantial visible clot burden at the oxygenator inlets. In contrast, FXII deficient rats all maintained low resistance flow without pharmacological anticoagulation (< 5mmHg/(mL/min) during the 1-hour ECMO timeframe.

The rapid circuit failure observed in WT rats on ECMO in the absence of pharmacological anticoagulation made meaningful comparisons between genotypes impossible. To address this, we pursued a separate experiment where 5 WT and 5 $FXII^{-/-}$ rats were placed on ECMO following the same protocol with the addition of argatroban. We also performed "sham" procedures on 3 WT and 5 $FXII^{-/-}$ rats that included intubation, cannulation and argatroban administration for the same timeframe as the ECMO cohort. All 10 rats placed on ECMO were maintained for the 1 hour timeframe without complications. Hemodynamic and metabolic parameters, including mean arterial pressure, heart rate, PaO_2, $PaCO_2$, pH and lactate remained stable throughout the 1-hour ECMO run and were similar between genotypes (Table 1). Blood flow resistance during ECMO was overall low in both genotypes anticoagulated with argatroban, but was lower in the $FXII^{-/-}$ rats relative to the WT cohort (Fig. 3C). Consistent with clinical observations,[1] CBCs obtained at the end of ECMO showed an ~50% diminution in platelet count relative to that in unchallenged rats that was similar between genotypes. No diminution in platelet count was observed in the sham cohort (Fig. 3D). Neither ECMO nor the sham surgery resulted in a significant change in plasma fibrinogen relative to unchallenged rats (Fig. 3D).

FXII promotes neutrophil margination/migration into liver tissue in ECMO

Following completion of ECMO, we performed analyses of the oxygenators as it is well established that leukocytes quickly adhere to the oxygenator membrane. The oxygenators were flushed using a non-enzymatic cell dissociation buffer to remove adherent cells. The isolated cells were enumerated, and a portion was analyzed by cytospins stained with Wright Giemsa. We obtained ~10 – 20 million cells from each oxygenator, and the number of adherent cells was similar between genotypes (Fig. 4A). Microscopic review of the cytospins revealed that most of the adherent cells (~95%) were neutrophils, regardless of rat genotype (Fig. 4B).

We harvested multiple organs from the argatroban anticoagulated $FXII^{-/-}$ and WT rats at the end of the ECMO and sham experiments. H&E-stained histological sections of lungs, livers and kidneys were reviewed with a pathologist blinded to animal genotype. Organs from both genotypes subjected to ECMO showed evidence of mild venous congestion, but no evidence of significant organ damage (Supplemental Fig. 2). We immunohistochemically stained tissues for myeloperoxidase (MPO), a marker of neutrophils MPO+ cells in the livers and kidneys were enumerated in 6 - 8 20X fields per tissue section by an investigator blinded to animal genotype. MPO+ pixels in the lungs were quantified using Aperios eSlide software as a function of the total tissue stained. MPO staining cells were few in the liver, lung and kidney tissues of the sham and unchallenged rats and similar between genotypes. ECMO resulted in a significant increase in MPO staining cells throughout the liver sinusoids that was significantly diminished by FXII deficiency (Fig. 5). ECMO also resulted in an increase in MPO+ staining cells in the kidneys and lungs, but

in contrast to the liver, deletion of FXII did not have a significant impact on this relative increase (Supplemental Fig. 3).

FXII is a major determinant of high molecular weight (HMW) kininogen cleavage and complement activation in ECMO.

We measured plasma levels of cytokines known to be altered in ECMO.[2] ECMO resulted in significant increases in the proinflammatory cytokines IL-6 and TNFα relative to unchallenged rats independently of FXII (Supplemental Fig. 4). IL-6 and TNFα levels tended to higher in ECMO-challenged rats relative to shams, but this did not reach statistical significance. The anti-inflammatory cytokine IL-10 was also increased in ECMO rats relative to unchallenged animals and WT shams with no FXII dependence. These data suggest that FXII is not a significant determinant of the generation of these cytokines in the context of ECMO.

Increased circulating bradykinin, a cleavage product of HMW kininogen, has been reported in humans undergoing cardiopulmonary bypass,[12] but there are limited studies of this peptide in VA-ECMO**.** To evaluate the impact of ECMO and FXII on HMW kininogen cleavage, a biomarker of bradykinin generation, we measured the intact (HK) and cleaved (cHK) species of HMW kininogen in plasma by Western blot using an antibody that recognizes both (Fig. 6A). The proportion of cHK was relatively low in unchallenged rats and similar between FXII genotypes (Fig. 6B&C). ECMO resulted in a significant decrease in HK and an increase in the ratio of cHK/HK in WT rats relative to unchallenged animals. This ECMO-dependent increase was significantly attenuated in $FXII^{-/-}$ rats (Fig. 6B&C). There was a trend toward increased cHK in WT rats after sham surgery relative to unchallenged animals, but there was no statistically significant difference between WT rats in the sham and ECMO cohorts. These data indicate that

FXII is a major driver of HMW kininogen cleavage in ECMO. They also suggest that the surgical procedure and catheterization may contribute to HMW kininogen cleavage in addition to ECMO in this context.

Previous studies have shown that ECMO is associate with rapid complement activation, which is thought to be caused by both the underlying disease processes and by blood contact with the ECMO circuit. We measured circulating markers of complement activation products; Terminal Complement Complex (TCC) and C3 fragments (C3b/iC3b/C3c) in unchallenged rats, and rats underwent the sham and ECMO procedures. Both complement activation markers were similarly low in WT and FXII$^{-/-}$ rats from the unchallenged and sham cohorts. ECMO resulted in a rapid increase in circulating markers of C3 activation (C3b/iC3b/C3c) and the TCC (Fig. 6D&E), suggesting that these sequelae are predominantly caused by the ECMO circuit and not the surgical procedure itself. Importantly, FXII deficiency essentially abrogated the ECMO-induced increase in C3b/iC3b/C3c and TCC.

