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Duval, C orcid.org/0000-0002-4870-6542, Ali, M, Chaudhry, WW et al. (3 more authors) (2016) Factor XIII A-Subunit V34L Variant Affects Thrombus Cross-Linking in a Murine Model of Thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology, 36 (2). pp. 308-316. ISSN 1079-5642

https://doi.org/10.1161/ATVBAHA.115.306695

Factor XIII A-subunit V34L variant affects thrombus cross-linking in a murine model of thrombosis

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ABSTRACT

Objectives: Factor XIII cross-links fibrin upon activation by thrombin. Activation involves cleavage at residue 37 by thrombin, releasing an activation peptide. A common polymorphism (V34L), located in the activation peptide, has been associated with increased activation rates and paradoxically a protective effect in cardiovascular disease. There is currently no data available on the effects of V34L from *in-vivo* models of thrombosis. We examined the effect of FXIII V34L on clot formation and cross-linking *in-vivo*.

Approach and Results: We generated a panel of full-length recombinant human FXIII-A_2 variants with amino-acid substitutions in the activation peptide to investigate the effect of these variants on activation rate, and we used wild-type, V34L, and G33A variants to study the effects of varying FXIII activation rate on thrombus formation in a murine model of $FeCl_3$ injury. FXIII activation assay showed that residues 29, 30, 33 and 34 play a critical role in thrombin interaction. Full-length rhFXIII-A_2 V34L has significant effects on clot formation, structure, and lysis *in-vitro*, using turbidity assay. This variant influenced fibrin cross-linking, but not size of the thrombus *in-vivo*.

Conclusions: Mutations in the activation peptide of full-length recombinant FXIII regulate activation rates by thrombin, and V34L influences *in-vivo* thrombus formation by increased cross-linking of the clot.

ABBREVIATIONS

α_2-AP	α_2-antiplasmin
CAD	Coronary Artery Disease
$FeCl_3$	Ferric Chloride
FXIII	Factor XIII
FXIIIa	Activated Factor XIII
FXIII-A	Factor XIII A-subunit
$FXIII^{-/-}$	Factor XIII knockout
G33A	Alanine to Glycine variant at residue 33
MI	Myocardial Infarction
rhFXIII	Recombinant Human Factor XIII
V34L	Valine to Leucine variant at residue 34
WT	Wild Type

INTRODUCTION

Coagulation factor XIII (FXIII) is a protransglutaminase consisting of two A-subunits, and two B-subunits which act as carrier for the A-subunits in plasma[1]. FXIII is activated by cleavage of an N-terminal activation peptide (residues 1-37) from the A-subunit by thrombin, and dissociation of the transglutaminase FXIII-A dimer from the B-subunits in the presence of calcium[2]. Activated FXIII (FXIIIa) plays an important role in clot stabilisation by cross-linking the α- and γ-chains of fibrin[3,4], and cross-linking α_2-antiplasmin (α_2-AP) to fibrin[5], resulting in increased resistance of the clot to mechanical and proteolytic challenges.
FXIII deficiency is a rare disorder, which leads to severe bleeding[6], recurrent miscarriage[7], and impaired wound healing[8]. The genes encoding for both A- and B-subunits are highly polymorphic[9]. The most common single nucleotide polymorphism, FXIII-A V34L, is located in the activation peptide and has been linked with an overall protective effect on thrombotic disease. Meta-analyses have shown that FXIII-A V34L is associated with decreased risk of myocardial infarction (MI)[10] and coronary artery disease (CAD)[11]. FXIII-A V34L has also been associated with a weak protective effect on venous thromboembolic disease[12].
The FXIII-A 34Leu variant purified from plasma has been shown to increase activation rates by thrombin, and alter fibrin structure *in-vitro*, when compared to 34Val[13]. The effects of V34L on fibrin structure depend on fibrinogen concentrations in the blood, with different effects at high compared with low fibrinogen[14]. However, the mechanisms by which these changes in fibrin clot structure relate to the risk of thrombosis are unclear, and no study has yet investigated the effects of FXIII V34L using *in-vivo* models of thrombosis.
The aims of this study were to produce recombinant human FXIII-A_2 (rhFXIII-A_2) variants, spanning residues downstream and upstream of the thrombin cleavage site, and to identify residues responsible for changes in FXIII activation rates. In addition, we investigated the effects of rhFXIII-A_2 V34L on clot structure and stability *in-vitro*, and thrombus formation using an *in-vivo* model. We found that FXIII residues 29, 30, 33 and 34 in particular play a critical role in thrombin interaction. FXIII-A_2 V34L has significant effects on clot structure *in-vitro* and influences fibrin cross-linking *in-vivo*.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Effect of FXIII-A variants on FXIII activation and cross-linking activity in-vitro

All rhFXIII-A_2 variants generated by site-directed mutagenesis (Fig. 1A) were successfully expressed, except P36A, which failed to transform and express after repeated attempts. Of these variants, T28A, L31A, Q32A, G38A, V39A, N40A, and L41A showed no change in the rate of FXIII activation peptide cleavage by thrombin compared to WT (Fig. 1B,C). Two variants, V34L and V34M, showed increased activation rate, 1.43- and 1.60-fold respectively, whereas five variants showed a

decrease in the rate of cleavage of the FXIII activation peptide by thrombin (V29A, x0.62; E30A, x0.60; G33A, x0.53; V34A, x0.78; and V35A, x0.82). Variant R37A could not be cleaved by thrombin, confirming the specificity for arginine at the thrombin cleavage site.
Variants that showed a significant change in activation rate where also analysed for FXIII activity rates in a kinetic pentylamine incorporation assay that is sensitive to the FXIII activation rate by thrombin (Fig. 1D). Those variants that had a decreased activation rate also exhibited a decrease in the kinetic pentylamine incorporation assay, reflected by a decrease in pentylamine incorporation onto casein (V29A, x0.86; E30A, x0.82; G33A, x0.74; V34A, x0.75), except V35A, which showed no change in FXIII activity. Variants V34L and V34M exhibited increased activity, 1.35- and 1.15-fold, in agreement with their increased activation rate. Note that previous studies have shown that this pentylamine incorporation assay is sensitive to the FXIII activation step[13,15], but that once fully activated, FXIII V34L shows normal substrate incorporation rates[16]. The variants with the highest increase (V34L) and decrease (G33A) in FXIII activities were used for further investigation.

