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(54) Titre : **MUTANTS DU FACTEUR X**  
(54) Title: **FACTOR X MUTANTS**

(57) **Abrégé/Abstract:**

The present invention concerns a protein comprising a mutated sequence of SEQ ID NO: 1, said mutated sequence of SEQ ID NO: 1 comprising at least one mutation A, A', B, C or C', in which: mutation A consists of substituting amino acids 43 to 52 of sequence SEQ ID NO: 1 with a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, mutation A' consists of substituting amino acids 47 to 52 of sequence SEQ ID NO: 1 with a sequence chosen from TSKLTR, FNDFTTR, LSSMTR, PPSLTR and LSCGQR, mutation B consists of inserting a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of sequence SEQ ID NO: 1, mutation C consists of inserting a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 of sequence SEQ ID NO: 1, and of deleting amino acids 4 to 13 from sequence SEQ ID NO: 1, mutation C' consists of inserting a sequence chosen from TSKLTR, FNDFTTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of sequence SEQ ID NO: 1, and of deleting amino acids 4 to 9 from sequence SEQ ID NO: 1.



**ABSTRACT****Factor X mutants**

- 5 The present invention concerns a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation A, A', B, C or C', in which:
- mutation A consists of substituting amino acids 43 to 52 of sequence SEQ ID No. 1 with a sequence chosen from DFLAEGGLTPR, KATN\*ATLSPR and KATXATLSPR,
- 10 mutation A' consists of substituting amino acids 47 to 52 of sequence SEQ ID No. 1 with a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR,
- mutation B consists of inserting a sequence chosen from DFLAEGGLTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of sequence SEQ ID No. 1,
- 15 mutation C consists of inserting a sequence chosen from DFLAEGGLTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 of sequence SEQ ID No. 1, and of deleting amino acids 4 to 13 from sequence SEQ ID No. 1,
- mutation C' consists of inserting a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of sequence SEQ ID
- 20 No. 1, and of deleting amino acids 4 to 9 from sequence SEQ ID No. 1.

**Factor X mutants**

The present invention relates to factor X mutants, and to the use thereof in treating blood coagulation disorders.

5

Factor X is a protein present in the blood. This protein plays an important role in the coagulation cascade. Blood coagulation is a complex process which makes it possible to prevent blood flow via damaged vessels. As soon as a vessel is broken, the elements responsible for coagulation interact with one another to form a plug, the hemostatic  
10 plug, at the site where the vessel is broken. The coagulation factors are required in order to hold the hemostatic plug in place and to stabilize the clot.

The formation of a normal clot occurs in four steps:

**Step 1** The blood vessel is damaged.

15 **Step 2** The blood vessel contracts so as to restrict the blood supply to the damaged zone.

**Step 3** The platelets adhere to the subendothelial space exposed during the damaging of the vessel and also to the stimulated blood vessel walls. The platelets spread, this is what is referred to as "platelet adhesion". These spread platelets release substances  
20 which activate other neighboring platelets such that they agglomerate at the seat of the lesion in order to form the hemostatic plug. This is what is referred to as "platelet aggregation".

**Step 4** The surface of the activated platelets thus constitutes a surface on which blood coagulation can take place. The coagulation proteins which circulate in the blood  
25 (including factor X) are activated at the surface of platelets and form a fibrin clot.

These coagulation proteins (i.e. factors I, II, V, VIII, IX, X, XI, XII and XIII, and also Von Willebrand factor) operate in a chain reaction, *i.e.* the coagulation cascade.

30 Factor X in activated form (Xa) is involved more particularly in the activation of prothrombin (factor II) to thrombin (factor IIa), in particular when it is complexed with

activated cofactor V so as to form the prothrombinase complex. This factor is an essential element in the coagulation cascade.

When this factor is lacking, bleeding occurs, such as epistaxis (nosebleeds),  
5 hemarthrosis (effusion of blood into a joint cavity) or gastrointestinal bleeding. Factor X deficiency is extremely rare. Its transmission is autosomal recessive, and its prevalence is 1/1 000 000.

FX activation occurs:

10 - either very early during the step of initiation of the coagulation cascade by the factor VIIa/tissue factor complex, in a relatively ineffective reaction which results in the formation of traces of thrombin;

- or during the step of amplification of the coagulation cascade resulting from positive feedback produced by the traces of thrombin, resulting in the activation of  
15 factors VIII and IX.

The latter two factors are missing in individuals suffering from hemophilia A and hemophilia B, thus causing a hemorrhagic disorder which can be fatal without treatment. The absence of these factors means that it is not possible to generate sufficient amounts of activated factor X to stop the hemorrhage.

20 Thus, there is a need for a modified factor X which can be activated by thrombin, and which would make it possible to have efficient coagulation in the absence of factor VIII and/or of factor IX, through the direct use of the traces of thrombin generated during the initiation of coagulation.

25 The inventors have identified specific factor X mutants (also called factor X variants), which are efficiently activated by thrombin, thus making it possible to restore coagulation in the absence of factor VIII, of factor IX and even of factor X. Indeed, as demonstrated in examples, these factor X mutants can be activated by thrombin, and allow efficient coagulation, even in the absence of endogenous factor VIII and/or factor  
30 IX and/or factor X.

The activation peptide cleavage sites generated in these factor X variants can also be the target of other coagulation proteases, such as factor VIIa, factor IXa, factor Xa, factor XIa, factor XIIa or kallikrein.

Moreover, a modification of the activation peptide of factor X can result in an additional  
5 modification of its pharmacological properties, other than the sole recognition by thrombin. This modification can confer on the factor X variant an improvement in specific activity, in stability, or in protease resistance, or else an increase in pharmacokinetics. In addition, the presence of additional glycosylations and phosphorylations, or on the contrary the absence of these modifications, compared with  
10 the wild-type molecule, may be caused by the modifications introduced into the activation peptide.

The present invention therefore relates to a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one  
15 mutation A, A', B, C or C', in which:

mutation A consists of the substitution of amino acids 43 to 52 of the sequence SEQ ID No. 1 with a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR,

mutation A' consists of the substitution of amino acids 47 to 52 of the sequence SEQ ID  
20 No. 1 with a sequence chosen from TSKLTR, FNDFTTR, LSSMTR, PPSLTR and LSCGQR,

mutation B consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1,

25 mutation C consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 13 of the sequence SEQ ID No. 1,

mutation C' consists of the insertion of a sequence chosen from TSKLTR, FNDFTTR,  
30 LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 9 of the sequence SEQ ID No. 1, where N\* is an optionally glycosylated asparagine.

Another subject of the invention is a polynucleotide encoding said protein.

Another subject of the invention is an expression vector comprising said polynucleotide.

Another subject of the invention is a host cell comprising said expression vector or said  
5 polynucleotide.

Another subject of the invention is the use of said protein as a medicament. In particular, said protein may be used for the treatment of blood coagulation disorders, in particular hemorrhagic disorders, such as hemophilias A, B and C (factor XI deficiency), factor X deficiencies, or even emergency coagulation needs in order to  
10 substitute for factor VIIa. When a powerful and rapid procoagulant response is required, said protein can be used in combination with other hemostatic molecules, such as factor VIIa and/or fibrinogen, or even in combination with procoagulant compounds (platelet transfusion, procoagulant mixture such as FEIBA, Kaskadil, Kanokad, etc.), which will be able to reinforce the efficacy of the treatment.

15

As used herein, the terms "protein" and "polypeptide" are used interchangeably and refer to an amino acid sequence having more than 100 amino acids. As used herein, the term "protein" comprises amino acid sequences having between 100 and 1000 amino acids, preferably between 120 and 500 amino acids.

20

The present invention relates to a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation A, A', B, C or C', in which:

mutation A consists of the substitution of amino acids 43 to 52 of the sequence SEQ ID  
25 No. 1 with a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR,

mutation A' consists of the substitution of amino acids 47 to 52 of the sequence SEQ ID No. 1 with a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR,

30 mutation B consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1,

mutation C consists of the insertion of a sequence chosen from DFLAEGGLTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 13 of the sequence SEQ ID No. 1,

- 5 mutation C' consists of the insertion of a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 9 of the sequence SEQ ID No. 1, where N\* is an optionally glycosylated asparagine.

Preferably, said protein comprises, preferably consists of, the sequence SEQ ID No. 7,  
10 with at least one mutation A, A', B, C or C' as described above.

The sequence SEQ ID No. 7 (500 amino acids) comprises all the sequence SEQ ID No. 1 (306 amino acids). More particularly, the sequence SEQ ID No. 7 comprises, in the N-terminal to C-terminal direction, a signal peptide and a propeptide (40 amino acids in total), the sequence SEQ ID No. 5, the sequence SEQ ID No. 1, then a tag (of  
15 the amino acids in position 489 to 500, i.e. a length of 12 amino acids), i.e. the HPC4 tag. The sequence SEQ ID No. 103 corresponds to the sequence SEQ ID No. 7 without signal peptide and without propeptide.

Said protein according to the invention is a mutated factor X which is effective in the  
20 treatment of coagulation disorders.

Factor X, also called Stuart-Prower factor, is encoded by the F10 gene and refers to the serine protease EC3.4.21.6. The factor X is composed of a heavy chain of 306 amino acids and of a light chain of 139 amino acids.

- 25 Factor X is a protein of 488 amino acids, consisting of a signal peptide, a propeptide, and light and heavy chains.

Human factor X can be found in UniProtKB under accession number P00742. Its native structure is illustrated in figure 1.

- 30 The protein is translated in prepropeptide form. After cleavage of the signal peptide, the propeptide is finally cleaved, resulting in a light chain and a heavy chain (respectively of 142 and 306 amino acids) (zymogen). Following the triggering of coagulation, the

heavy chain is finally activated by cleavage of the activation peptide, so as to contain only 254 amino acids (the first 52 amino acids are cleaved during the treatment): this is the heavy chain of factor Xa (SEQ ID No. 6).

The prepropeptide of human factor X corresponds to SEQ ID No. 4. The heavy chain corresponds to SEQ ID No. 1, and the light chain corresponds to SEQ ID No. 5. The activation peptide of the heavy chain corresponds to SEQ ID No. 3, and comprises 52 amino acids.

SEQ ID No. 2 is identical to amino acids 1 to 182 of SEQ ID No. 4.

SEQ ID No. 1 is identical to amino acids 183 to 488 of SEQ ID No. 4.

10 The heavy chain of factor Xa (SEQ ID No. 6) corresponds to SEQ ID No. 1, in which the peptide SEQ ID No. 3 has been cleaved.

The proteins according to the invention comprise mutated factor X proteins, in zymogen form, comprising, depending on the constructions, an activation peptide:

15 -modified by substitution of an identical number of residues at the same site included at the widest between residues 42 and 52; or  
 -having received an insertion of residues between residues 52 and 53; or  
 -having received an insertion of residues at the widest between residues 52 and 53, coupled to a deletion of an identical number of residues at the widest between residues 4  
 20 and 13.

The protein according to the invention may be a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation A, in which mutation A consists of the substitution of amino acids 43 to 52 of  
 25 the sequence SEQ ID No. 1 with a sequence chosen from DFLAEGGLTPR, KATN\*ATLSPR and KATXATLSPR, where N\* is an optionally glycosylated asparagine. Preferably, the protein comprises the sequence SEQ ID No. 7 with at least one mutation A.

Further preferably, in this case, the mutated protein comprises a sequence chosen from  
 30 SEQ ID No. 9, SEQ ID No. 10 and SEQ ID No. 11.

The protein according to the invention may be a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation A', in which mutation A' consists of the substitution of amino acids 47 to 52 of the sequence SEQ ID No. 1 with the sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR. Preferably, the protein comprises the sequence SEQ ID No. 7 with at least one mutation A'.

Further preferably, in this case, the mutated protein comprises a sequence chosen from SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16.

10 The protein according to the invention may be a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation B, in which mutation B consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, 15 where N\* is an optionally glycosylated asparagine. Preferably, the protein comprises the sequence SEQ ID No. 7 with at least one mutation B.

Further preferably, in this case, the mutated protein comprises a sequence chosen from SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24 and SEQ ID No. 25.

20

The protein according to the invention may be a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation C, in which mutation C consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 25 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 13 of the sequence SEQ ID No. 1, where N\* is an optionally glycosylated asparagine. Preferably, the protein comprises the sequence SEQ ID No. 7 with at least one mutation C.

Further preferably, in this case, the mutated protein comprises a sequence chosen from SEQ ID No. 27, SEQ ID No. 28 and SEQ ID No. 29.

30

The protein according to the invention may be a protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one

mutation C', in which mutation C' consists of the insertion of a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 9 of the sequence SEQ ID No. 1. Preferably, the protein comprises the sequence SEQ ID No. 7  
5 with at least one mutation C'.

Further preferably, in this case, the mutated protein comprises a sequence chosen from SEQ ID No. 30, SEQ ID No. 31, SEQ ID No. 32, SEQ ID No. 33 and SEQ ID No. 34.

Preferably, the protein according to the invention comprises, preferably consists of, a  
10 sequence chosen from SEQ ID No. 9 to 16, 18 to 25, 27 to 34, 105 to 112, 114 to 121 and 123 to 130.

The sequences described in the present application can be summarized as follows:

| SEQ ID No. | Protein                                                                                                                                                                                                   |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1          | Human factor X heavy chain (306 amino acids),<br>comprising the activation peptide                                                                                                                        |
| 2          | Human factor X signal peptide, propeptide and light chain<br>(182 amino acids)                                                                                                                            |
| 3          | Heavy chain activation peptide (52 amino acids)                                                                                                                                                           |
| 4          | Human factor X prepropeptide (488 amino acids)                                                                                                                                                            |
| 5          | Human factor X light chain (142 amino acids)                                                                                                                                                              |
| 6          | Activated human factor X (FXa) heavy chain (254 amino<br>acids)                                                                                                                                           |
| 7          | FX-WT (corresponds to a human FX of which the<br>nucleotide sequence has been optimized)                                                                                                                  |
| 8          | FX-control+ (mutant of SEQ ID No. 7 comprising the<br>substitution of 10 amino acids with a sequence<br>corresponding to the thrombin recognition site on<br>fibrinogen = fibrinopeptide A) (comparative) |
| 9          | FX-IIa (mutant of SEQ ID No. 7 comprising a mutation<br>A: insertion of the thrombin cleavage consensus site)                                                                                             |

|    |                                                                                                                                                                           |
|----|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 10 | FX-PAR1 (mutant of SEQ ID No. 7 comprising a mutation A: insertion of the modified thrombin cleavage site on the PAR1 receptor)                                           |
| 11 | FX-PAR1M (mutant of SEQ ID No. 7 comprising a mutation A: insertion of the modified thrombin cleavage site on the PAR1 receptor without glycosylation site)               |
| 12 | FX-FXIIa1 (mutant of SEQ ID No. 7 comprising a mutation A': insertion of FXIIa cleavage site 1 on FIX)                                                                    |
| 13 | FX-FXIIa2 (mutant of SEQ ID No. 7 comprising a mutation A': insertion of FXIIa cleavage site 2 on FIX)                                                                    |
| 14 | FX-Kal1 (mutant of SEQ ID No. 7 comprising a mutation A': insertion of kallikrein cleavage site 1 on FXII)                                                                |
| 15 | FX-Kal2 (mutant of SEQ ID No. 7 comprising a mutation A': insertion of kallikrein cleavage site 2 on FXII)                                                                |
| 16 | FX-Kal3 (mutant of SEQ ID No. 7 comprising a mutation A': insertion of kallikrein cleavage site 3 on FXII)                                                                |
| 17 | FX-control+ (mutant of SEQ ID No. 7 comprising the insertion of a sequence corresponding to the thrombin recognition site on fibrinogen = fibrinopeptide A) (comparative) |
| 18 | FX-IIa (mutant of SEQ ID No. 7 comprising a mutation B: insertion of the thrombin cleavage consensus site)                                                                |
| 19 | FX-PAR1 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of the modified thrombin cleavage site on the PAR1 receptor)                                           |
| 20 | FX-PAR1M (mutant of SEQ ID No. 7 comprising a mutation B: insertion of the modified thrombin cleavage site on the PAR1 receptor without glycosylation site)               |
| 21 | FX-FXIIa1 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of the FXIIa cleavage site 1 on FIX)                                                                 |
| 22 | FX-FXIIa2 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of the FXIIa cleavage site 2 on FIX)                                                                 |

|           |                                                                                                                                                                                                               |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 23        | FX-Kal1 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of kallikrein cleavage site 1 on FXII)                                                                                                     |
| 24        | FX-Kal2 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of kallikrein cleavage site 2 on FXII)                                                                                                     |
| 25        | FX-Kal3 (mutant of SEQ ID No. 7 comprising a mutation B: insertion of kallikrein cleavage site 3 on FXII)                                                                                                     |
| 26        | FX-control+ (mutant of SEQ ID No. 7 comprising the insertion of a sequence corresponding to the thrombin recognition site on fibrinogen = fibrinopeptide A, and the deletion of 10 amino acids) (comparative) |
| 27        | FX-IIa (mutant of SEQ ID No. 7 comprising a mutation C: insertion of the thrombin cleavage consensus site)                                                                                                    |
| 28        | FX-PAR1 (mutant of SEQ ID No. 7 comprising a mutation C: insertion of the modified thrombin cleavage site on the PAR1 receptor)                                                                               |
| 29        | FX-PAR1M (mutant of SEQ ID No. 7 comprising a mutation C: insertion of the modified thrombin cleavage site on the PAR1 receptor without glycosylation site)                                                   |
| 30        | FX-FXIa1 (mutant of SEQ ID No. 7 comprising a mutation C': insertion of FXIa cleavage site 1 on FIX)                                                                                                          |
| 31        | FX-FXIa2 (mutant of SEQ ID No. 7 comprising a mutation C': insertion of FXIa cleavage site 2 on FIX)                                                                                                          |
| 32        | FX-Kal1 (mutant of SEQ ID No. 7 comprising a mutation C': insertion of kallikrein cleavage site 1 on FXII)                                                                                                    |
| 33        | FX-Kal2 (mutant of SEQ ID No. 7 comprising a mutation C': insertion of kallikrein cleavage site 2 on FXII)                                                                                                    |
| 34        | FX-Kal3 (mutant of SEQ ID No. 7 comprising a mutation C': insertion of kallikrein cleavage site 3 on FXII)                                                                                                    |
| 35        | Nucleic sequence encoding FX-WT (SEQ ID No. 7)                                                                                                                                                                |
| 36 to 75  | Primers                                                                                                                                                                                                       |
| 76 to 102 | Nucleic sequences encoding respectively the proteins SEQ ID No. 8 to 34                                                                                                                                       |

|            |                                                                                                                               |
|------------|-------------------------------------------------------------------------------------------------------------------------------|
| 103 to 130 | Protein sequences corresponding respectively to the proteins SEQ ID No. 7 to 34 without signal peptide and without propeptide |
| 131 to 158 | Nucleic sequences encoding respectively the proteins SEQ ID No. 103 to 130                                                    |

A subject of the present invention is also a protein complex comprising:

- a protein comprising a mutated sequence of SEQ ID No. 1 according to the invention, and
  - 5       - at least one protein of sequence SEQ ID No. 2,
- said proteins being linked to one another by a disulfide bridge.

Another subject of the invention is a nucleic acid (polynucleotide) encoding said protein. Preferably, the nucleic acid is chosen from the sequences SEQ ID No. 77 to 84,  
10       86 to 93, 95 to 102, 133 to 140, 142 to 149 and 151 to 158.

Another subject of the invention is an expression vector comprising said polynucleotide encoding said protein, or an expression cassette comprising said polynucleotide. According to the invention, the expression vectors appropriate for use according to the  
15       invention may comprise at least one expression-controlling element functionally linked to the nucleic acid sequence. The expression-controlling elements are inserted into the vector and make it possible to regulate the expression of the nucleic acid sequence. Examples of expression-controlling elements include in particular lac systems, the lambda phage promoter, yeast promoters or viral promoters. Other functional elements  
20       may be incorporated, such as a leader sequence, stop codons, polyadenylation signals and sequences required for the subsequent transcription and translation of the nucleic acid sequence in the host system. It will be understood by those skilled in the art that the correct combination of expression-controlling elements depends on the host system chosen. It will also be understood that the expression vector must contain the additional  
25       elements required for the subsequent transfer and replication of the expression vector containing the nucleic acid sequence into and in the host system.

Such vectors are easily constructed using conventional or commercially available methods.

Another subject of the invention is a recombinant cell comprising an expression vector  
5 as described above, or a polynucleotide as described above. According to the invention,  
examples of host cells which can be used are eukaryotic cells, such as animal, plant,  
insect and yeast cells; and prokaryotic cells, such as E. coli. The means via which the  
vector carrying the gene can be introduced into the cells comprise in particular  
10 microinjection, electroporation, transduction or transfection by means of DEAE-  
dextran, lipofection, calcium phosphate or other procedures known to those skilled in  
the art. In one preferred embodiment, eukaryotic expression vectors which function in  
eukaryotic cells are used. Examples of such vectors include viral vectors, such as  
retroviruses, adenoviruses, herpes viruses, vaccinia virus, smallpox virus, poliovirus or  
lentiviruses, bacterial expression vectors or plasmids such as pcDNA5. The preferred  
15 eukaryotic cell lines include COS cells, CHO cells, HEK cells, BHK cells, PerC6 cells,  
HeLa cells, NIH/3T3 293 cells (ATCC # CRL1573), T2 cells, dendritic cells or  
monocytes.

The protein according to the invention may be produced in the milk of the transgenic  
20 animals.

In this case, according to a first aspect, the expression of a DNA sequence containing a  
gene encoding the protein according to the invention is controlled by a mammalian  
casein promoter or a mammalian whey promoter, said promoter not naturally  
controlling the transcription of said gene, and the DNA sequence also containing a  
25 sequence for secretion of the protein. The secretion sequence comprises a secretion  
signal interposed between the gene and the promoter.

The transgenic animal used is capable not only of producing the desired protein, but  
also of transmitting this capacity to its descendants. The secretion of the protein into the  
milk facilitates purification and avoids the use of blood products. The animal can thus  
30 be chosen from goats, doe rabbits, ewes or cows.

The protein according to the invention can be used as a medicament. Consequently, the protein according to the invention can be introduced into a pharmaceutical composition. In particular, the protein according to the invention can be used for the treatment of coagulation disorders, in particular hemorrhagic disorders.