DISCUSSION

These studies represent the first to evaluate the role of FXII in thrombotic and inflammatory pathologies related to ECMO. The genetic deletion of FXII resulted in a major prolongation in ECMO circuit life in the absence of pharmacological anticoagulation. Moreover, FXII$^{-/-}$ rats anticoagulated with argatroban had significantly lower blood flow resistance values relative to WT rats similarly anticoagulated, suggesting that limiting FXII could further enhance ECMO circuit function when combined with traditional anticoagulation approaches. FXII was also shown to be a major factor promoting neutrophil margination/migration into the liver in ECMO. The data also

307 indicate that FXII plays a key role in driving HMW kininogen cleavage and complement activation
308 in ECMO. See Figure 7 for a graphical summary.
309
310 Our data are consistent with previous studies in rabbit models of ECMO showing that an antibody
311 targeting FXII, a peptide FXII inhibitor, and an mRNA approach that limits hepatic FXII synthesis
312 prolong ECMO circuit lifespan and limit thromboembolism.[7-9] Although the use of protein and
313 heparin covalent bonded circuit components have limited the need for anticoagulation in certain
314 circumstances, thromboembolism remains a major problem during ECMO, particularly in infants
315 and young children where thrombotic complications occur in up to 37.5% of patients.[13] The
316 antithrombotic efficacy of targeting FXII in ECMO remains to be determined in clinical settings,
317 but given that FXII plays no role in surgical hemostasis, any improvement in antithrombotic
318 efficacy that comes with targeting FXII would carry no bleeding risk. Moreover, our data indicate
319 that targeting FXII could come with the dual benefit of limiting ECMO-associated inflammatory
320 sequelae.
321
322
323 It is notable that all the preclinical studies of FXII in ECMO, including the studies presented here,
324 were carried out in healthy animals. It remains to be determined how effective targeting FXII
325 would be in preventing mechanical circulation associated thromboembolism when there are
326 additional initiators of thrombin generation other than the ECMO circuit. Patients requiring ECMO
327 are often critically ill, and factors such as circulating TF-expressing extracellular vesicles, cell free
328 DNA, and endothelial cell activation are all potential contributors to the prothrombotic state
329 present in these patients.[14] While the mechanical circuit used in cardiopulmonary bypass is similar

to an ECMO circuit, the sternotomy and manipulation of cardiac tissue are likely to result in significant release of circulating TF.[15] In these contexts, targeting FXII alone may be insufficient to prevent thromboembolism. Future studies in animal models where inflammatory and/or additional surgical challenges are imposed are needed. The data presented here showing that FXII deficiency together with argatroban lowered blood flow resistance more than argatroban alone suggest that targeting FXII could augment the antithrombotic efficacy of standard anticoagulation approaches. Moreover, the anti-inflammatory mechanisms associated with targeting FXII could also limit thrombotic complications by limiting downstream immune-mediated hemostatic system activation. For example, both bradykinin generation and complement activation are associated with vascular changes known to promote thromboembolism.[16, 17]

ECMO resulted in a significant increase in neutrophil margination/migration into the liver, lung, and kidneys. FXII significantly attenuated this phenomenon in the liver, but not in the kidneys or lungs. It is unclear why the role of FXII in ECMO-induced accumulation of neutrophils is most prominent in the liver. The mechanisms promoting neutrophil migration are likely tissue specific, with the key receptors promoting neutrophil migration being distinct between different organs.[18] The selectin dependent mechanisms that primarily regulate neutrophil adhesion and migration into the lung and kidney appear to be less important in liver.[18] An intriguing observation that might offer a clue is the finding that hyaluronan is disproportionately expressed in the liver versus other organs under both basal and inflammatory conditions.[18] These studies suggested that hyaluronan promotes neutrophil migration via interaction with CD44. Interestingly, previous studies have also suggested a link between hyaluronan/CD44 interactions and upregulation of uPAR in other contexts.[19] FXII zymogen has been shown to promote neutrophil migration by binding to uPAR.[20]

Together, these data suggest the intriguing hypothesis that the relatively hyaluronan rich environment of the liver may upregulate neutrophil uPAR expression, making them more sensitive to FXII zymogen activation.

A significant increase in HMW kininogen cleavage, a marker of bradykinin generation, was observed in our rat model of ECMO. ECMO-induced HMW kininogen cleavage was essentially abrogated by deletion of FXII. Clinical studies have shown that cardiopulmonary bypass (CPB) results in increased plasma bradykinin,[12] but this has not been specifically studied in ECMO. Given that the mechanical circuits used in ECMO and CPB are similar, it would seem likely that bradykinin generation is a factor in both. Consistent with the relatively short ECMO timeframe, we did not see any evidence of vascular leak or organ damage in our histological analyses. Previous studies from our group suggested that limiting FXII expression in a 4 hour long rabbit model of ECMO results in less pulmonary edema.[9] Based on the data presented here, it is conceivable that FXII-mediated bradykinin generation may promote tissue edema in ECMO. Notably, circulation in complete VA-ECMO largely bypasses the pulmonary arterial system, which is responsible for inactivation of the majority of circulating bradykinin.[21] Whether FXII plays any role in bradykinin clearance remains to be determined. Future studies of FXII-deficient rats in ECMO that span longer timeframes will better elucidate these mechanisms.

Previous clinical studies have shown that ECMO results in rapid complement activation that peaks within 1 – 2 hours.[2] This was recapitulated in our rat model and was entirely driven by FXII. FXII could drive complement activation in this context through multiple non-mutually exclusive mechanisms. FXII is a multifunctional protease that can proteolytically activate FXI, leading to

thrombin generation. FXII also activates prekallikrein to the active protease, kallikrein. Kallikrein has been shown to activate the complement component C3.[4] FXII has also been shown to interact with the globular C1q receptor and the complement regulators C1INH and FH.[22-24] Recently, FXI was also shown to neutralize the complement regulator, factor H[25]. Future mechanistic studies can readily be envisioned where genetic or pharmacological targeting of FXI and/or prekallikrein are combined with FXII deficiency to better define the role of these pathways in ECMO related complement activation and other pathologies.

It is notable that ECMO resulted in a significant increase in tissue neutrophil infiltration and complement activation compared to shams. In contrast, proinflammatory cytokine elaboration and cleavage of HK were clearly increased in ECMO, but they were not statically significant compared to the sham group. This suggests that vascular access manipulation and catheter insertion may play a role in these specific inflammatory responses. These data highlight the fact that multiple factors can influence the inflammatory response to ECMO, including surgical tissue injury, circuit design, duration of support, and the catheterization materials and techniques used.

In this study we evaluated the impact of genetic FXII deletion on ECMO-induced inflammatory responses. It remains to be determined if targeting FXII clinically could replicate these findings. As noted, FXII proteolytic functions can activate FXI, cleave HMW kininogen and activate prekallikrein. Additionally, FXII zymogen has been shown to promote neutrophil activation through engagement of uPAR.[20] Conceivably, antibodies that clear FXII and mRNA-based approaches limiting FXII production could target all these mechanisms. FXII is made by hepatocytes and neutrophils, it is unclear to what extent emerging antibody therapies and ASO

