

Coagulation Factor XII Haploinsufficiency is Protective Against Venous Thromboembolism in a Population-scale Multidimensional Analysis

Corresponding Author: Dr Pavan Bendapudi

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript entitled “Coagulation Factor XII (F12) Haploinsufficiency is Protective Against Venous Thromboembolism in a Population-scale Multidimensional Analysis”, Haj et al. perform a large population-scale analysis examining genetic variations in the coding region of F12 locus of participants in the UK biobank and NIH All of Us repository. FXII was identified as potential safe target in antithrombotic therapy while not increasing the bleeding risk leading to the (clinical) development of several FXII inhibitors. Still, the main focus of translating FXII to clinics is on its inhibition in HAE.

Surprisingly, human data on FXII role in (venous) thrombosis is still limited. While FXII deficiency is typically associated with reduced blood clotting and a lower risk of thrombotic events, some evidence suggests that heterozygous mutations in the F12 gene may slightly increase the risk of venous thromboembolism (VTE) (Cochery-Nouvellon et al., 2007). Further research is needed to better understand FXII's precise role in thrombosis development.

In this study, the authors provide a population-scale analysis to assess the role of FXII in VTE. For this, they include whole exome sequencing data from more than 400,000 participants in the UK Biobank and whole genome sequencing data from approximately 200,000 participants in the NIH All of Us biorepository within total 55% recruited female carriers and a mean age at enrollment of 57 y of the UK biobank. The vast majority of F12 variant carriers of the UK biobank was of European ancestry (>95%), while more evenly distributed in the All of Us biorepository. Sex distribution and mean age was similar in the All of Us biorepository.

Their main result is that heterozygous F12 variant carrier is strongly associated with a protection against VTE.

The data are robust and conclusive, backed by stringent design and supported by mouse in vivo data. The reviewer has no major comment to make, and would only like to point out minor aspects:

- I could not assess Video 1, since the file seems to be damaged and could not be opened.

- The data on ESS1 is striking, exhibiting an elevated basal FXIIa activity albeit an reduced total FXII level not leading to protection against VTE. How does this splice site variant alter the activation? Change in structure?

Minor:

- L 140: to appear is doubled

- L. 277 ff: Published data on F12[±] mice is limited, mouse studies mainly focused on homozygous ko mice. Renné et al. studied F12[±] mice in one arterial thrombosis model, where they did not observe any protection from thrombus formation. Still, this might be different to the venous thrombosis model and should be stated here more clearly.

- Fig 1B: please check the Legend: It seems that there's an annotation missing (next to FN2)?

- Fig 5A: Plasma FXII level in WT mice are 4 times higher compared to the literature (Beck et al., JTH) and approx. twice as high as in humans (Fig. 2H). How do you explain this discrepancy?

Reviewer #2

(Remarks to the Author)

In this study, Amelia and colleagues explored how heterozygous FXII deficiency affects the risk of venous thromboembolism (VTE). This groundbreaking research was the first to show that people with these specific genetic changes (High-confidence

loss-of-function variants in the F12 gene) are less likely to develop VTE in a large population. The authors found that having half the normal amount of FXII reduces the procoagulant potential, as shown in thrombin generation test activated through the intrinsic pathway. Additionally, heterozygous F12 knockout mice were also found to be less prone to forming blood clots in an intravital electrolytic femoral vein injury model. Here are some thoughts and questions for the authors:

1. The study highlights that heterozygous F12 deficiency lowers VTE risk and suggests this could be a new clinical phenotype. While severe FXII deficiency is already known to protect against thrombosis formation, if the protective effect varies only by degree between individuals harboring heterozygous or homozygous mutations in F12 gene, should the heterozygous F12 deficiency with decreased VTE risk warrant a distinct new clinical phenotype of its own?
2. The research mainly discussed about type I FXII deficiencies, which are deleterious for FXII expression, but lacks data on type II deficiencies of FXII. For instance, the ESS2 splice-site mutation is considered by the authors as type II deficiency because FXII levels are normal and the model predicts lower risk for thrombosis. However, further characterization of the mutation is needed to confirm this classification.
3. The impact of heterozygous FXII deficiency on the status of global coagulation function was evaluated by thrombin generation tests in the study, and samples with 50% of normal FXII levels were shown to have reduced thrombin generation potential. However, the thrombin generation curves of plasma samples containing 75%, 90%, and 100% of normal FXII levels showed almost no difference and overlap substantially. According to these findings, there seems no linear relationship between the coagulation potential with FXII levels, and 50% decrease from normal FXII level constitutes a threshold for global coagulation activity reduction. Any possible coagulation function mechanisms underlying these unexpected findings?
4. In the animal experiments, F12 heterozygous knockout mice were shown to have less thrombosis formation upon femoral vein injury, which, however, contradicts with earlier findings that heterozygous deficiency of FXII did not protect mouse model from thrombosis formation in Reference [20]. How to reconcile the seemingly conflicts? Could the differences be attributed to the methods used for thrombosis induction in animal model? The issue shall be addressed in the paper.