5

The pharmaceutical composition of the invention can be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, for forming a therapeutic composition.

The pharmaceutical composition of the present invention can be administered orally, 10 sublingually, subcutaneously, intramuscularly, intravenously, intra-arterially, intrathecally, intraocularly, intracerebrally, transdermally, locally or rectally. The active ingredient, alone or in combination with another active ingredient, can then be administered in unit administration form, as a mixture with conventional pharmaceutical carriers. Unit administration forms comprise oral forms, such as tablets, gel capsules, 15 powders, granules and oral solutions or suspensions, sublingual and buccal administration forms, aerosols, subcutaneous implants, transdermal, topical, intraperitoneal, intramuscular, intravenous, subcutaneous and intrathecal administration forms, intranasal administration forms and rectal administration forms.

Preferably, the pharmaceutical composition contains a pharmaceutically acceptable 20 vehicle for a formulation capable of being injected. This may involve in particular sterile, isotonic formulae, saline solutions (with monosodium or disodium phosphate, sodium chloride, potassium chloride, calcium chloride or magnesium chloride and the like, or mixtures of such salts), or lyophilized compositions, which, when sterilized water or physiological saline is added, as appropriate, enable the constitution of 25 injectable solutes.

The pharmaceutical forms appropriate for injectable use comprise sterile aqueous solutions or dispersions, oily formulations, including sesame oil, and peanut oil, and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In any event, the form must be sterile and must be fluid since it must be 30 injected using a syringe. It must be stable under the manufacturing and storage conditions and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The dispersions according to the invention can be prepared in glycerol, liquid polyethylene glycols or mixtures thereof, or in oils. Under normal conditions of storage and use, these preparations contain a preservative for preventing microorganism growth. The pharmaceutically acceptable vehicle may be a solvent or dispersion medium  
5 containing, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, polyethylene glycol, and the like), appropriate mixtures thereof, and/or vegetable oils. Suitable fluidity may be maintained, for example, through the use of a surfactant, such as lecithin. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example parabens, chlorobutanol,  
10 phenol, sorbic acid or else thimerosal. In many cases, it will be preferable to include isotonic agents, for example sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about through the use, in the compositions, of absorption-delaying agents, for example aluminum monostearate or gelatin.

The sterile injectable solutions are prepared by incorporating the active substances in  
15 the required amount into the appropriate solvent with several of the other ingredients listed above, where appropriate followed by filtration sterilization. As a general rule, the dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the other ingredients required among those listed above. In the case of sterile powders for the preparation of  
20 sterile injectable solutions, the preferred preparation processes are drying under vacuum and lyophilization. During formulation, the solutions will be administered in a manner compatible with the dosage-regimen formulation and in a therapeutically effective amount. The formulations are easily administered in a variety of pharmaceutical forms, such as the injectable solutions described above, but drug-release capsules and the like  
25 can also be used. For parenteral administration in an aqueous solution for example, the solution must be suitably buffered and the liquid diluent made isotonic with a sufficient amount of saline solution or of glucose. These particular aqueous solutions are particularly suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this regard, the sterile aqueous media which can be used are known to  
30 those skilled in the art. For example, a dose can be dissolved in 1 ml of isotonic NaCl solution and then added to 1000 ml of appropriate liquid, or injected on the proposed

site of the infusion. Certain dosage-regimen variations will necessarily have to occur according to the condition of the subject treated.

The pharmaceutical composition of the invention may be formulated in a therapeutic mixture comprising approximately 0.0001 to 1.0 milligrams, or approximately 0.001 to 0.1 milligrams, or approximately 0.1 to 1.0 milligrams, or even approximately 10 milligrams per dose or more. Multiple doses can also be administered. The level of therapeutically effective dose specific for a particular patient will depend on a variety of factors, including the disorder which is treated and the seriousness of the disease, the activity of the specific compound used, the specific composition used, the age, body weight, general health, sex and diet of the patient, the time of the administration, the route of administration, the excretion rate of the specific compound used, the duration of the treatment, or else the medicaments used in parallel.

The following examples are given for the purpose of illustrating various embodiments of the invention.

### **Figure legends**

**Figure 1: Structure of native human factor X**

### **Figure 2: Mutant development strategies**

Family 1 encompasses the mutants comprising mutations A or A'.

Family 2 groups together the mutants comprising mutation B.

Family 3 groups together the mutants comprising mutation C or C'.

### **Figure 3: Level of expression of the variant factors produced in CHO**

The three FX families were expressed following transfection in CHO. The day-7 supernatants were analyzed in triplicate with the Zymutest FX kit (Hyphen). The concentrations ( $\mu\text{g/ml}$ ) are indicated along the y-axis. The standard deviations are indicated above the histograms. Family 1, grey bar; family 2, black bar; family 3, white bar.

**Figure 4: Level of expression of factors X and variants produced in HEK**

The three FX families were expressed following transfection in HEK293S. The day-7 supernatants were analyzed in triplicate with the Zymutest FX kit (Hyphen). The concentrations ( $\mu\text{g/ml}$ ) are indicated along the y-axis. The standard deviations are indicated above the histograms. Family 1, grey bar; family 2, black bar; family 3, white bar.

**Figure 5: Chronometric activity of factors X and variants produced in CHO in factor-X-deficient plasma**

The three FX families were expressed following transfection in CHO-S. The day-7 supernatants were analyzed at least in duplicate by means of a TP test on a Star automated device (Stago). The coagulation times made it possible to calculate a specific activity (in  $\text{s}/(\mu\text{g/ml})$ ) and were then converted into percentage wild-type recombinant factor X activity. These values are given along the y-axis. The standard deviations are indicated above the histograms. Family 1, grey bar; family 2, black bar; family 3, white bar. \* FX-Kal2 family 3, not done.

**Figure 6: Activity of factor X variants of family 1 produced in CHO after activation by the RVV-X fraction**

The FX variants of family 1 were expressed following transfection in CHO-S. The day-7 supernatants were incubated at two FX concentrations (at least in duplicate) in the presence of the RVV-X fraction of Russell's viper venom. The appearance of FXa was measured by monitoring the hydrolysis of the pNAPEP 1025 substrate at 405 nM. The initial conversion rates ( $\text{mODU}/\text{min}/\text{nM}$ ) were compared to that of FX-WT, fixed at 100%. The mean of the values at two concentrations is established and given on the y-axis. The standard deviations are indicated above the histograms.

**Figure 7: Thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor VIII-deficient plasma following activation by tissue factor (1 pM)**

- along the x-axis: time (in minutes)

- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the factor FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 5 µg/ml except for FX-Kal2 and 3 which, for technical reasons, were used at 3.5 and 1.65 µg/ml respectively.

**Figure 8: Thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor VIII-deficient plasma following activation by cephalin**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the factor FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 5 µg/ml except for FX-Kal2 and 3 which, for technical reasons, were used at 3.5 and 1.65 µg/ml respectively.

**Figure 9: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor VIII-deficient plasma, following activation by tissue factor or cephalin**

The values of the velocities (in nM/min of thrombin generated) arising from figures 7 and 8 are presented. It should be noted that FX-Kal2 and 3 (\*) were used at 3.5 and 1.65 µg/ml respectively instead of 5 µg/ml for the other FX and variants. White bars,

values obtained by activation with tissue factor (1 pM); black bars, values obtained by activation with cephalin.

**Figure 10: Thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor IX-deficient plasma, following activation by tissue factor (1 pM)**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the factor FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 5 µg/ml except for FX-Kal2 and 3 which, for technical reasons, were used at 3.5 and 1.65 µg/ml respectively.

**Figure 11: Thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor IX-deficient plasma, following activation by cephalin**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the factor FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 5 µg/ml except for FX-Kal2 and 3 which, for technical reasons, were used at 3.5 and 1.65 µg/ml respectively.

**Figure 12: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor IX-deficient plasma, following activation by tissue factor (1 pM) or cephalin.**

5 The values of the velocities (in nM/min of thrombin generated) arising from figures 10 and 11 are presented. It should be noted that FX-Kal2 and 3 (\*) were used at 3.5 and 1.65 µg/ml respectively instead of 5 µg/ml for the other FX and variants. The results are presented in logarithmic scale. For greater readability, the values below 1 nM/min of thrombin generated are not represented (+). White bars, values obtained by activation  
10 with tissue factor (1 pM); black bars, values obtained by activation with cephalin.

**Figure 13: Thrombin activation of FX-WT and variants of family 2.**

The activation of FX-WT and certain variants thereof of family 2 was carried out in the presence of thrombin (10 nM) in Hepes buffer containing Pefachrome FXa 8595  
15 substrate. The appearance of the para-nitroanilide released by the FXa generated was monitored at 405 nm over time. Activated factor X, at various concentrations, was used as a positive control.

**Figure 14: Thrombinograms obtained from the addition of FX or variants thereof  
20 of family 1 to a pool of factor VIII-deficient plasma, following activation by tissue factor**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the  
25 deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or  
30 variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

**Figure 15: Thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor VIII-deficient plasma, following activation by cephalin**

- 5                   - along the x-axis: time (in minutes)  
                   - along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

**Figure 16: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor VIII-deficient plasma, following activation by tissue factor (1 pM) or cephalin.**

20   The values of the velocities (in nM/min of thrombin generated) arising from figures 14 and 15 are presented. It should be noted that the FX-Kal3 (\*) was used at 1.65 µg/ml instead of 7.5 µg/ml for the other FX and variants. The value of the FX-WT is the mean of the two experiments. For greater readability, the values below 1 nM/min of thrombin generated are not represented (+). White bars, values obtained by activation with tissue factor (1 pM); black bars, values obtained by activation with cephalin.

**Figure 17: Thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor IX-deficient plasma, following activation by tissue factor**

- 30                   - along the x-axis: time (in minutes)  
                   - along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FIX-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FIX are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△),  
 5 FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

10

**Figure 18: Thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor IX-deficient plasma, following activation by cephalin**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

15 The normal plasma pool sample is represented by the black curve (●) and the FIX-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FIX are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△),  
 20 FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

25 **Figure 19: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor IX-deficient plasma, following activation by tissue factor (1 pM) or cephalin.**

The values of the velocities (in nM/min of thrombin generated) arising from figures 17 and 18 are presented. It should be noted that FX-Kal3 (\*) was used at 1.65 µg/ml  
 30 instead of 7.5 µg/ml for the other FX and variants. The value of the FX-WT is the mean of the two experiments. For greater readability, the values below 1 nM/min of thrombin

generated are not represented (+). White bars, values obtained by activation with tissue factor (1 pM); black bars, values obtained by activation with cephalin.

**Figure 20: Thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor VIII-deficient plasma, following activation by tissue factor**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆ and ---), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

**Figure 21: Thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor VIII-deficient plasma, following activation by cephalin**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FVIII-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FVIII are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆ and ---), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

**Figure 22: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor VIII-deficient plasma, following activation by tissue factor (1 pM) or cephalin**

- 5 The values of the velocities (in nM/min of thrombin generated) arising from figures 20 and 21 are presented. It should be noted that FX-Kal3 (\*) was used at 1.65 µg/ml instead of 7.5 µg/ml for the other FX and variants. The value of the FX-WT is the mean of the two experiments. White bars, values obtained by activation with tissue factor (1 pM); black bars, values obtained by activation with cephalin.

10

**Figure 23: Thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor IX-deficient plasma, following activation by tissue factor**

- along the x-axis: time (in minutes)
- 15 - along the y-axis: maximum concentration of thrombin observed (in nM)

The normal plasma pool sample is represented by the black curve (●) and the FIX-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FIX are represented by the symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), 20 FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (†), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

25

**Figure 24: Thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor IX-deficient plasma, following activation by cephalin**

- along the x-axis: time (in minutes)
- along the y-axis: maximum concentration of thrombin observed (in nM)
- 30 The normal plasma pool sample is represented by the black curve (●) and the FIX-deficient plasma pool sample is represented by (○). The curves corresponding to the deficient plasma overloaded with 1 U/ml or 0.1 U/ml of FIX are represented by the

symbols ■ and □ respectively. The symbols representing the wild-type FXs and the variants are the following: FX-WT (◆), FX- control+ (◇), FX-IIa (▲), FX-PAR1 (△), FX-PAR1M (× in solid line), FX-FIXa1 (\*), FX-FIXa2 (× with dashed curve), FX-Kal1 (+), FX-Kal2 (○) and FX-Kal3 (solid line without symbol). The factor X or  
 5 variants thereof were used at 7.5 µg/ml except for FX-Kal3 which, for technical reasons, was used at 1.65 µg/ml.

**Figure 25: Velocities of the thrombograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor IX-deficient plasma, following  
 10 activation by tissue factor (1 pM) or cephalin**

The values of the velocities (in nM/min of thrombin generated) arising from figures 23 and 24 are presented. It should be noted that FX-Kal3 (\*) was used at 1.65 µg/ml instead of 7.5 µg/ml for the other FX and variants. The value of the FX-WT is the mean of the two experiments. White bars, values obtained by activation with tissue factor  
 15 (1 pM); black bars, values obtained by activation with cephalin.

## Examples

### 20 Example 1: Generation of the complementary DNAs of the factor X variants

#### 1- General observations

The nucleotide and protein sequences of the various constructs are provided in the sequence listing, and are summarized in the table of the description. The wild-type FX  
 25 molecule is called FX-WT (SEQ ID No. 7), it corresponds to a human FX, the nucleotide sequence of which has been optimized. This molecule will be used as a control for the three families of mutated molecules.

The mutated molecules are named according to the cleavage site placed upstream of the heavy chain. FX-control+ corresponds to the thrombin recognition site on fibrinogen, or  
 30 fibrinopeptide A. The mutated molecules according to the invention are named, respectively, FX-IIa (thrombin cleavage consensus site), FX-PAR1 (modified thrombin cleavage site on the PAR1 receptor), FX-PAR1M (modified thrombin cleavage site on

the PAR1 receptor without glycosylation site), FX-FXla1 (FXIa cleavage site 1 on FIX), FX-FXla2 (FXIa cleavage site 2 on FIX), FX-Kal1 (kallikrein cleavage site 1 on FXII), FX-Kal2 (kallikrein cleavage site 2 on FXII) and FX-Kal3 (kallikrein cleavage site 3 on FXII).

5 As shown in figure 2, each of these sites is present in a different environment (family), namely:

- either in family 1 which consists of the substitution of 6 or 10 amino acids upstream of the activation peptide cleavage site (mutation A or A');
- or in family 2 which consists of the insertion of the same sequences upstream of the
- 10 activation peptide cleavage site (mutation B);
- or in family 3 which consists of the insertion of these sequences upstream of the activation peptide cleavage site, coupled to the deletion of a part of the activation peptide (mutation C or C').

Thus, the proteins of sequence SEQ ID No. 9 to 16 correspond to the sequence SEQ ID  
15 No. 7, into which a mutation A or A' has been inserted. These proteins therefore belong to family 1.

The proteins of sequence SEQ ID No. 18 to 25 correspond to the sequence SEQ ID No. 7, into which a mutation B has been inserted. These proteins therefore belong to family 2.

20 Finally, the proteins of sequence SEQ ID No. 27 to 34 correspond to the sequence SEQ ID No. 7, into which a mutation C or C' has been inserted. These proteins therefore belong to family 3.

The sequences SEQ ID No. 8, 17 and 26 correspond to an FX-control+, and are comparative.

25

## **2- Experimental protocol**

The sequences specific to each variant are introduced, by assembly or Infusion PCR, using primers judiciously designed so as to allow the insertion and/or the deletion of nucleotide sequences, into a synthetic nucleotide sequence encoding FX optimized for  
30 expression in *Homo sapiens* (SEQ ID No. 35).

### **2.1. Preparation of the pCEP4-FXWT4HS-gs vector encoding human FX**

The pUC57 cloning vector containing the synthetic gene optimized for expression in *Homo sapiens* and prepared by Genescript is digested, like the pCEP4 expression vector (Life Technologies), with the BamHI and HindIII enzymes. The insert corresponding to the FX gene (FXWT4HSgs) and the digested pCEP4 vector are purified using  
 5 Nucleospin extract II (Clontech Laboratories) before being ligated together using T4 ligase. The ligation product is used to transform Top10 bacteria (Life Technologies). The presence of the insert in the bacterial colonies is determined by digesting the plasmid with the BamHI and HindIII enzymes and passing the digestion product through agarose gel in order to detect therein a band of 1519 bp. The cDNA is verified  
 10 by sequencing using the CMVs1 (5'-GGGACTTTCCTACTTGGCAGT-3' SEQ ID No. 36) and SV40-3'UTR (5'-TTCAC TGCATTCTAGTTGTGGT-3' SEQ ID No. 37) primers.

## 2.2- Preparation of the OptiCHO FXWT4HS vector encoding human FX

15 For optimal expression of the variants, the latter and also the wild-type molecule are prepared in the OptiCHO vector.

Using the pCEP4-FXWT4HS-gs vector, the cDNA of the FXWT4HS sequence is amplified by PCR (kapa Hifi; Biosystems) with the 5'FXWT and 3'FX-SwaI primers.

20 The 1551-bp PCR product is purified using Nucleospin extract before being digested with the NheI and SwaI enzymes, just like the OptiCHO destination vector. They are again purified on Nucleospin extract after digestion.

The insert and the vector are ligated together using T4 ligase before the ligation product is integrated into competent Top10 bacteria. After bacterial amplification in the  
 25 presence of ampicillin, bacterial colonies are sampled from a Petri dish and screened by PCR to search for a 296-bp amplicon with the 5'ef1a and 3FX primers, said amplicon being a sign of the presence of the insert encoding FX in the OptiCHO vector. The PCR screening is supplemented by a screening of the purified vectors by enzymatic digestion with the Nhe I and SwaI enzymes in order to search for a 1538-bp fragment on agarose  
 30 gel. The OptiCHO-FXWT4HS vector is sequenced with the primers:

- 5'ef1a: 5'-GTGGAGACTGAAGTTAGGCCAG-3' (SEQ ID No. 38)
- 3FX: 5'-CTTCATTTCTCCAGGAAAGAGTTGGC-3' (SEQ ID No. 39)
- 2BGHPA: 5'-CAGATGGCTGGCAACTAGAA-3' (SEQ ID No. 40)

### 2.3. Preparation of the family 1 variants

The preparation of the inserts encoding the cDNAs of the family 1 variants is carried out according to table 1 by means of assembly PCR and ligation or by means of the Infusion technique using the primers listed in table 2. The template used for PCR1 and 2 is the OptiCHO-FXWT4HS vector. The PCR products are treated with DpnI in order to digest the parental DNA. The amplicons of interest are purified using Nucleospin extract.

- 10 For the OptiCHO-FX WTF1D, OptiCHO-FX WTF1G and OptiCHO-FX WTF1I molecules, the purified amplicons from PCR 1 and 2 were used as template and assembled by assembly PCR according to table 1. The purified PCR3 amplicons and also the vector digested with NheI and SmaI are assembled by ligation with T4 ligase.

15 **Table 1**

| Variant     | PCR1          |               | PCR2             |               | Assembly PCR 3 after digestion of PCR1 and 2 |               |
|-------------|---------------|---------------|------------------|---------------|----------------------------------------------|---------------|
|             | Primers       | Amplicon size | Primers          | Amplicon size | Primers                                      | Amplicon size |
| FXWT4HS F1D | 5'FXWT+3'FX1d | 720           | 5'FX1d+3'FX SmaI | 842           | 5'FX WT+3'Fx SmaI                            | 1551          |
| FXWT4HS F1G | 5'FXWT+3'FX1g | 716           | 5'FX1g+3'FX SmaI | 841           | 5'FX WT+3'Fx SmaI                            | 1551          |
| FXWT4HS F1I | 5'FXWT+3'FX1i | 716           | 5'FX1i+3'FX SmaI | 838           | 5'FX WT+3'Fx SmaI                            | 1551          |