FXIII-A_2 G33A and V34L variants affect fibrin polymerisation and lysis time

Compared to the WT (0.254±0.003), rhFXIII-A_2 V34L and G33A variants showed decreased (0.238±0.002, $p<0.01$) and increased (0.265±0.003, $p<0.05$) maximum absorbency in a turbidity assay of fibrin polymerisation by thrombin (Fig. 2A) respectively, indicative of an effect on fibre diameter, with clots formed in the presence of rhFXIII-A_2 V34L demonstrating thinner fibres. No difference was observed in terms of polymerisation rates, indicating that rhFXIII-A_2 variants have no effect on the initial steps of fibrin formation. The lysis profile was investigated in a purified system, in the absence and presence of α_2-AP. In the absence of α_2-AP (Fig. 2B,D), time to half-lysis was significantly increased for V34L (1.22-fold, $p<0.05$), but not G33A, indicating that fibrin clots cross-linked with V34L are harder to lyse that those made with WT. In the presence of α_2-AP (Fig. 2C,D), time to half-lysis was significantly increased for WT (2.79-fold, $p<0.005$), compared to that of the absence of α_2-AP. Variants V34L and G33A significantly increased (1.25, $p<0.05$) and decreased (0.83, $p<0.05$) time to half-lysis, respectively (Fig. 2C,D), indicating a potential difference in α_2-AP cross-linking within the clot by these variants.

FXIII-A_2 V34L and G33A variants affect α_2-antiplasmin cross-linking to fibrin, and fibrin α- and γ-chains cross-linking

The effects of the rhFXIII-A_2 variants on the lysis profile in the presence of α_2-AP were further investigated by analysing their effects on α_2-AP cross-linking to fibrin. Using an α_2-AP incorporation assay, V34L significantly increased α_2-AP cross-linking to fibrin compared to WT (1.63, $p<0.01$), whereas G33A induced a slight decrease which however was not significant (Fig. 2E). The role of these variants in fibrin α- and γ-chains was also investigated by SDS-PAGE (Supplemental Figure). Within 2 minutes of the initiation of clot formation, the percentage of fibrin γ-chain cross-linking was higher for V34L (52%), and lower for G33A (24%) compared to WT (30%), and virtually all γ-chains were cross-linked at 10 minutes for all variants. After 5 minutes, α-chain cross-linking was less than 20% for all variants, with V34L showing a higher, and G33A a lower, cross-linking compared to WT. After 10 minutes, the amount of cross-linking markedly increased, with the highest increase for V34L (66%), intermediate increase for WT (52%) and a lesser increase for G33A (41%). The

appearance of α-α cross-linking products further increased over time, with V34L showing a higher (85%), and G33A a lower (66%), amount of cross-linking compared to WT (74%) at 30 minutes. These data confirm that fibrin γ-chains are cross-linked more rapidly than α-chains, and that V34L increases, whilst G33A decreases, the cross-linking rate for both chains, compared to WT.

FXIII-A_2 G33A and V34L activation rates in-vivo

The optimum amount of rhFXIII-A_2 to be injected in FXIII$^{-/-}$ mice was determined by injecting various quantities of FXIII, collecting the blood after 15 minutes and performing a pentylamine incorporation assay on the plasma. Injection of 54μg of rhFXIII-A_2 WT in FXIII$^{-/-}$ mice, was found to reproduce the phenotype of CBA/129 mice (98±14% and 100±15% FXIII activity, respectively; data not shown). The remaining experiments were performed using age- and weight-matched mice for consistency. FXIII assay was performed on the plasma from CBA/129 mice, or FXIII$^{-/-}$ mice injected with the different FXIII-A_2 variants. As per optimisation, FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT had a similar FXIII activity (97±22%) compared to that of CBA/129 mice (100±26%). Injection of rhFXIII-A_2 V34L resulted in increased (168±17%, $p<0.05$) FXIII activation rates (measured by a kinetic pentylamine incorporation assay sensitive to the FXIII activation step), compared to both CBA/129 and FXIII$^{-/-}$ mice injected with WT, whilst G33A resulted in decreased (55±16%) FXIII activation rate (Fig. 3). Plasma from FXIII$^{-/-}$ mice injected with saline showed no FXIII activity (4±2%, $p<0.05$ compared to all other conditions).

FXIII-A_2 G33A and V34L variants do not affect fibrin thrombus size in-vivo

Fibrin thrombus size was investigated by measuring the AlexaFluor488 (green) pixels area size. The thrombus area was unchanged over an hour for both CBA/129 injected with saline and FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT (Fig. 4A), indicating that the thrombus was already fully formed in terms of size 5 minutes (first measurement time-point) after $FeCl_3$ injury, and that the injection of 54μg of rhFXIII-A_2 WT in FXIII$^{-/-}$ mice reproduces the phenotype of CBA/129 mice, in terms of fibrin clot formation. Injection of rhFXIII-A_2 V34L and G33A in FXIII$^{-/-}$ mice did not induce any changes in thrombus size over time, whilst FXIII$^{-/-}$ mice injected with saline showed an increase (2.75-fold, $p<0.01$) in thrombus size (Fig. 4B). Immediately after injury (5 minutes), the thrombus size in FXIII$^{-/-}$ mice injected with saline was lower (0.58-fold, $p<0.05$) than that of FXIII$^{-/-}$ injected with rhFXIII-A_2 WT and CBA/129 mice injected with saline (Fig. 4C). However, 65 minutes following injury, the thrombus size was higher (1.43-fold, $p<0.05$) in FXIII$^{-/-}$ injected with saline, compared to those injected with rhFXIII-A_2 WT and to CBA/129 mice injected with saline (Fig. 4C). Figure 4D shows an example visualisation of the clots at 65 minutes, before background subtraction.