Reviewer #3

(Remarks to the Author)

The importance of factor (F) XII in thrombosis is uncertain. To address this uncertainty, Haj and colleagues interrogated UK and NIH biobank databases. They demonstrated protection against venous thromboembolism (VTE) in subjects heterozygous for FXII variants associated with a 50% reduction in FXII levels, compared with subjects without FXII deficiency, and no increase in bleeding or infection. They also demonstrated reduced silica-induced, but not tissue factor-induced, thrombin generation in plasma with 50% FXII levels compared with 100% FXII, as well as attenuated thrombus formation in response to electrolytic injury in heterozygous FXII-deficient mice compared with wild-type mice. The authors conclude that because heterozygous FXII deficiency protects against VTE and is not associated with bleeding, FXII represents a promising target for new anticoagulants.

Although the findings are intriguing, major and minor concerns must be addressed.

MAJOR

1. The strength of the population data conclusion that FXII deficiency protects against VTE is limited by the small sample size (Figure 2A).
2. The authors demonstrate an association between FXII levels and VTE; however, the possibility of residual confounding cannot be ruled out. For example, were pregnant women, women taking estrogens, and subjects with recurrent VTE or cancer excluded from the analysis?
3. Reduced silica-induced thrombin generation with FXII deficiency is a well-described phenomenon. However, the relevance of this finding to the risk of thrombosis remains uncertain.
4. The finding that compared with wild-type mice, thrombosis in response to electrolytic injury is reduced in mice heterozygous for FXII deficiency is not new. Furthermore, previous studies have shown that thrombosis in mice is FXII-driven, but it is unknown whether the same is true in humans.

MINOR

1. Lifelong anticoagulation is only recommended for patients with unprovoked VTE or persistent major risk factors, such as active cancer (page 3). Consequently, the statement that "current guidelines recommend lifelong therapy for most patients" needs to be modified accordingly.
2. Page 2: What is a presumed qualitative difference? Was this not validated?
3. The population data findings would be more substantial if the authors demonstrated a link between low FXII levels and a reduced risk of both arterial and venous thrombosis, as well as a link between high FXII levels and an increased risk of both venous and arterial thrombosis. FXII would be a less appealing target if it were only implicated in venous thrombosis.
4. Tissue factor is the likely driver of most cases of VTE. Back activation of FXI by tissue factor-induced thrombin generation could bypass FXIIa inhibition. The authors need to address this possibility.
5. Figure 1E: Why were the two gene sets separated? The only difference is the inclusion of FV. Why wasn't FXI included, given its current focus as a novel target for safer anticoagulants?
6. Were there any individuals with biallelic FXII FIS = 1? Do these individuals exhibit an increased risk of bleeding?
7. Figure 2B: Data for F10, F2, and F11 should be added to calibrate the analysis with known outcomes in patients receiving

DOACs.

8. Figure 3: Is the expected bleeding observed in patients with FIS = 1 in FIX and FVIII?

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all my previous concerns satisfactorily. I have no further comments. The video S1 was now accessible and fully functioning.

Reviewer #2

(Remarks to the Author)

All concerns have been adequately addressed, no additional comments on the revised manuscript.

Reviewer #3

(Remarks to the Author)

The revised paper is much improved. However, I still have concerns.