**Table 2**

| Primers | SEQ ID No. | Sequences                                                |
|---------|------------|----------------------------------------------------------|
| 3'FX1a  | 41         | 5'-TCCTTCTGCCAGGAAGTCCTGTGTCTGGTTGAAGTCCAGCAGGTCA-3'     |
| 3'FX1b  | 42         | 5'-TCAGGCCCTCGGCCAGGAAGTCCTGTGTCTGGTTGAAGTCCAGCAGGTCA-3' |
| 3'FX1c  | 43         | 5'-TGGCGTTGGTGGCCTTCTGTGTCTGGTTGAAGTCCAGCAGGTCAA-3'      |
| 3'FX1d  | 44         | 5'-AGGGTGGCCTGGGTGGCCTTCTGTGTCTGGTTGAAGTCCAGCAGGTCA-3'   |
| 3'FX1e  | 45         | 5'-TGGTCAGCTTGCTGGTGCCTCTTTCAGGCTGTGTCTGGTTGA-3'         |
| 3'FX1f  | 46         | 5'-TGGTGAAGTCGTTGAAGCCTCTTTCAGGCTGTGTCTGGTTGAAG-3'       |
| 3'FX1g  | 47         | 5'-TGGTCATGCTGCTCAGGCCTCTTTCAGGCTGTGTCTGGTTGAAGT-3'      |
| 3'FX1h  | 48         | 5'-TGGTCAGGCTGGGAGGGCCTCTTTCAGGCTGTGTCTGGTTGAAG-3'       |
| 3'FX1i  | 49         | 5'-TCTGGCCGCAGGACAGGCCTCTTTCAGGCTGTGTCTGGTTGAAG-3'       |
| 3FX-2a  | 50         | 5'-CTCCTTCTGCCAGGAAGTCCCTAGTCAGATTGTTATCGCCTCTTTCAGGC-   |

|                |    |                                                                       |
|----------------|----|-----------------------------------------------------------------------|
|                |    | 3'                                                                    |
| 3FX-2b         | 51 | 5'-<br>GTCAGGCCCTCGGCCAGGAAGTCCCTAGTCAGATTGTTATCGCCTCTTTCA<br>GGC-3'  |
| 3FX-2c         | 52 | 5'-<br>AGGGTGGCGTTGGTGGCCTTCCTAGTCAGATTGTTATCGCCTCTTTCAGGC<br>-3'     |
| 3FX-2d         | 53 | 5'-<br>AGGGTGGCCTGGGTGGCCTTCCTAGTCAGATTGTTATCGCCTCTTTCAGGC<br>-3'     |
| 3FX-2e         | 54 | 5'-TCAGCTTGCTGGTCCTAGTCAGATTGTTATCGCCTCTTTCAGGC-3'                    |
| 3FX-2f         | 55 | 5'-GGTGAAGTCGTTGAACCTAGTCAGATTGTTATCGCCTCTTTCAGGC-3'                  |
| 3FX-2g         | 56 | 5'-ATGCTGCTCAGCCTAGTCAGATTGTTATCGCCTCTTTCAGGC-3'                      |
| 3FX-2h         | 57 | 5'-GTCAGGCTGGGAGGCCTAGTCAGATTGTTATCGCCTCTTTCAGGC-3'                   |
| 3FX-2i         | 58 | 5'-TCTGGCCGCAGGACAGCCTAGTCAGATTGTTATCGCCTCTTTCAGGC-3'                 |
| 5'FX1a         | 59 | 5'-GCAGAAGGAGGAGGAGTGAGGATCGTGGGAGGACAGGAGTGCA-3'                     |
| 5'FX1b         | 60 | 5'-AGGGCCTGACCCCTAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                     |
| 5'FX1cbis      | 61 | 5'-<br>AGGCCACCAACGCCACCCTGTCCCCTAGGATCGTGGGAGGACAGGAGTG<br>CAAGGA-3' |
| 5'FX1d         | 62 | 5'-CAGGCCACCCTGAGCCCTAGGATCGTGGGAGGACAGGAGTGCAAG-3'                   |
| 5'FX1e         | 63 | 5'-ACCAGCAAGCTGACCAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                    |
| 5'FX1f         | 64 | 5'-CAACGACTTCACCAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                      |
| 5'FX1g         | 65 | 5'-TGAGCAGCATGACCAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                     |
| 5'FX1h         | 66 | 5'-CCTCCCAGCCTGACCAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                    |
| 5'FX1i         | 67 | 5'-TGTCCTGCGGCCAGAGGATCGTGGGAGGACAGGAGTGCAAGGA-3'                     |
| 5'fusion<br>FX | 68 | 5'-TCTTCCATTTCAGCTAGCAAGCTTGCCGCCAC-3'                                |
| 3fusionF<br>X  | 69 | 5'-AGCTCTAGACAATTGATTAAATGGATCCTCACTTGCCGTC-3'                        |
| 5'FXWT         | 70 | 5'-ACCAGCTGCTAGCAAGCTTGCCG-3'                                         |
| 3'FX-<br>SwaI  | 71 | 5'-GAAACTATTTAAATGGATCCTCACTTGCCGTCAATCAGC-3'                         |
| 3FXF3          | 72 | 5'-CCAGGTAATGCTATCAGCCACTGACCTTTTGCGCCTCTC-3'                         |
| 5FXF3          | 73 | 5'-TCAGTGGCTGATAGCATTACCTGGAAACCTTATGACGC-3'                          |
| 3FXF3bis       | 74 | 5'-GCTAGTTGCCTGAGCCACTGACCTTTTG-3'                                    |
| 5FXF3bis       | 75 | 5'-GCTCAGGCAACTAGCGATAGCATTACCTGGAAACCTTATGACGC-3'                    |
| 5'efla         | 38 | 5'-GTGGAGACTGAAGTTAGGCCAG-3'                                          |
| 3FX            | 39 | 5'-CTTCATTTCTCCAGGAAAGAGTTGGC-3'                                      |
| 2BGHPA         | 40 | 5'-CAGATGGCTGGCAACTAGAA-3'                                            |

For the OptiCHO-FX WTF1a, OptiCHO-FX WTF1b, OptiCHO-FX WTF1c, OptiCHO-FX WTF1e, OptiCHO-FX WTF1f and OptiCHO-FX WTF1h molecules, the purified amplicons from PCR 1 and 2 were generated by PCR according to the conditions of table 3. The purified amplicons of PCR 1 and 2 are assembled by Infusion with the vector predigested with NheI and SwaI and purified using Nucleospin Extract.

**Table 3:**

| Variant  | PCR1                |               | PCR2                  |               |
|----------|---------------------|---------------|-----------------------|---------------|
|          | Primers             | Amplicon size | Primers               | Amplicon size |
| FX4hsF1a | 5'fusionFX + 3'FX1a | 718           | 5'FX1A + 3fusionFX    | 842           |
| FX4hsF1b | 5'fusionFX3'F + X1b | 722           | 5'FX1b + 3fusionFX    | 838           |
| FX4hsF1c | 5'fusionFX + 3'FX1c | 718           | 5'FX1cbis + 3fusionFX | 859           |
| FX4hsF1e | 5'fusionFX + 3'FX1e | 714           | 5'FX1e + 3fusionFX    | 839           |
| FX4hsF1f | 5'fusionFX + 3'FX1f | 716           | 5'FX1f + 3fusionFX    | 814           |
| FX4hsF1h | 5'fusionFX + 3'FX1h | 716           | 5'FX1h+ 3fusionFX     | 839           |

For each variant, the final vector is inserted by bacterial transformation into Top10 bacteria. After bacterial amplification in the presence of ampicillin, bacterial colonies  
5 are sampled from a Petri dish and screened by PCR in order to search for a 296-bp amplicon with the 5'efla and 3FX primers, said amplicon being a sign of the presence of the insert encoding FX in the OptiCHO vector. The OptiCHO-FX WTF1a to OptiCHO-FX WTF1i vectors are sequenced with the primers:

- 5'efla
- 10 - 2BGHPA.

#### **2.4- Preparation of the family 2 variants**

The preparation of the inserts encoding the cDNAs of the family 2 variants is carried out according to table 4 by means of the technique of PCR and assembly by Infusion  
15 using the primers listed in table 2. The template used for PCR1 and 2 is the OptiCHO-FXWT4HS vector. The PCR products are treated with DpnI in order to digest the parental DNA. The amplicons are purified using Nucleospin extract. The purified amplicons of PCR 1 and 2 are assembled by Infusion with the OptiCHO vector predigested with NheI and SwaI and purified using Nucleospin Extract.

**Table 4:**

|      | PCR1           |            |               | PCR 2           |            |               |
|------|----------------|------------|---------------|-----------------|------------|---------------|
|      | 5' primers     | 3' primers | Amplicon size | 5' primers      | 3' primers | Amplicon size |
| Fx-a | 5'fusionF<br>X | 3FX-2a     | 781           | 5'F<br>X-<br>1a | 3fusion FX | 851           |
| Fx-b | 5'fusionF<br>X | 3FX-2b     | 758           | 5'F<br>X-<br>1b | 3fusion FX | 847           |
| Fx-c | 5'fusionF<br>X | 3FX-2c     | 755           | 5'F<br>X-<br>1c | 3fusion FX | 850           |
| Fx-d | 5'fusionF<br>X | 3FX-2d     | 755           | 5'F<br>X-<br>1d | 3fusion FX | 851           |
| Fx-e | 5'fusionF<br>X | 3FX-2 e    | 748           | 5'F<br>X-<br>1e | 3fusion FX | 848           |
| Fx-f | 5'fusionF<br>X | 3FX-2f     | 750           | 5'F<br>X-<br>1f | 3fusion FX | 846           |
| Fx-g | 5'fusionF<br>X | 3FX-2g     | 746           | 5'F<br>X-<br>1g | 3fusion FX | 847           |
| Fx-h | 5'fusionF<br>X | 3FX-2h     | 749           | 5'F<br>X-<br>1h | 3fusion FX | 848           |
| Fx-i | 5'fusionF<br>X | 3FX-2i     | 751           | 5'F<br>X-<br>1i | 3fusion FX | 847           |

For each variant, the final vector is inserted by bacterial transformation into Top10 bacteria. After bacterial amplification in the presence of ampicillin, bacterial colonies are sampled from a Petri dish and screened by PCR in order to search for a 296-bp amplicon with the 5'ef1a and 3FX primers, said amplicon being a sign of the presence of the insert encoding FX in the OptiCHO vector. The OptiCHO-FX WTF2a to OptiCHO-FX WTF2i vectors are sequenced with the primers:

- 5'ef1a
- 2BGHPA.

### 2.5.- Preparation of the family 3 variants

Family 3 contains deletions in the activation peptide that it is necessary to prepare before being able to insert therein the enzymatic cleavage sites. In this respect, two intermediate vectors are prepared, OptiCHO FXWT F3AD for the family 3 variants F3a to F3d, and OptiCHO FXWT F3EI for the family 3 variants F3e to F3i.

The inserts of the intermediate vectors are constructed by assembly PCR according to table 5 using the OptiCHO FXWT4HS-gs vector as template and the 5'FXWT, 3'FX-SwaI, 3FXF3, 5FXF3, 3FXF3bis and 5FXF3bis primers listed in table 2.

**Table 5:**

|                      | PCR1              |               | PCR2                |               | Assembly PCR 3 after digestion of PCR1 and 2 |               |
|----------------------|-------------------|---------------|---------------------|---------------|----------------------------------------------|---------------|
| Inter-mediate vector | Primers           | Amplicon size | Primers             | Amplicon size | Primers                                      | Amplicon size |
| OptiCHO FXWT F3AD    | 5'FXWT + 3FXF3    | 598           | 3FX-SwaI + 5FXF3    | 947           | 5'FXWT + 3FX-SwaI                            | 1521          |
| OptiCHO FXWT F3EI    | 5'FXWT + 3FXF3bis | 595           | 3FX-SwaI + 5FXF3bis | 953           | 5'FXWT + 3FX-SwaI                            | 1533          |

The assembly PCR products are purified using Nucleospin extract before being digested, just like the OptiCHO destination vector, with the NheI and SwaI enzymes.

5 They are again purified on Nucleospin extract after digestion.

The insert and the vector are ligated together using T4 ligase before the ligation product is integrated into competent Top10 bacteria. After bacterial amplification in the presence of ampicillin, bacterial colonies are sampled from a Petri dish and screened by PCR in order to search for a 296-bp amplicon with the 5'efl<sub>a</sub> and 3FX primers, said  
10 amplicon being a sign of the presence of the insert encoding the FX variant in the OptiCHO vector.

The OptiCHO FXWT F3AD and OptiCHO FXWT F3EI intermediate vectors are sequenced with the primers:

- 5'efl<sub>a</sub>
- 15 - 3FX
- 2BGHPA.

With the exception of the OptiCHO FXWT F3A molecule, the preparation of the inserts encoding the cDNAs of the series 3 variants is carried out according to table 6 by assembly PCR using the primers listed in table 2. The template used for PCR1 and 2 is  
20 the OptiCHO FXWT F3AD vector for the family 3 variants F3a to F3d, and OptiCHO FXWT F3EI for the family 3 variants F3e to F3i. The amplicons are purified using Nucleospin extract. The purified amplicons of PCR 1 and 2 are assembled by assembly PCR with the OptiCHO vector predigested with NheI and SwaI and purified using Nucleospin extract.

**Table 6:**

|      | PCR1            |               | PCR2                           |               | Assembly PCR 3 after digestion of PCR1 and 2 |               |
|------|-----------------|---------------|--------------------------------|---------------|----------------------------------------------|---------------|
|      | Primers         | Amplicon size | Primers                        | Amplicon size | Primers                                      | Amplicon size |
| Fx-b | 5'FXWT + 3FX-2b | 723           | 5FX-1b + 3'FX-SwaI             | 838           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-c | 5'FXWT + 3FX-2c | 720           | 5FX-1c + 3'FX-SwaI             | 841           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-d | 5'FXWT + 3FX-2d | 720           | 5FX-1d + 3'FX-SwaI             | 842           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-e | 5'FXWT + 3FX-2e | 713           | 5FX-1 <sup>e</sup> + 3'FX-SwaI | 725           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-f | 5'FXWT + 3FX-2f | 715           | 5FX-1f + 3'FX-SwaI             | 727           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-g | 5'FXWT + 3FX-2g | 711           | 5FX-1g + 3'FX-SwaI             | 723           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-h | 5'FXWT + 3FX-2h | 714           | 5FX-1h + 3'FX-SwaI             | 726           | 5'FXWT + 3'FX-SwaI                           | 1551          |
| Fx-i | 5'FXWT + 3FX-2i | 716           | 5FX-1i + 3'FX-SwaI             | 728           | 5'FXWT + 3'FX-SwaI                           | 1551          |

The assembly PCR products are purified using Nucleospin extract before being digested, just like the OptiCHO destination vector, with the NheI and SwaI enzymes.

- 5 They are again purified on Nucleospin extract after digestion.

The insert and the vector are ligated together using T4 ligase before the ligation product is inserted into competent Top10 bacteria. After bacterial amplification in the presence of ampicillin, bacterial colonies are sampled from a Petri dish and screened by PCR in order to search for a 296-bp amplicon with the 5'efla and 3FX primers, said amplicon being a sign of the presence of the insert encoding the FX variant in the OptiCHO vector.

The final family 3 vectors are sequenced with the primers:

- 5'efla
- 2BGHPA

15

## **Example 2: Production of recombinant factors X**

### **1 – Experimental protocol**

20

#### **1.1 – Reagents**

ProCHO4 (Lonza) and Freestyle™ F17 (Gibco) culture medium.

L-glutamine (Gibco).

CHO-S cell transfection medium: Opti-Pro SFM (Gibco).

HEK cell transfection medium: Opti-MEM (Gibco).

Vitamin K1 (Sigma).

5

## 1.2 - Protocol

The wild-type factor X and variants thereof are produced in CHO-S or HEK-293-Freestyle eukaryotic cells (Invitrogen) in transient expression.

10 The CHO-S cells are cultured in ProCHO4 medium and the HEK 293F cells in F17 medium, supplemented respectively with 4 mM and 8 mM of L-glutamine. The 2 cell lines are cultured under conditions shaken at 135 rpm in a controlled atmosphere (8% CO<sub>2</sub>) at 37°C. On the day before the day of transfection, the cells are seeded at a density of  $7 \times 10^5$  cells/ml.

15 On the day of the transfection, the DNA (20-30 µg) and 30 µg of transfection agent (TA) are preincubated separately in Opti-Pro medium for CHO-S and Opti-MEM for HEK 293F for 5 minutes and then mixed and incubated for 20 minutes in order to allow the formation of the DNA/TA complex. The whole thing is added to a cell preparation of  $1 \times 10^6$  cells/ml in a volume of 30 ml.

20 In the case of the co-transfections of the FXs with native vitamin K epoxide reductase (VKOR), the 2 vectors are added at various ratios in order to obtain a total amount of DNA of 20-30 µg. The VKOR enzyme enables production of active FX in HEK while optimizing gamma-carboxylation. Immediately after transfection, vitamin K1 (5 µg/ml) is added to the medium. The transfection levels are evaluated the day after transfection, using a control plasmid expressing GFP (Green Fluorescent Protein). The productions  
25 are carried out in "batch" mode for 7 days. At the end of production, the cells and the supernatant are separated by centrifugation. The cells are removed and the supernatant is filtered and then frozen.

## 30 Example 3: Measurement of factor X concentration

### 1- Experimental protocol

The factor X concentration is measured by means of the commercial ELISA Zymutest Factor X (Hyphen BioMed) according to the manufacturer's recommendations. The concentrations are measured in triplicate using antigen values located in the linear zone of detection of the assay. In order to be sure that the mutations introduced do not disrupt the measurement of the concentration, the FXs are deposited in identical amount and revealed by immunoblotting with a polyclonal antibody different than the one used in the ELISA (anti-human FX polyclonal antibody (Cryopep cat No. PAHFX-S)) or by staining after SDS-PAGE (data not shown).

## 10            **2 – Results**

### **2.1– Expression of factor X variants transiently expressed in CHO**

The concentrations of the factor X (FX) variants present in the supernatants of the CHO-S cells transfected with the cDNAs encoding families 1 to 3 were measured by using the commercial ELISA Zymutest Factor X (figure 3).

15    As expected, the supernatants of non-transfected (control) CHO cells do not contain FX. The transfection with the vectors encoding the various FXs makes it possible to obtain levels ranging from 0.5 to 3.06 µg/ml. There is no major difference in expression of the FXs of the various families. At most, FX-IIa of family 3 is expressed 2.1X more strongly (2.64 µg/ml) than that of family 1 (1.26 µg/ml). Some constructs make it possible to obtain factor X at a higher concentration than the wild-type factor X: these are the FX-IIa (family 3) and FX-PAR1 (family 1 and 3) constructs. FX-Kal3 appears to reduce FX production.

### **2.2– Expression of the factor X variants transiently expressed in HEK**

25    The concentrations of the factor X variants present in the supernatants of the HEK293S cells transfected with the cDNAs encoding families 1 to 3 were measured using the commercial ELISA Zymutest Factor X (figure 4). As expected, the supernatants of non-transfected (control) HEK293S cells do not contain FX. The family 1 molecules were produced at a level close to that of FX-WT (from 0.14 to 1.64 µg/ml). Only the FX-PAR1 molecule is produced at a higher level. The FX-Kal3 molecule remains the least produced molecule. Family 2 was produced in a similar manner for all the constructs, with expression values of from 1.79 to 2.54 µg/ml except for the FX-Kal3 construct

produced at a lower level (0.66 µg/ml). Family 3 was produced at lower levels of from 0.2 to 1.2 µg/ml.

*In conclusion, certain factor X variant constructs make it possible to produce FX at higher levels than FX-WT:*

*- these are, in CHO cells, the FX-IIa (family 3) and FX-PAR1 (families 1 and 3) constructs and*

*- in HEK cells, FX-PAR1 of family 1.*

10

#### **Example 4: Measurement of the coagulant activity of the factor X variants produced in CHO-S**

##### **1 – Experimental protocol**

15 The chronometric activity of the FX variants produced by the CHO-S cells was measured using a Star automated device (Stago) in the presence of FX-deficient plasma. The FX-deficient plasma, the neoplastin and the Owren-Koller buffer come from Stago (Asnières, France).

The concentrated culture supernatant is diluted to 1/10 in Owren-Koller buffer before  
20 being added to the FX-deficient plasma. The mixture is incubated for 240 seconds at 37°C and then the prothrombin time (PT) is initiated by adding 100 µl of neoplastin. The coagulation times (in s.) are converted into specific activity of the FX.

##### **2– Results**

25 The concentrated supernatants resulting from the various transfections in CHO were evaluated for their capacity to compensate for a factor X deficiency. The supernatants were incubated in an FX-deficient plasma and a PT test was carried out. The coagulation times (in s.) were converted into specific activity (SA; in seconds per µg of protein) then the percentage of specific activity compared with the wild-type FX was  
30 calculated (figure 5). The results are coherent between the various families. This result indicates that the differences in behavior originate mainly from the cleavage sites and not from the way in which they are cloned.

The constructs can be classified into three categories: a first category, the SA of which is similar to that of FX-WT (contains FX-control+), a second category, the SA of which is decreased compared with that of the control (contains FX-IIa, FX-PAR1, FX-PAR1M and FX-Kal3) and a third category, the activity of which in the absence of FX is greater than that of FX-WT (contains FX-FXIa1 and 2, FX-Kal1 and 2). It should be noted that the FX-Kal2 family 3 construct could not be analyzed for technical reasons (\* on the graph).

*These results indicate that the modifications introduced and which form family 3 (FX-FXIa1 and 2, FX-Kal1 and 2) confer, on the factor X molecules, a more effective capacity to coagulate than the wild-type factor X in the absence of endogenous factor X.*

#### **Example 5: Measurement of the activation of the factor X variants produced in CHO-S by the RVV-X venom fraction**

##### **1 – Experimental protocol**

The activation of the FX variants produced by the CHO-S cells was measured following incubation of the culture supernatants in the presence of Russell's viper venom anti-factor X fraction (RVV-X). The control activated factor X, the venom fraction X (RVV-X) and the pNAPEP 1025 substrate come from Haematologic Technologies (Cryo pep Montpellier, France).

The activation was studied at 37°C in the following buffer: 25 mM HEPES, pH 7.4, 0.175 M NaCl, 5 mM CaCl<sub>2</sub> and 5 mg/ml BSA. For concentrations of 0 to 100 nM of FX, a concentration of 200 mU/ml of RVV-X was used. After incubation for 5 min, the reaction is stopped in 50 mM Tris buffer, pH 8.8, 0.475 M NaCl, 9 mM EDTA. The amount of FXa generated is monitored by measuring the rate of hydrolysis of the pNAPEP 1025 substrate (250 µM) at 405 nm.

##### **2 – Results**

The supernatants of CHO expressing the family 1 variants were incubated with the RVV-X. The generation of FXa was measured following this treatment using various

concentrations of FX. The presence of FXa is quantified by the rate of appearance of the product of pNAPEP 1025 in solution (in mODU/min). This generation is the reflection of the recognition and cleavage of the FXs by RVV-X and also of the capacity of the FXa generated to recognize the FX substrate. The mean of the appearance rates is  
 5 calculated for the various initial concentrations of FX and this value is related to the percentage of the value of the FX-WT.

The analysis of family 1 shows in part results that are similar to the results obtained by PT, in particular for FX-control+, FX-IIa, FX-Kal1, FX-Kal2 and FX-Kal3 (figure 6). The FX-XIa1 and 2 molecules show an activity, but which this time is not greater than  
 10 FX-WT. On the other hand, the FX-PAR1 and FX-PAR1M molecules demonstrate a significant activity greater than that observed by PT.

*These results indicate that FX-PAR1M and FX-Kal2 of family 1 are more effectively activated by RVV-X than FX-WT.*

15

**Example 6: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 2: Activation of the extrinsic coagulation pathway (TF 1pM/PL 4  $\mu$ M) in FVIII-deficient plasma**

20

## **1 – Experimental protocol**

### **1.1 - Reagents**

Thrombin calibrator, PPP reagent low, CK-Prest, Fluca Kit (Fluo-buffer + Fluo-substrate), PNP and FIX-deficient plasma come from Stago (Asnières, France). The FX-deficient plasma comes from Cryopep (Montpellier, France). The FVIII-deficient plasma comes from Siemens Healthcare (Marburg, Germany). The human FX (cat No. HCX-0050), and the human FXa (cat No. HCXA-0060) come from Haematologic Technologies Inc. (Burlington, Vt, USA). The control recombinant human factor VIII comes from Baxter (Recombinate) (Maurepas, France).

