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Title: Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of  $\alpha$ 2-plasmin inhibitor to fibrin and its consequences on fibrinolysis

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**Abstract:** Introduction: It has been shown that thrombomodulin (TM) considerably delays factor XIII (FXIII) activation and this effect is abrogated by Factor V Leiden (FVLeiden) mutation. The aim of the study was to explore the effect of TM on the cross-linking of  $\alpha$ 2-plasmin inhibitor ( $\alpha$ 2-PI) to fibrin in plasma samples of different FV genotypes and how this effect is related to the impaired fibrinolysis of FVLeiden carriers. Methods: In the plasma samples of fifteen individuals with different FV genotypes and in FV deficient plasma supplemented with wild type FV or FVLeiden coagulation was initiated by recombinant human tissue factor and phospholipids with or without recombinant human TM (rhTM). In the recovered clots the extent of  $\alpha$ 2-PI-fibrin cross-linking was evaluated by Western blotting and quantitative densitometry. The effect of rhTM on tissue plasminogen activator (tPA) induced clot lysis was measured by turbidimetric method. Results: rhTM significantly delayed the formation of  $\alpha$ 2-PI-fibrin  $\alpha$ -chain heterodimers/oligomers in plasma samples containing wild type FV. This effect of rhTM was impaired in the presence of FVLeiden. rhTM delayed tPA-induced clot lysis and this effect of rhTM was more pronounced in plasma containing FVLeiden. When TAFIa was inhibited by potato carboxypeptidase inhibitor, rhTM accelerated clot lysis in the presence of wild type FV, which is explained by the delayed  $\alpha$ 2-PI-fibrin cross-linking. This effect of rhTM did not prevail in the presence of FVLeiden. Conclusion: FVLeiden abrogates the delaying effect of rhTM on  $\alpha$ 2-PI-fibrin cross-linking, which contributes to the impaired fibrinolysis observed in FVLeiden carriers.

**Conflict of Interest Form**

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**Regular article**

**Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of  $\alpha_2$ -plasmin inhibitor to fibrin and its consequences on fibrinolysis**

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## **ABSTRACT**

*Introduction:* It has been shown that thrombomodulin (TM) considerably delays factor XIII (FXIII) activation and this effect is abrogated by Factor V Leiden (FV<sub>Leiden</sub>) mutation. The aim of the study was to explore the effect of TM on the cross-linking of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin in plasma samples of different FV genotypes and how this effect is related to the impaired fibrinolysis of FV<sub>Leiden</sub> carriers. *Methods:* In the plasma samples of fifteen individuals with different FV genotypes and in FV deficient plasma supplemented with wild type FV or FV<sub>Leiden</sub> coagulation was initiated by recombinant human tissue factor and phospholipids with or without recombinant human TM (rhTM). In the recovered clots the extent of  $\alpha_2$ -PI-fibrin cross-linking was evaluated by Western blotting and quantitative densitometry. The effect of rhTM on tissue plasminogen activator (tPA) induced clot lysis was measured by turbidimetric method. *Results:* rhTM significantly delayed the formation of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimers/oligomers in plasma samples containing wild type FV. This effect of rhTM was impaired in the presence of FV<sub>Leiden</sub>. rhTM delayed tPA-induced clot lysis and this effect of rhTM was more pronounced in plasma containing FV<sub>Leiden</sub>. When TAFIa was inhibited by potato carboxypeptidase inhibitor, rhTM accelerated clot lysis in the presence of wild type FV, which is explained by the delayed  $\alpha_2$ -PI-fibrin cross-linking. This effect of rhTM did not prevail in the presence of FV<sub>Leiden</sub>. *Conclusion:* FV<sub>Leiden</sub> abrogates the delaying effect of rhTM on  $\alpha_2$ -PI-fibrin cross-linking, which contributes to the impaired fibrinolysis observed in FV<sub>Leiden</sub> carriers.

**Keywords:**

$\alpha_2$ -plasmin inhibitor, factor V Leiden mutation, factor XIII, fibrinolysis, thrombin  
activatable fibrinolysis inhibitor.

**List of abbreviations:**

$\alpha_2$ -PI:	$\alpha_2$ -plasmin inhibitor
APC:	activated protein C
APCE:	antiplasmin cleaving enzyme
CPI:	carboxypeptidase inhibitor
FVa:	activated factor V
FV <sub>Leiden</sub> :	factor V Leiden mutation
FXIII:	factor XIII
FXIIIa:	active factor XIII
FXIII-A:	factor XIII A subunit
FXIII-A <sup>'</sup> :	proteolytically activated factor XIII A subunit
PDP:	platelet depleted plasma
PPACK:	D-phenylalanyl-L-prolyl-L-arginin chloromethyl ketone
rhTM:	recombinant human thrombomodulin
T <sub>1/2</sub> :	time required for half maximal cross-linking of $\alpha_2$ -plasmin inhibitor to fibrin $\alpha$ -chain
TAFI:	thrombin activatable fibrinolysis inhibitor
TAFIa:	active thrombin activatable fibrinolysis inhibitor
TF:	tissue factor

TM: thrombomodulin

tPA: tissue plasminogen activator

## Introduction

p.506R>Q mutation in factor V (FV) heavy chain (FV<sub>Leiden</sub>), which is common among Caucasians, is associated with 5-8-fold increased risk of deep vein thrombosis in heterozygotes and 50-80-fold risk among homozygotes [1, 2]. This amino acid replacement eliminates the primary cleavage site of activated protein C (APC) in FV and, consequently compromises the APC-induced down-regulation of thrombin generation through a double mechanism [3-6]. It impairs the down-regulation of the procoagulant activity of activated FV (FVa) by APC and also FV cofactor activity involved in APC-catalyzed inactivation of activated factor VIII [6-8]. These effects lead to increased thrombin generation, a key event in the increased thrombosis risk of individuals with FV<sub>Leiden</sub> [6, 9]. The downstream consequences of FV<sub>Leiden</sub>, which might be connected to the increased thrombin generation and are very likely important in the increased thrombotic risk, have only partially been revealed. It has been shown that APC also exerts a profibrinolytic effect and this mechanism, at least in part, operates through the down-regulation of thrombin-induced activation of thrombin activatable fibrinolysis inhibitor (TAFI) [10, 11]. TAFI, also known as procarboxypeptidase U, procarboxypeptidase B2 or procarboxypeptidase R, is activated by thrombin, a process that is more than 1,000-fold enhanced in the presence of thrombomodulin (TM) [10]. Activated TAFI (TAFIa) cleaves off C-terminal lysines from fibrin fragments; these residues in partially digested fibrin are essential for efficient plasminogen activation (see reviewed in references [12-15]). FV<sub>Leiden</sub> also impairs the profibrinolytic response to APC [16]. In subjects with FV<sub>Leiden</sub>, the impaired fibrinolysis is associated with the lack of

APC effect on thrombin generation and, consequently, on TAFI activation [16]. The increased activation of TAFI in the presence of FV<sub>Leiden</sub> depends on TM concentration [17].

Blood coagulation factor XIII (FXIII) is another thrombin substrate with an important role in the regulation of fibrinolysis [18, 19]. FXIII transformed into an active transglutaminase (FXIIIa) by thrombin and Ca<sup>2+</sup> cross-links fibrin chains and  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin. This way FXIIIa makes fibrin more resistant to shear stress and protects it from fibrinolytic degradation. It has been shown that TM considerably inhibited the activation of FXIII in purified systems [20, 21]. In the plasma from FV wild type individuals recombinant human TM (rhTM) significantly delayed FXIII activation and the suppression of thrombin generation by APC seemed to play a primary role in this process [22]. This effect of rhTM was strongly diminished in the plasma from FV<sub>Leiden</sub> carriers [22]. In the present study we investigated how the effect of rhTM and FV<sub>Leiden</sub> on FXIII activation influences the fibrinolytic process through the cross-linking of  $\alpha_2$ -PI to fibrin. It was also explored how the effects on FXIII and TAFI activation are related to the impaired fibrinolysis caused by FV<sub>Leiden</sub> in the presence of rhTM.

## **Material and methods**

### *Subjects*

Fifteen apparently healthy individuals of known FV<sub>Leiden</sub> genotypes (n=5 for each genotype) without the history of thrombotic disorders were recruited for the study (3 men



and 12 women; age 17-67 years). All individuals had normal coagulation screening tests; their fibrinogen level, FXIII activity and antigen were in the reference interval. They did not take any medication for at least 2 weeks prior to blood sampling. As the rate of FXIII activation and the cross-linking of  $\alpha_2$ -PI to fibrin are modified by FXIII-A Val34Leu polymorphism [23-26], only subjects being wild type for this polymorphism were selected for the study. Genotyping for FV<sub>Leiden</sub> mutation and FXIII-A Val34Leu polymorphism was carried out by standard methods [27, 28]. The study protocol was approved by the Regional Ethics Committee of the University of Debrecen. Informed consent was given by all subjects involved in the study.

#### *Preparation of plasma samples*

18 mL blood was collected by vein puncture into 2 mL 0.105 M trisodium citrate using 21 gauge, 0.8 x 38 mm needle (Becton Dickinson, Franklin Lakes, NJ). Contact activation of coagulation was suppressed by the immediate addition of 50  $\mu$ g/mL corn trypsin inhibitor (Gentaur, Brussels, Belgium) to the blood sample [29]. Platelet-depleted plasma (PDP) was obtained by double centrifugation. First, citrated blood was centrifuged at 1,400 g for 20 min, and then the platelet poor plasma was removed and centrifuged again. In PDP no platelet was detected by platelet counting using Sysmex XE-2100 hematology analyzer (Kobe, Japan).