399 approaches would limit neutrophil-derived FXII. Comparison of these modalities together with
400 genetic deletion of FXII and emerging small molecule inhibitors of FXII proteolytic function could
401 be mechanistically informative.
402
403 It should be noted that the small amount of FXII protein detected by ELISA in $FXII^{-/-}$ rats could
404 represent a truncated FXII protein with no function, or rare readthrough events of the premature
405 stop codon resulting in small amounts of full length FXII. It would seem unlikely that such small
406 amounts of FXII would significantly contribute to thrombin generation. However, it is conceivable
407 that even small amounts of zymogen FXII could significantly impact neutrophil functions.
408 Analyses in rats with a large deletion of the FXII gene rather than a premature stop codon could
409 be informative.
410
411 The potential for FXII to limit multiple inflammatory pathways in ECMO raises the question of
412 whether targeting FXII could increase infection risk or impair wound healing. Recent studies
413 suggest that FXII evolved, at least in part, to deal with soil contamination of wounds.[26] Here FXII
414 has been proposed to bridge activation of hemostasis and inflammatory pathways, leading to
415 maintenance of vascular integrity and pathogen clearance. Interestingly, studies in mice showed
416 that FXII deletion had no impact on survival following systemic infection with *Streptococcus*
417 *pneumoniae*, and FXII deletion improved survival following infection with *Klebsiella*
418 *pneumoniae*.[27] Furthermore, FXII deletion in mice resulted in impaired migration of neutrophils
419 into skin wounds, but deletion of FXII resulted in more rapid wound closure.[20] Taken together, the
420 available data suggest that targeting FXII in ECMO would not impair wound healing or

fundamentally impair the response to bacterial infection, but additional preclinical studies and clinical trials are needed.

In summary, the data presented demonstrate, for the first time, that FXII plays a multifaceted role in ECMO, promoting both thrombotic complications that limit circuit life, and deleterious inflammatory events capable of damaging end organs. The current study suggests that pharmacological targeting of FXII in ECMO could have the dual benefit of limiting thromboembolism without incurring any bleeding risk and reducing inflammatory-mediated organ damage.

REFERNCES CITED

1. Thomas J, Kostousov V, Teruya J. Bleeding and Thrombotic Complications in the Use of Extracorporeal Membrane Oxygenation. *Semin Thromb Hemost.* 2018;44:20-29.

2. Al-Fares A, Pettenuzzo T, Del Sorbo L. Extracorporeal life support and systemic inflammation. *Intensive Care Med Exp.* 2019;7:46.

3. Foley JH, Conway EM. Cross Talk Pathways Between Coagulation and Inflammation. *Circ Res.* 2016;118:1392-1408.

4. Renne T, Stavrou EX. Roles of Factor XII in Innate Immunity. *Front Immunol.* 2019;10:2011.

5. Renne T. The procoagulant and proinflammatory plasma contact system. *Semin Immunopathol.* 2012;34:31-41.

6. Tillman BF, Gruber A, McCarty OJT, Gailani D. Plasma contact factors as therapeutic targets. *Blood Rev.* 2018;32:433-448.

7. Larsson M, Rayzman V, Nolte MW, et al. A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk. *Sci Transl Med.* 2014;6:222ra217.

8. Wilbs J, Kong XD, Middendorp SJ, et al. Cyclic peptide FXII inhibitor provides safe anticoagulation in a thrombosis model and in artificial lungs. *Nat Commun.* 2020;11:3890.

9. Tweddell JS, Kharnaf M, Zafar F, et al. Targeting the contact system in a rabbit model of extracorporeal membrane oxygenation. *Blood Adv.* 2023;7:1404-1417.

10. Renne T, Pozgajova M, Gruner S, et al. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med.* 2005;202:271-281.

11. Kleinschnitz C, Stoll G, Bendszus M, et al. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J Exp Med.* 2006;203:513-518.

12. Cugno M, Nussberger J, Biglioli P, Giovagnoni MG, Gardinali M, Agostoni A. Cardiopulmonary bypass increases plasma bradykinin concentrations. *Immunopharmacology.* 1999;43:145-147.

13. Dalton HJ, Reeder R, Garcia-Filion P, et al. Factors Associated with Bleeding and Thrombosis in Children Receiving Extracorporeal Membrane Oxygenation. *American journal of respiratory and critical care medicine.* 2017;196:762-771.

14. Murphy DA, Hockings LE, Andrews RK, et al. Extracorporeal membrane oxygenation-hemostatic complications. *Transfus Med Rev.* 2015;29:90-101.

15. Edmunds LH, Jr., Colman RW. Thrombin during cardiopulmonary bypass. *Ann Thorac Surg.* 2006;82:2315-2322.

16. Shariat-Madar Z, Mahdi F, Warnock M, et al. Bradykinin B2 receptor knockout mice are protected from thrombosis by increased nitric oxide and prostacyclin. *Blood.* 2006;108:192-199.

17. Subramaniam S, Jurk K, Hobohm L, et al. Distinct contributions of complement factors to platelet activation and fibrin formation in venous thrombus development. *Blood.* 2017;129:2291-2302.

18. Maas SL, Soehnlein O, Viola JR. Organ-Specific Mechanisms of Transendothelial Neutrophil Migration in the Lung, Liver, Kidney, and Aorta. *Front Immunol.* 2018;9:2739.

19. Kobayashi H, Suzuki M, Kanayama N, Nishida T, Takigawa M, Terao T. CD44 stimulation by fragmented hyaluronic acid induces upregulation of urokinase-type plasminogen activator and its receptor and subsequently facilitates invasion of human chondrosarcoma cells. *Int J Cancer.* 2002;102:379-389.

20. Stavrou EX, Fang C, Bane KL, et al. Factor XII and uPAR upregulate neutrophil functions to influence wound healing. *J Clin Invest.* 2018;128:944-959.

21. Campbell DJ. Neprilysin Inhibitors and Bradykinin. *Front Med (Lausanne).* 2018;5:257.

22. Luo S, Hu D, Wang M, Zipfel PF, Hu Y. Complement in Hemolysis- and Thrombosis-Related Diseases. *Front Immunol.* 2020;11:1212.

23. Didiasova M, Wujak L, Schaefer L, Wygrecka M. Factor XII in coagulation, inflammation and beyond. *Cell Signal.* 2018;51:257-265.

24. Thangaraj SS, Christiansen SH, Graversen JH, et al. Contact activation-induced complex formation between complement factor H and coagulation factor XIIa. *J Thromb Haemost.* 2020;18:876-884.

25. Puy C, Pang J, Reitsma SE, et al. Cross-Talk between the Complement Pathway and the Contact Activation System of Coagulation: Activated Factor XI Neutralizes Complement Factor H. *J Immunol.* 2021;206:1784-1792.

26. Juang LJ, Mazinani N, Novakowski SK, et al. Coagulation factor XII contributes to hemostasis when activated by soil in wounds. *Blood Adv.* 2020;4:1737-1745.

27. Stroo I, Zeerleder S, Ding C, et al. Coagulation factor XI improves host defence during murine pneumonia-derived sepsis independent of factor XII activation. *Thromb Haemost.* 2017;117:1601-1614.