1. The observation that partial factor XII deficiency is associated with a reduced risk of venous thromboembolism is unexpected because such a link has not been demonstrated in observational studies (Johnson CY, et al. Am J Epidemiol. 201; 173: 136-44) or GWAS analyses (Klarin D, et al. Nat Genet. 2019; 51:1574-1579), and contrary to the results reported in this paper with venous thrombosis, arterial thrombosis in response to injury was reported to be normal in mice with heterozygous deficiency of factor XII (Renné T, et al. J Exp Med. 2005; 18;202:271-81). Therefore, if the findings of the current paper are correct, they are potentially paradigm-shifting. Because the stakes are high, I want assurance that the methodology works. As far as I can tell, this technology has not been used previously to study coagulation factors or venous thromboembolism. Unless data supports its use, I would like to see a similar analysis with factor XI loss-of-function alleles, which are known to be associated with a reduced risk of venous thromboembolism, to confirm that the methodology works.

2. The impact of the findings would be enhanced if the authors demonstrated a link between factor XII deficiency and arterial thrombosis. Mechanistically, why would the effect of factor XII be localized to veins, and why are the current results in a mouse model of venous thrombosis different from those reported in a murine model of arterial thrombosis?

Reviewer #4

(Remarks to the Author)

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Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors have addressed my concerns.

Reviewer #4

(Remarks to the Author)

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RESPONSE TO REVIEWERS

Reviewer #1 (Remarks to the Author):

In this manuscript entitled “Coagulation Factor XII (F12) Haploinsufficiency is Protective Against Venous Thromboembolism in a Population-scale Multidimensional Analysis”, Haj et al. perform a large population-scale analysis examining genetic variations in the coding region of F12 locus of participants in the UK biobank and NIH All of Us repository. FXII was identified as potential safe target in antithrombotic therapy while not increasing the bleeding risk leading to the (clinical) development of several FXII inhibitors. Still, the main focus of translating FXII to clinics is on its inhibition in HAE. Surprisingly, human data on FXII role in (venous) thrombosis is still limited. While FXII deficiency is typically associated with reduced blood clotting and a lower risk of thrombotic events, some evidence suggests that heterozygous mutations in the F12 gene may slightly increase the risk of venous thromboembolism (VTE) (Cochery-Nouvellon et al., 2007). Further research is needed to better understand FXII's precise role in thrombosis development.

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- I could not assess Video 1, since the file seems to be damaged and could not be opened.

We apologize for this issue. Should the journal decide to publish our manuscript, we will work with the editorial team to ensure that Video 1 is accessible and fully functioning.

- The data on ESS1 is striking, exhibiting an elevated basal FXIIa activity albeit an reduced total FXII level not leading to protection against VTE. How does this splice site variant alter the activation? Change in structure?

We thank the reviewer for this comment and agree that the ESS1 is a very interesting variant. Although to date no complete structure of FXII has been solved, conformation of the FXII heavy chain (AA 1-353) has been shown to protect the FXII activation loop and regulate conversion of the zymogen to the active enzyme. Given the location of ESS1, it is plausible that conformation of the heavy chain may have been disrupted by this variant, leading to enhanced autocatalytic cleavage and/or cleavage by plasma kallikrein. However, we also stress that ESS variants often have more complex and unpredictable effects than coding missense variants, as disruption of essential splice sites can result in truncation, single exon skipping, multiple exon skipping, or the generation of an entirely novel transcript via noncanonical (cryptic) alternative splicing. The characterization of ESS1 and ESS2 is currently the subject of future investigations in our laboratory.

Minor:

- L 140: to appear is doubled

We are grateful to the Reviewer for pointing out this error, and we have corrected it.

- L. 277 ff: Published data on F12+/- mice is limited, mouse studies mainly focused on homozygous ko mice. Renné et al. studied F12+/- mice in one arterial thrombosis model, where they did not observe any protection from thrombus formation. Still, this might be different to the venous thrombosis model and should be stated here more clearly.