#### *Preparation of crude factor V from pooled plasma*

Partial purification of FV from the pooled plasma of wild type individuals or FV<sub>Leiden</sub> homozygotes was carried out using the slightly modified method of Dahlback [22, 30]. Briefly, PDP samples of the same genotypes were pooled and one tablet of protease inhibitor cocktail (Complete, Mini, EDTA-free, Roche Diagnostics, Indianapolis, IN) was added to each 10 mL of pooled plasma. After absorption by barium citrate, crude FV was isolated by differential PEG-6,000 (Sigma-Aldrich, St. Louis, MO) precipitation. The FV containing precipitate was washed with 120 g/L PEG-6,000 in dist. water and it was dissolved in immunodepleted FV deficient plasma (Technoclone, Vienna, Austria). The FV activity of dissolved preparation was determined by one-stage clotting assay, then it was diluted in FV deficient plasma to set the activity to 0.9 U/mL.

#### *Cross-linking of $\alpha_2$ -plasmin inhibitor to fibrin in plasma samples*

In 150  $\mu$ L PDP samples and 10  $\mu$ L physiological saline coagulation was induced by 40  $\mu$ L activator cocktail as described earlier [22]. The activator cocktail consisted of Technothrombin<sup>®</sup> TGA RC Low (Technoclone GmbH, Vienna, Austria) dissolved in 62.5 mM CaCl<sub>2</sub>, with or without 7.5 nM rhTM (American Diagnostica, Stamford, CT). The dissolved Technothrombin<sup>®</sup> TGA RC Low contained ~5 pmol/L recombinant human TF, low concentration of phospholipid micelles in Tris-Hepes-NaCl buffer. An rhTM concentration, 7.5 nM in the activator cocktail, 1.5 nM final concentration, that inhibits thrombin generation in the plasma of FV wild type individuals by approximately 50% was recommended by Dielis et al. to study the effect of changes in thrombin generation [31]. We tested the recommended rhTM concentration in the plasma of FV wild type

individuals and 45.4% mean inhibition (range 40-61%) by rhTM was obtained as calculated from the AUC (area under the curve; also referred to as endogen thrombin potential) values. After various intervals the reaction was stopped by an equal volume of inhibitor cocktail containing 20 mM benzamidine, 0.1 mM D-phenylalanyl-L-prolyl-L-arginin chloromethyl ketone (PPACK), 2 mM iodoacetamide, 50 mM  $\epsilon$ -aminocaproic acid, 100 mM NaCl, 50 mM EDTA, 50 mM HEPES, pH 7.5 to block thrombin and FXIIIa, and to prevent fibrinolysis [25]. Fibrin clots, if present, were recovered by centrifugation, exhaustively washed with physiological NaCl and dissolved in SDS PAGE sample buffer. No free, non-cross-linked  $\alpha_2$ -PI remained in the clot after extensive washing.

#### *SDS-PAGE and Western blotting*

The dissolved fibrin samples were subjected to SDS-PAGE in 7.5% gel and the cross-linking of  $\alpha_2$ -PI to fibrin was monitored by Western blotting using affinity purified goat anti-human  $\alpha_2$ -PI as primary antibody (Affinity Biologicals, Ancaster, Canada). The immunoreaction was developed by biotinylated rabbit anti-goat IgG and avidin-biotinylated peroxidase complex (components of Vectastain ABC kit, Vector, Burlingame, CA). The immune reaction was visualized by enhanced chemiluminescence detection (ECL Plus+, Amersham, Little Chalfont, UK) according to the manufacturer's instructions. The extent of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer formation and the relative amount of total cross-linked  $\alpha_2$ -PI were determined by quantitative densitometry and

expressed as percentage of maximal amount of the respective cross-linked products.  $T_{1/2}$  values, the intervals required for 50% of  $\alpha_2$ -PI cross-linking, were calculated.

#### *Clot lysis assay*

Tissue plasminogen activator (tPA) induced clot lysis was investigated in a turbidimetric assay system [16, 17]. Fibrinolysis was induced by mixing 10  $\mu$ L tPA (American Diagnostica, Stamford, CT; final concentration: 5 nM), to the plasma immediately before the addition of activator cocktail with or without 7.5 nM rhTM. The process of clotting and fibrinolysis was monitored for two hours in a temperature-controlled microplate reader (405 nm, 37 °C). 50% clot lysis times were determined as the time elapsed between the maximum turbidity and the midpoint of the maximum turbid-to-clear transition [17]. To inhibit TAFIa, certain experiments were carried out in the presence 25  $\mu$ g/mL carboxypeptidase inhibitor (CPI) from potato tubers (Sigma, St. Louis, MO), as recommended by Mosnier et al. [17]. When indicated, FXIIIa activity was blocked by the addition of 2 mM iodoacetamide to the reaction mixture.

#### *Statistical analysis*

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Kruskal-Wallis one-way analysis of variance was used to determine differences among groups. The extent of significance was determined by Mann-Whitney U test;  $p < 0.05$  was considered as significant.

## Results

The covalent incorporation of  $\alpha_2$ -PI into fibrin proceeds in two steps. First, there is a quick formation of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer which is followed by the much slower incorporation of the heterodimer into fibrin  $\alpha$ -chain oligomers/polymers [32]. The effect of rhTM on the cross-linking of  $\alpha_2$ -PI to fibrin in the plasma from a representative FV wild type individual is demonstrated on Fig. 1 and on Fig. 3. In the presence of rhTM the formation of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer starts and becomes intensive significantly later than in the absence of rhTM. There is also a significant difference in the appearance of high Mr  $\alpha_2$ -PI containing polymers. In contrast, the difference between samples with and without rhTM disappears in patients homozygous for FV<sub>Leiden</sub> mutation (Fig. 2 and Fig. 3) and even the presence of one mutant allele significantly decreases the difference (Fig. 3). Fig. 3A demonstrates the kinetics of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer formation in the plasma of a representative individual of each FV genotype. The ineffectiveness of rhTM in both groups of FV<sub>Leiden</sub> carriers is evident. The average differences in the  $T_{1/2}$  values for  $\alpha$ -chain heterodimer formation measured in the absence and presence of rhTM are shown on Fig. 3B. The difference between wild type individuals and heterozygotes or homozygotes is statistically significant. The two FV<sub>Leiden</sub> carrier groups did not differ significantly. Similar FV<sub>Leiden</sub> mutation-dependence was obtained when the formation of total cross-linked  $\alpha_2$ -PI ( $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimers plus oligomers) was measured in the presence and absence of rhTM (not shown).

FV<sub>Leiden</sub> homozygotes, wild type for FXIII Val34Leu polymorphism and not on anticoagulant treatment, were very hard to recruit and five was the maximum number which we were able to select for the study from over three thousands genotyped individuals. For this reason, and in order to eliminate individual variations that influence clot formation, a different approach was also followed. FV was isolated from pooled plasma of individuals either wild type or homozygous for FV<sub>Leiden</sub> and these isolated crude FV preparations were used to supplement FV deficient plasma. The supplemented plasma samples, which differed only in the genotype of FV, were used to study how FV<sub>Leiden</sub> mutation influences the effect of rhTM on  $\alpha_2$ -PI cross-linking (Figs. 4-6). In FV deficient plasma supplemented with wild type FV rhTM significantly delayed  $\alpha_2$ -PI cross-linking (Fig. 4 and Fig. 6). In the plasma supplemented with FV<sub>Leiden</sub> the effect of rhTM was strongly diminished (Fig. 5 and Fig. 6).

As the cross-linking of  $\alpha_2$ -PI to fibrin has a major impact on clot lysis, in the followings we investigated the effect of rhTM on tPA induced clot lysis (Fig. 7). In the absence of rhTM there was no difference between the two genotypes. rhTM significantly prolonged the clot lysis time in both genotypes, i.e., the fibrinolysis was significantly decreased if rhTM was present (Fig. 7A). However, in the case of FV<sub>Leiden</sub> homozygotes rhTM was significantly more effective than in the case of wild type individuals. In the former case 50% lysis occurred 40 minutes later than in the plasma of FV wild type individuals. A major effect of TM on fibrinolysis is exerted through affecting the activation of TAFI. To prove that its effect on  $\alpha_2$ -PI cross-linking also plays a role in the regulation of fibrinolysis, experiments were also carried out in the presence of CPI, a TAFIa inhibitor and also in condition when both TAFIa and FXIIIa were inhibited. In

sharp contrast with the results obtained without TAFIa inhibitor, when TAFIa was inhibited rhTM resulted in accelerated fibrinolysis in FV wild type plasma (Fig. 7B) and rhTM did not accelerated fibrinolysis in plasma samples from individuals with FV<sub>Leiden</sub> mutation. It seemed very likely that when TAFIa was blocked, the acceleration of fibrinolysis by rhTM in wild type plasma samples was due to the delay in the cross-linking of  $\alpha_2$ -PI, which did not occur in plasma from FV<sub>Leiden</sub> mutants. To prove the role of FXIII, i.e.,  $\alpha_2$ -PI cross-linking, in the effect of rhTM in TAFIa blocked plasma samples, the clot lysis assay was also carried out in the presence of FXIIIa inhibitor (Fig. 7C). In this case clot lysis was equally and highly accelerated in all plasma samples and the difference between wild type plasma samples with and without rhTM disappeared.