FIGURE LEGENDS

Figure 1. Generation and characterization of gene-targeted FXII deficient rats. A) Shown are the nucleotide and amino acid sequences of the wild-type (WT) and mutant FXII allele. Note that the mutant animals carry a premature stop codon in exon 4 (*) of the *f12* gene. Shown are quantitation of liver mRNA expression normalized to $FXII^{+/+}$ (WT) rats (B), and ELISA quantitation of plasma FXII protein (C). The aPTT was significantly prolonged in $FXII^{-/-}$ rats (D) and FXII activity based on a one-stage clotting assay was below the lower limit of detection (E). F) Thrombin generation assays initiated by 1 pM tissue factor were similar between genotypes. (Data represents mean ± SEM.) $FXII^{-/-}$ FXII deficient, $FXII^{+/+}$ FXII sufficient, $FXII^{+/-}$ FXII Heterozygous mutants.

Figure 2. Description of rat VA-EMCO model. A) Shown is a typical example of a rat on the VA-ECMO circuit. Note the venous line (1), peristaltic pump (2), oxygenator (3), arterial line (4), femoral arterial line used for blood pressure monitoring (5), and the pre- and post-pressure transducers (*). Also shown are summaries of the timelines for the two experimental paradigms used. The first (B) compared WT and $FXII^{-/-}$ rats without anticoagulation The second (C) compared WT and $FXII^{-/-}$ rats anticoagulated with argatroban. $FXII^{-/-}$-FXII deficient, WT-Wild type, $CaCl_2$-Calcium Chloride, ABG-Arterial blood gases.

Figure 3. FXII promotes thrombosis in ECMO. A) FXII-deficiency significantly prolonged circuit life compared to rats with normal FXII expression (WT) in ECMO without any pharmacological anticoagulation. B) Circuit failure in WT rats was preceded by a rapid exponential

rise in blood flow resistance across the oxygenator. C) Shown is the blood flow resistance across the oxygenator from a cohort of 5 WT and 5 $FXII^{-/-}$ rats placed on ECMO with argatroban anticoagulation. Note that blood flow resistance was relatively low and stable in both cohorts, but significantly lower in the $FXII^{-/-}$ rats. D) Platelet counts and fibrinogen were measured at the end of the 1 hour ECMO and sham procedures in the argatroban anticoagulated cohort. The dotted line denotes the mean obtained from analyses of unchallenged rats. Note that ECMO, but not the sham surgery, resulted in an ~50% diminution in circulating platelet count that was similar between genotypes. WT-Wild type, $FXII^{-/-}$ FXII deficient.

Figure 4. FXII is not a determinant of early leukocyte adhesion to the oxygenator in ECMO. A) Data represent the quantitation of total leukocytes adherent to the oxygenators. B) Shown are representative images of cytospins of leukocytes adherent to the oxygenators from WT and $FXII^{-/-}$ rats harvested at the end of the ECMO run. Note that the majority of the leukocytes (~95%) were neutrophils. Data shown represent mean ± SEM. WT-Wild type, $FXII^{-/-}$ FXII deficient.

Figure 5. FXII promotes neutrophil margination/migration into liver tissue in ECMO. Shown are representative photomicrographs of myeloperoxidase-stained (MPO) liver sections from WT and $FXII^{-/-}$ rats that were unchallenged or harvested following sham or ECMO experiments. Note that ECMO but not sham surgery resulted in a significant increase in MPO+ staining cells (brown). Deletion of FXII significantly attenuated MPO+ cells (a marker for neutrophils) into the liver. Size bars represent 25 μm. Quantitative data are shown to the right of the photomicrographs. (Data shown represent mean ± SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. All comparisons were

545 analyed, but only statistically significant P values are shown. P values were generated using a one-
546 way Anova, multiple comparisons.) WT-Wild type, $FXII^{-/-}$ FXII deficient.
547
548 **Figure 6. FXII promotes HMW kininogen cleavage and complement activation in ECMO.**
549 A) Intact (HK) and cleaved (cHK) HMW kininogen were quantitated using a Western blot with an
550 antibody that recognizes both species of the protein. Shown are quantitation of intact HK (B)
551 (expressed as a percentage of HK in WT unchallenged rats) and (C) the percentage of cleaved HK
552 relative to total HK (intact HK and cleaved HK combined). Note that ECMO resulted in a
553 significant increase in HMW kininogen cleavage relative to unchallenged rats that was attenuated
554 by FXII deficiency. Sham surgery did not result in a significant increase in HK cleavage relative
555 to unchallenged rats. Markers of complement activation were measured in plasma from
556 unchallenged rats and rats following 1 hour of ECMO or sham surgery. Shown are analyses of C3
557 fragments C3b/iC3b/C3c (D) and Terminal Complement Complex (TCC) (E). ECMO resulted in
558 a significant increase in both markers of complement activation that was not observed in animals
559 following sham surgery. FXII deletion completely abrogated the increase in complement activation
560 associated with ECMO. (Data shown represent mean ± SEM. * $P < 0.05$, ** $P < 0.05$, *** $P <$
561 0.005, **** $P < 0.001$. All comparisons were analyzed, but only statistically significant P values
562 are shown. P values were generated using a one-way Anova, multiple comparisons). $FXII^{-/-}$-FXII
563 deficient, WT-Wild type, HK-Intact high molecular weight kininogen, cHK-cleaved high
564 molecular weight kininogen.
565
566 Figure 7. Graphical Abstract.
567

568 **SUPPLEMENTARY FIGURE LEGENDS**

569

570 **SUPPLEMENTARY FIGURE 1. Complete blood counts and plasma fibrinogen levels in**
571 **FXII-deficient rats.** The circulating white blood cells count (A), hemoglobin (B), platelet count
572 (C), and plasma fibrinogen levels were similar in FXII-sufficient and -deficient rats. Note that
573 there were no statistically significant differences in any comparisons. Data shown represent mean
574 ± SEM. $FXII^{-/-}$ FXII deficient, $FXII^{+/+}$ FXII sufficient, $FXII^{+/-}$ FXII Heterozygous mutants.

575

576 **SUPPLEMENTARY FIGURE 2. Histological analyses of lung, liver and kidney following**
577 **ECMO.** Shown are representative photo micrographs of lung, liver and kidney tissue harvested
578 from WT and $FXII^{-/-}$ immediately following sham and ECMO. There was no significant pathology
579 in any of the organs analyzed. There was evidence of modest venous congestion in organs from
580 EMCO challenged rats, but no evidence of organ damage or significant cell death. Size bars
581 represent 50 μm. WT-Wild type, $FXII^{-/-}$ FXII deficient.