30

### **1.2 – Protocol**

The thrombin generation test consists in activating coagulation *ex vivo* either by means of a mixture of tissue factor and phospholipids (activation of the extrinsic pathway), or by means of cephalin (activation of the intrinsic pathway), and in then measuring the concentration of thrombin generated over time.

The reaction is initiated by adding 20  $\mu$ l of Fluca-kit (substrate +  $\text{CaCl}_2$ ) which constitutes the beginning of the measurement of the appearance of thrombin. The appearance of fluorescence is measured on a Fluoroskan Ascent fluorometer (ThermoLabsystems) at an excitation wavelength of 390 nm and at an emission length of 460 nm. The thrombinograms (curves representing the fluorescence intensity as a function of time) are then analyzed by means of the Thrombinoscope™ software which converts the fluorescence value into nM of thrombin by comparative calculation.

The supernatants were concentrated approximately 20-fold on a Sartorius VivaSpin20-30 kDa at 2500 g for at least 1 h until the desired concentration was obtained. The Unicalibrator plasmas and also the FVIII-deficient plasmas reconstituted with 0, 0.1 or 1 U/ml are used as controls.

25 As expected, following the activation of coagulation with tissue factor, the FVIII-deficient plasma gives the weakest signal, corresponding to the background noise of the experiment. The Unicalibrator plasma gives a signal that is weaker than the FVIII-deficient plasma reconstituted with the FVIII concentrations (0.1 or 1 U/ml). On the other hand, the reconstitution of an FVIII-deficient plasma with FX-WT does not make  
30 it possible to generate sufficient amounts of thrombin. This reconstitution is barely greater than the deficient plasma alone (figure 7, table 7).

| Product Name | Unicalibrator | FVIII Deficient | FVIII Def + 1 U/ml rFVIII | FVIII Def + 0,1 U/ml rFVIII | FVIII Def + FX-WT (5 µg/ml) | FVIII Def + FX-control+ (5 µg/ml) | FVIII Def + FX-Ila (5 µg/ml) |
|--------------|---------------|-----------------|---------------------------|-----------------------------|-----------------------------|-----------------------------------|------------------------------|
| Lagtime      | 7.36          | 8.19            | 6.19                      | 8.03                        | 7.86                        | 7.36                              | 7.19                         |
| ETP          | 1271          | 314             | 1850.5                    | 1415                        | 1278                        | 1539.5                            | 1618                         |
| Peak         | 135.8         | 11.89           | 329.1                     | 154.3                       | 75.96                       | 153.28                            | 184.11                       |
| ttPeak       | 11.87         | 20.88           | 8.19                      | 12.03                       | 13.37                       | 11.2                              | 10.7                         |
| StartTail    | 40            | 56              | 31                        | 41.5                        | 70.5                        | 44                                | 38.5                         |
| Velocity     | 30.11         | 0.94            | 164.55                    | 38.58                       | 13.79                       | 39.92                             | 52.45                        |

| Product Name | FVIII Def + FX-PAR1 (5 µg/ml) | FVIII Def + FX-PAR1M (5 µg/ml) | FVIII Def + FX-FXla1 (5 µg/ml) | FVIII Def + FX-FXla2 (5 µg/ml) | FVIII Def + FX-FXla3 (5 µg/ml) | FVIII Def + FX-Kal1 (3,5 µg/ml) | FVIII Def + FX-Kal2 (1,65 µg/ml) |
|--------------|-------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|---------------------------------|----------------------------------|
| Lagtime      | 6.86                          | 7.36                           | 6.69                           | 7.69                           | 7.19                           | 7.36                            | 7.69                             |
| ETP          | 1604                          | 1534                           | 1582                           | 1467.5                         | 1504                           | 1408                            | 1438                             |
| Peak         | 201.35                        | 169.82                         | 190.67                         | 151.76                         | 167.8                          | 118.53                          | 162.52                           |
| ttPeak       | 10.03                         | 10.87                          | 10.03                          | 11.37                          | 10.53                          | 11.87                           | 11.03                            |
| StartTail    | 36.5                          | 41.5                           | 38                             | 45                             | 42                             | 52                              | 41.5                             |
| Velocity     | 63.52                         | 48.38                          | 57.09                          | 41.24                          | 50.24                          | 26.28                           | 48.66                            |

Table 7: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor VIII-deficient plasma, following activation with tissue factor (1 pM)

5 The kinetic parameters of the thrombinograms of figure 7 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

All the FX variant molecules give a greater signal than FX-WT, suggesting that their capacity to generate thrombin in the absence of FVIII is significantly increased compared with FX-WT (table 7). The speeds of the FX mutants of family 2 oscillate between 2 and 4.5X that of FX-WT (figure 9, table 7). It should be noted that a speed of 2X is obtained with an amount of FX-Kal2 that is 30% lower (3.5 µg/ml instead of 5 µg/ml).

*The mutants which make it possible to generate the largest amounts of thrombin in the absence of FVIII are, in order, FX-PAR1, FX-FXIa1, FX-IIa and FX-Kal1.*

10

**Example 7: Measurement, in terms of the thrombin generation time (TGT), of the procoagulant capacity of the factor X variants: intrinsic coagulation pathway (cephalin alone) in FVIII-deficient plasma**

15

#### **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

The thrombin generation tests are carried out on 80 µl of a pool of normal plasma optionally containing the cell supernatants and the controls in the presence of 20 µl of cephalin (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of 20 µl of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are a normal plasma and a factor VIII-deficient plasma.

25

#### **2 – Results**

The supernatants used in example 6 were analyzed by TGT after activation with cephalin using the same controls.

The controls behave expectedly, the FVIII-deficient plasma does not allow any generation of IIa and a gradient of effectiveness is found when increasing the dose of FVIII (table 8).

30

| Product Name | Unicalibrator | FVIII Deficient | FVIII Def + 1 U/ml rFVIII | FVIII Def + 0,1 U/ml rFVIII | FVIII Def + FX-WT (5 µg/ml) | FVIII Def + FX-control+ (5 µg/ml) | FVIII Def + FX-IIa (5 µg/ml) |
|--------------|---------------|-----------------|---------------------------|-----------------------------|-----------------------------|-----------------------------------|------------------------------|
| Lagtime      | 19.04         | 35.73           | 8.69                      | 20.54                       | 23.88                       | 16.37                             | 13.03                        |
| ETP          | 1149.5        | 0               | 1417.5                    | 1089.5                      | 589                         | 1134.5                            | 1284.5                       |
| Peak         | 271.07        | 3.48            | 292.17                    | 171.4                       | 35.98                       | 102.19                            | 235.25                       |
| ttPeak       | 21.04         | 70.46           | 10.53                     | 24.05                       | 34.4                        | 24.05                             | 16.04                        |
| StartTail    | 36            | 0               | 29.5                      | 41.5                        | 67.5                        | 47                                | 33.5                         |
| Velocity     | 135.54        | 0.10            | 158.79                    | 48.83                       | 3.42                        | 13.31                             | 78.16                        |

FVIII Def

| Product Name | FVIII Def + FX-PAR1 (5 µg/ml) | FVIII Def + FX-PAR1M (5 µg/ml) | FVIII Def + FX-FXIa1 (5 µg/ml) | FVIII Def + FX-FXIa2 (5 µg/ml) | FVIII Def + FX-FXIa3 (5 µg/ml) | FVIII Def + FX-Kal1 (3,5 µg/ml) | FVIII Def + FX-Kal2 (1,65 µg/ml) |
|--------------|-------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|---------------------------------|----------------------------------|
| Lagtime      | 15.53                         | 17.54                          | 16.37                          | 19.54                          | 18.37                          | 20.71                           | 19.88                            |
| ETP          | 950.5                         | 892.5                          | 968                            | 805                            | 869                            | 844.5                           | 814                              |
| Peak         | 87.25                         | 71.5                           | 80.35                          | 59.78                          | 68.08                          | 57.78                           | 59.2                             |
| ttPeak       | 22.88                         | 25.72                          | 24.05                          | 28.22                          | 26.39                          | 30.23                           | 28.89                            |
| StartTail    | 46.5                          | 51.5                           | 49.5                           | 57.5                           | 54.5                           | 61                              | 58.5                             |
| Velocity     | 11.87                         | 8.74                           | 10.46                          | 6.89                           | 8.49                           | 6.07                            | 6.57                             |

Table 8: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor VIII-deficient plasma, following activation with cephalin

The kinetic parameters of the thrombinograms of figure 8 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

The analysis of family 2 shows, as expected, that the wild-type FX is the least active FX molecule, showing a weak residual activity (3.4 nM/s). All the other molecules show a greater capacity to generate thrombin (table 8). The FX-Kal-1 to 3, FX-FXI-2 and FX-PAR1M molecules show an activity which is a little stronger (6.6 to 8.7 nM/s) but less  
 5 than the FX-control+, FX-PAR1 and FX-FXI-1 constructs (10.5-13.3 nM/s). On the other hand, the FX-IIa molecule makes it possible to obtain a signal which is between the values obtained for 0.1 and 1 U/ml of FVIII (78.2 nM/s), very effectively correcting the FVIII deficiency. These differences in speeds show, for the mutants, a thrombin-generating effectiveness which is 1.8X greater (but for the FX-Kal2 mutant at  
 10 3.5 µg/ml) to 22.8X greater (for the FX-IIa mutant).

*In conclusion, several FX mutants have the capacity to restore coagulation in the absence of FVIII following activation of the plasma with tissue factor and cephalin.*

15

**Example 8: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 2: activation of the extrinsic coagulation pathway (TF 1pM/PL 4 µM) in FIX-deficient plasma**

#### 20           **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

The thrombin generation tests are carried out on 80 µl of a pool of normal plasma optionally containing the cell supernatants and the controls in the presence of 1 pM of  
 25 tissue factor (TF) and 4 µM of phospholipids (PL) and of 20 µl of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are either normal or factor IX-deficient.

#### **2 – Results**

The supernatants used in example 6 were analyzed by TGT after activation with tissue  
 30 factor using, as controls, a normal plasma (Unicalibrator) or an FIX-deficient plasma reconstituted with 0, 0.1 or 1 U/ml of plasma FIX (figure 10, table 9).

The FIX-deficient plasma is negative (it does not enable any generation of IIa) and the reconstitution thereof with FIX allows it to generate large amounts of thrombin. However, there is no quantitative difference between the two concentrations of FIX used, thereby suggesting that they both make it possible to form a maximum amount of IIa (table 9).

| Product Name | Unicalibrator | FIX Deficient | FIX Def + FIX 1 U/ml | FIX Def + FIX 0.1 U/ml | FIX Def + FX-WT (5 µg/ml) | FIX Def + FX-control+ (5 µg/ml) | FIX Def + FX-IIa (5 µg/ml) |
|--------------|---------------|---------------|----------------------|------------------------|---------------------------|---------------------------------|----------------------------|
| Lagtime      | 7.33          | 4.5           | 4.83                 | 5                      | 6.67                      | 6.67                            | 6.67                       |
| ETP          | 1216          | 296           | 1645                 | 1623.5                 | 1251                      | 1533                            | 1540.6                     |
| Peak         | 97.46         | 9.74          | 292.04               | 283.36                 | 69.18                     | 112.48                          | 138.17                     |
| ttPeak       | 12.83         | 19            | 7.67                 | 7.83                   | 14                        | 12.83                           | 12.5                       |
| StartTail    | 47            | 68            | 23.5                 | 24.5                   | 66                        | 46.5                            | 38                         |
| Velocity     | 17.72         | 0.67          | 102.83               | 100.13                 | 8.07                      | 18.26                           | 23.70                      |

| Product Name | FIX Def + FX-PAR1 (5 µg/ml) | FIX Def + FX-PAR1M (5 µg/ml) | FIX Def + FX-FXla1 (5 µg/ml) | FIX Def + FX-FXla2 (5 µg/ml) | FIX Def + FX-FXla3 (5 µg/ml) | FIX Def + FX-Kal1 (3.5 µg/ml) | FIX Def + FX-Kal2 (1.65 µg/ml) |
|--------------|-----------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-------------------------------|--------------------------------|
| Lagtime      | 6.67                        | 6.67                         | 6.5                          | 7                            | 6.67                         | 6.33                          | 7                              |
| ETP          | 1483                        | 1506                         | 1580.5                       | 1446                         | 1457.5                       | 1523.5                        | 1483                           |
| Peak         | 160.46                      | 125.64                       | 126.16                       | 97.1                         | 123.59                       | 85.83                         | 103.73                         |
| ttPeak       | 11                          | 11.83                        | 11.83                        | 12.67                        | 11.67                        | 13.33                         | 12.83                          |
| StartTail    | 35.5                        | 43                           | 43.5                         | 64.5                         | 43                           | 61.5                          | 60                             |
| Velocity     | 37.06                       | 24.35                        | 23.48                        | 17.13                        | 24.72                        | 12.26                         | 17.79                          |

9: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor IX-deficient plasma, following activation with tissue factor (1 pM)

The kinetic parameters of the thrombinograms of figure 10 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

In this assay, all the mutants make it possible to generate greater amounts of IIa than FX-WT. However, the differences are less substantial than in the absence of FVIII: thus, the speed ranges from 1.50X (for FX-Kal2 at 3.5 µg/ml) to 4.60X (for FX-PAR1). In addition to this mutant, the FX-IIa, FX-PAR1M, FX-FXIa1 and FX-Kal1 molecules are  
 5 the most effective (figure 12, table 9).

**Example 9: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 2: activation of the  
 10 intrinsic coagulation pathway (cephalin) in FIX-deficient plasma**

#### **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

15 The thrombin generation tests are carried out on 80 µl of a pool of normal plasma optionally containing the cell supernatants and the controls in the presence of cephalin (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of 20 µl of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are either normal or factor IX-deficient.

#### **20 2 – Results**

The supernatants used in example 6 were analyzed by TGT after activation with cephalin using, as controls, a normal plasma (Unicalibrator) and an FIX-deficient plasma reconstituted with 0, 0.1 or 1 U/ml of plasma FIX (figure 11, table 10).

The FIX-deficient plasma is negative (it does not allow any generation of IIa) and the  
 25 reconstitution thereof with FIX allows it to generate large amounts of thrombin. However, there is no quantitative difference between the two concentrations of FIX used, thereby suggesting that they form a maximum amount of IIa (table 10).

| Product Name | Unicalibrator | FIX Deficient | FIX Def + FIX 1 U/ml | FIX Def + FIX 0.1 U/ml | FIX Def + FX-WT (5 µg/ml) | FIX Def + FX-control+ (5 µg/ml) | FIX Def + FX-IIa (5 µg/ml) |
|--------------|---------------|---------------|----------------------|------------------------|---------------------------|---------------------------------|----------------------------|
| Lagtime      | 29.17         | 0             | 13                   | 13.33                  | 36.67                     | 28                              | 22.17                      |
| ETP          | 1209          | 0             | 1539.5               | 1521.5                 | 0                         | 734                             | 1528.5                     |
| Peak         | 252.9         | 0             | 378.46               | 370.94                 | 12.79                     | 62.02                           | 240.9                      |
| ttPeak       | 31.67         | 0             | 14.67                | 15                     | 51.83                     | 36.83                           | 26.83                      |
| StartTail    | 47            | 0             | 30                   | 30.5                   | 0                         | 57                              | 40                         |
| Velocity     | 101.16        | 0.00          | 226.62               | 222.12                 | 0.94                      | 7.02                            | 51.70                      |

| Product Name | FIX Def + FX-PAR1 (5 µg/ml) | FIX Def + FX-PAR1M (5 µg/ml) | FIX Def + FX-FXla1 (5 µg/ml) | FIX Def + FX-FXla2 (5 µg/ml) | FIX Def + FX-FXla3 (5 µg/ml) | FIX Def + FX-Kal1 (3.5 µg/ml) | FIX Def + FX-Kal2 (1.65 µg/ml) |
|--------------|-----------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-------------------------------|--------------------------------|
| Lagtime      | 22.17                       | 25.33                        | 25.5                         | 29.5                         | 26.67                        | 29.33                         | 28.5                           |
| ETP          | 452.5                       | 407                          | 370.5                        | 327.5                        | 385                          | 319.5                         | 310.5                          |
| Peak         | 31.86                       | 25.82                        | 22.34                        | 17.99                        | 22.39                        | 17.05                         | 17.53                          |
| ttPeak       | 32.33                       | 36.17                        | 37                           | 42                           | 38                           | 41.67                         | 39.83                          |
| StartTail    | 54.5                        | 62.5                         | 63                           | 70                           | 67.5                         | 70                            | 69.5                           |
| Velocity     | 3.14                        | 2.38                         | 1.94                         | 1.44                         | 1.98                         | 1.38                          | 1.55                           |

Table 10: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 2 to a pool of factor IX-deficient plasma, following activation with cephalin

The kinetic parameters of the thrombinograms of figure 11 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

Following the induction of coagulation with cephalin, only three molecules make it possible to significantly generate more thrombin than FX-WT, these are FX-IIa, FX-control+ and FX-PAR1 with a speed which is 61X, 8.3X and 3.8X greater than that of FX-WT respectively (figure 12, table 10). However, the other mutants have speeds greater than that of FX-WT.

*In conclusion, all the mutants studied make it possible to generate thrombin in the absence of FLX, whatever the induction used. The FX-IIa, FX-control+ and FX-PAR1 are the most effective.*

#### **Example 10: Thrombin activation of the factor X variants of family 2 and wild-type factor Xs produced in HEK**

##### **1 – Experimental protocol**

The activation of the FX variants produced by the HEK cells in the presence of vitamin K epoxide reductase (VKOR) was measured following incubation of the culture supernatants in the presence of thrombin. The control activated factor X, the thrombin and the Pefachrome FXa8595 substrate comes from Haematologic Technologies (Cryo pep, Montpellier, France).

The phospholipids come from Diagnostica Stago (Asnières, France).

The activation was studied at 37°C in the following buffer: 25 mM HEPES, pH 7.4, 0.175 M NaCl, 5 mM CaCl<sub>2</sub> and 5 mg/ml BSA. For concentrations of 42.5 and 85 nM of FX, a concentration of 10 nM of thrombin and a concentration of 4 µM of phospholipids were used. After incubation for 1 hour at 37°C, the amount of FXa generated is monitored by measuring the rate of hydrolysis of the Pefachrome FXa8595 substrate (250 µM) at 405 nm.

##### **2 – Results**

In order to verify that the sites intended to be cleaved by thrombin are indeed recognized by this enzyme, the concentrated supernatants of FX-WT, FX-control+ and FX-IIa were incubated in the presence of thrombin. The appearance of FXa was

measured by the appearance of the substrate degradation product (figure 13). Plasma FXa was used as a positive control. The kinetics of appearance of the product with the mutants cannot be directly compared with those of FXa since these molecules need to be activated before releasing the activity. Moreover, since the cleavage sites for thrombin are composites, the activation of molecules is not necessarily optimal. Indeed, all the molecules conserved the original zone downstream of the cleavage site, only the upstream parts are modified. The kinetics of FXa substrate degradation by the recombinant molecules are consequently different than that by the FXa.

The results obtained confirm the data obtained by TGT with, as expected, the plasma and WT FXs being negative since they do not have a thrombin recognition site. On the other hand, FX-IIa and FX-control+ are capable of being activated by thrombin and of releasing FXa. As in the TGT measurement, FX-IIa is more effectively activated than FX-control+.

15

**Example 11: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 1: extrinsic coagulation pathway (TF 1pM/PL 4  $\mu$ M) in FVIII-deficient plasma**

## 20           1 – Experimental protocol

The thrombin generation tests are carried out on 80  $\mu$ l of a pool of plasma optionally containing the cell supernatants or the controls, in the presence of 20  $\mu$ l of PPP reagent (Stago) containing a final concentration of 1 pM of tissue factor (TF) and 4  $\mu$ M of phospholipids (PL). Various plasmas are used, normal and factor VIII-deficient.

25 The reaction is initiated by adding 20  $\mu$ l of Fluca-kit (substrate +  $\text{CaCl}_2$ ) which constitutes the beginning of the measurement of the appearance of thrombin. The appearance of fluorescence is measured on a Fluoroskan Ascent fluorimeter (ThermoLabsystems) at an excitation wavelength of 390 nm and at an emission length of 460 nm. The thrombinograms (curves representing the fluorescence intensity as a function of time) are then analyzed by means of the Thrombinoscope™ software which

30 converts the fluorescence value into nM of thrombin by comparative calculation.

## 2 – Results

The family 1 supernatants resulting from a transfection of HEK293F cells in the presence of VKOR were analyzed by TGT after activation with tissue factor using the controls already described. The controls (normal plasma, FVIII-deficient plasma reconstituted with recombinant FVIII or not reconstituted with recombinant FVIII) behave expectedly. On the other hand, FX-WT gives a signal which is moderate but greater than expected (figures 14 and 16; table 11). Its mean velocity (59 nM/min) is however exceeded by those of several mutants, in particular FX-FXIa1/2, FX-Ka11/2 and FX-control+ and FX-IIa which have a velocity of at least 130% that of the control.

| Group name | Unicalibrator | FVIII Deficient | FVIII Def + Recombinate<br>1 U/ml | FVIII Def + Recombinate<br>0.1 U/ml | FVIII Def + FX-WT<br>7.5 µg/ml | FVIII Def + FX-control+<br>7.5 µg/ml | FVIII Def + FX-IIa<br>7.5 µg/ml |
|------------|---------------|-----------------|-----------------------------------|-------------------------------------|--------------------------------|--------------------------------------|---------------------------------|
| Lagtime    | 8             | 9.33            | 7.17                              | 9.33                                | 7.83                           | 7                                    | 7.83                            |
| ETP        | 1325.5        | 0               | 1798                              | 1334.5                              | 1549.5                         | 1619                                 | 1652                            |
| Peak       | 136.55        | 12.25           | 291.64                            | 125.53                              | 175.6                          | 230.11                               | 240.61                          |
| ttPeak     | 12.5          | 29.67           | 9.5                               | 13.67                               | 11.17                          | 10                                   | 10.83                           |
| StartTail  | 40            | 0               | 33                                | 46.5                                | 40                             | 33                                   | 33.5                            |
| Velocity   | 30.34         | 0.60            | 125.17                            | 28.92                               | 52.57                          | 76.70                                | 80.20                           |

| Group name | FVIII Def + FX-<br>PAR1 7.5<br>µg/ml | FVIII Def + FX-PAR1M<br>7.5 µg/ml | FVIII Def + FX-FXIa1<br>7.5 µg/ml | FVIII Def + FX-FXIa2<br>7.5 µg/ml | FVIII Def + FX-Ka11<br>7.5 µg/ml | FVIII Def + FX-Ka12<br>7.5 µg/ml | FVIII Def + FX-Ka13<br>1.65 µg/ml | FVIII Def + FX-WT<br>7.5 µg/ml |
|------------|--------------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|----------------------------------|----------------------------------|-----------------------------------|--------------------------------|
| Lagtime    | 8.5                                  | 7.5                               | 6.17                              | 5.83                              | 6.67                             | 6                                | 5.67                              | 7.83                           |
| ETP        | 1337                                 | 1562                              | 1704                              | 1678.5                            | 1628                             | 1642.5                           | 1667                              | 1569.5                         |
| Peak       | 112.83                               | 196.06                            | 310.73                            | 308.24                            | 252.3                            | 274.07                           | 289.45                            | 199.2                          |
| ttPeak     | 13                                   | 10.67                             | 7.17                              | 7.67                              | 9.17                             | 8.33                             | 7.83                              | 10.93                          |
| StartTail  | 50                                   | 36.5                              | 28.5                              | 29                                | 32                               | 30.5                             | 29                                | 36.5                           |
| Velocity   | 25.07                                | 61.85                             | 155.37                            | 167.52                            | 100.92                           | 117.63                           | 134.00                            | 66.40                          |

**Table 11: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor VIII-deficient plasma, following activation with tissue factor**

The kinetic parameters of the thrombinograms of figure 14 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 12: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 1: intrinsic coagulation pathway (cephalin alone) in FVIII-deficient plasma**

5

**1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

10 The thrombin generation tests are carried out on 80  $\mu$ l of a pool of plasma optionally containing the cell supernatants and the controls in the presence of 20  $\mu$ l of cephalin (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of 20  $\mu$ l of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are a normal plasma and a factor VIII-deficient plasma.