## **Discussion**

One of the prothrombotic effect of FV<sub>Leiden</sub> is the inhibition of fibrinolysis, which has been demonstrated both in vitro [16] and in vivo in transgenic mice [33]. Two key down-regulators of fibrinolysis are  $\alpha_2$ -PI and TAFI.  $\alpha_2$ -PI, the main physiological inhibitor of plasmin, is synthesized in the liver and becomes secreted as a protein of 491 amino acids starting with a methionine (Met1- $\alpha_2$ -PI) [32]. In the plasma a protease, antiplasmin cleaving enzyme (APCE), cleaves off the N-terminal propeptide of 12 amino acid residues [34] and transforms approximately 70% of Met1- $\alpha_2$ -PI into Asn1- $\alpha_2$ -PI. The latter is an excellent substrate of FXIIIa [35, 36] and during the formation of the fibrin clot, its Gln2 site becomes cross-linked to Lys303 in fibrin  $\alpha$ -chain [37].  $\alpha_2$ -PI cross-linked to fibrin retains its full inhibitory activity and makes fibrin strikingly resistant to

digestion by plasmin [34, 38]. The covalent incorporation of  $\alpha_2$ -PI into the clot protects fibrin from the prompt elimination by the fibrinolytic system. Most recently it has been revealed that the antifibrinolytic effect of FXIII is exclusively expressed through  $\alpha_2$ -PI cross-linking [39].

In the plasma containing wild type FV the onset of FXIII activation [22] and the cross-linking of  $\alpha_2$ -PI to fibrin was significantly delayed in the presence of rhTM. In theory, this effect could be due to the direct inhibition of thrombin-induced FXIII activation [20, 21] or, indirectly to the inhibition of thrombin generation exerted through the APC pathway. The finding that the delaying effect was not present in plasma samples from individuals with FV<sub>Leiden</sub> mutation favor the latter mechanism that operates through decreased thrombin generation. This delay could give time for the initial elimination of newly formed thrombus by the fibrinolytic enzyme in wild type individuals. The abrogation of the delaying effect of TM by FV<sub>Leiden</sub> could represent a novel molecular mechanism that contributes to the antifibrinolytic effect of FV<sub>Leiden</sub> mutation, and this way to the increased risk of thrombosis in these individuals.

Concerning the effect of rhTM on the incorporation of  $\alpha_2$ -PI into fibrin, the difference between wild type and heterozygous individuals in  $T_{1/2}$  of  $\alpha_2$ -PI cross-linking was considerably higher than between heterozygous and homozygous individuals and there was no statistically significant difference between FV<sub>Leiden</sub> heterozygotes and homozygotes. It is likely that the relationship between thrombin generation and  $\alpha_2$ -PI cross-linking is not linear, and the lack of significance might be due to the lack of sufficient statistical power. Alternatively, gene dosage effect might not operate in this



situation and a single mutant allele was sufficient to bring about close to maximal effect in the in vitro set-up used for the experiments.

The regulation of fibrinolysis by TM is a complex mechanism, which involves both TAFI and  $\alpha_2$ -PI (Fig. 8). Its concentration-dependent effect on TAFI activation is complex in itself and implicates two opposing mechanisms [17, 40, 41]. On one hand TM can down-regulate fibrinolysis by stimulating thrombin-induced TAFI activation, on the other hand this effect is diminished by the TM-promoted activation of PC, which decreases the generation of thrombin required for TAFI activation. The latter mechanism also delays FXIII activation and, consequently, the cross-linking of  $\alpha_2$ -PI to fibrin resulting in a profibrinolytic effect. In the plasma of FV wild type individuals, at the rhTM concentration selected for the experiments the opposing mechanisms cannot overcome the stimulation of TAFIa production and the net result is the inhibition of fibrinolysis, as demonstrated by the prolonged clot lysis time (Fig. 7A). In the plasma with FV<sub>Leiden</sub> the down-regulation of thrombin generation is strongly diminished and the opposing mechanisms do not operate. As a result, TAFI activation is not inhibited and the cross-linking of  $\alpha_2$ -PI to fibrin is not delayed, which leads to the increased prolongation of clot lysis time, i.e., in an even more effective antifibrinolytic effect. To prove that FXIII activation and  $\alpha_2$ -PI cross-linking indeed play a role in the effect of rhTM on fibrinolysis, additional experiments were carried out in the presence of a TAFIa inhibitor, which eliminated the mechanisms operating through TAFI activation. When TAFIa was inhibited in plasma samples from FV wild type individuals,  $\alpha_2$ -PI cross-linking was delayed and fibrinolysis was accelerated in the presence of rhTM (Fig. 7B). In the case of FV<sub>Leiden</sub> mutants this delay of  $\alpha_2$ -PI cross-linking was abrogated and there was no

significant difference between the clot lysis times measured in the presence and absence of rhTM. The fact that additional inhibition of  $\alpha_2$ -PI cross-linking by a FXIIIa inhibitor highly accelerated fibrinolysis and eliminated the differences among TAFIa inhibited samples (Fig. 7C) underlines the importance of  $\alpha_2$ -PI cross-linking in the antifibrinolytic effect of FV<sub>Leiden</sub> mutation.

In summary, TM delays thrombin-induced FXIII activation and  $\alpha_2$ -PI cross-linking to fibrin by FXIIIa in FV wild type individuals by down-regulating thrombin generation through the APC pathway. The abrogation of these effects of TM by FV<sub>Leiden</sub> contributes to the antifibrinolytic effect of the mutation.

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### **Disclosure of Conflict of Interests**

The authors state that they have no conflict of interest.

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## Figure legends

**Fig. 1.** The cross-linking of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin  $\alpha$ -chain in plasma samples of a FV wild type individual in the absence and presence of recombinant human thrombomodulin (rhTM). (A) Washed plasma clots recovered at various time after the activation of coagulation were analyzed by Western blotting for  $\alpha_2$ -PI. Numbers under the individual bands on the blots indicate the time elapsed after the initiation of coagulation. Bands representing  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer, and  $\alpha_2$ -PI-fibrin  $\alpha$ -chain oligomers are indicated. (B) Densitometric tracings of Western blots obtained at selected times after the initiation of coagulation.

**Fig. 2.** The cross-linking of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin  $\alpha$ -chain in plasma samples of an individual homozygous for FV<sub>Leiden</sub> mutation in the absence and presence of recombinant human thrombomodulin (rhTM). (A) Washed plasma clots recovered at various time after the activation of coagulation were analyzed by Western blotting for  $\alpha_2$ -PI. Numbers under the individual bands on the blots indicate the time elapsed after the initiation of coagulation. Bands representing  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer, and  $\alpha_2$ -PI-fibrin  $\alpha$ -chain oligomers are indicated. (B) Densitometric tracings of Western blots obtained at selected times after the initiation of coagulation.

**Fig. 3.** The effect of rhTM on the time course of  $\alpha_2$ -PI cross-linking to fibrin in the plasma of individuals of different FV R506Q genotype. (A) Results of quantitative

densitometric analysis of Western blots obtained with representative plasma clots. The percentage of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer as compared to the maximal amount of heterodimer formed in the clot was calculated for each time points. Results obtained in the presence of rhTM (+rhTM) are depicted by solid symbols, while empty symbols represent results obtained in the absence of rhTM (-rhTM). The respective curves are also indicated by arrows. One representative sample of each genotype is shown. Broken lines demonstrate 50% heterodimer formation. (B) The effect of FV<sub>Leiden</sub> genotype on the difference in the lag time required for 50%  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer formation ( $T_{1/2}$ ) in the presence and absence of rhTM. The bars represent differences in  $T_{1/2}$  values measured in the presence and absence of rhTM: ( $T_{1/2}$ rhTM+)-( $T_{1/2}$ rhTM-). Error bars depict SEM, n=5 for each genotype, \* $p < 0.05$ , \*\* $p < 0.01$ . +/+ : wild type, +/- : FV<sub>Leiden</sub> heterozygotes, -/- : FV<sub>Leiden</sub> homozygotes.

**Fig. 4.** The cross-linking of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin  $\alpha$ -chain in FV deficient plasma supplemented with crude FV prepared from pooled plasma of FV wild type individuals. Washed plasma clots were recovered at various times after the activation of coagulation in the absence or presence of rhTM. Cross-linked  $\alpha_2$ -PI in the recovered clot was detected by Western blotting and quantified by quantitative densitometry. Numbers under the individual bands on the blots indicate the time elapsed after the initiation of coagulation. Bands representing  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer, and  $\alpha_2$ -PI-fibrin  $\alpha$ -chain oligomers are indicated. (B) Densitometric tracings of Western blots obtained at selected times after the initiation of coagulation.

**Fig. 5.** The cross-linking of  $\alpha_2$ -plasmin inhibitor ( $\alpha_2$ -PI) to fibrin  $\alpha$ -chain in FV deficient plasma supplemented with FV prepared from plasma of individuals homozygous for  $FV_{Leiden}$  mutation. Washed plasma clots were recovered at various times after the activation of coagulation in the absence or presence of rhTM. Cross-linked  $\alpha_2$ -PI in the recovered clot was detected by Western blotting and quantified by quantitative densitometry. Numbers under the individual bands on the blots indicate the time elapsed after the initiation of coagulation. Bands representing  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer, and  $\alpha_2$ -PI-fibrin  $\alpha$ -chain oligomers are indicated. (B) Densitometric tracings of Western blots obtained at selected times after the initiation of coagulation.