582

583 **SUPPLEMENTARY FIGURE 3. Myeloperoxidase staining of lung and kidney.** Shown are
584 representative photomicrographs of myeloperoxidase-stained (MPO) tissue sections of kidney and
585 lung harvested from unchallenged WT and $FXII^{-/-}$ rats as well as rats following sham or ECMO
586 experiments. ECMO resulted in a significant increase in MPO+ staining cells (brown staining) in
587 both organs relative to unchallenged and sham surgical rats, but there was no FXII genotype
588 dependence. Size bars represent 25 μm. Quantitative data are shown to the right of the
589 photomicrographs. (Data shown represent mean ± SEM. * $P < 0.05$, ** $P < 0.005$. P values were

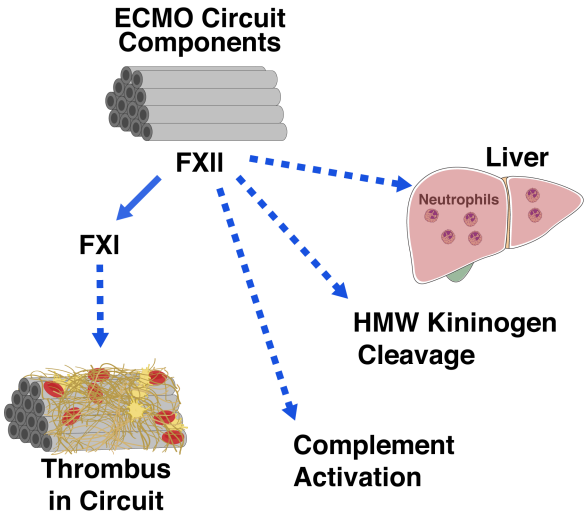
generated using a one-way Anova, multiple comparisons. All comparisons were analyzed, but only statistically significant P values are shown.) WT-Wild type, $FXII^{-/-}$ FXII deficient.

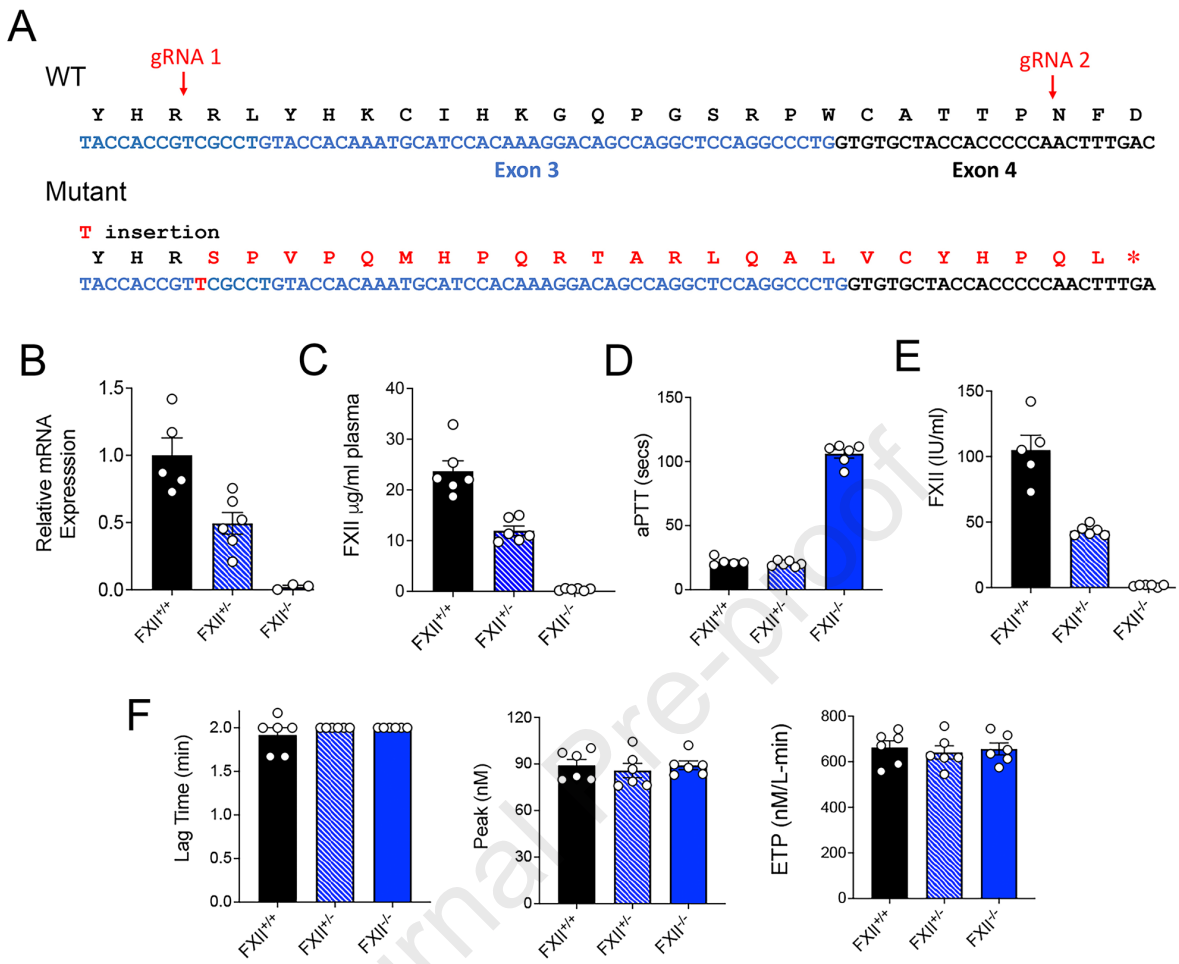
SUPPLEMENTARY FIGURE 4. Plasma cytokine measurements. Shown are quantitations of plasma cytokines in unchallenged WT and $FXII^{-/-}$ rats as well as rats following 1 hour sham or ECMO experiments. There were no genotype dependent differences in cytokine levels. (Data shown represent mean ± SEM. * $P < 0.05$, ** $P < 0.005$. P values were generated using a one-way Anova, multiple comparisons. All comparisons were analyzed, but only statistically significant P values are shown.) WT-Wild type, $FXII^{-/-}$ FXII deficient.

Table 1. Hemodynamic and gas exchange variables. Shown are measures of MAP (mean arterial blood pressure), HR (heart rate), PaO_2 (partial pressure of oxygen), $PaCO_2$ (partial pressure of carbon dioxide), pH, and lactate measured prior to (baseline) and during sham surgeries and VA-ECMO (veno-arterial extracorporeal membrane oxygenation) experiments. Note that all rats in these analyses were anticoagulated with argatroban. (Data represent mean ± SD).