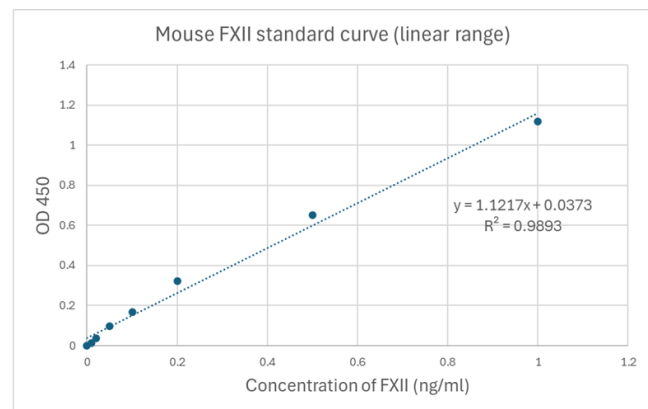
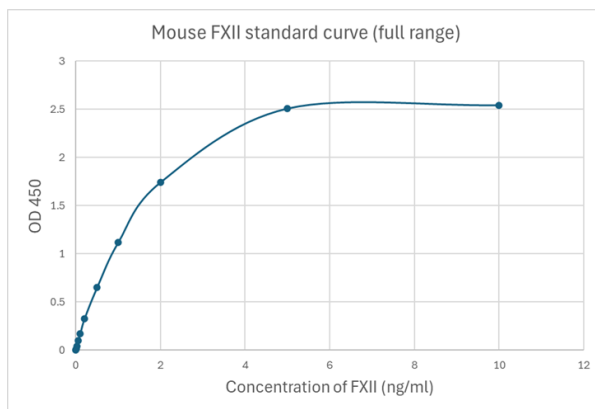
The reviewer correctly notes that the prior data for thrombosis in F12+/- mice are extremely sparse. To our knowledge, only one experiment has been reported to date, and this work was performed in a murine mesenteric arteriole thrombosis model (Renne, et al. *JEM* 2005; Figure 3). No data have been published on venous thrombosis. In fact, previous murine studies have largely ignored the F12+/- genotype. We suspect that the observed discrepancy between our data and the result in the 2005 *JEM* paper is due to differences in the models and vascular beds studied. We have updated the Discussion section to include this point (page 12).

- Fig 1B: please check the Legend: It seems that there's an annotation missing (next to FN2)?

We thank the Reviewer for bringing this error to our attention. The box that the Reviewer references was an artifact left over by the software we used to generate the figure. In fact, AA 1-19 comprise the signal peptide of FXII, which the software was unable to correctly annotate. We have modified the figure and noted the designation for AA 1-19 in the caption.

- Fig 5A: Plasma FXII level in WT mice are 4 times higher compared to the literature (Beck et al., JTH) and approx. twice as high as in humans (Fig. 2H). How do you explain this discrepancy?

Thank you for this important comment. We too were struck by the higher than expected FXII levels found in our samples, which we attributed to the specific ELISA kit that was used (IHUFXIIKTT, Innovative Research, Novi, MI). We reviewed the standard curve we generated (below) as well as the raw data for the assay in detail and were unable to identify any errors. Despite this issue, the data do appear to accurately reflect the relative differences in circulating FXII levels between the genotypes.



Reviewer #2 (Remarks to the Author):

In this study, Amelia and colleagues explored how heterozygous FXII deficiency affects the risk of venous thromboembolism (VTE). This groundbreaking research was the first to show that people with these specific genetic changes (High-confidence loss-of-function variants in the F12 gene) are less likely to develop VTE in a large population. The authors found that having half the normal amount of FXII reduces the procoagulant potential, as shown in thrombin generation test activated through the intrinsic pathway. Additionally, heterozygous F12 knockout mice were also found to be less prone to forming blood clots in an intravital electrolytic femoral vein injury model. Here are some thoughts and questions for the authors:

1. The study highlights that heterozygous F12 deficiency lowers VTE risk and suggests this could be a new clinical phenotype. While severe FXII deficiency is already known to protect against thrombosis formation, if the protective effect varies only by degree between individuals harboring heterozygous or homozygous mutations in F12 gene, should the heterozygous F12 deficiency with decreased VTE risk warrant a distinct new clinical phenotype of its own?

We thank the reviewer for this insightful question. We contend that the clinical phenotype of factor XII deficiency (and possibly other autosomal coagulation factor deficiencies) is more nuanced than previously appreciated. Rather than simple binary Mendelian inheritance in which wild-type and heterozygous individuals are thought to be unaffected while homozygous loss of function (LOF) variant carriers express a phenotype, our data for *F12* point to a novel haploinsufficient state with measurable clinical impacts. That said, homozygous *F12* LOF has never been shown to protect against thrombosis in humans, only in animal models. Therefore, whether the heterozygous state constitutes a distinct clinical phenotype relative to homozygous *F12* LOF remains unknown. For example, homozygous *F12* LOF may be more protective but could also have unforeseen consequences (e.g. increased infection risk), making it a distinct clinical phenotype. We have included language in the Discussion section (page 13) that touches on this issue and would be happy to further modify the manuscript if the Reviewer recommends any specific changes.