**Fig. 6.** The effect of rhTM on the time course of  $\alpha_2$ -PI cross-linking to fibrin in the FV deficient plasma supplemented with FV prepared from the plasma of FV wild type individuals or  $FV_{Leiden}$  homozygotes. Based on quantitative densitometric analysis of Western blots the percentage of  $\alpha_2$ -PI-fibrin  $\alpha$ -chain heterodimer as compared to the maximal amount of heterodimer formed in the clot was calculated for each time points. The symbols represent the means of two parallel experiments with practically identical results. Results obtained in the presence of rhTM (+rhTM) are depicted by solid symbols, while empty symbols represent results obtained in the absence of rhTM (-rhTM). The respective curves are also indicated by arrows. Broken lines demonstrate 50% heterodimer formation.

**Fig. 7.** The effect of rhTM on tPA induced clot lysis in plasma samples of FV wild type and  $FV_{Leiden}$  homozygous individuals. (A) in the absence of inhibitors (B) in the presence of TAFIa inhibitor, (C) in the presence of TAFIa and FXIIIa inhibitors. 50% clot lysis



times were determined as the time between the maximum turbidity and the midpoint of the maximum turbid-to-clear transition. TM- without thrombomodulin, TM+ in the presence of thrombomodulin. Error bars depict SEM, n=5 for each genotype. Horizontal lines with the asterisks above the columns indicate the groups, which were compared and between which significant difference was found. \* $p < 0.05$ , \*\* $p < 0.01$

**Fig. 8.** Schematic diagram demonstrating the effect of thrombomodulin on clot lysis in FV wild type individuals and in individuals with FV<sub>Leiden</sub> mutation.

## Response to reviewers

We are indebted to the referees for reviewing our manuscript and suggesting improvements. We accepted their suggestions and revised the manuscript accordingly. Changes in the manuscript are shown in red color.

### Reviewer 1

We thank the reviewer for considering the study as well done and clearly presented and are indebted for the suggestions.

1. *What concentration of TM has been used for clot lysis experiments (page 10, line 4)?*

The concentration of TM in the activator cocktail (7.5 nM) was added to the text.

2. *Why 7.5 nM concentration of TM was chosen for cross linking experiments?*

The aim was to select a TM concentration that inhibits thrombin generation by approximately 50% in the plasma of wild type FV individuals (Dielis et al. Haematologica 2008; 93: 1351-7). At 50% inhibition the system is sensitive enough to study the effect of changes in thrombin generation. Dielis et al. found that 1.5 nM rhTM (7.5 nM in the activator cocktail) inhibits thrombin generation in the FV wild type individuals by 50%. We tested the recommended concentration in our assay system and obtained inhibition in the range of 40-61%. Thus, the concentration recommended by Dielis et al. was adapted for the experiments. The selection of rhTM concentration is now explained in the revised manuscript in more details.

3. *Discussion; page 14, last two lines: the same sentence appears in the last paragraph on page 16, and therefore could be deleted.*

The sentence was deleted.

4. *Figures: Figure 8 does not clarify further the statements in the text on page 15. I suggest deleting it.*

This is a complicated system and it is our experience from presenting the results on scientific congresses, that readers less familiar with the topic need some visual help in understanding the impact of the results. For this reason we would rather keep the figure. However, if the reviewer insists, we will delete it.

*In Figure 7 I suggest deleting the crossed over TAFI in B and crossed over TAFI, FXIII in C, since their absence is sufficiently explained in the legend to the figure.*

Crossed over and non-crossed over TAFI and FXIII were removed from Figure 7.

### Reviewer 2

We thank the reviewer for finding our manuscript interesting and well written.

### Reviewer 3

We are grateful to the reviewer for considering our study valuable and the manuscript well-written.

1. *Figure 3:*

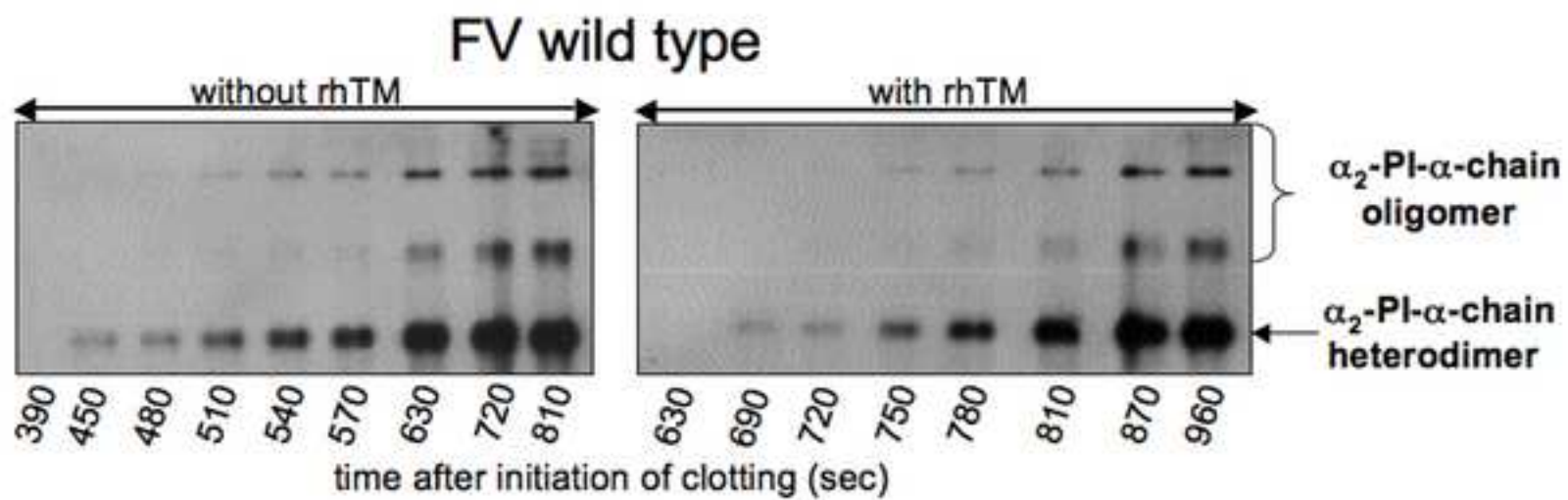
*It would be helpful for the reader of the manuscript if the difference between the "solid symbols" and "empty symbols" (presence or absence of rhTM, as correctly explained in the figure legend) could be directly marked in the figure for example by labelled small arrows pointing on the curves at least on the first graph of figure 3A.*

*2. Figure 6:*

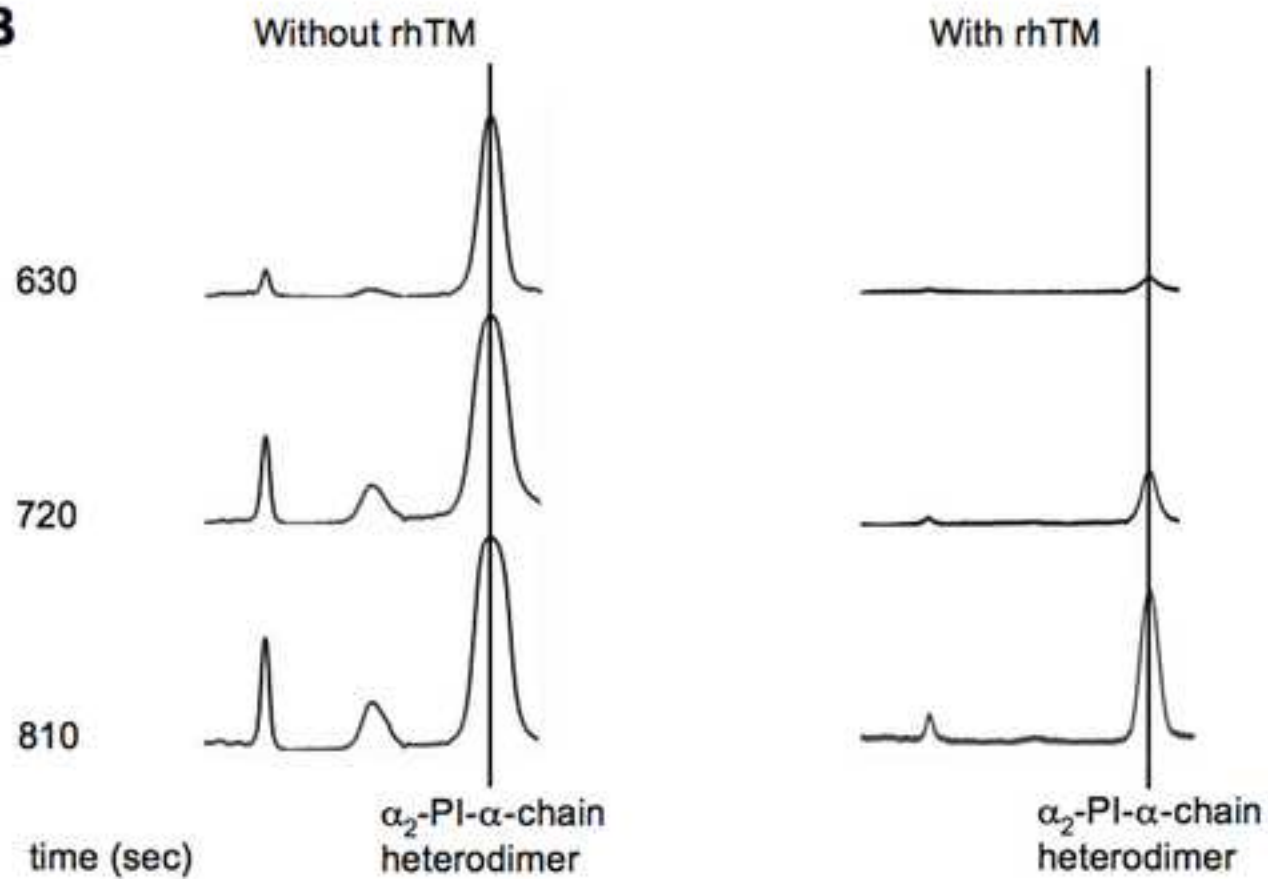
*The reviewer suggests the same labelling for figure 6 as mentioned for figure 3.*

Labeled small arrows were put on both Figures as suggested by the reviewer.