FXIII-A_2 V34L, but not G33A, increases fibrin cross-linking in-vivo

FXIII cross-linking activity *in-vivo* was investigated by measuring the level of α_2-AP-based A15 peptide incorporation within the thrombus, reflected by the colocalization of AlexaFluor488 (green, fibrin) and AlexaFluor680 (red, A15) pixels. FXIII$^{-/-}$ mice injected with saline showed no FXIII activity throughout the duration of the experiment (Fig. 5B). In FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT, the incorporation of A15 within the thrombus increased over the hour of data collection, in a similar manner to that of CBA/129 mice injected with saline (1.47- and 1.59-fold, respectively) (Fig. 5A), confirming that the injection of 54μg of rhFXIII-A_2 WT in FXIII$^{-/-}$

mice reproduces the phenotype of CBA/129 mice, in terms of overall cross-linking activity. Injection of rhFXIII-A_2 G33A in FXIII$^{-/-}$ mice induced a lower increase (1.42-fold) in A15 cross-linking over time compared to CBA/129 mice injected with saline (1.59-fold) and FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT (1.47-fold) (Fig. 5B). Interestingly, FXIII$^{-/-}$ injected with rhFXIII-A_2 V34L showed an increase (1.81-fold, $p<0.01$) in A15 incorporation over the 60 minutes period (Fig. 5B). In these mice, the level of A15 incorporation immediately after injury (5 minutes time point) was similar to that of FXIII$^{-/-}$ injected with rhFXIII-A_2 WT and CBA/129 mice injected with saline, but this was increased by 1.46- ($p<0.01$) and 1.42-fold respectively at 65 minutes (Fig. 5C). Figure 5D shows an example visualisation of the clots at 65 minutes, before background substraction.

DISCUSSION

This study provides novel insights into the effects of FXIIIa modulation on thrombus formation *in-vivo*. We found that mutations of full-length FXIII-A proteins in the region of the activation peptide close to the cleavage site by thrombin had significant effects on FXIII-A activation rates. The G33A and V34L substitutions were responsible for the highest decrease and increase (respectively) in activation rate by thrombin. These variants were shown to affect clot formation and lysis *in-vitro*. *In-vivo*, V34L increased cross-linking within the thrombus, but did not affect the thrombus size, suggesting that the effect of this single nucleotide polymorphism in human thrombotic disease is related to the cross-linking of proteins within the thrombus rather than an effect on the actual thrombus size. However, further studies using other, longer term models of thrombosis are required to further investigate this.

We investigated effects of mutations downstream (N-terminal) and upstream (C-terminal) of the cleavage site by thrombin (R37). None of the mutations upstream of this site were found to influence FXIII-A activation rates. Few mutations downstream of the thrombin cleavage site were shown to increase (V34L, V34M) or decrease (V29A, G33A, V34A) activation rates. The G33A variant triggered the largest decrease, whereas V34L showed the largest increase in activation rates. Interestingly, altering the nature of the amino-acid at position 34 triggered opposing effects on activation rate, with V34A decreasing and V34L or M increasing this rate. These findings highlight the importance of this particular amino-acid for the interaction of FXIII with thrombin.

Previous studies using short synthetic peptides have focused on the importance of the FXIII amino-acid sequence downstream of the thrombin cleavage site. Peptides containing residues 28 to 41 exhibited an increase in k_{cat} and a decrease in K_m, resulting in an increased k_{cat}/K_m when 34Val was replaced by 34Leu, indicating that the 34Leu peptide has a higher affinity for thrombin, and is more efficiently cleaved[17]. Changes in the peptide sequence at positions V29F and V34L affected the interaction with thrombin, with residue 34 playing a more important role than residue 29[18]. The importance of residue 34 was further highlighted by a V34A substitution showing a decrease in k_{cat}/K_m, indicating a decrease in hydrolysis by thrombin[19]. Our new data, using full-length rhFXIII-A_2 protein, support some of these earlier findings based on synthetic peptides. Amino-acid substitutions at position 29 and 34 triggered effects on the efficiency thrombin cleavage and initial pentylamine-incorporation rates. Together, these data show the importance of residues 34 and 29 in the kinetics

of FXIII activation and show that differences in k_{cat}/K_m values at peptide level translate into altered function of FXIII at the protein and whole organism level.
The effects of rhFXIII-A_2 V34L on activation rate are similar to those of plasma-purified[13] and platelet-purified[16] V34L. These data demonstrate that the effects on FXIII activation are indeed caused by the Val to Leu substitution, and indicate that rhFXIII-A_2 behaves similarly with regards to clot formation and stabilisation as compared with the protein purified from plasma or platelets. The recombinant V34L variant decreased the maximum absorbency of the clot formed *in-vitro* (-6.3%) in a similar manner to that of V34L variant purified from human plasma (-5.2%)[13] compared with WT, indicating that both recombinant and plasma-purified FXIII-A V34L formed denser clots with thinner fibres[20], thought to be more resistant to lysis[21-23]. Additionally, a non-naturally occurring variant (G33A) showed decreased FXIII-A activation rate and increased maximum absorbency (+4.3%), indicating the formation of less dense clots with thicker fibres[20]. *In-vitro* clot lysis experiments, in the absence of α_2-AP, showed that the recombinant V34L variant increased time to half lysis compared with WT, whereas G33A did not change time of half-lysis. These data indicate that different levels of FXIIIa cross-linking activity affect clot lysis in the absence of α_2-AP, and are in agreement with the hypothesis that increased FXIII activation rates lead to denser clots that are resistant to lysis, whereas reduced FXIII activation rates lead to looser clots that are more amenable to lysis. This was further confirmed by the fact that V34L induced fibrin γ-γ and α-α cross-links at a higher rate than WT, whereas G33A cross-linking rates were decreased. When α_2-AP was added to the lysis experiments, the resistance to lysis was further increased for all 3 variants, with V34L having the largest effect, and G33A the smallest. The rate of cross-linking of α_2-AP onto fibrin was shown to be significantly higher for V34L compared to WT, whereas this was marginally decreased for G33A. The increase or decrease in α_2-AP cross-linking further explains the differences observed for the lysis experiments in the presence of α_2-AP.
When both WT and V34L recombinant FXIII-A were injected into mice lacking FXIII-A expression, plasma collected from these mice showed similar pentylamine-incorporation to that of human subjects possessing the different alleles. The plasma from mice injected with the V34L variant showed increased FXIII pentylamine-incorporation compared to mice injected with rhFXIII-A_2 WT, reproducing the pentylamine-incorporation levels observed in the plasma from subjects with the 34Leu and 34Val alleles, respectively[9,24].
We next used a murine model of $FeCl_3$ vessel injury to investigate the role of FXIII variants in thrombus formation. In the presence of FXIII, the clot was formed rapidly (within the first 5 minutes), and was stable afterwards. Similar observations were made with FXIII-A variants, indicating that FXIII activation rates do not affect clot area during thrombus formation. Interestingly, in the absence of FXIII, clot area was initially smaller than that in the presence of FXIII, as observed by Ali *et al.*[25]. From 10 minutes following injury, clot area increased in the absence of FXIII to become larger than that observed in presence of FXIII (55 minutes onwards). We stipulate that the amount of fibrinogen converted to fibrin by thrombin is likely the same, but that at the early time-points the clot is unstable without FXIII, resulting in clots of smaller size. The fact that the clot area in the absence of FXIII continued to increase over 60 minutes may be attributed to a certain level of plasticity and remodelling of the clot. We hypothesise that the clot area may increase due to a flattening of the clot without cross-linking under the pressure exhibited by the blood flow. Chernysh *et al.* showed that fibrin clots without FXIII can be remodelled *in-vitro* without proteolytic digestion[26].