2. The research mainly discussed about type I FXII deficiencies, which are deleterious for FXII expression, but lacks data on type II deficiencies of FXII. For instance, the ESS2 splice-site mutation is considered by the authors as type II deficiency because FXII levels are normal and the model predicts lower risk for thrombosis. However, further characterization of the mutation is needed to confirm this classification.

We completely agree that ESS2 could be of significant clinical and biological interest and warrants further characterization. Unfortunately, UKB plasma samples are not available to us, limiting the work that could be done within the scope of this manuscript. ESS variants often have more complex and unpredictable effects than coding missense variants, as disruption of essential splice sites can result in truncation, single exon skipping, multiple exon skipping, or the generation of an entirely novel transcript via noncanonical (cryptic) alternative splicing. Here, we hypothesize that ESS2 is disrupting the catalytic function of FXII without impairing the production of a mature protein. This would lead to a Type II (qualitative) FXII deficiency. However, as we have not been able to confirm a Type II deficiency, we have softened the language used to describe ESS2 (page 7). The characterization of ESS1 and ESS2 will be the subject of future investigations in our laboratory.

3. The impact of heterozygous FXII deficiency on the status of global coagulation function was evaluated by thrombin generation tests in the study, and samples with 50% of normal FXII levels were shown to have reduced thrombin generation potential. However, the thrombin generation curves of plasma samples containing 75%, 90%, and 100% of normal FXII levels showed almost no difference and overlap substantially. According to these findings, there seems no linear relationship between the coagulation

potential with FXII levels, and 50% decrease from normal FXII level constitutes a threshold for global coagulation activity reduction. Any possible coagulation function mechanisms underlying these unexpected findings?

The Reviewer makes an important point. Global assays of coagulation (e.g. the aPTT and thrombin generation assays) are commonly known to involve threshold effects for peak thrombin generation, which are likely due to the minimum amount of thrombin required to activate FV/FVIII and generate the “thrombin burst” under specific assay conditions (see, for example: Sidonio, et al. *RPTH* 2022 or Wada, et al. *Ann Lab Med* 2023). Our data show that in a TGA using typical/standard conditions, thrombin generation appears to have an inflection point at around 50% FXII levels, consistent with our observations in the human and mouse data.

4. In the animal experiments, F12 heterozygous knockout mice were shown to have less thrombosis formation upon femoral vein injury, which, however, contradicts with earlier findings that heterozygous deficiency of FXII did not protect mouse model from thrombosis formation in Reference [20]. How to reconcile the seemingly conflicts? Could the differences be attributed to the methods used for thrombosis induction in animal model? The issue shall be addressed in the paper.

This is an excellent question. To our knowledge, only one experiment using F12+/- mice has ever been reported (Renne, et al. *JEM* 2005; Figure 3), and this work used a mesenteric arteriole thrombosis model. No data have been published on venous thrombosis. In fact, our data and the 2005 *JEM* result do not necessarily contradict. As the Reviewer suggests, the divergence is likely due to significant differences in the thrombosis models and vascular beds studied in the two papers. We did not evaluate arterial thrombosis and employed sophisticated readouts of venous thrombosis that are highly sensitive to coagulation factor deficiencies (Grover, et al. *JTH* 2020), which we believe allowed us to corroborate the phenotype observed in our human-level data. We have updated the Discussion section to include these points (page 12).

Reviewer #3 (Remarks to the Author):

The importance of factor (F) XII in thrombosis is uncertain. To address this uncertainty, Haj and colleagues interrogated UK and NIH biobank databases. They demonstrated protection against venous thromboembolism (VTE) in subjects heterozygous for FXII variants associated with a 50% reduction in FXII levels, compared with subjects without FXII deficiency, and no increase in bleeding or infection. They also demonstrated reduced silica-induced, but not tissue factor-induced, thrombin generation in plasma with 50% FXII levels compared with 100% FXII, as well as attenuated thrombus

formation in response to electrolytic injury in heterozygous FXII-deficient mice compared with wild-type mice. The authors conclude that because heterozygous FXII deficiency protects against VTE and is not associated with bleeding, FXII represents a promising target for new anticoagulants.

Although the findings are intriguing, major and minor concerns must be addressed.