	WT						FXII$^{-/-}$					
	Sham			VA-ECMO			Sham			VA-ECMO		
Time Point	**Baseline**	**30 mins**	**60 mins**	**Baseline**	**30 mins**	**60 mins**	**Baseline**	**30 mins**	**60 mins**	**Baseline**	**30 mins**	**60 mins**
MAP (mm Hg)	66.5± 18	64.95± 14	59.05± 16	69.96± 21	99.9± 20	93.34± 20	67.6± 16	60.9± 14	53.7± 5	58.74± 10	108.2± 2	84.68± 2
HR (beat/min)	327.6± 31	338± 29	340± 27	327.5± 40	321± 17	330.8± 16	321± 45	326± 36	329± 25	323.4± 55	355± 21	345.2± 1
PaO2 (mm Hg)	297± 71	308± 91	272± 53	309± 44	448± 94	299± 50	267± 33	236± 73	208± 49	366± 61	444.6± 6	411± 139
PaCO2 (mm Hg)	43.9± 21	36± 12	32.8± 2	51.84± 2	23.86± 17	42.24± 12	49± 21	42± 12	40± 2	49.22± 14	25.42± 8	38.06± 15
pH	7.4± 0.1	7.4± 0.1	7.32± 0.1	7.32± 0.1	7.6± 0.1	7.47± 0.1	7.28± 0.1	7.36± 0.1	7.3± 0.1	7.39± 0.0	7.5± 0.2	7.44± 0.1
Lactate (mmol/L)	2.7± 1.0	3.2± 1.0	3.3± 0.9	2.2± 0.7	3.0± 0.2	3.0± 0.5	2.13± 0.4	2.32± 0.7	2.34± 0.8	1.6± 0.7	2.5± 0.6	3.3± 0.9

WT - Wild type; FXII$^{-/-}$ - FXII-deficient.





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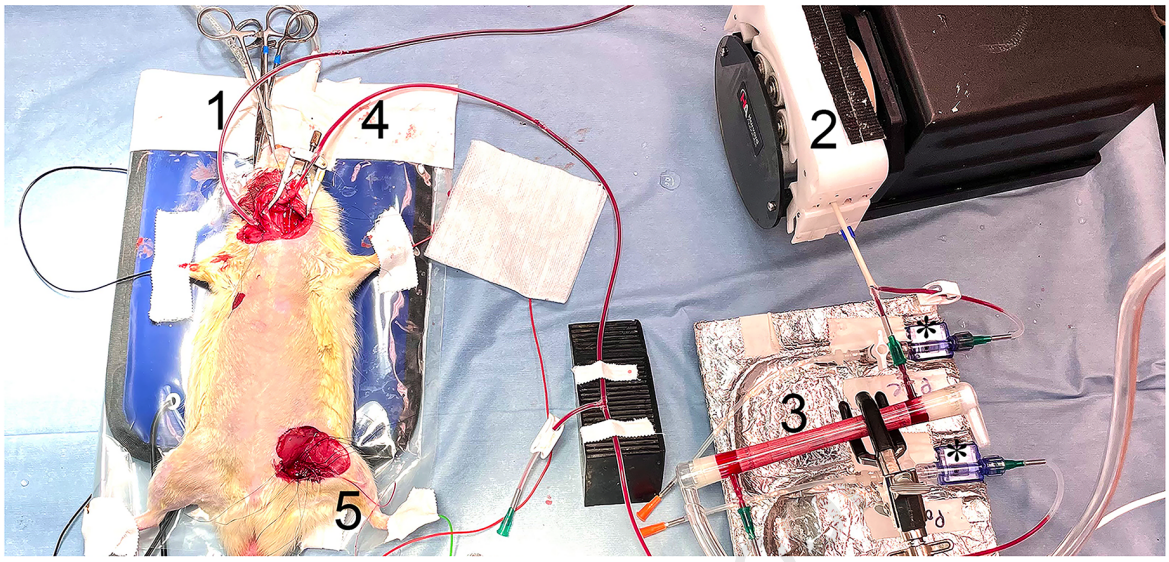

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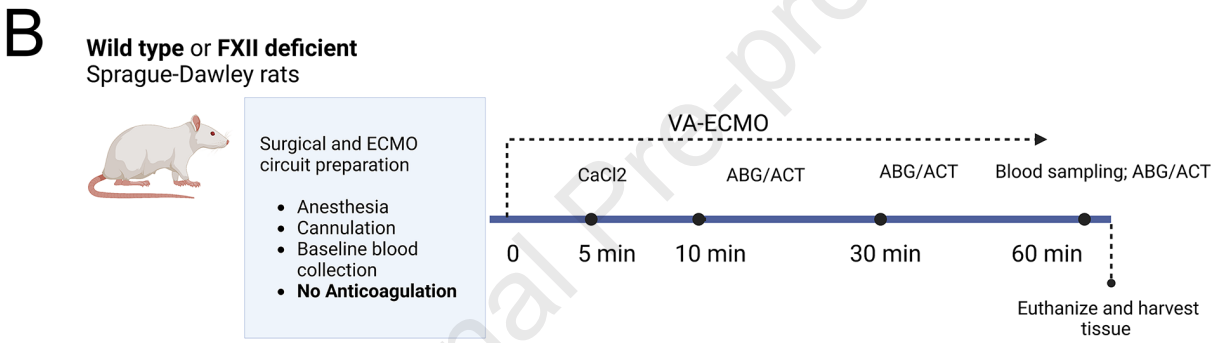

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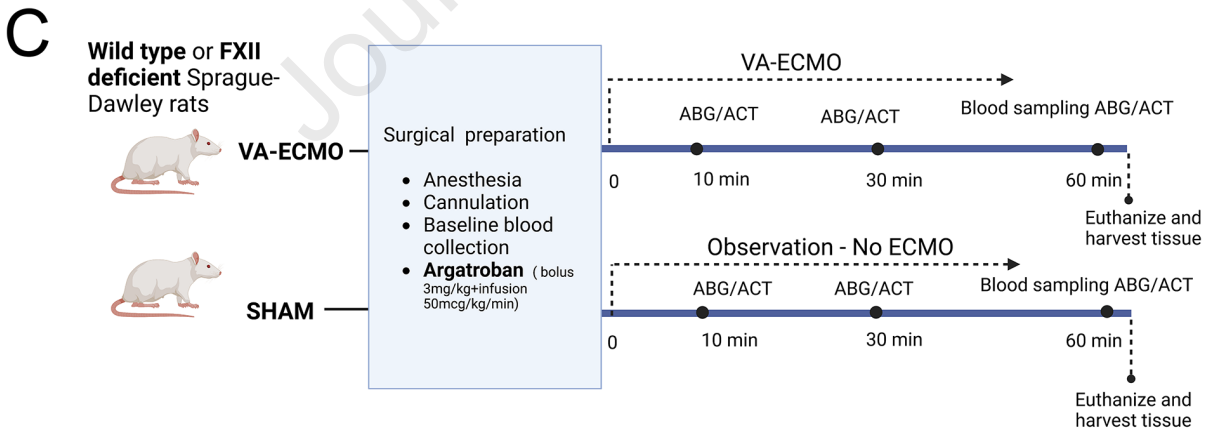