The authors found that in the absence FXIII, fibrin monomers and oligomers dissociate and re-associate from fibrin fibres. They estimated the proportion of mobile fibrin to be around 14% of the total fibrin incorporated into the fibres. Also, they showed that the addition of peptide GPRP (competing with fibrin fibre formation) to a preformed clot resulted in clot dissolution. We hypothesise that similar mechanisms may play a role *in-vivo* leading to increased plasticity of the thrombus in the absence of FXIII.

Cross-linking of the *in-vivo* thrombi was analysed using a labelled peptide substrate for FXIIIa (A15). We found that at initial time-points, the level of A15 incorporation was similar between different FXIII-A variants. However, over the course of our experiments, rhFXIII-A_2 V34L showed a greater increase in A15 incorporation compared with WT. Our *in-vitro* studies showed that the initial rate of α_2-AP incorporation by FXIII-A was significantly increased for V34L, in agreement with the increased activation rate for this variant. These data are in agreement with our *in-vitro* data showing that V34L significantly increases incorporation of α_2-AP onto fibrin, A15 corresponding to the N-terminal sequence of α_2-AP. This difference in A15 incorporation, *in-vivo*, cannot be attributed to a difference in FXIII activity once it has been fully activated, as Wartiovaara *et al.* showed that there is no difference in specific activity (activity per mg enzyme) between FXIII-A WT and V34L[16]. We also exclude differences in FXIII-A concentrations as identical amounts of protein were injected in age- and weight-matched mice. We hypothesise that the difference in A15 incorporation is due to the intrinsic difference in FXIII-A activation rate and the effects of blood flow. Blood circulation results in a constant supply of FXIII. Due to the presence of thrombin at the site of thrombus formation, FXIII is activated, with the V34L variant being activated faster than WT. Once activated, the hydrophobic A-subunit dissociates from the plasma carrier B-subunit, and the activated A-subunit binds its substrate fibrin[27]. Our data suggest that, with a constant supply of FXIII, and with a faster activation rate for FXIII V34L, more V34L than WT FXIII will be activated and bind to the thrombus over time, resulting in an increase in cross-linking in animals injected with V34L, compared to WT. It is therefore plausible that V34L could have a different effect on thrombus compared to WT, affecting the structure of the clot by increased cross-linking of the fibrin fibres, and potentially reducing fibrinolysis by increased incorporation of α_2-AP, as shown *in-vitro* in this study.

Since the original report by Kohler *et al.*[28], several studies have investigated the relationship between FXIII-A V34L and cardiovascular disease. The relationship between FXIII-A V34L and MI has been summarised in a meta-analysis of 28 studies[10], indicating that V34L is associated with a small but significant decreased risk of MI. This meta-analysis highlighted some differences between ethnicity and gender, indicating that other factors may influence the association between FXIII-A V34L and MI. The relationship of the FXIII V34L with CAD had previously been reported in a meta-analysis of 16 studies, concluding with a small protective effect of this polymorphism on CAD[11]. Our current data from an *in-vivo* model of thrombosis due to endothelial injury suggest that any association between FXIII V34L and DVT, CAD or MI is more complex than merely a reduction in thrombosis . Indeed, we found increased cross-linking of the thrombi and a normal thrombus size in FXIII V34L mice compared with WT. Furthermore, cross-linking by FXIIIa *in-vitro* leads to the formation of denser clot with thinner fibres, which are slower to lyse[21-23]. However, our model has limitations in that we did not assess thrombus size by physical measures of the extracted thrombus. Furthermore, $FeCl_3$ injury of the endothelium is relatively severe and may not represent (venous or arterial) thrombosis accurately.

The pathogenesis of both CAD and MI involves inflammation of the coronary artery, macrophage infiltration, lipid deposition and the generation of an atherosclerotic plaque. Thrombosis and subsequent MI is triggered by plaque rupture which activates platelets and coagulation[29]. We hypothesise that any protective effect of FXIII-A V34L on atherothrombotic disease may be related to plaque stability rather than effects on thrombosis. The development of the plaque include deposition of fibrin(ogen)[30] and extracellular matrix proteins such as collagens[31,32], fibronectin[31,32], and vitronectin[33], which are all known substrates of FXIIIa[34-37]. Increased cross-linking of these elements of the plaque in subjects possessing the FXIII-A V34L allele could have protective effects on atherothrombosis by stabilising the plaque and preventing its rupture. Further studies will be required to study the role of FXIII V34L in models of plaque stability and atherothrombosis.