MAJOR

1. The strength of the population data conclusion that FXII deficiency protects against VTE is limited by the small sample size (Figure 2A).

Although perhaps we misunderstand the Reviewer, we would respectfully disagree that our conclusions are based on a small sample size. Figure 2A features data from nearly 600,000 individuals, making our work approximately 250-fold larger than all prior general population studies of FXII deficiency combined (see refs 26, 27). In addition, 100% of participants in our study underwent germline sequencing via leading-edge NGS technology, which allowed us to extensively evaluate genetically-defined FXII deficiency for the first time. Therefore, we have employed unique, state-of-the-art, population-scale datasets to make novel discoveries with respect to FXII deficiency that previously would have been impossible (for other examples, see: Ryu, et al. *Blood* 2024, Haj, et al. *JTH* 2024, and Chaudhry, et al. *JAMA* 2025).

Nevertheless, in response to the Reviewer's comments we have extensively reworked our analyses to include over 100,000 additional participants (N=703,745). This was possible because during the intervening period since we first submitted our manuscript, the NIH All of Us program made available their "414K" data release, which added a substantial number of participants to the cohort. This larger re-analysis has slightly strengthened the observed association between heterozygous F12 loss of function and protection against VTE with respect to both statistical significance ($P=0.002 \rightarrow P=0.001$) and effect size ($HR=0.651 \rightarrow HR=0.648$). Adding to the robustness of our findings, effect size estimates for protection against VTE are nearly identical between UKB and AoU despite these cohorts being drawn from demographically and geographically distinct populations.

2. The authors demonstrate an association between FXII levels and VTE; however, the possibility of residual confounding cannot be ruled out. For example, were pregnant women, women taking estrogens, and subjects with recurrent VTE or cancer excluded from the analysis?

We thank the reviewer for raising this point and agree that residual confounding is always a concern in case/control analyses. We included several known VTE risk factors for which data were available (Table S4) and found that doing so did not meaningfully impact effect size estimates for *F12* loss of function (LOF). This is not surprising given that risk factors such as smoking status, BMI, blood group, and CRP are very unlikely to affect *F12* variant carrier status (in contrast, for example, to ancestry) and thus are not true statistical confounders. The relative lack of statistical confounders in germline genetic analyses compared to studies that rely on plasma FXII levels is a strength of our approach.

In light the Reviewer's request, we took a closer look at OCP and cancer status in our cohort. *F12* LOF variant carriers and non-carriers had very similar rates of OCP use and cancer diagnoses (Table S1). As expected, including these covariates did not appreciably alter the effect size estimate for *F12* LOF, and therefore our conclusions about the association between *F12* LOF and VTE risk remain unchanged:

Basic Primary Analysis (Table S3)

UK Biobank			NIH All of Us		
HR	95% CI	P	HR	95% CI	P
0.645	0.466-0.864	0.003	0.657	0.366-1.074	0.098

Primary Analysis Adjusted for OCP Use and Cancer Status

UK Biobank			NIH All of Us		
HR	95% CI	P	HR	95% CI	P
0.641	0.464-0.860	0.002	0.639	0.355-1.043	0.075

Unfortunately, the datasets we used do not contain information on recurrent VTE, and our conclusions are limited to the effects of heterozygous *F12* LOF on a first thrombosis event. We have updated the language throughout the manuscript to clarify that we are only examining the risk of a first VTE event.

3. Reduced silica-induced thrombin generation with FXII deficiency is a well-described phenomenon. However, the relevance of this finding to the risk of thrombosis remains uncertain.

We agree that considered in isolation, reduced *in vitro* thrombin generation in FXII-deficient plasma is not surprising. These data are intended to be viewed holistically in the context of the entire manuscript and show that in a sensitive TGA using typical/standard conditions, thrombin generation appears to have an inflection point at around 50% FXII levels, consistent with our observations in the human and mouse data.

Moreover, higher thrombin generation in global assays of coagulation such as the aPTT have been shown to associate with increased VTE risk (see, for example, Zakai, et al. Am J of Med 2008 or Tripoldi, et al. Blood 2004), further buttressing our conclusions.

4. The finding that compared with wild-type mice, thrombosis in response to electrolytic injury is reduced in mice heterozygous for FXII deficiency is not new. Furthermore, previous studies have shown that thrombosis in mice is FXII-driven, but it is unknown whether the same is true in humans.