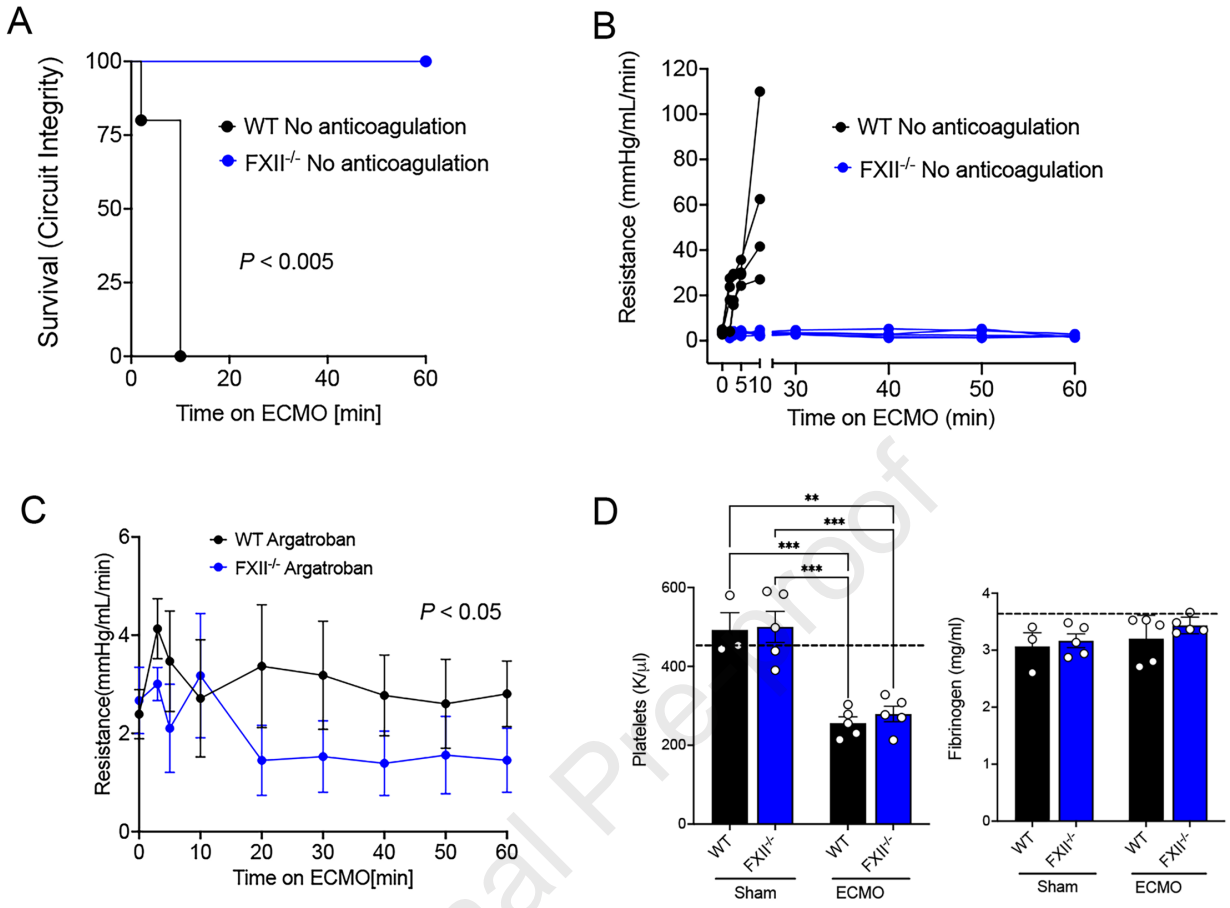

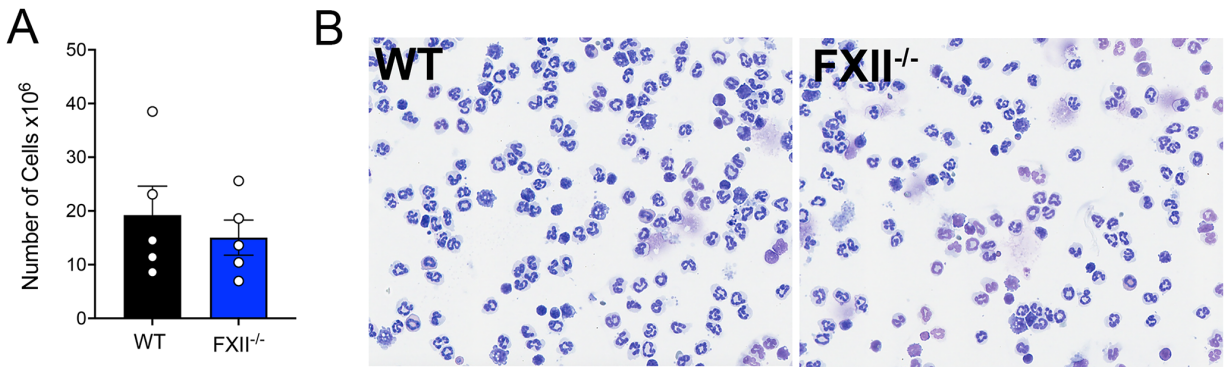

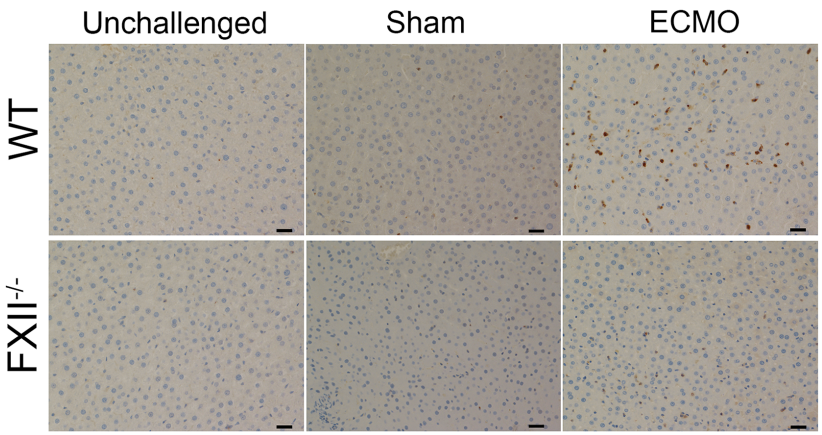

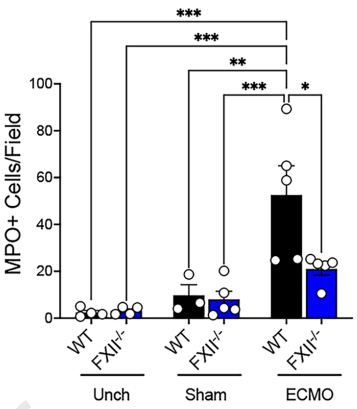

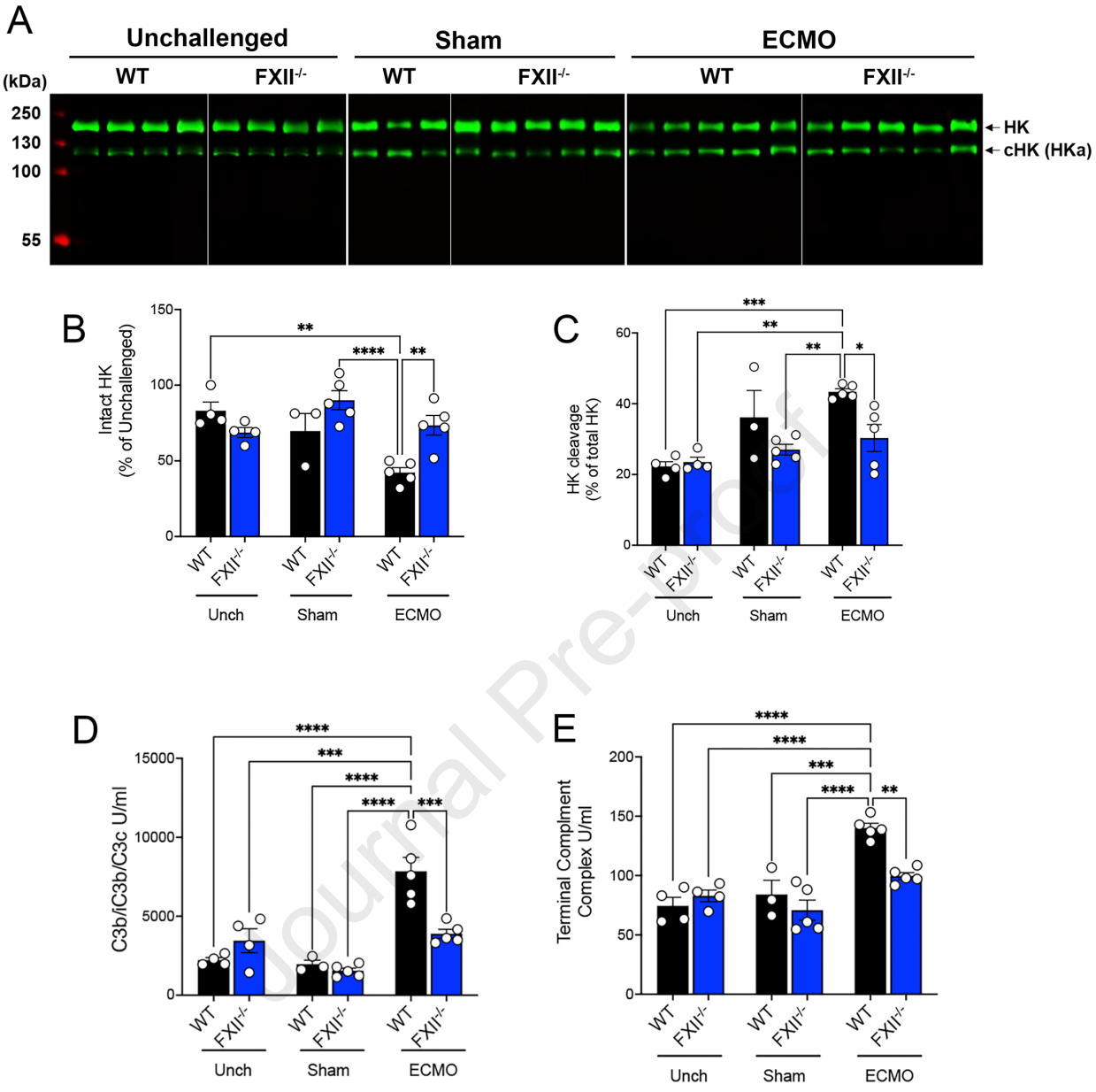

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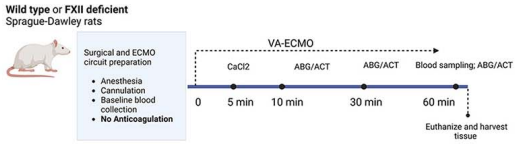
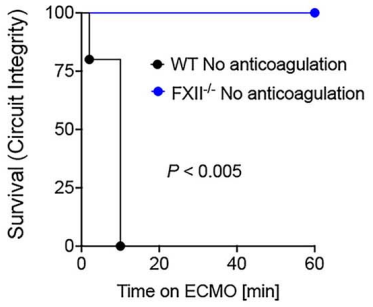
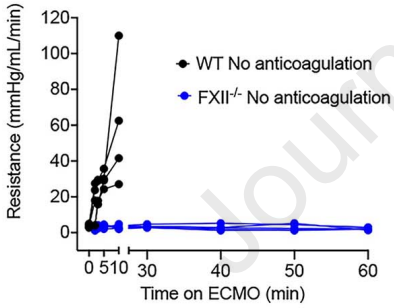
What is the role of factor XII in ECMO associated thromboinflammation?

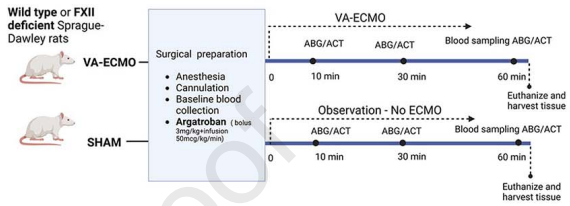
THROMBOSIS

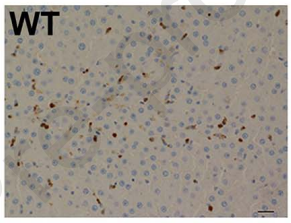