FXIII V34L has previously also been associated with a weak protective effect in venous thromboembolic disease[12,38-40], despite a couple of studies not finding an association[41,42]. The pathogenesis of venous disease is different from arterial thrombosis and involves immobilisation, stasis, inflammation and hypercoagulability[43]. Parts of thrombi in the leg or arm may break and cause emboli downstream in the circulation by travelling to other blood vessels, particularly in the lung (pulmonary embolism). It is possible that increased thrombus cross-linking by FXIII V34L as we have observed in our *in-vivo* model may stabilise the thrombus and prevent embolization. In addition, increased cross-linking of inhibitors of fibrinolysis such as α_2-AP by FXIII V34L may further stabilise the thrombus. However, further studies will be required to investigate this hypothesis in more detail.

In conclusion, we have shown that FXIII-A V34L influences *in-vivo* thrombus formation by increased cross-linking of the clot, without apparently affecting clot size in the first hour of thrombus development. The effects of this polymorphism on cardiovascular diseases may therefore be related to clot structure and cross-linking, and may include longer-term vascular effects that play a role in cardiovascular disease. Further studies using different of thrombosis and cardiovascular disease are warranted.

ACKNOWLEDGEMENTS

C.D. performed research, analysed data, and wrote the paper. M.A., W.C. performed research. V.C.R. and H.P. designed research. R.A.S.A. designed research, helped writing the paper. All authors have contributed to revision of the manuscript.

SOURCES OF FUNDING

This study was funded by the Medical Research Council (G0901546) and the British Heart Foundation (RG/13/3/30104).

DISCLOSURES

None.

SIGNIFICANCE

Thrombosis is a complication of cardiovascular diseases and is responsible for a large number of deaths world-wide. Factor XIII is a transglutaminase that cross-links the fibrin clot. A naturally occurring FXIII variant, V34L, influences thrombosis. Previous functional studies of V34L have been based on plasma-purified proteins or short synthetic peptides of FXIII-A. We have now for the first time characterised recombinant full-length human FXIII V34L, and demonstrated that this is a functional mutation. We provide insight into the role of the amino acid residues of the activation peptide and its interaction with thrombin. Moreover, we show that V34L changes the thrombotic response in a novel *in-vivo* model of thrombosis, by increasing the rate of cross-linking within the clot rather than the actual clot size. These novel findings demonstrate that FXIII plays a critical role in thrombosis, and that V34L is a functional variant which changes the response to thrombotic disease.

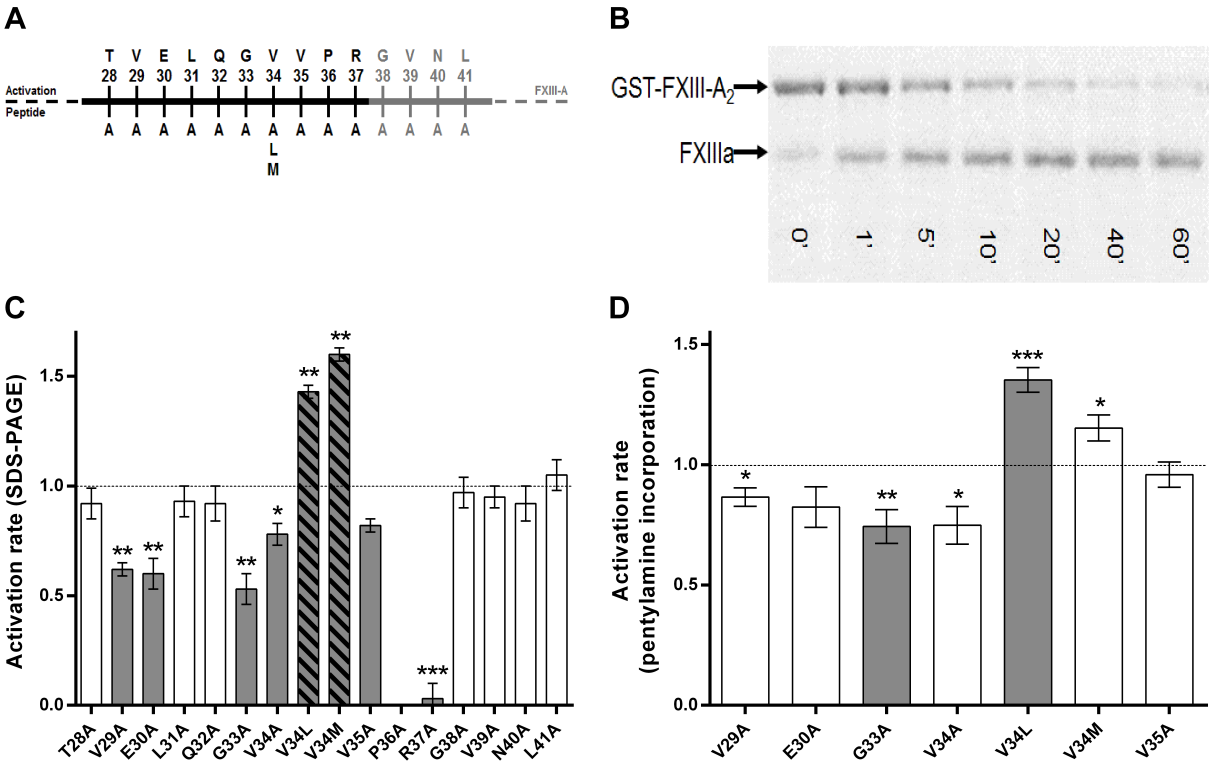

Figure 1: Effects of rhFXIII-A_2 variants on activation peptide cleavage by thrombin. Activation of GST-rhFXIII-A_2 fusion protein variants (T28 to L41; A) by thrombin (B,C) and kinetic analysis of incorporation of pentylamine onto casein by GST-free rhFXIII-A_2 (D) were measured relative to the WT protein. V34L and V34M showed increased activation by thrombin and increased biotin incorporation rates, compared to WT (---). V39A, E30A, G33A, V34A, and V35A showed a decrease in both activation and biotin incorporation rates. T28A, L31A, Q32A, G38A, V39A, N40A, and L41A activation by thrombin was not affected, whereas R37A showed no activation at all, due to its location at the activation peptide cleavage site. P36A construct was successfully generated, but the transformation step repeatedly failed. * $p<0.05$, ** $p<0.01$, *** $p<0.005$ compared to WT. n=3.