**2 – Results**

15 The family 1 supernatants resulting from a transfection of HEK293F cells in the presence of VKOR were analyzed by TGT after activation with cephalin using the already described controls.

20 The controls behave expectedly, the FVIII-deficient plasma is negative (it does not allow any generation of IIa) and a gradient of effectiveness is found when increasing the dose of FVIII (figure 15; table 12). In this assay, the supplementation with FX (2 assays were carried out) does not make it possible to generate significant amounts of thrombin (velocities of 6.06 and 7.65 nM/min). On the other hand, the FX-IIa, FX-FXIa1 and 2 and FX-control+ mutants of family 1 make it possible to generate significantly greater amounts of thrombin of at least 3.5X that of the control (figures 15 and 16; table 12).

| Group name | Unicalibrator | Deficient FVIII | FVIII Def + Recombinant<br>1 U/ml | FVIII Def + Recombinant<br>0.1 U/ml | FVIII Def + FX-WT<br>7.5 µg/ml | FVIII Def + FX-control+<br>7.5 µg/ml | FVIII Def + FX-IIa<br>7.5 µg/ml |
|------------|---------------|-----------------|-----------------------------------|-------------------------------------|--------------------------------|--------------------------------------|---------------------------------|
| Lagtime    | 19.33         | 15.5            | 7.87                              | 18                                  | 28.87                          | 18.17                                | 13.83                           |
| ETP        | 1347.5        | 0               | 1803                              | 1442.5                              | 938                            | 1369                                 | 1543.5                          |
| Peak       | 304.16        | 0.48            | 336.48                            | 278.12                              | 56.51                          | 167.22                               | 296.77                          |
| ttPeak     | 21.5          | 38.33           | 9.33                              | 20.17                               | 38                             | 23.67                                | 16.17                           |
| StartTail  | 37            | 0               | 32                                | 41.5                                | 71                             | 44                                   | 36.5                            |
| Velocity   | 140.17        | 0.02            | 202.70                            | 128.17                              | 6.06                           | 30.40                                | 126.40                          |

| Group name | FVIII Def + FX-PAR1<br>7.5 µg/ml | FVIII Def + FX-PAR1M<br>7.5 µg/ml | FVIII Def + FX-FXIIa1<br>7.5 µg/ml | FVIII Def + FX-FXIIa2<br>7.5 µg/ml | FVIII Def + FX-Kal1<br>7.5 µg/ml | FVIII Def + FX-Kal2<br>7.5 µg/ml | FVIII Def + FX-WT<br>7.5 µg/ml |
|------------|----------------------------------|-----------------------------------|------------------------------------|------------------------------------|----------------------------------|----------------------------------|--------------------------------|
| Lagtime    | 30.83                            | 25.5                              | 14                                 | 15.17                              | 21.33                            | 18                               | 26.17                          |
| ETP        | 853                              | 1052.5                            | 1359.5                             | 1318.5                             | 1144                             | 1225                             | 1035                           |
| Peak       | 50.44                            | 76.26                             | 161.43                             | 145.34                             | 89.5                             | 106.56                           | 67.55                          |
| ttPeak     | 41.17                            | 33.83                             | 19                                 | 21                                 | 28.5                             | 24.5                             | 35                             |
| StartTail  | 71                               | 63                                | 41.5                               | 44                                 | 57.5                             | 52                               | 67                             |
| Velocity   | 4.88                             | 9.15                              | 32.29                              | 24.93                              | 12.88                            | 16.39                            | 7.65                           |

Table 12: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor VIII-deficient plasma, following activation with cephalin

The kinetic parameters of the thrombinograms of figure 15 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 13: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 1: extrinsic coagulation pathway (TF 1pM/PL 4  $\mu$ M) in FIX-deficient plasma**

**5                    1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in the example 6.

The thrombin generation tests are carried out on 80  $\mu$ l of a pool of plasma optionally containing the cell supernatants and the controls in the presence of 20  $\mu$ l of PPP reagent  
 10 (Stago) containing a final concentration of 1 pM of tissue factor (TF) and 4  $\mu$ M of phospholipids (PL) and of 20  $\mu$ l of fluca-kit (substrate +  $\text{CaCl}_2$ ). The plasmas used are a normal plasma and a factor IX-deficient plasma.

**2 – Results**

15 The family 1 supernatants resulting from a transfection of HEK293F cells in the presence of VKOR were analyzed by TGT in FIX-deficient plasma after activation with tissue factor using the already described controls. The controls (normal plasma, FIX-deficient plasma reconstituted with plasma FIX (at 10% or 100%) not reconstituted with plasma FIX) behave expectedly. On the other hand, FX-WT gives a signal which is  
 20 moderate but greater than expected (figure 17; table 13). Its mean velocity (23 nM/min) is however exceeded by those of all the mutants except FX-PAR1. Said mutants are, in particular, FX-FXIa1/2, FX-Ka12/3 and FX-control+ and FX-IIa, which have a velocity of at least 150% that of the control.

| Group name | Unicallibrator              | FIX Deficient               | FIX Def + FIX 1 U/ml         | FIX Def + FIX 0.1 U/ml        | FIX Def + FX-WT 7.5 µg/ml   | FIX Def + FX-control+ 7.5 µg/ml | FIX Def + FX-IIa 7.5 µg/ml  |                           |
|------------|-----------------------------|-----------------------------|------------------------------|-------------------------------|-----------------------------|---------------------------------|-----------------------------|---------------------------|
| Lagtime    | 8.33                        | 5.33                        | 5.33                         | 7.17                          | 7.33                        | 6.83                            | 7.5                         |                           |
| ETP        | 1345.5                      | 238.5                       | 1641                         | 1063.5                        | 1471                        | 1550                            | 1543                        |                           |
| Peak       | 134.27                      | 7.23                        | 256.66                       | 82.84                         | 110.99                      | 173.16                          | 197.8                       |                           |
| ttPeak     | 12.67                       | 22.5                        | 8.33                         | 13.33                         | 12.67                       | 11.83                           | 12.33                       |                           |
| StartTail  | 41                          | 70                          | 26                           | 46                            | 48.5                        | 32.5                            | 31                          |                           |
| Velocity   | 30.94                       | 0.42                        | 88.89                        | 13.45                         | 20.78                       | 34.63                           | 40.95                       |                           |
|            |                             |                             |                              |                               |                             |                                 |                             |                           |
| Group name | FIX Def + FX-PAR1 7.5 µg/ml | FIX Def+ FX-PAR1M 7.5 µg/ml | FIX Def+ FX-FXIIa1 7.5 µg/ml | FIX Def + FX-FXIIa2 7.5 µg/ml | FIX Def + FX-Ka11 7.5 µg/ml | FIX Def + FX-Ka12 7.5 µg/ml     | FIX Def + FX-Ka13 1.6 µg/ml | FIX Def + FX-WT 7.5 µg/ml |
| Lagtime    | 7.5                         | 7.17                        | 5.5                          | 6.17                          | 6.5                         | 6.5                             | 6                           | 7.33                      |
| ETP        | 1366                        | 1472.5                      | 1589.5                       | 1583                          | 1543                        | 1549                            | 1594                        | 1472                      |
| Peak       | 67.56                       | 133.76                      | 267.69                       | 257.72                        | 179.29                      | 220                             | 242.54                      | 126.29                    |
| ttPeak     | 14.17                       | 11.67                       | 8.33                         | 8.83                          | 10.5                        | 9.83                            | 9                           | 12.17                     |
| StartTail  | 71                          | 40.5                        | 27                           | 27.5                          | 33                          | 29.5                            | 28.5                        | 43                        |
| Velocity   | 10.13                       | 29.72                       | 94.59                        | 96.89                         | 44.82                       | 66.07                           | 80.85                       | 26.09                     |

Table 13: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor IX-deficient plasma, following activation with tissue factor

The kinetic parameters of the thrombinograms of figure 17 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 14: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 1: intrinsic coagulation pathway (cephalin) in FIX-deficient plasma**

5           **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

The thrombin generation tests are carried out on 80 µl of a pool of plasma optionally containing the cell supernatants and the controls in the presence of 20 µl of cephalin  
10 (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of 20 µl of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are a normal plasma and a factor IX-deficient plasma.

**2 – Results**

The family 1 supernatants resulting from a transfection of HEK293F cells in the  
15 presence of VKOR were analyzed by TGT after activation with cephalin using the already described controls.

The controls behave expectedly, the FIX-deficient plasma is negative (it does not allow any generation of IIa) and a gradient of effectiveness is found when increasing the dose of FIX (figure 18; table 14). In this assay, the supplementation with FX (2 assays were  
20 carried out) does not make it possible to generate significant amounts of thrombin (velocities of 1.24 and 1.53 nM/min). On the other hand, the FX-IIa, FX-FXIa1 and 2 and FX-control+ mutants of family 1 make it possible to generate significantly greater amounts of thrombin of at least 4.7X that of the control to 55X (figures 18 and 19; table 14).

| Group name | Unicalibrator | FIX Deficient | FIX Def + FIX 1 U/ml | FIX Def + FIX 0,1 U/ml | FIX Def + FX-WT 7.5 µg/ml | FIX Def + FX-control+ 7.5 µg/ml | FIX Def + FX-IIa 7.5 µg/ml |
|------------|---------------|---------------|----------------------|------------------------|---------------------------|---------------------------------|----------------------------|
| Lagtime    | 18.33         | 42.5          | 13.67                | 31.83                  | 30.5                      | 22.33                           | 16.33                      |
| ETP        | 1383.5        | 0             | 1529                 | 899.5                  | 316.5                     | 1147                            | 1448.5                     |
| Peak       | 311.19        | 0.49          | 369.87               | 107.63                 | 16.16                     | 120.28                          | 279.02                     |
| ttPeak     | 20.33         | 57.33         | 15.33                | 36.33                  | 43.5                      | 29                              | 20                         |
| StartTail  | 36.5          | 0             | 31                   | 55                     | 71                        | 44.5                            | 32.5                       |
| Velocity   | 155.60        | 0.03          | 222.81               | 23.90                  | 1.24                      | 18.03                           | 76.03                      |

| Group name | FIX Def + FX-PAR1 7.5 µg/ml | FIX Def + FX-PAR1M 7.5 µg/ml | FIX Def + FX-FXIIa1 7.5 µg/ml | FIX Def + FX-FXIIa2 7.5 µg/ml | FIX Def + FX-Kal1 7.5 µg/ml | FIX Def + FX-Kal2 7.5 µg/ml | FIX Def + FX-Kal3 1.6 µg/ml | FIX Def + FX-WT 7.5 µg/ml |
|------------|-----------------------------|------------------------------|-------------------------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------|
| Lagtime    | 37.17                       | 28.17                        | 15.67                         | 17                            | 23                          | 18.5                        | 16                          | 27.5                      |
| ETP        | 237                         | 402.5                        | 686                           | 626.5                         | 429.5                       | 498.5                       | 496                         | 341                       |
| Peak       | 12.51                       | 24.06                        | 59.45                         | 53.54                         | 28.73                       | 36.12                       | 38.66                       | 18.68                     |
| ttPeak     | 51.17                       | 39.67                        | 23.5                          | 25.17                         | 33                          | 28.17                       | 25.33                       | 39.67                     |
| StartTail  | 71                          | 68.5                         | 44.5                          | 45.5                          | 55                          | 48.5                        | 51                          | 70                        |
| Velocity   | 0.89                        | 2.09                         | 7.59                          | 6.55                          | 2.87                        | 3.74                        | 4.14                        | 1.53                      |

Table 14: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 1 to a pool of factor IX-deficient plasma, following activation with cephalin

The kinetic parameters of the thrombinograms of figure 18 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM)l; Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 15: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 3: extrinsic coagulation pathway (TF 1pM/PL 4  $\mu$ M) in FVIII-deficient plasma**

**5                    1 – Experimental protocol**

The thrombin generation tests are carried out on 80  $\mu$ l of a pool of plasma optionally containing the cell supernatants or the controls, in the presence of 20  $\mu$ l of PPP reagent (Stago) containing a final concentration of 1 pM of tissue factor (TF) and 4  $\mu$ M of phospholipids (PL). Various plasmas are used, normal and factor VIII-deficient.

10    The reaction is initiated by adding 20  $\mu$ l of Fluca-kit (substrate +  $\text{CaCl}_2$ ) which constitutes the beginning of the measurement of the appearance of thrombin. The appearance of fluorescence is measured on a Fluoroskan Ascent fluorimeter (ThermoLabsystems) at an excitation wavelength of 390 nm and at an emission length of 460 nm. The thrombinograms (curves representing the fluorescence intensity as a  
15    function of time) are then analyzed by means of the Thrombinoscope™ software which converts the fluorescence value into nM of thrombin by comparative calculation.

**2 – Results**

The family 3 supernatants resulting from a transfection of HEK293F cells in the  
20    presence of VKOR were analyzed by TGT after activation with tissue factor using the already described controls. The controls (normal plasma, FVIII-deficient plasma reconstituted with recombinant FVIII or not reconstituted with recombinant FVIII) behave expectedly. On the other hand, FX-WT gives a signal that is moderate but greater than expected (figures 20 and 21; table 15). Its mean velocity (59 nM/min) is  
25    however exceeded by those of several mutants, in particular FX-FXIa1/2, FX-Ka11/2/3 and FX-control+ and FX-IIa, the velocities of which are above 78 nM/min (i.e. at least 130% of the control).

| Group name | Unicalibrator | FVIII Deficient | FVIII Def + Recombinant U/ml | FVIII Def + FX-WT 7.5 µg/ml | FVIII Def + FX-control+ 7.5 µg/ml | FVIII Def + FX-IIa 7.5 µg/ml |
|------------|---------------|-----------------|------------------------------|-----------------------------|-----------------------------------|------------------------------|
| Lagtime    | 8             | 9.33            | 7.17                         | 7.83                        | 7.17                              | 7.5                          |
| ETP        | 1325.5        | 0               | 1795                         | 1549.5                      | 1641                              | 1683.5                       |
| Peak       | 136.55        | 12.25           | 291.64                       | 175.6                       | 233.75                            | 234.31                       |
| ttPeak     | 12.5          | 29.67           | 9.5                          | 11.17                       | 10.17                             | 10.5                         |
| StartTail  | 40            | 0               | 33                           | 40                          | 34.5                              | 34.5                         |
| Velocity   | 30.34         | 0.60            | 125.17                       | 52.57                       | 77.92                             | 78.10                        |

| Group name | FVIII Def + FX-PAR1 7.5 µg/ml | FVIII Def + FX-PAR1M 7.5 µg/ml | FVIII Def + FX-FXIa1 7.5 µg/ml | FVIII Def + FX-FXIa2 µg/ml | FVIII Def + FX-Ka11 7.5 µg/ml | FVIII Def + FX-Ka12 7.5 µg/ml | FVIII Def + FX-Ka13 1.65 µg/ml | FVIII Def + FX-WT 7.5 µg/ml |
|------------|-------------------------------|--------------------------------|--------------------------------|----------------------------|-------------------------------|-------------------------------|--------------------------------|-----------------------------|
| Lagtime    | 6.5                           | 8.5                            | 5.67                           | 6.33                       | 8.33                          | 6                             | 6.33                           | 7.83                        |
| ETP        | 1665                          | 1565                           | 1724                           | 1724                       | 1560                          | 1803                          | 1715.5                         | 1569.5                      |
| Peak       | 225.48                        | 189.25                         | 301.86                         | 281.98                     | 213.57                        | 303.58                        | 270.6                          | 199.2                       |
| ttPeak     | 9.5                           | 11.83                          | 7.67                           | 8.5                        | 11                            | 8                             | 9                              | 10.83                       |
| StartTail  | 35                            | 39.5                           | 31                             | 31                         | 37                            | 31                            | 32                             | 36.5                        |
| Velocity   | 75.16                         | 56.83                          | 150.93                         | 129.94                     | 79.99                         | 151.79                        | 101.35                         | 66.40                       |

Table 15: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor VIII-deficient plasma, following activation with tissue factor

The kinetic parameters of the thrombinograms of figure 20 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 14: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 3: intrinsic coagulation pathway (cephalin alone) in FVIII-deficient plasma**

5           **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6. The thrombin generation tests are carried out on 80 µl of a pool of plasma optionally containing the cell supernatants and the controls in the presence of 20 µl of cephalin (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of 20 µl of  
10   fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are a normal plasma and a factor VIII-deficient plasma.

**2 – Results**

The family 3 of supernatants resulting from a transfection of HEK293F cells in the  
15   presence of VKOR were analyzed by TGT after activation with cephalin using the already described controls.

The controls behave expectedly, the FVIII-deficient plasma is negative (it does not allow any generation of IIa) and a gradient of effectiveness is found when increasing the dose of FVIII (figure 21; table 16). The supplementation with FX (2 assays were carried  
20   out) does not make it possible to generate significant amounts of thrombin (velocities of 6.06 and 7.65 nM/min). On the other hand, the FX-IIa, FX-FXIa1 and 2 and FX-control+ mutants of family 3 make it possible to generate significant amounts of thrombin with velocities greater than 26 nM, i.e. at least 3.8X faster than FX-WT (figure 23; table 16).

| Group name | Unicalibrator                 | FVIII Deficient                | FVIII Def + Recombinant 1 U/ml | FVIII Def + Recombinant 0,1 U/ml | FVIII Def + FX-WT 7,5 µg/ml   | FVIII Def + FX-control+ 7,5 µg/ml | FVIII Def + FX-IIa 7,5 µg/ml  |                             |
|------------|-------------------------------|--------------------------------|--------------------------------|----------------------------------|-------------------------------|-----------------------------------|-------------------------------|-----------------------------|
| Lagtime    | 19.33                         | 15.5                           | 7.67                           | 18                               | 28.67                         | 18.67                             | 14.67                         |                             |
| ETP        | 1347.5                        | 0                              | 1803                           | 1442.5                           | 938                           | 1491.5                            | 1636.5                        |                             |
| Peak       | 304.16                        | 0.48                           | 336.48                         | 278.12                           | 56.51                         | 167.62                            | 288.52                        |                             |
| ttPeak     | 21.5                          | 38.33                          | 9.33                           | 20.17                            | 38                            | 24.17                             | 17                            |                             |
| StartTail  | 37                            | 0                              | 32                             | 41.5                             | 71                            | 46                                | 38                            |                             |
| Velocity   | 140.17                        | 0.02                           | 202.70                         | 128.17                           | 6.06                          | 30.48                             | 123.83                        |                             |
|            |                               |                                |                                |                                  |                               |                                   |                               |                             |
| Group name | FVIII Def + FX-PAR1 7,5 µg/ml | FVIII Def + FX-PAR1M 7,5 µg/ml | FVIII Def + FX-FXIa1 7,5 µg/ml | FVIII Def + FX-FXIa2 7,5 µg/ml   | FVIII Def + FX-Ka11 7,5 µg/ml | FVIII Def + FX-Ka12 7,5 µg/ml     | FVIII Def + FX-Ka13 7,5 µg/ml | FVIII Def + FX-WT 7,5 µg/ml |
| Lagtime    | 19.83                         | 26.17                          | 14.17                          | 15.17                            | 24.5                          | 14.6                              | 17.67                         | 26.17                       |
| ETP        | 1335.5                        | 1054                           | 1396.5                         | 1426.5                           | 1033                          | 1464                              | 1365.5                        | 1036                        |
| Peak       | 113.21                        | 74.28                          | 160.63                         | 149.07                           | 69.4                          | 163.57                            | 119.67                        | 67.55                       |
| ttPeak     | 27.33                         | 35                             | 19.5                           | 20.83                            | 33                            | 20                                | 24.67                         | 35                          |
| StartTail  | 53.5                          | 65                             | 43.5                           | 44.5                             | 65.5                          | 42.5                              | 50.5                          | 67                          |
| Velocity   | 15.09                         | 8.41                           | 30.14                          | 26.34                            | 8.16                          | 29.74                             | 17.10                         | 7.65                        |

Table 16: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor VIII-deficient plasma, following activation with cephalin

5 The kinetic parameters of the thrombinograms of figure 23 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 15: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 3: extrinsic coagulation pathway (TF 1pM/PL 4  $\mu$ M) in FIX-deficient plasma**

5                   **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6.

The thrombin generation tests are carried out on 80  $\mu$ l of a pool of plasma optionally containing the cell supernatants and the controls in the presence of 20  $\mu$ l of PPP reagent  
10 (Stago) containing a final concentration of 1 pM of tissue factor (TF) and 4  $\mu$ M of phospholipids (PL) and of 20  $\mu$ l of fluca-kit (substrate +  $\text{CaCl}_2$ ). The plasmas used are a normal plasma and a factor IX-deficient plasma.