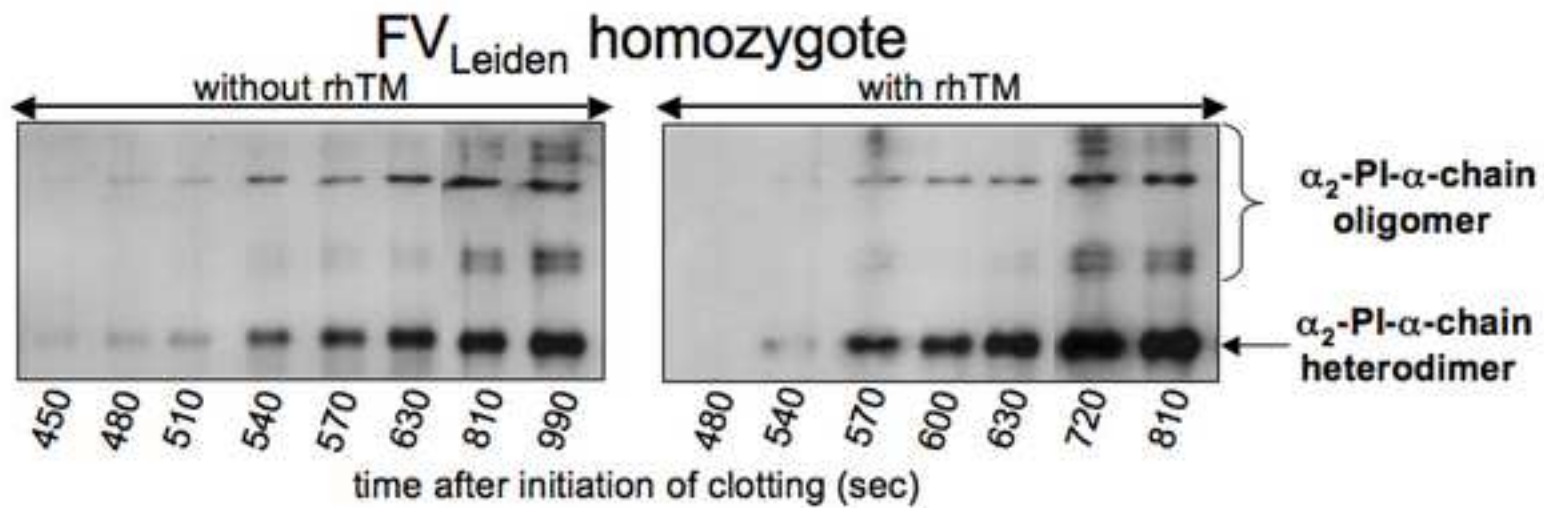
**Fig 1 A**



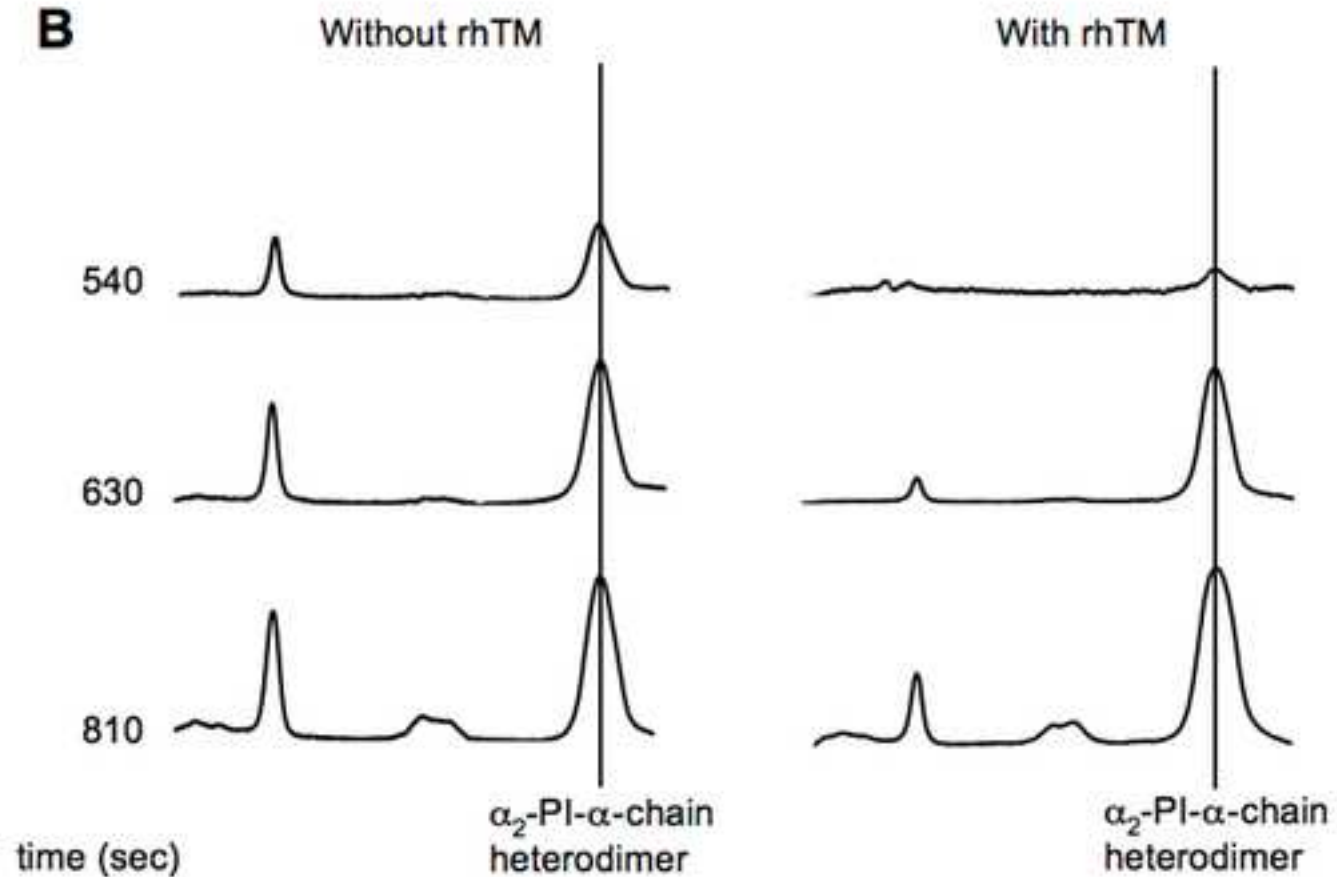
**B**

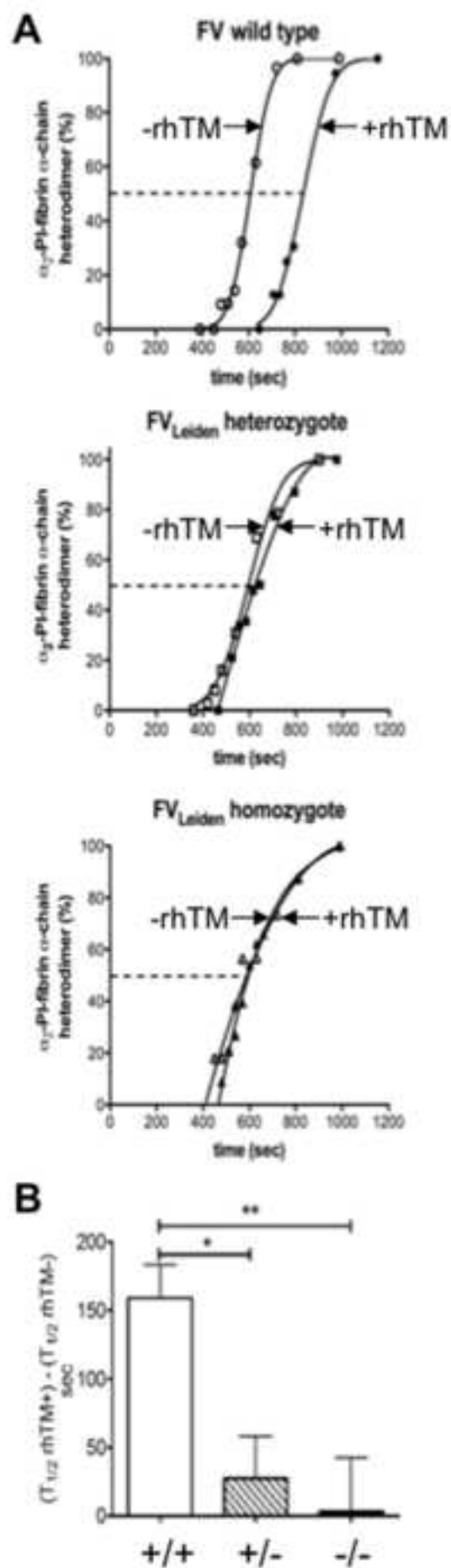


**Fig 2 A**

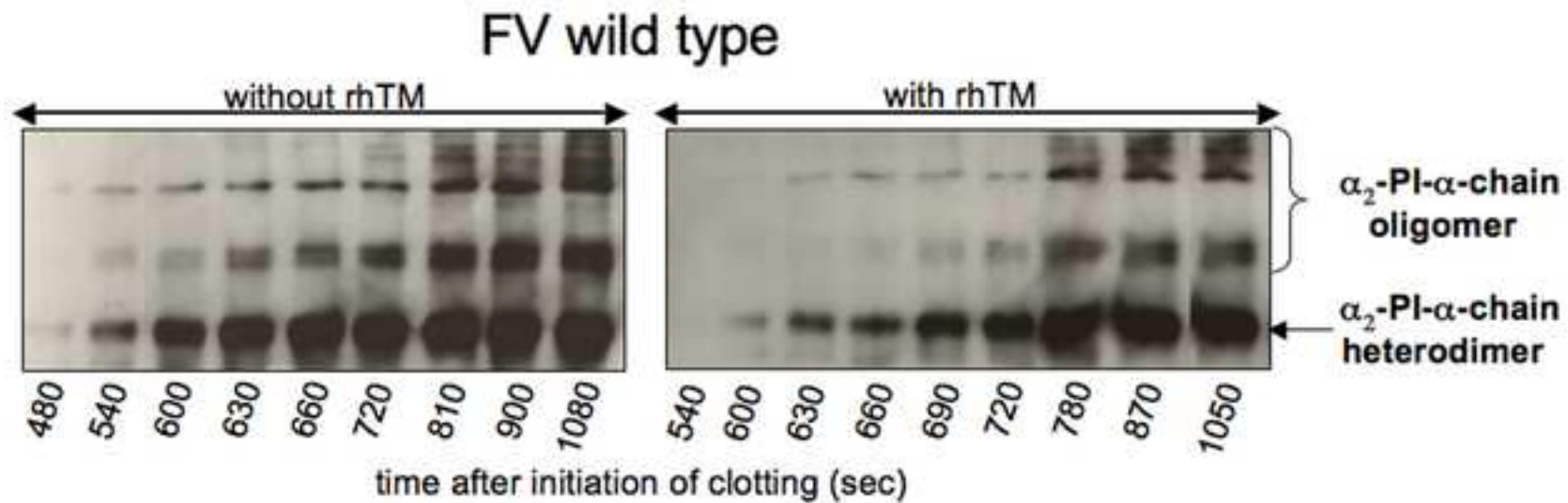


**B**

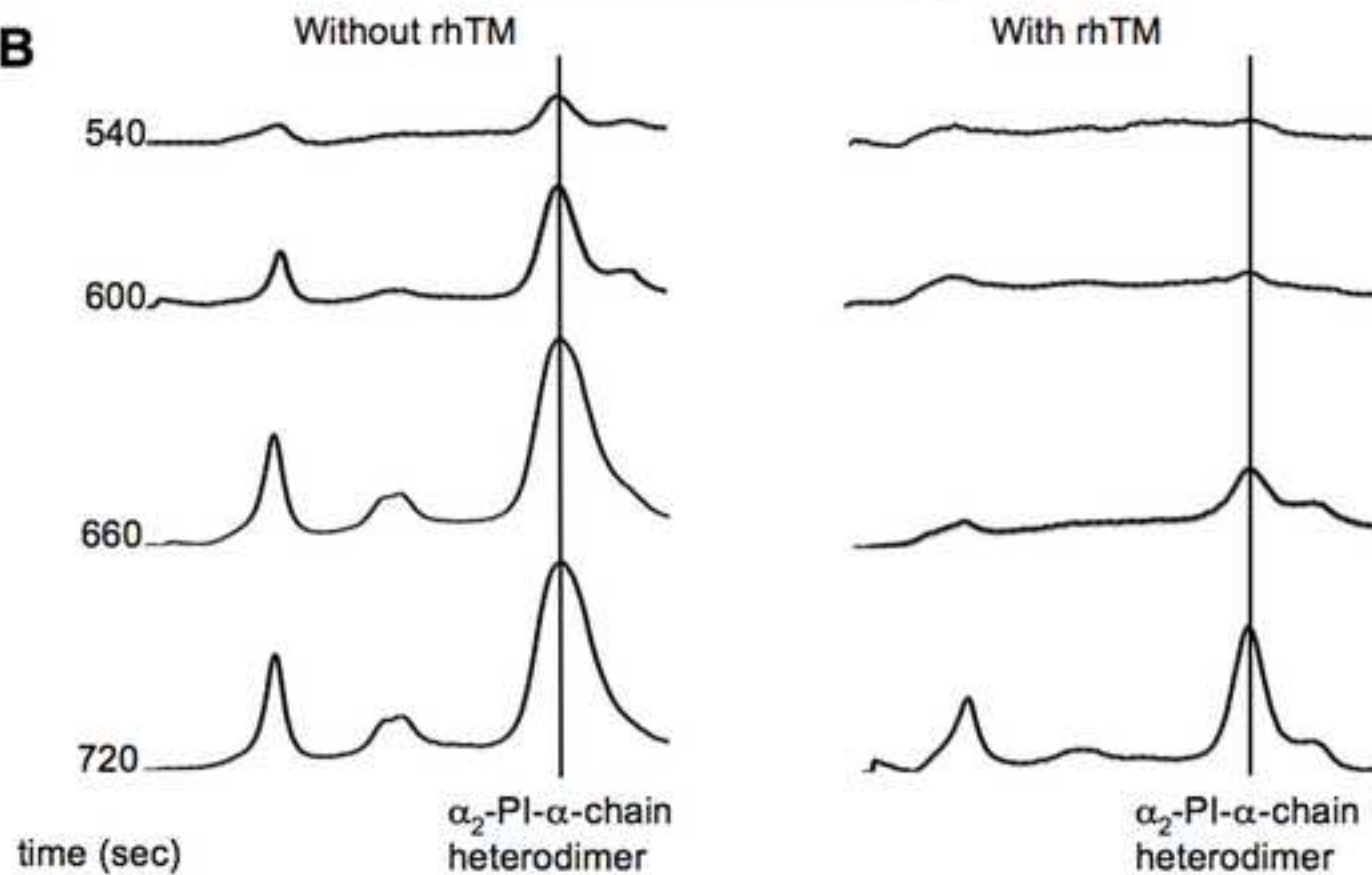


**Fig 3**

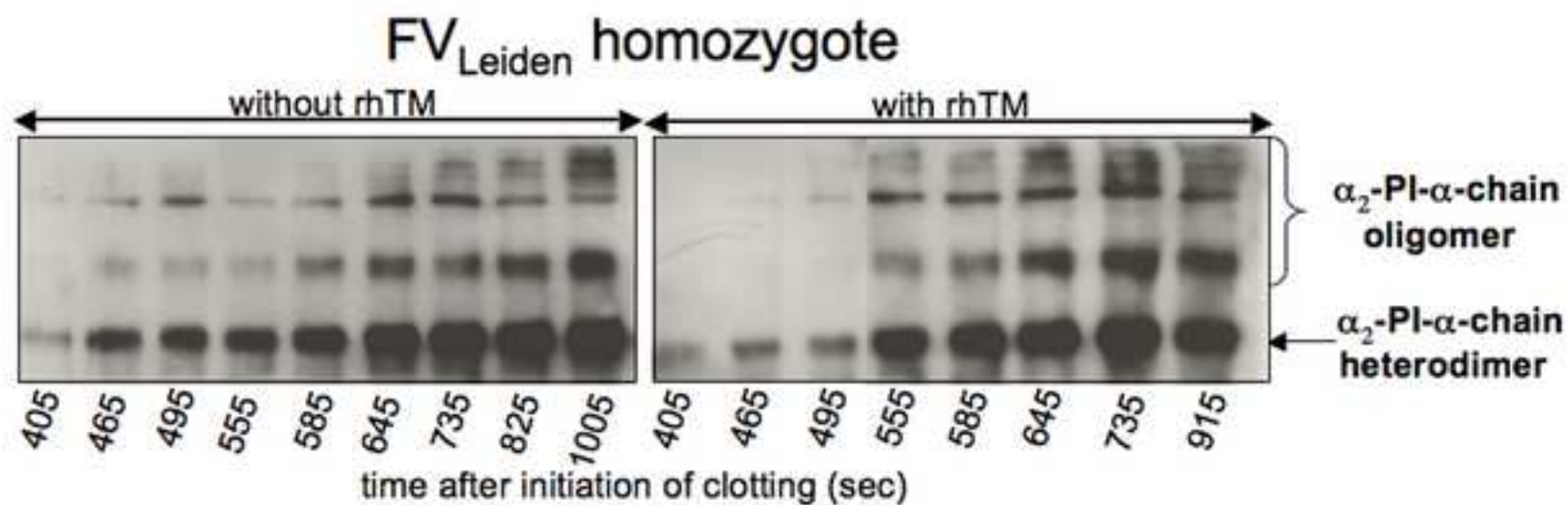