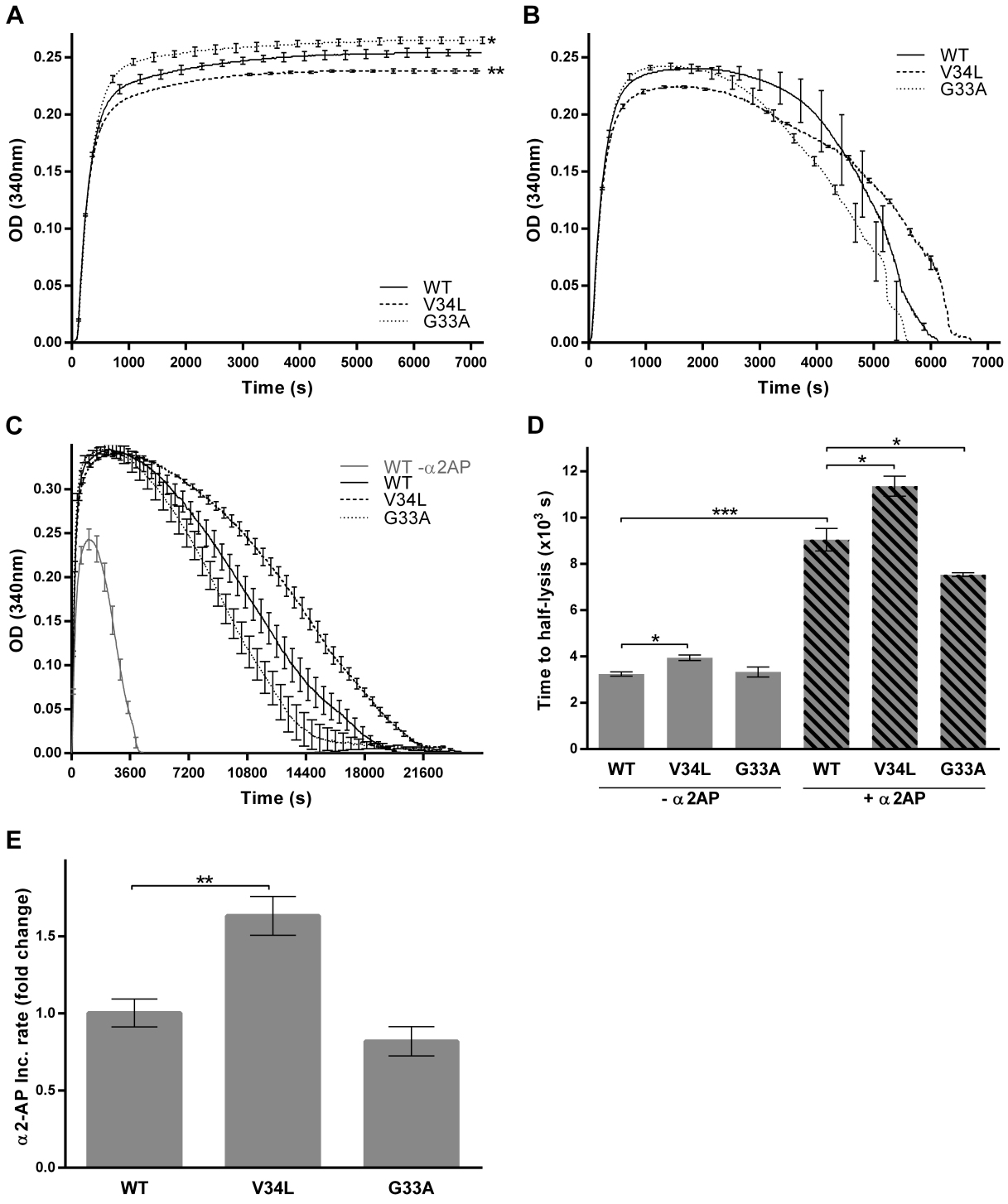

Figure 2: rhFXIII-A_2 V34L and G33A affect clot formation, lysis, and α_2-antiplasmin incorporation. Turbidity (A) and turbidity/lysis in the absence (B) or presence of α_2-AP (C) were performed using 0.5mg/ml fibrinogen, 0.1U/ml thrombin, 5mM CaCl2 and 3.7μg/ml rhFXIII-A_2 variants. 100pM tPA, 0.24μM plasminogen and 7.5μg/ml α_2-AP (when applicable) were added for the lysis experiments. Compared to WT, rhFXIII-A_2 V34L and G33A variants showed significantly decreased and increased maximum absorbency (A) respectively, reflecting an effect on fibre thickness. Time to half lysis was significantly increased for V34L both in the absence (B,D) or presence (C,D) of α_2-AP. G33A only showed a significant decrease in time to half-lysis in the presence of α_2-AP (C,D). Rate of incorporation of α_2-AP onto fibrin (E) was increased for V34L, compared to WT, but unchanged for G33A. * p<0.05, ** p<0.01, p<0.005. n=3.