**2 – Results**

15 The family 3 supernatants resulting from a transfection of HEK293F cells in the presence of VKOR were analyzed by TGT after activation with tissue factor using the already described controls.

The controls behave expectedly, the FIX-deficient plasma is negative (it does not allow any generation of IIa) and a gradient of effectiveness is found when increasing the dose  
20 of FIX (figure 23; table 17). The supplementation with FX (2 assays were carried out) does not make it possible to generate significant amounts of thrombin (mean velocity of 23 nM/min). On the other hand, all the mutants of family 3, except FX-PAR1M, make it possible to generate significant amounts of thrombin with velocities greater than 33 nM, i.e. at least 140% faster than FX-WT (figure 23; table 17).

| Group name | Unicalibrator              | FIX Deficient               | FIX Def + FIX 1 U/ml         | FIX Def + FIX 0.1 U/ml       | FIX Def + FX-WT 7.5 µg/ml   | FIX Def +FX-control+ 7.5 µg/ml | FIX Def + FX-Ila 7.5 µg/ml |
|------------|----------------------------|-----------------------------|------------------------------|------------------------------|-----------------------------|--------------------------------|----------------------------|
| Lagtime    | 8.33                       | 5.33                        | 5.33                         | 7.17                         | 7.33                        | 7.17                           |                            |
| ETP        | 1345.5                     | 238.5                       | 1641                         | 1063.5                       | 1471                        | 1632                           | 1660.5                     |
| Peak       | 134.27                     | 7.23                        | 266.66                       | 82.84                        | 110.99                      | 185.83                         | 190.32                     |
| ttPeak     | 12.67                      | 22.5                        | 8.33                         | 13.33                        | 12.67                       | 11.67                          | 12.17                      |
| StartTail  | 41                         | 70                          | 26                           | 46                           | 48.5                        | 33.5                           | 33                         |
| Velocity   | 30.94                      | 0.42                        | 88.89                        | 13.45                        | 20.78                       | 41.30                          | 38.06                      |
|            |                            |                             |                              |                              |                             |                                |                            |
| Group name | FIX Def+ FX-PAR1 7.5 µg/ml | FIX Def+ FX-PAR1M 7.5 µg/ml | FIX Def + FX-FXla1 7.5 µg/ml | FIX Def + FX-FXla2 7.5 µg/ml | FIX Def + FX-Kal1 7.5 µg/ml | FIX Def + FX-Kal2 7.5 µg/ml    | FIX Def + FX-WT 7.5 µg/ml  |
| Lagtime    | 6.33                       | 8                           | 6                            | 6.67                         | 8.6                         | 6                              | 7.33                       |
| ETP        | 1606                       | 1451                        | 1599                         | 1646                         | 1428.5                      | 1740                           | 1472                       |
| Peak       | 173.4                      | 126.89                      | 250.41                       | 240.75                       | 143.97                      | 260.48                         | 126.29                     |
| ttPeak     | 11.5                       | 12.67                       | 9                            | 10                           | 12.5                        | 9                              | 12.17                      |
| StartTail  | 34                         | 43.5                        | 28                           | 30                           | 41                          | 28                             | 43                         |
| Velocity   | 33.54                      | 27.17                       | 83.47                        | 72.30                        | 35.99                       | 86.83                          | 26.09                      |

Table 17: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor IX-deficient plasma, following activation in the table. Lagtime (min); ETP, total thrombin generated (nM); The kinetic parameters of the thrombinograms of figure 23 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**Example 16: Measurement, in terms of thrombin generation time (TGT), of the procoagulant capacity of the factor X variants of family 3: intrinsic coagulation pathway (cephalin) in FIX-deficient plasma**

5           **1 – Experimental protocol**

The reagents, the automated device and the experimental protocol are identical to those described in example 6. The thrombin generation tests are carried out on 80 µl of a pool of normal plasma optionally containing the cell supernatants and the controls in the presence of 20 µl of cephalin (CK-Prest reconstituted with 1 ml of distilled H<sub>2</sub>O) and of  
10   20 µL of fluca-kit (substrate + CaCl<sub>2</sub>). The plasmas used are a normal plasma and a factor IX-deficient plasma.

**2 – Results**

The family 3 supernatants resulting from a transfection of HEK293F cells in the  
15   presence of VKOR were analyzed by TGT after activation with cephalin using the already described controls.

The controls behave expectedly, the FIX-deficient plasma is negative (it does not allow any generation of IIa) and a gradient of effectiveness is found when increasing the dose of FXI (figure 24; table 18). In this assay, the supplementation with FX (2 assays were  
20   carried out) does not make it possible to generate significant amounts of thrombin (velocities of 1.24 and 1.53 nM/min). On the other hand, the FX-IIa, FX-FXIa1 and 2, FX-Kal 2 and 3, and FX-control+ mutants of family 1 make it possible to generate significantly greater amounts of thrombin of at least 3.4X that of the control to 52X (figures 24 and 25; table 18). Moreover, all the mutants, except the FX-PAR1M and  
25   FX-Kal2 mutants, are more active than FX-WT.

| Group name | Unicalibrator               | Deficient FIX                | FIX Def + FIX 1 U/ml          | FIX Def + FIX 0,1 U/ml        | FIX Def + FX-WT 7.5 µg/ml   | FIX Def + FX-control 7.5 µg/ml | FIX Def + FX-IIa 7.5 µg/ml |
|------------|-----------------------------|------------------------------|-------------------------------|-------------------------------|-----------------------------|--------------------------------|----------------------------|
| Lagtime    | 18.33                       | 42.5                         | 13.67                         | 31.83                         | 30.5                        | 22.5                           | 17.5                       |
| ETP        | 1383.5                      | 0                            | 1529                          | 899.5                         | 316.5                       | 1233.5                         | 1495.5                     |
| Peak       | 311.19                      | 0.49                         | 369.87                        | 107.53                        | 16.16                       | 125.06                         | 265.59                     |
| ttPeak     | 20.33                       | 57.33                        | 15.33                         | 36.33                         | 43.5                        | 29.67                          | 21.17                      |
| StartTail  | 36.5                        | 0                            | 31                            | 55                            | 71                          | 46                             | 35                         |
| Velocity   | 155.60                      | 0.03                         | 222.81                        | 23.90                         | 1.24                        | 17.44                          | 72.37                      |
|            |                             |                              |                               |                               |                             |                                |                            |
| Group name | FIX Def + FX-PAR1 7.5 µg/ml | FIX Def + FX-PAR1M 7.5 µg/ml | FIX Def + FX-FXIIa1 7.5 µg/ml | FIX Def + FX-FXIIa2 7.5 µg/ml | FIX Def + FX-Kal1 7.5 µg/ml | FIX Def + FX-Kal2 7.5 µg/ml    | FIX Def + FX-WT 7.5 µg/ml  |
| Lagtime    | 22                          | 29.67                        | 15                            | 17.67                         | 27.67                       | 16.5                           | 27.5                       |
| ETP        | 527                         | 424.5                        | 752.5                         | 618                           | 400                         | 772.5                          | 341                        |
| Peak       | 38.39                       | 24                           | 59.55                         | 48.93                         | 21.04                       | 61.27                          | 18.68                      |
| ttPeak     | 32.67                       | 42                           | 23.67                         | 26.83                         | 39.83                       | 24.67                          | 39.67                      |
| StartTail  | 57                          | 71                           | 45.5                          | 46                            | 70                          | 49                             | 70                         |
| Velocity   | 3.60                        | 1.95                         | 6.88                          | 5.34                          | 1.73                        | 7.50                           | 1.53                       |

Table 18: Kinetic parameters resulting from the thrombinograms obtained from the addition of FX or variants thereof of family 3 to a pool of factor IX-deficient plasma, following activation in the table. Lagtime (min); ETP, total thrombin generated (nM); The kinetic parameters of the thrombinograms of figure 24 are presented in the table. Lagtime (min); ETP, total thrombin generated (nM); Peak, height of the thrombin peak (nM); ttPEAK, time of the peak maximum (min); Start Tail, velocity start time (min); Velocity, maximum rate of thrombin generation (nM/min).

**CLAIMS**

1. A protein comprising a mutated sequence of SEQ ID No. 1, said mutated sequence of SEQ ID No. 1 comprising at least one mutation A, A', B, C or C', in which: mutation A  
5 consists of the substitution of amino acids 43 to 52 of the sequence SEQ ID No. 1 with a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, mutation A' consists of the substitution of amino acids 47 to 52 of the sequence SEQ ID No. 1 with a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR,  
10 mutation B consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR, KATXATLSPR, TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR between amino acids 52 and 53 of the sequence SEQ ID No. 1, mutation C consists of the insertion of a sequence chosen from DFLAEGLTTPR, KATN\*ATLSPR and KATXATLSPR, between amino acids 52 and 53 of the sequence  
15 SEQ ID No. 1, and of the deletion of amino acids 4 to 13 of the sequence SEQ ID No. 1, mutation C' consists of the insertion of a sequence chosen from TSKLTR, FNDFTR, LSSMTR, PPSLTR and LSCGQR, between amino acids 52 and 53 of the sequence SEQ ID No. 1, and of the deletion of amino acids 4 to 9 of the sequence SEQ ID No. 1,  
20 where N\* is an optionally glycosylated asparagine.
2. The protein as claimed in claim 1, characterized in that it comprises, preferably consists of, the sequence SEQ ID No. 7, with at least one mutation A, A', B, C or C'.
- 25 3. The protein as claimed in claim 1 or 2, characterized in that it comprises a sequence chosen from SEQ ID No. 9 to 16, 18 to 25, 27 to 34, 105 to 112, 114 to 121 and 123 to 130.
4. A protein complex comprising a protein as claimed in one of claims 1 to 3, and at  
30 least one protein of sequence SEQ ID No. 2, said proteins being linked to one another by a disulfide bridge.

5. A nucleic acid characterized in that it encodes the protein as claimed in one of claims 1 to 4.

6. The nucleic acid as claimed in claim 5, characterized in that it is chosen from the  
5 sequences SEQ ID No. 77 to 84, 86 to 93, 95 to 102, 133 to 140, 142 to 149 and 151 to 158.

7. An expression cassette comprising the nucleic acid as claimed in claim 5 or 6.

10 8. An expression vector characterized in that it comprises the expression cassette as claimed in claim 7.

9. A recombinant cell comprising the nucleic acid as claimed in claim 5 or 6, said nucleic acid preferentially being included in the vector as claimed in claim 8.

15

10. The protein as claimed in one of claims 1 to 4, for use as a medicament.

11. The protein as claimed in one of claims 1 to 4, for use in treating hemorrhagic disorders.

20

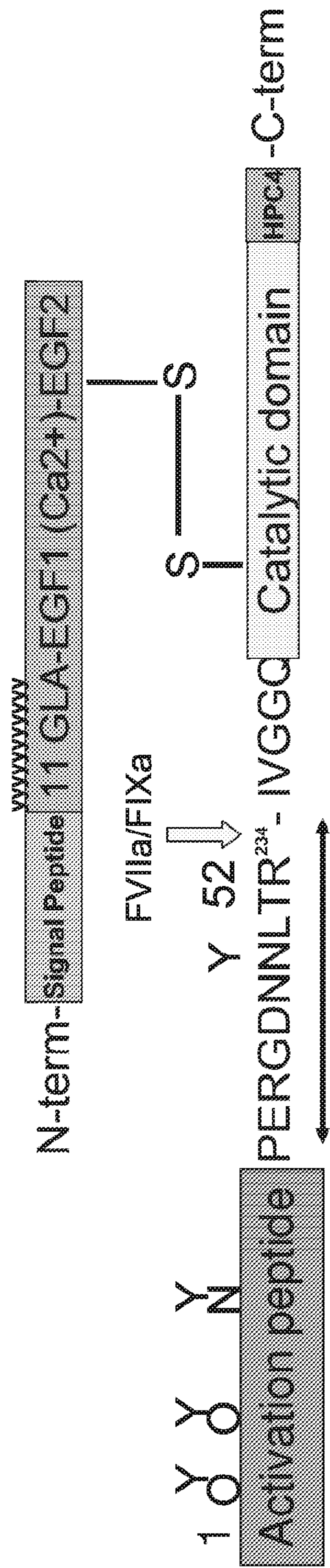
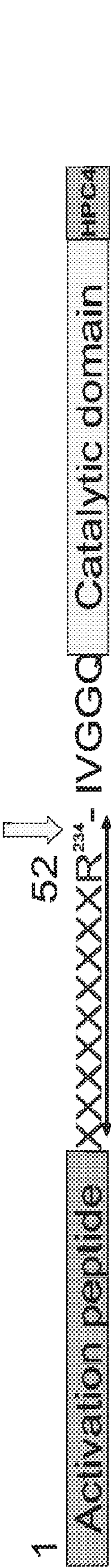