**Fig 4 A**



**B**



**Fig 5 A**



**B**

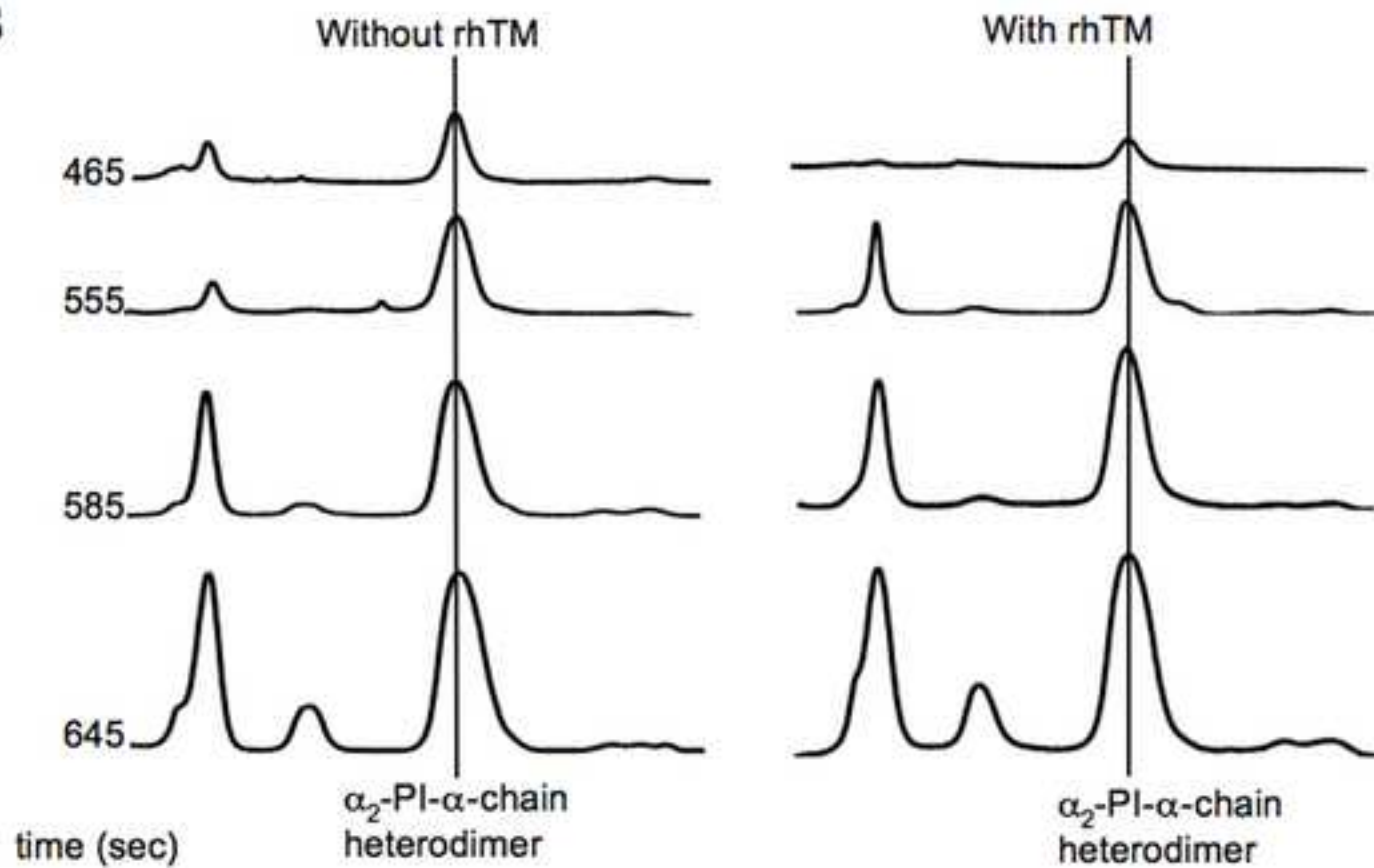




Fig 6

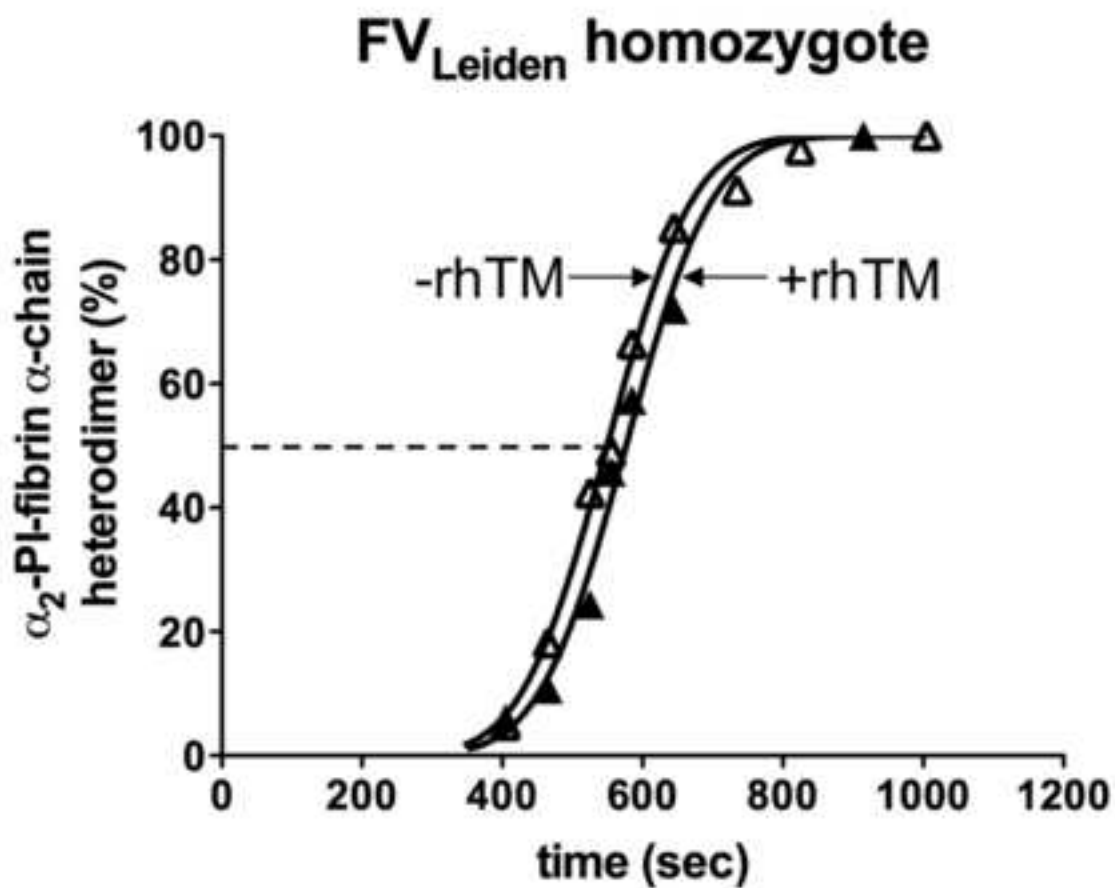
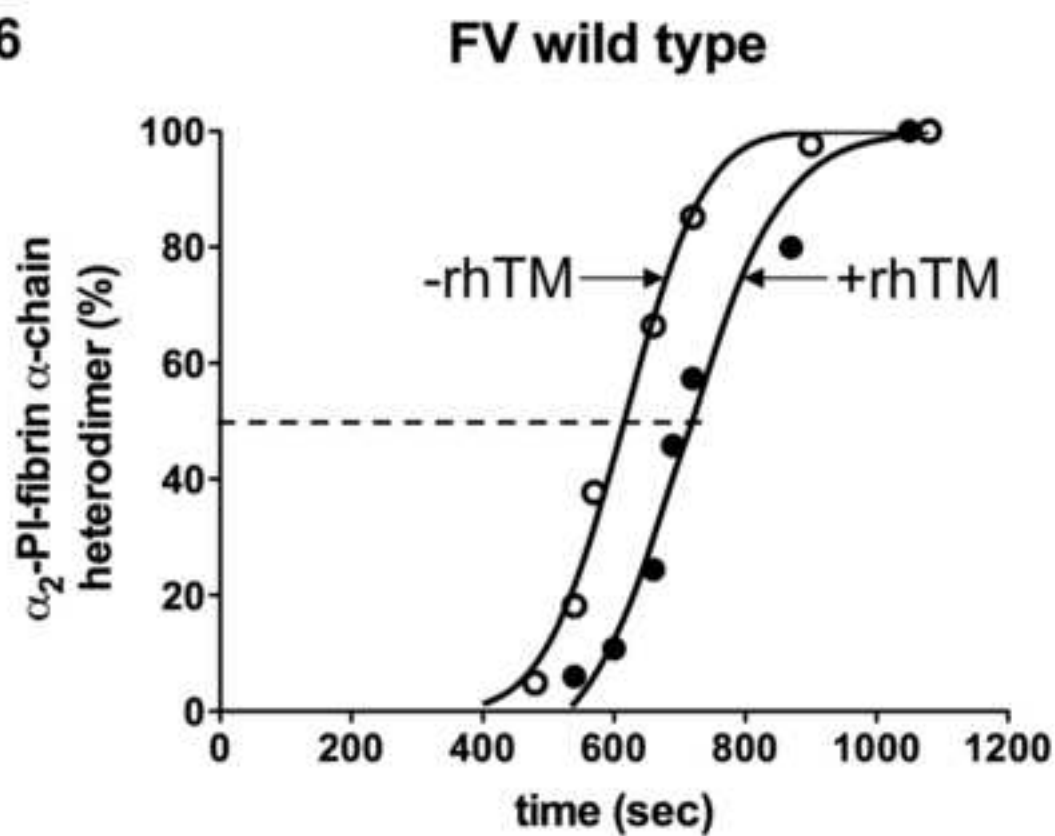
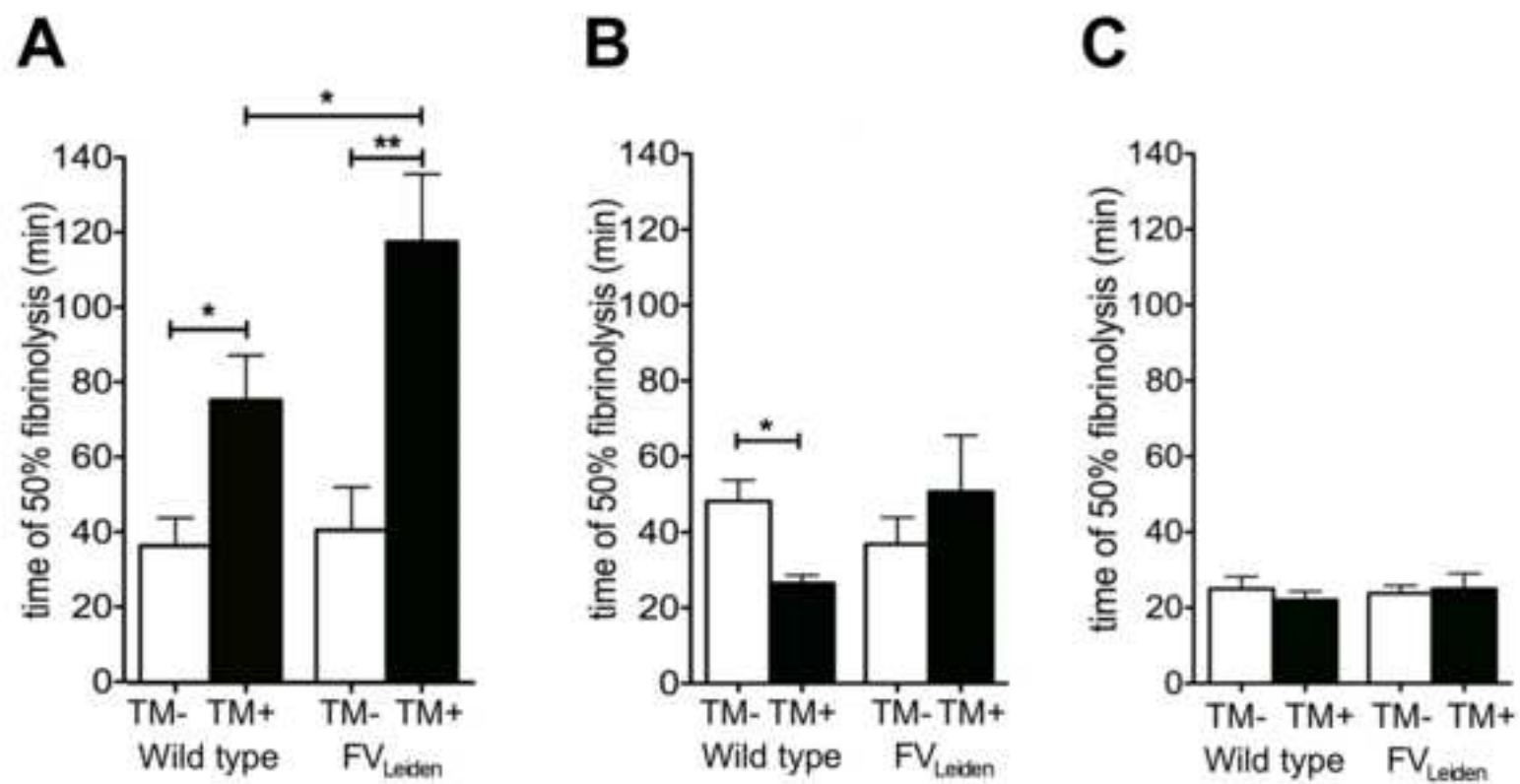
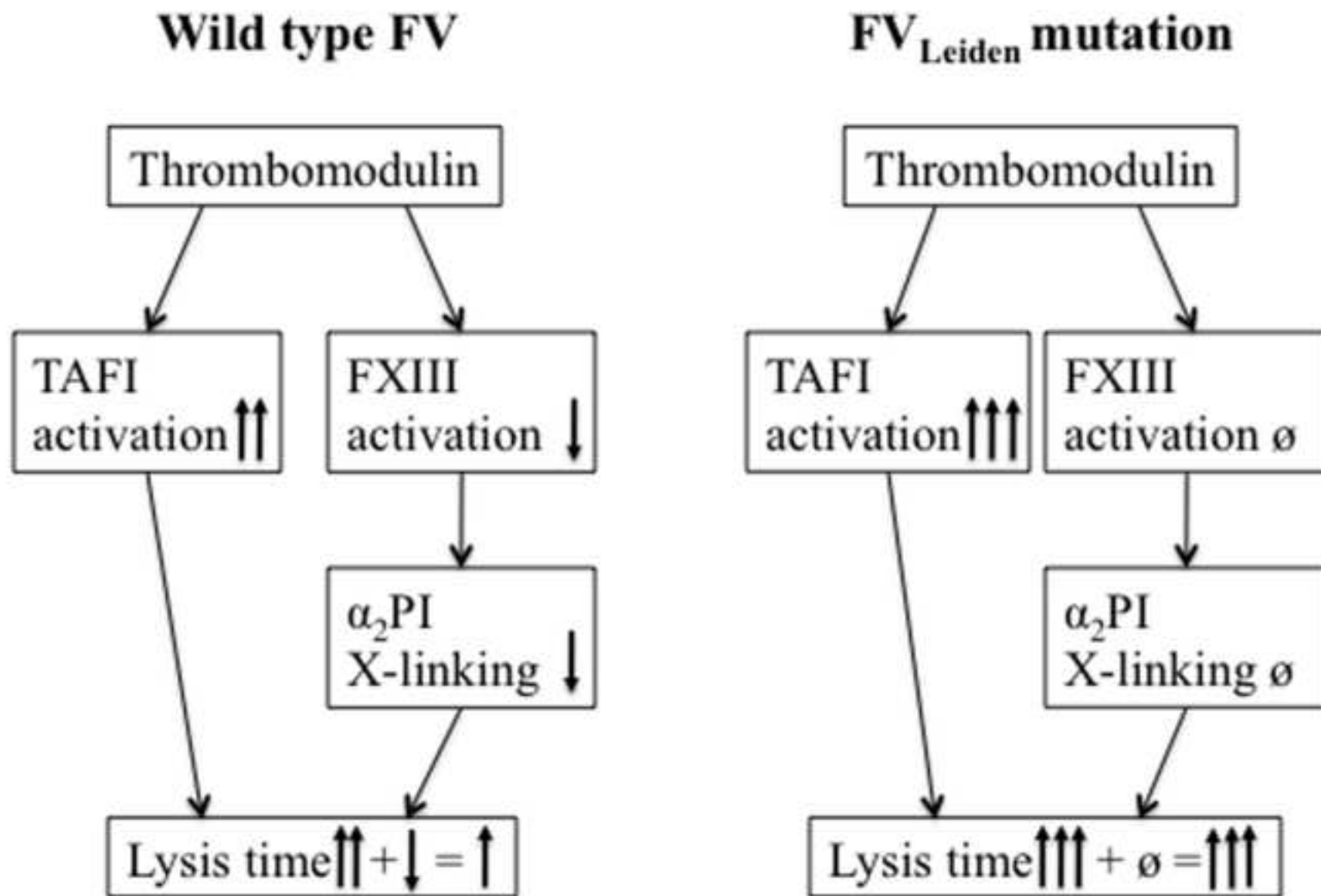


Fig 7



# Fig 8



Dear Professor Sandset,

We are submitting the revised version of our manuscript (Ms. No. TR-D-12-00157) entitled "Thrombomodulin-dependent effect of factor V Leiden mutation on the cross-linking of  $\alpha$ 2-plasmin inhibitor to fibrin and its consequences on fibrinolysis". The revision was carried out as requested by the reviewers and are indicated in red color in the manuscript. We addressed the issues raised by the reviewers and a point to point reply was provided. We hope that the introduced changes would satisfy the reviewers.

Thank you for your assistance,  
Yours sincerely,

Prof. László Muszbek MD, PhD