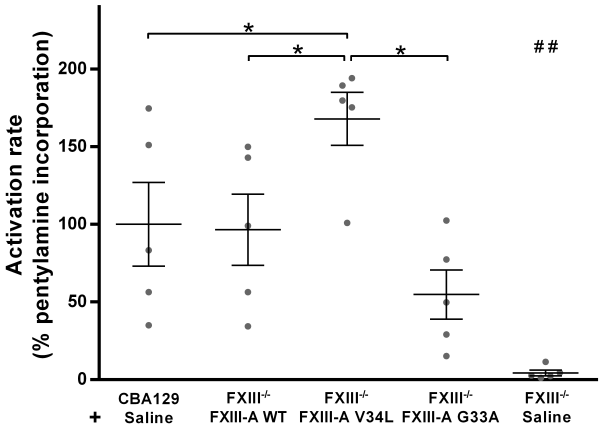

Figure 3: FXIII$^{-/-}$ mice injected with rhFXIII-A_2 V34L and G33A show increased and decreased (respectively) FXIII activation in murine plasma. A kinetic biotinylated pentylamine incorporation assay sensitive to FXIII activation rates was performed on plasma from CBA/129 mice, FXIII$^{-/-}$ mice injected with 54μg of rhFXIII-A_2 variants or saline. Injection of rhFXIII-A_2 WT in FXIII$^{-/-}$ mice restored the FXIII activation level observed in CBA/129 mice. Injection of V34L significantly increased, whereas G33A decreased, FXIII activation compared to CBA/129 mice and FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT. FXIII$^{-/-}$ mice injected with saline showed no FXIII activation. Kruskal-Wallis between all data (panel C) p=0.001, post-hoc Mann-Whitney between pairs * $p<0.05$. $^{\#\#}$ $p<0.05$. n=5.

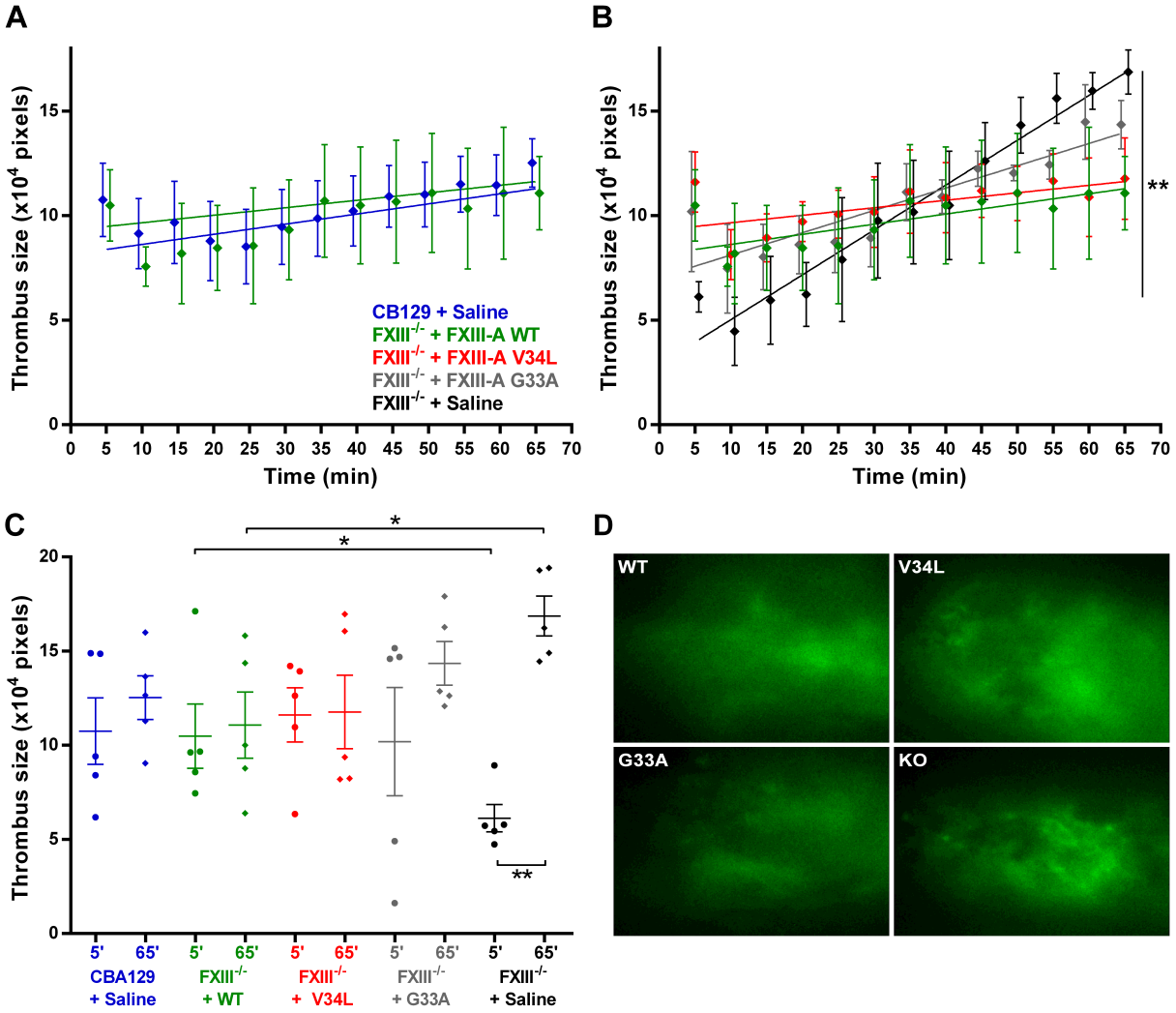

Figure 4: rhFXIII-A_2 variants do not affect clot size. Clot size growth was measured in CBA/129 mice, and mice injected with 54μg of rhFXIII-A_2 variants or saline. Clot size was determined as a combination of area and intensity of green (fibrinogen) pixels. A) The phenotype of FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT was identical to that of CBA/129 mice injected with saline. B) Over 60 minutes following injury, clot size was unchanged for FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT, V34L, and G33A, whereas FXIII$^{-/-}$ mice injected with saline showed a significant increase of clot size. C) Comparison of the initial and final time points shows that 5 minutes after induction of injury, CBA/129 and FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT, V34L, and G33A had similar clot size, whereas FXIII$^{-/-}$ mice injected with saline had a significantly smaller clot size. 65 minutes after induction of injury, clot formed in FXIII$^{-/-}$ mice injected with saline showed a significant increase in clot size compared to the 65 minutes time points for CBA/129 and FXIII$^{-/-}$ mice injected with rhFXIII-A_2 WT, and to the 5 minute time point of FXIII$^{-/-}$ mice injected with saline. D) Example visualisation of the clots at 65 minutes, before background subtraction. Kruskal-Wallis between all data p=0.189 (5') and p=0.104 (65'), post-hoc Mann-Whitney between pairs * p<0.05, ** p<0.01. n=5.