Figure 1

■ Famille 1: Substitution de séquence



■ Famille 2: Insertion de séquence



■ Famille 3: Insertion de séquence en raccourcissant le peptide d'activation

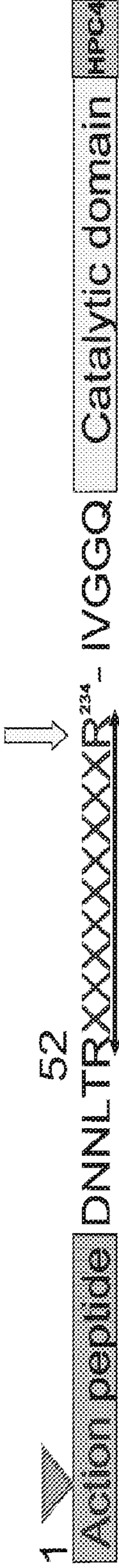


Figure 2

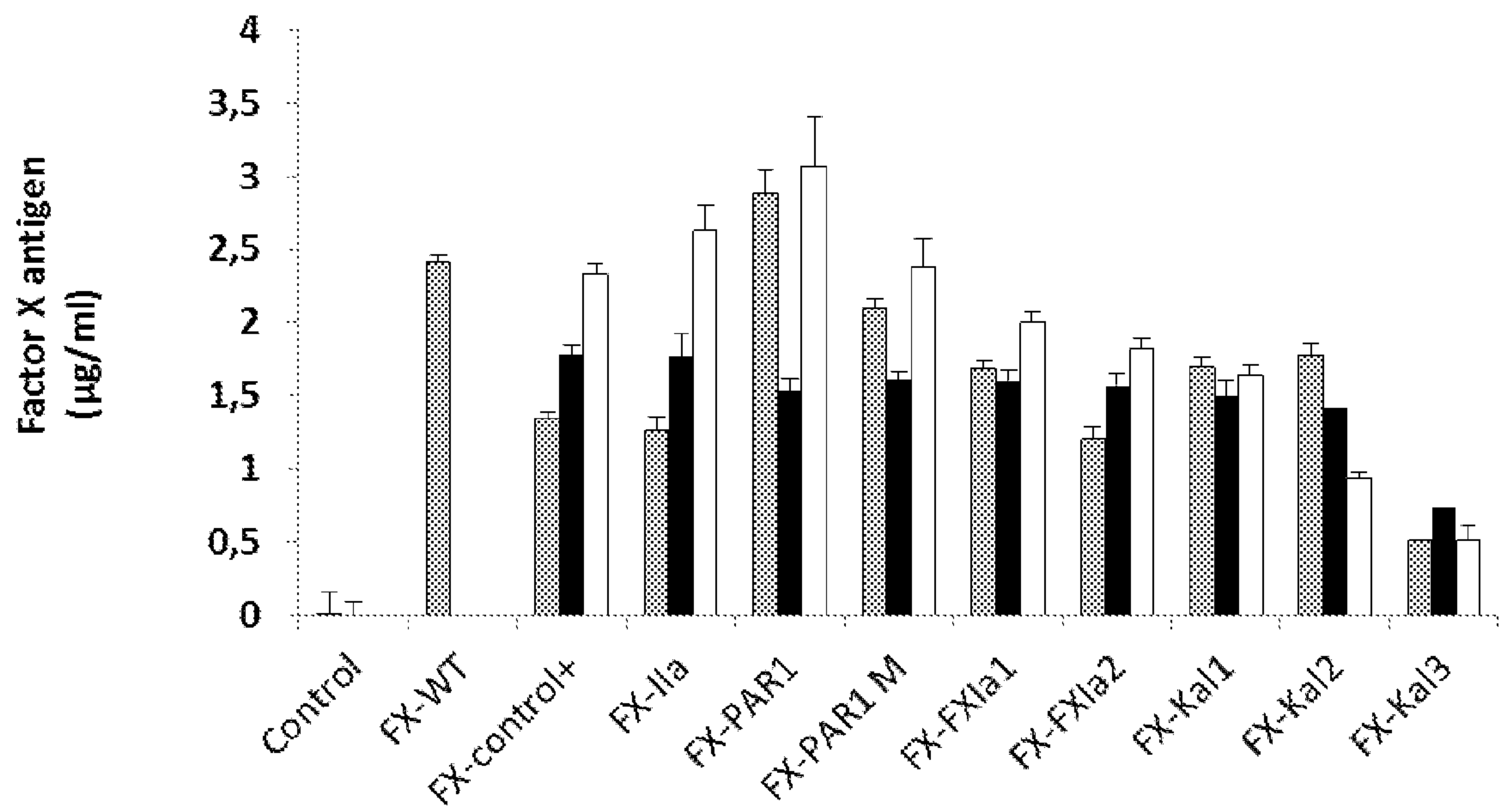


Figure 3

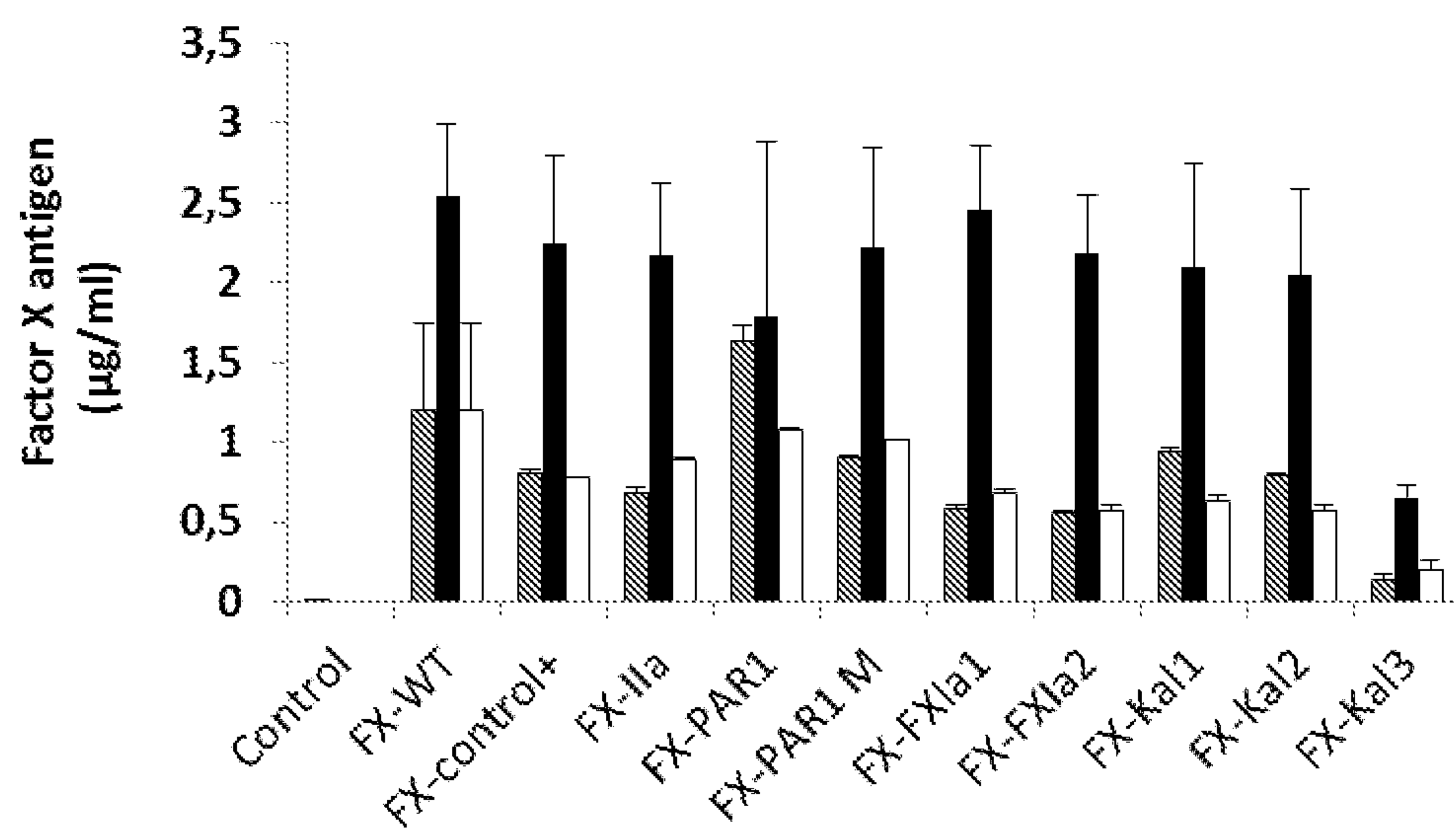


Figure 4

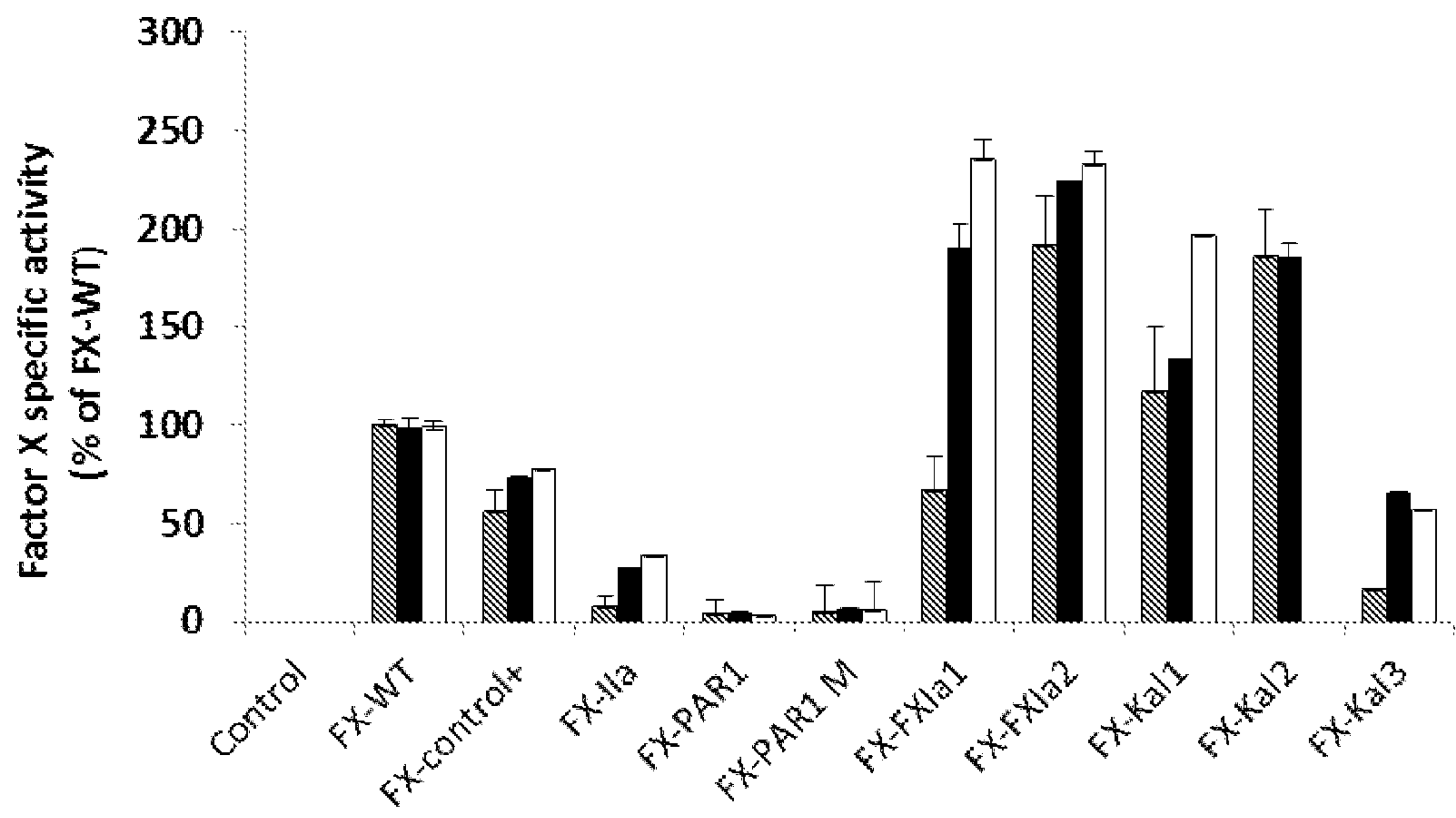


Figure 5

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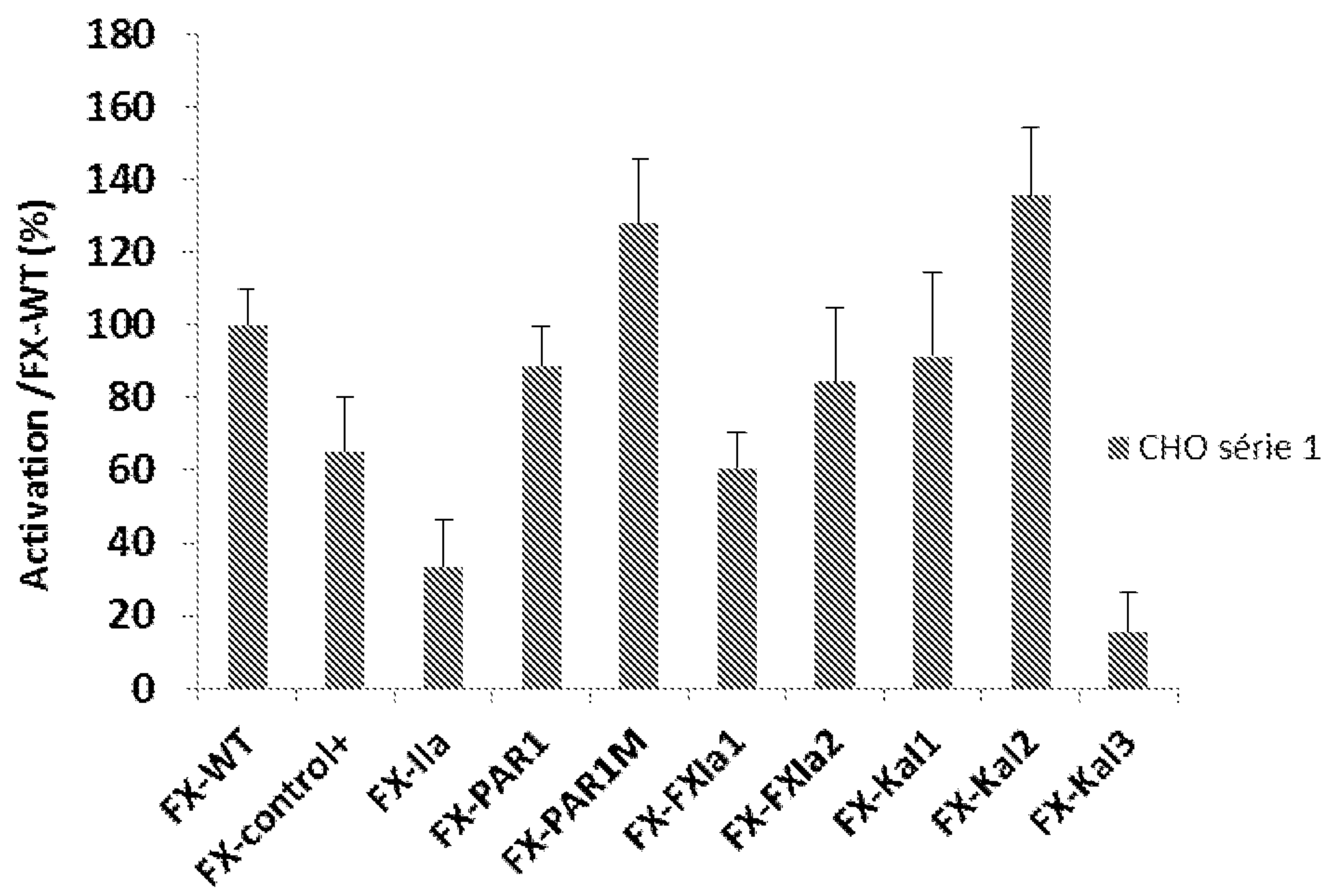