We respectfully disagree. In fact, previous murine studies have consistently ignored the F12+/- genotype, which contributes to the novelty of our work. We know of only one experiment using F12+/- mice reported in the literature (Renne, et al. *JEM* 2005; Figure 3), and this work used a ferric chloride mesenteric arteriole thrombosis model. To our knowledge, the FXII heterozygous state has never been evaluated in a mouse model of venous thrombosis. Moreover, we stress that our experiments were specifically intended to assess for a haploinsufficient state associated with heterozygous *F12* LOF, something that has never been done before. Our findings in the mouse venous thrombosis model corroborate the large-scale human data presented herein showing a protective effect from *F12* LOF.

MINOR

1. Lifelong anticoagulation is only recommended for patients with unprovoked VTE or persistent major risk factors, such as active cancer (page 3). Consequently, the statement that “current guidelines recommend lifelong therapy for most patients” needs to be modified accordingly.

We thank the Reviewer for raising this point and have modified the manuscript accordingly.

2. Page 2: What is a presumed qualitative difference? Was this not validated?

Functional validation of “presumed” Type II (qualitative) FXII deficiencies was not performed and we believe is beyond the scope of this manuscript. In the text, we have moved away from the word “presumed,” which we agree may be confusing, and replaced it with the word “possible.” (Page 2, abstract) Specifically, characterization of the possible Type II variants ESS1 and ESS2 will be the subject of future investigations in our laboratory.

3. The population data findings would be more substantial if the authors demonstrated a link between low FXII levels and a reduced risk of both arterial and venous thrombosis, as well as a link between high FXII levels and an increased risk of both venous and

arterial thrombosis. FXII would be a less appealing target if it were only implicated in venous thrombosis.

We respectfully disagree. VTE is a major worldwide cause of morbidity and mortality, with vast resources spent on its diagnosis, prevention, and management (see, for example, Khan, et al. *Lancet* 2021). Assessment of potential associations between *F12* LOF and other thrombosis phenotypes is the subject of ongoing research in our group. Furthermore, given significant concern that analyses relying on spot plasma FXII levels are heavily confounded by the multitude of variables that may affect circulating protein levels (e.g. renal function, diurnal cycle, endocrine function, liver function, nutritional status, medications, recent hemostatic challenges, etc.), we believe that correlating plasma FXII levels to outcomes is unlikely to be informative. By contrast, our analysis was designed to circumvent the well-known limitations of spot plasma protein levels by using germline genetics to assess the impact of lifelong FXII deficiency.

4. Tissue factor is the likely driver of most cases of VTE. Back activation of FXI by tissue factor-induced thrombin generation could bypass FXIIa inhibition. The authors need to address this possibility.

We agree that TF-mediated thrombin generation and back activation of FXI has been proposed as a contributor to VTE. Nevertheless, our data indicate that FXII-mediated thrombin generation remains an important contributor to VTE risk in addition to any effects that may be occurring via FXI. We have modified the Discussion section to clarify this point (page 12).

5. Figure 1E: Why were the two gene sets separated? The only difference is the inclusion of FV. Why wasn't FXI included, given its current focus as a novel target for safer anticoagulants?

We thank the reviewer for raising this point. This figure attempts to compare FXII to well-established groupings of coagulation factors that are necessary for physiologic hemostasis and would be familiar to an audience in the field (i.e., vitamin K dependent factors and essential humoral coagulation factors). With respect to FXI, there is no consensus that it is a "necessary" coagulation factor that is indispensable for physiologic hemostasis. We felt it better to avoid including FXI in order to ensure clarity and simplicity and because FXI is beyond the scope of our manuscript.

6. Were there any individuals with biallelic FXII FIS = 1? Do these individuals exhibit an increased risk of bleeding?

There were 2 individuals suspected to have biallelic *F12* mutations. None demonstrated bleeding. However, meaningful analysis was precluded given the lack of participants with biallelic *F12* LOF.

7. Figure 2B: Data for *F10*, *F2*, and *F11* should be added to calibrate the analysis with known outcomes in patients receiving DOACs.