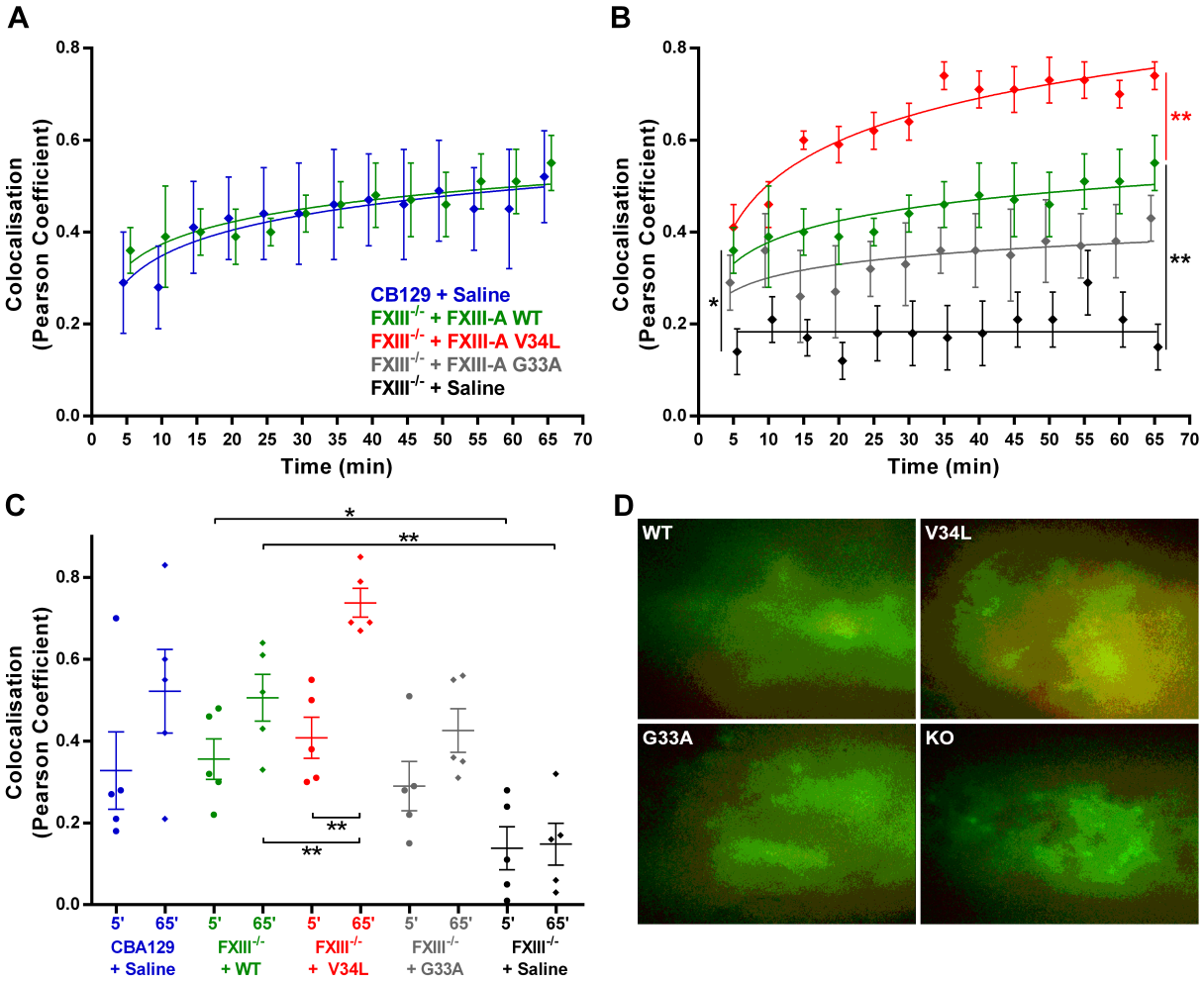

Figure 5: FXIII$^{-/-}$ mice injected with rhFXIII-A$_2$ V34L variants show a significant increase in A15 incorporation within the clot. A15 incorporation within the clot was measured in CBA/129 mice, and mice injected with 54μg of rhFXIII-A$_2$ variants or saline. A15 incorporation was determined by colocalisation of red (A15) and green (fibrinogen) pixels. A) The phenotype of FXIII$^{-/-}$ mice injected with rhFXIII-A$_2$ WT was identical to that of CBA/129 mice injected with saline. B) Over 60 minutes following injury, A15 incorporation was significantly increased in FXIII$^{-/-}$ mice injected with rhFXIII-A$_2$ V34L compared to those injected with WT, whereas mice injected with G33A showed a non-significant decrease in A15 incorporation. FXIII$^{-/-}$ mice injected with saline showed no A15 incorporation. C) Comparison of the initial and final time points shows that 5 minutes after induction of injury, CBA/129 and FXIII$^{-/-}$ mice injected with rhFXIII-A$_2$ WT, V34L, and G33A had similar levels of A15 incorporation within the clot, whereas FXIII$^{-/-}$ mice injected with saline had no A15 incorporation. 65 minutes after induction of injury, only clot formed in FXIII$^{-/-}$ mice injected with rhFXIII-A$_2$ V34L showed a significant increase in A15 incorporation compared to both 5 minute time point for V34L and 65 minutes time point for WT. D) Example visualisation of the clots at 65 minutes, before background subtraction. Kruskal-Wallis between all data (panel C) p=0.032 (5') and p=0.002 (65'), post-hoc Mann-Whitney between pairs * p<0.05, ** p<0.01. n=5.

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