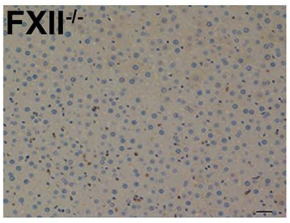


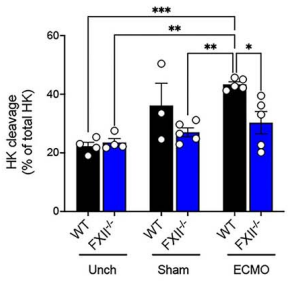


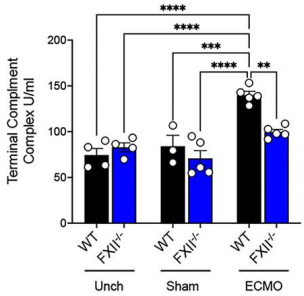
INFLAMMATION











FXII deletion prevents thrombosis in the ECMO circtuit, limits neutrophil migration into the liver, attenuates HMW kininogen cleavage and prevents complement activation.

Abbreviations: VA-ECMO - veno-arterial extracorporeal membrane oxygenation; FXII - factor XII; FXII$^{-/-}$ - factor XII deficient; WT - wild type; ABG - arterial blood gas; $CaCl_2$ - calcium chloride; HK - high molecular weight kininogen