We respectfully disagree. The objective of this panel is simply to show that *F12* variant deleteriousness is associated with protection against VTE but not with bleeding—that comparison is achieved in the current form of Figure 2B. Both the bleeding and VTE phenotypes have been extensively validated. For example, the thrombosis phenotype we are using is sensitive to classic thrombophilias such as factor V Leiden and protein S deficiency (see Ryu, et al. *Blood* 2024 and Chaudhry, et al. *JAMA* 2025). We also show data that the VTE phenotype is linked to known VTE associations such as BMI, blood group, CRP and smoking status (Table S4). Likewise, the bleeding phenotype we use has also been validated previously (see Haj, et al. *JTH* 2024). We believe that repeating these analyses with several additional genes that are not the focus of this paper would reduce clarity and could potentially be misleading.

8. Figure 3: Is the expected bleeding observed in patients with FIS = 1 in *FIX* and *FVIII*?

Unfortunately, it was not possible to evaluate the impact of germline loss of *F8* or *F9* on bleeding risk due to the lack of participants with high-confidence LOF variants in these genes. However, we have previously shown that the coagulopathic bleeding phenotype is associated with loss of function in the gene encoding von Willebrand factor (*VWF*) (please see Haj, et al. *JTH* 2024).

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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RESPONSE TO REVIEWERS

Reviewer #3 (Remarks to the Author):

The revised paper is much improved. However, I still have concerns.

1. The observation that partial factor XII deficiency is associated with a reduced risk of venous thromboembolism is unexpected because such a link has not been demonstrated in observational studies (Johnson CY, et al. *Am J Epidemiol.* 201; 173: 136-44) or GWAS analyses (Klarin D, et al. *Nat Genet.* 2019; 51:1574-1579), and contrary to the results reported in this paper with venous thrombosis, arterial thrombosis in response to injury was reported to be normal in mice with heterozygous deficiency of factor XII (Renné T, et al. *J Exp Med.* 2005; 18;202:271-81). Therefore, if the findings of the current paper are correct, they are potentially paradigm-shifting. Because the stakes are high, I want assurance that the methodology works. As far as I can tell, this technology has not been used previously to study coagulation factors or venous thromboembolism. Unless data supports its use, I would like to see a similar analysis with factor XI loss-of-function alleles, which are known to be associated with a reduced risk of venous thromboembolism, to confirm that the methodology works.

We greatly appreciate the Reviewer's comment that our work is potentially paradigm-shifting and completely agree that such findings deserve rigorous evaluation. At the outset, we note that both the Johnson and Klarin papers employed common variant approaches (observational and genotyping array GWAS, respectively) that lack the acuity necessary to detect a phenotype associated with rare loss of function variants in *F12*. Therefore, our distinctive methodology contributes to the novelty and impact of our work. We can reassure the Reviewer that our approach is well-established and has been successfully applied in our hands to study bleeding and clotting disorders, including VTE (see, for example, Chaudhry, et al. *JAMA* 2025; Ryu, et al. *Blood* 2024; Haj, et al. *JTH* 2024). A detailed description of the methodology developed and implemented by our group can be found here: Jurgens, et al. *Nat Genetics* 2024.

Redacted by the editor due to the description of preliminary data that have not yet been published

Finally, we appreciate the Reviewer bringing the Johnson paper (Am J Epidemiol. 2011; 173: 136-44) to our attention. We read it with interest and have added it to our citations for this manuscript.

2. The impact of the findings would be enhanced if the authors demonstrated a link between factor XII deficiency and arterial thrombosis. Mechanistically, why would the effect of factor XII be localized to veins, and why are the current results in a mouse model of venous thrombosis different from those reported in a murine model of arterial thrombosis?

We fully agree with the Reviewer that it would be of interest to show an impact from *F12* haploinsufficiency on arterial thrombosis. However, we are unable to change the scope and overall structure of this manuscript, which is specifically focused on VTE at the levels of human genomic data, *in vitro* validation, and mouse models. The impact of germline loss of function in *F12* on the risk of arterial thrombosis phenotypes is the subject of a separate ongoing study in our lab. Further, we note that there are many examples of humoral coagulation factors that play a role in venous but not arterial thrombosis despite underlying biology suggesting that both vascular beds should be affected (e.g. protein C, protein S, antithrombin, factor V Leiden, prothrombin gene mutation G20210A, etc.). Some of these examples have been confirmed in our own

hands using population-scale datasets (see Chaudhry, et al. *JAMA* 2025 or Ryu, et al. *Blood* 2024). The mechanism underlying this observation remains incompletely understood.