Figure 6

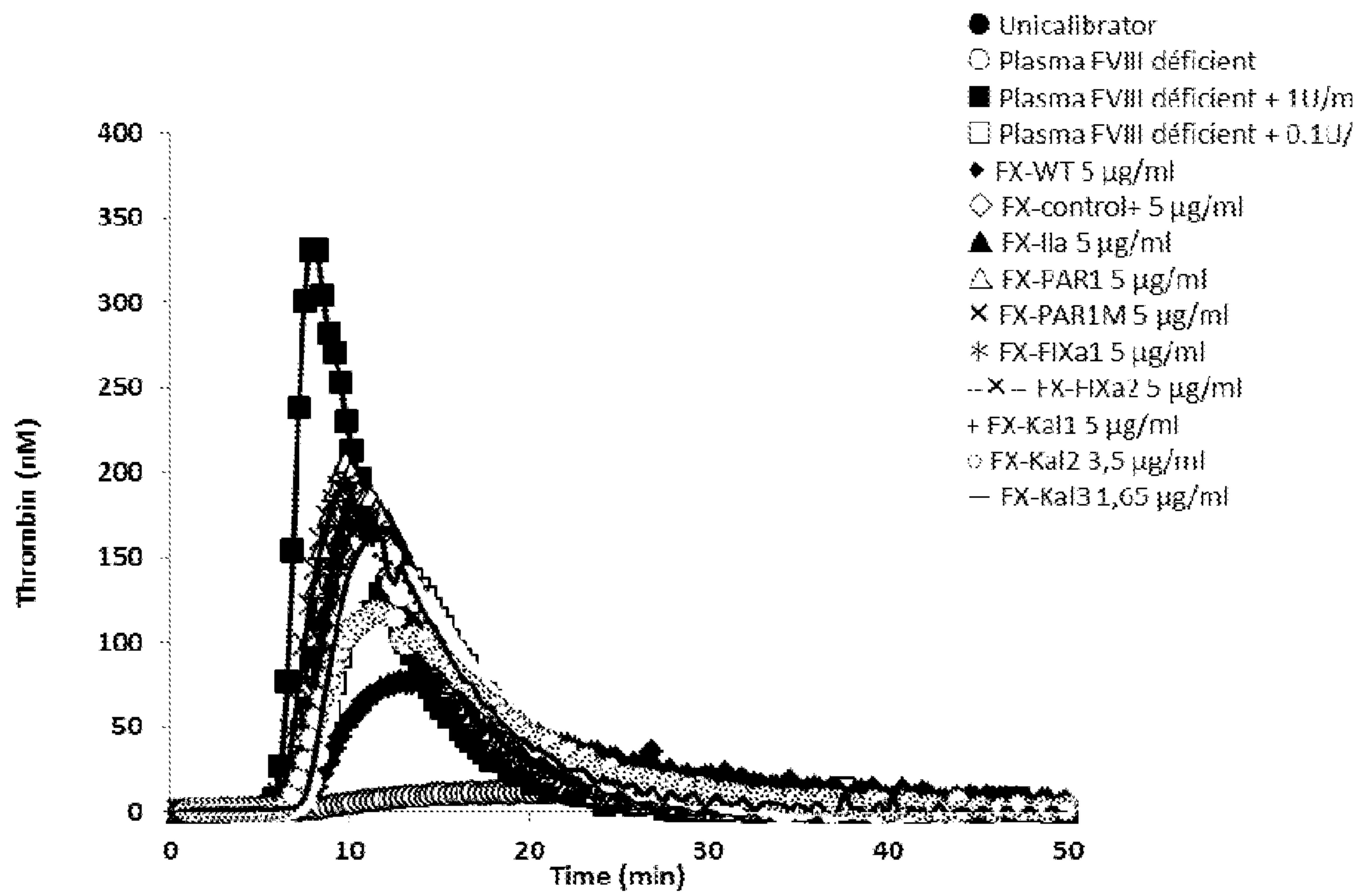


Figure 7

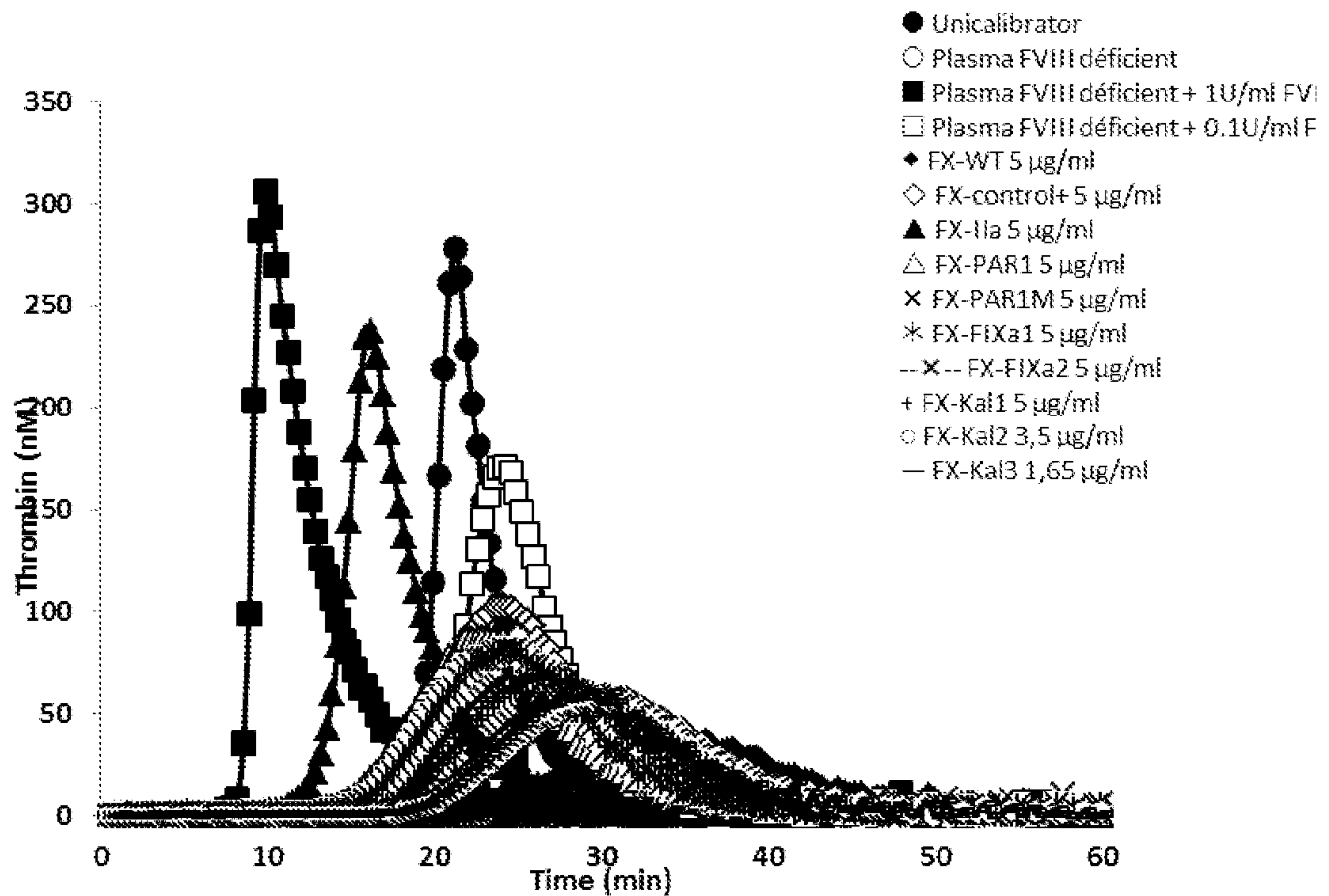


Figure 8

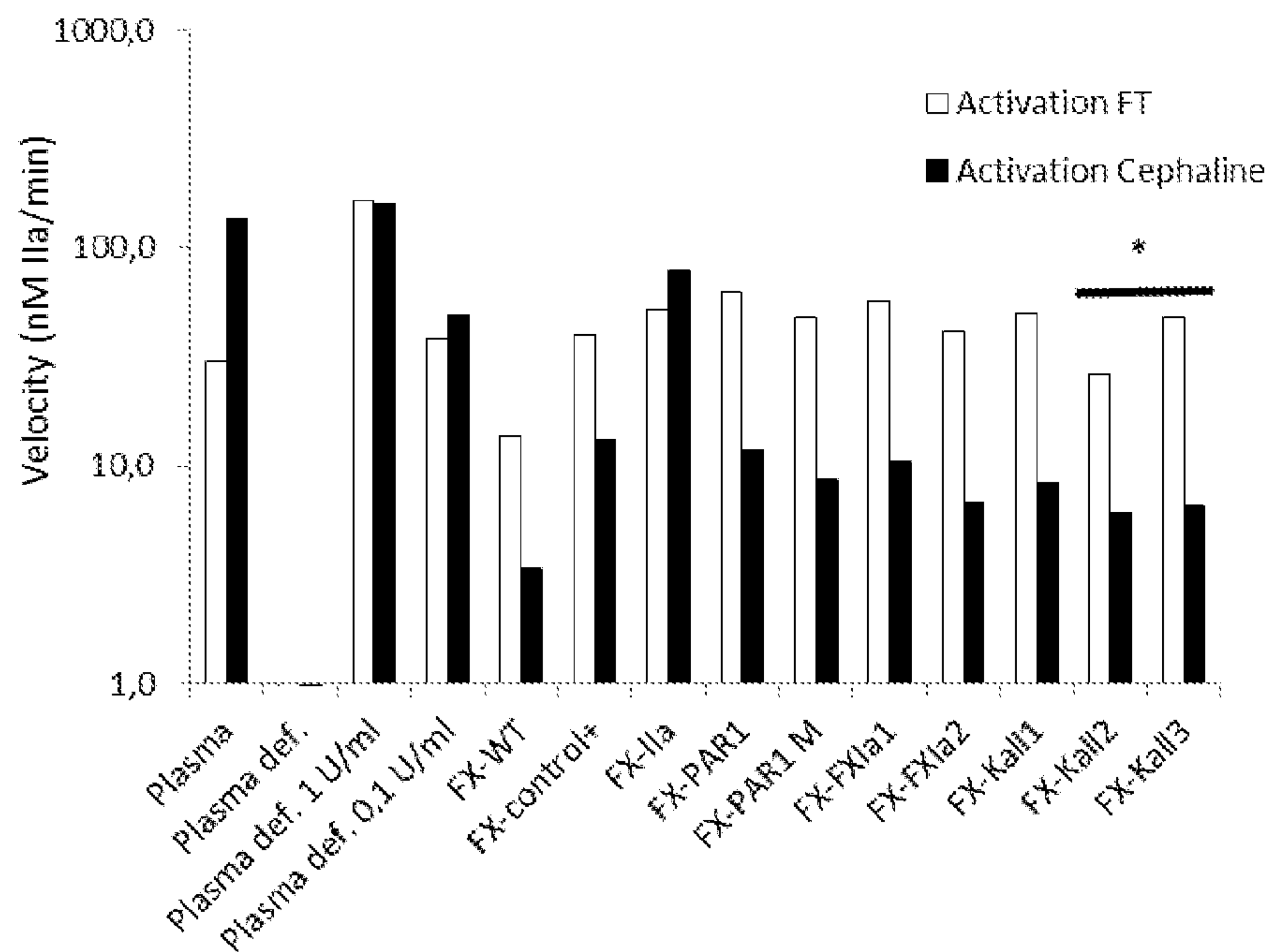


Figure 9

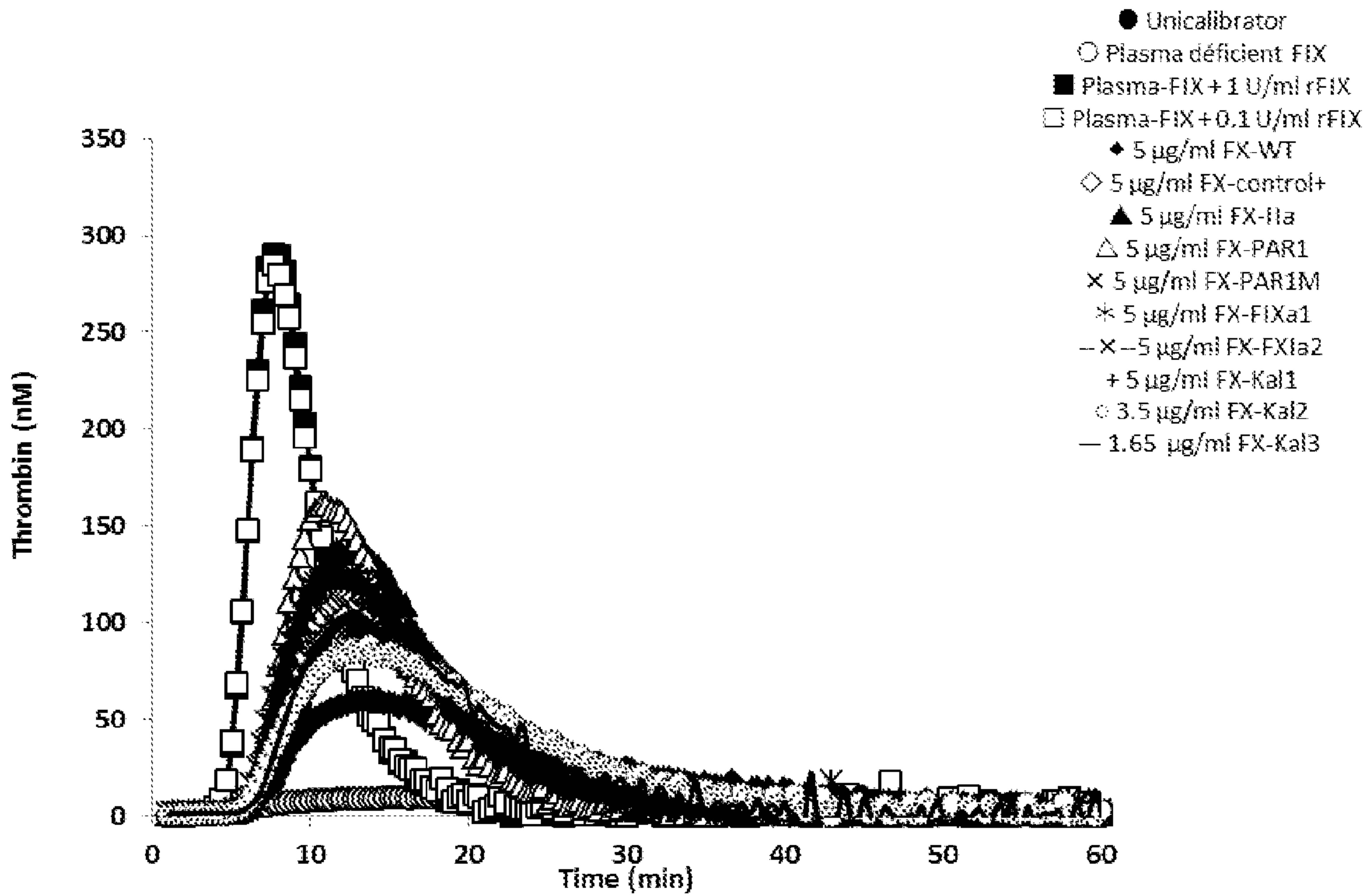


Figure 10

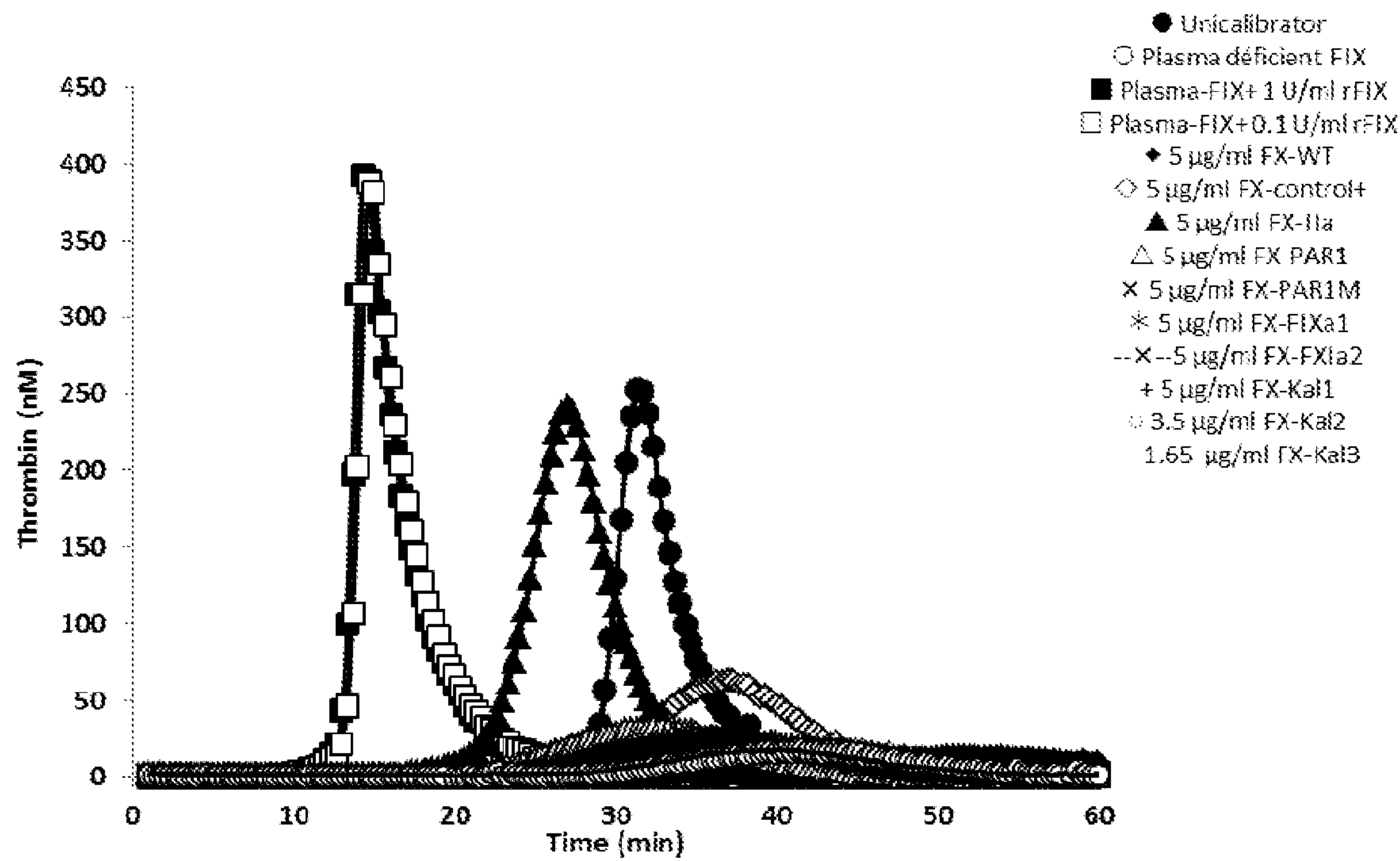


Figure 11

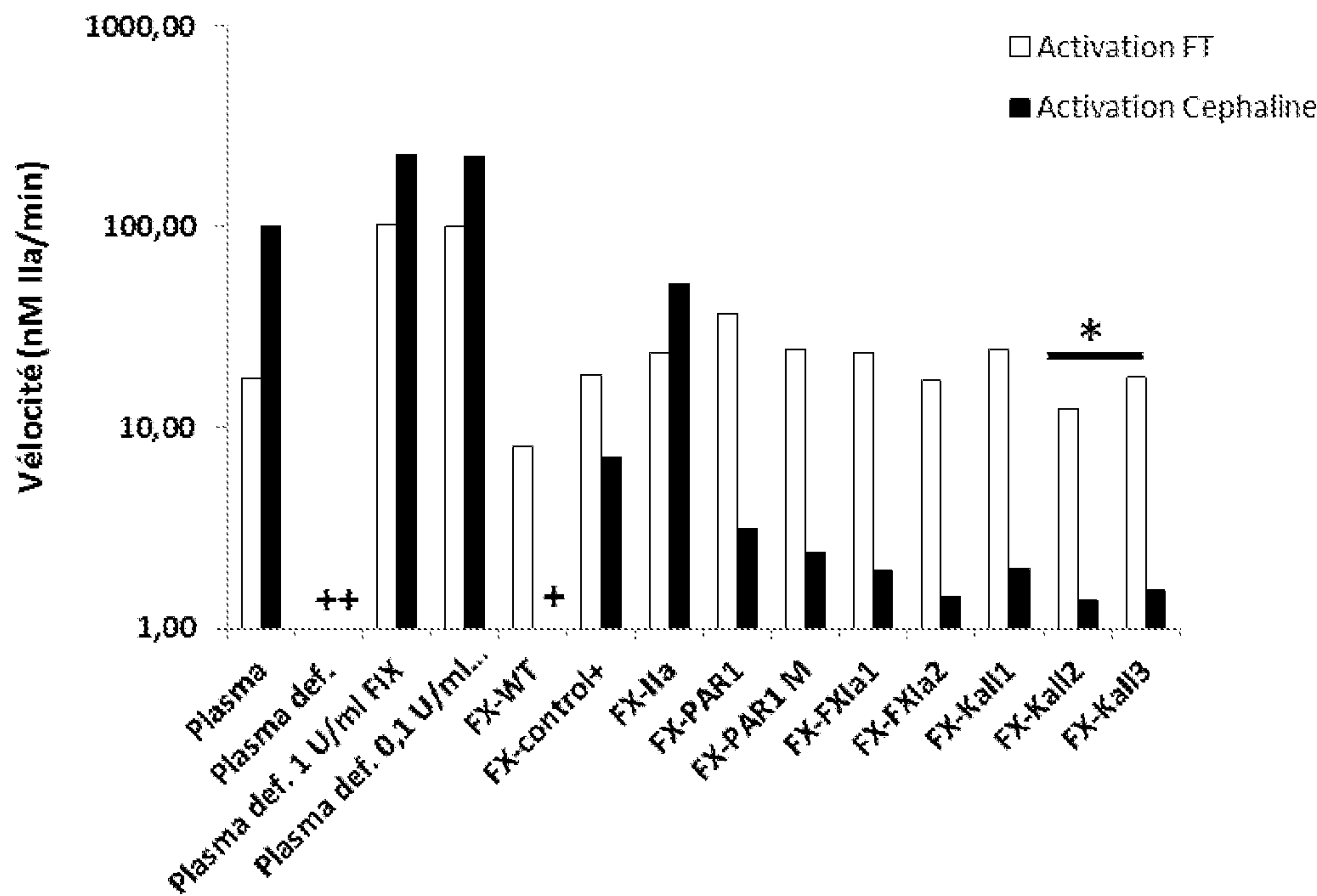


Figure 12

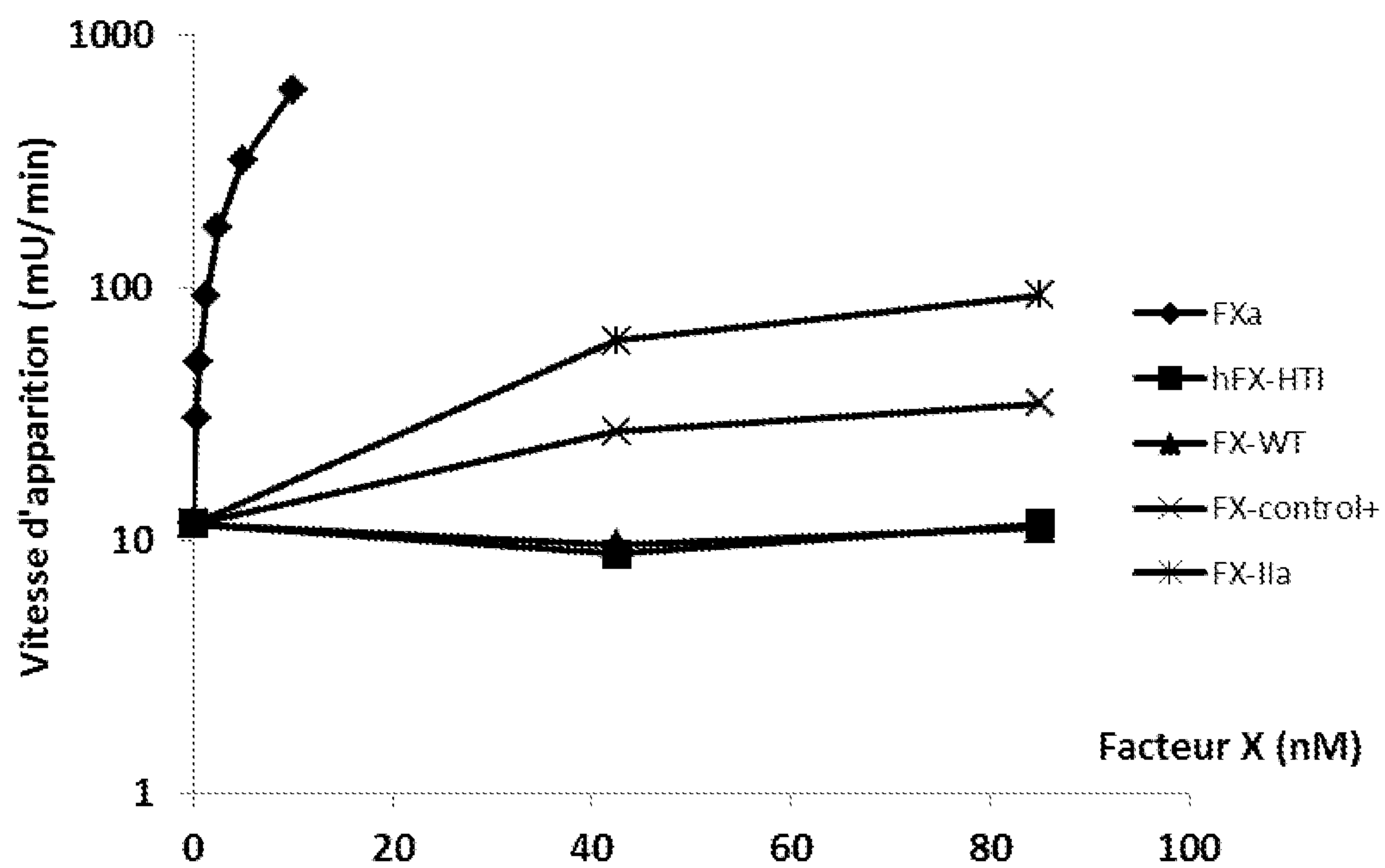


Figure 13

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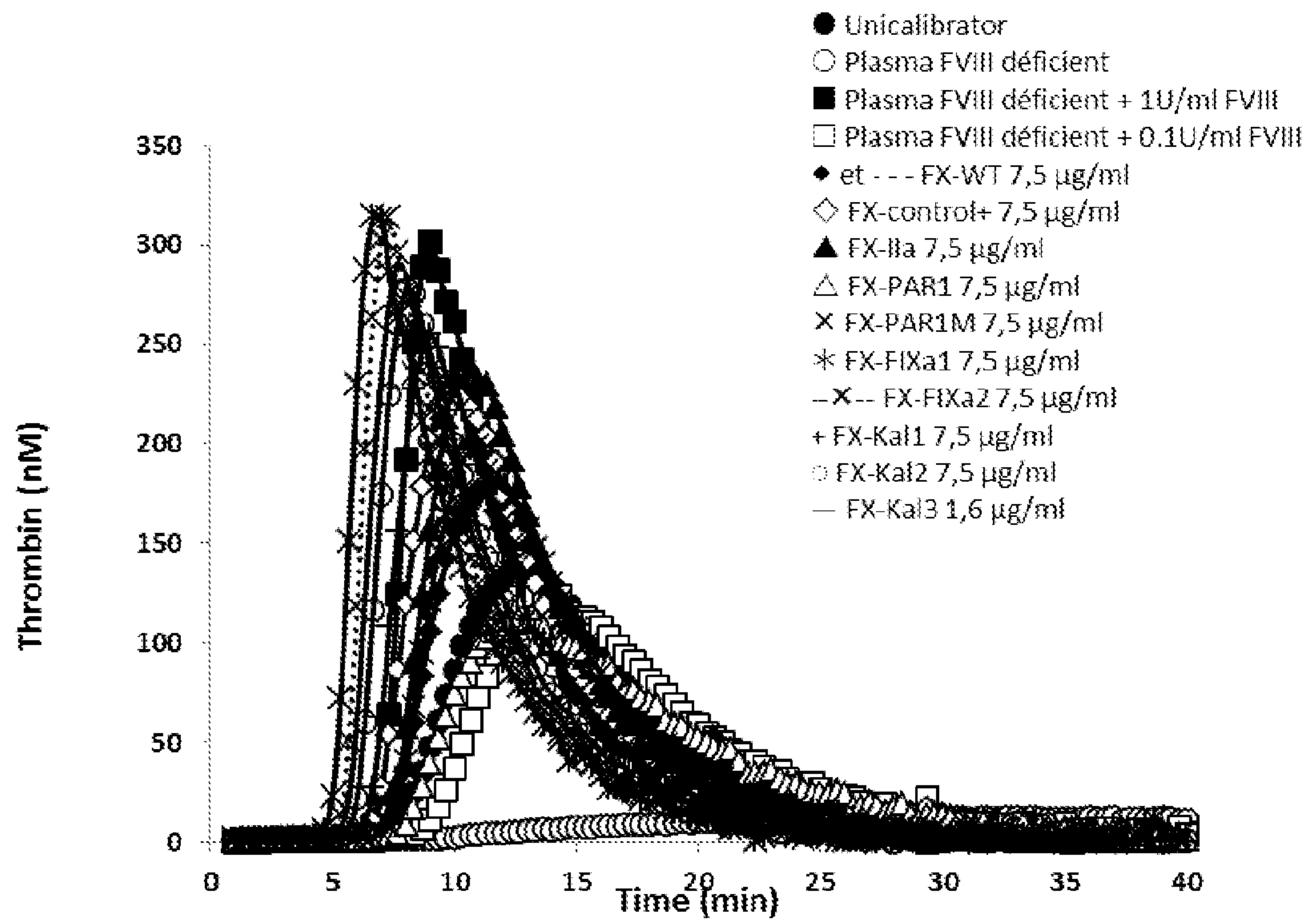


Figure 14

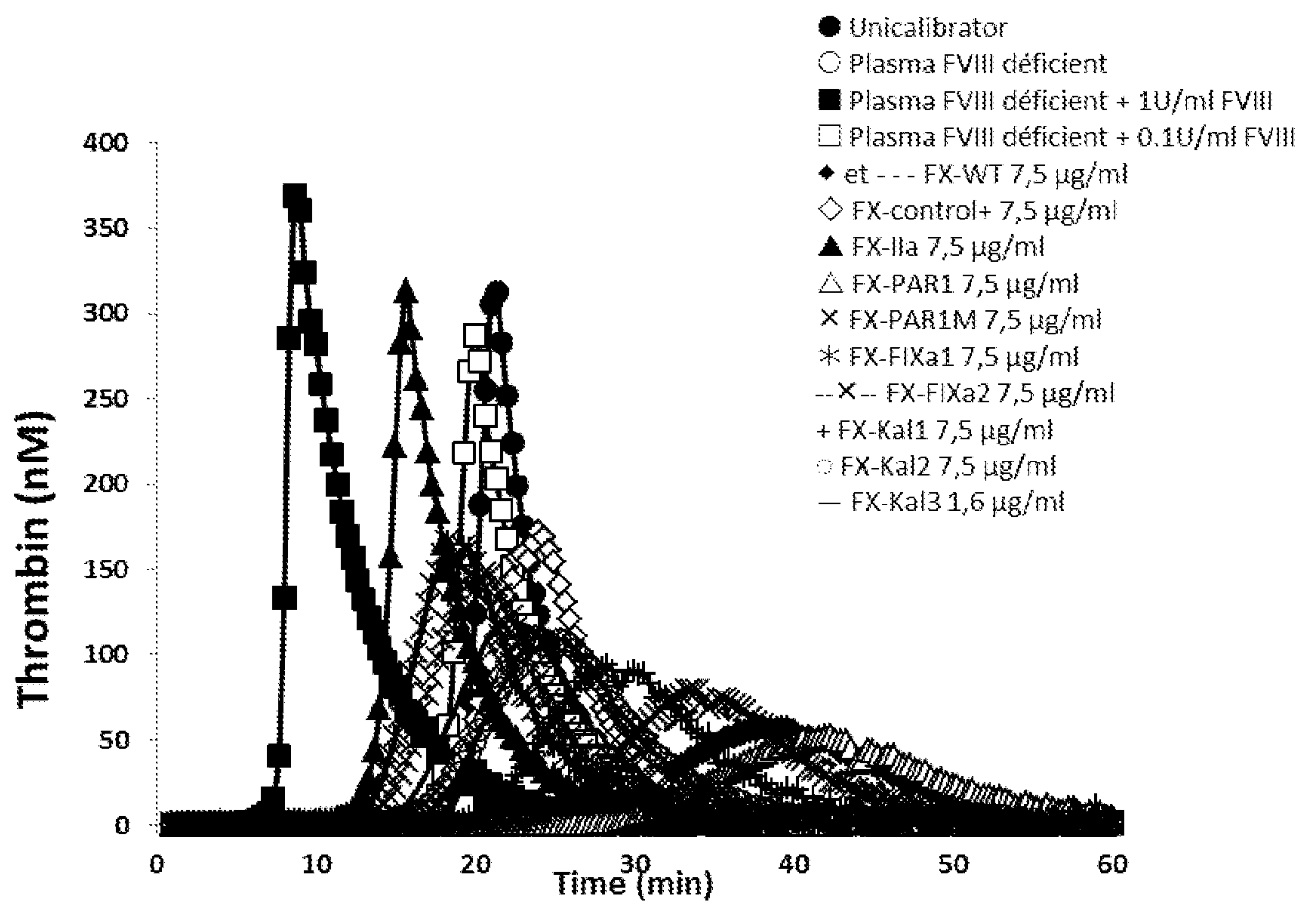


Figure 15

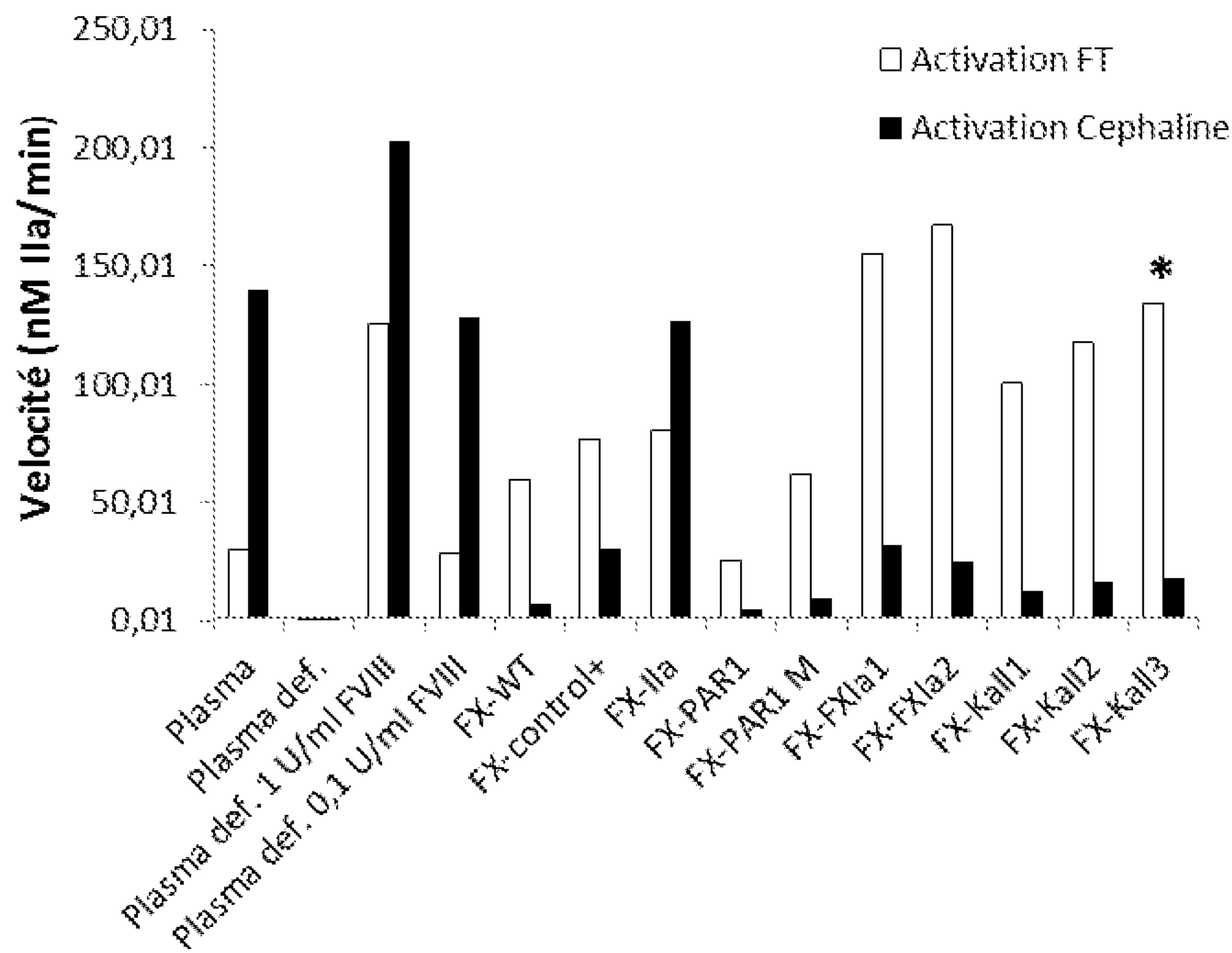


Figure 16

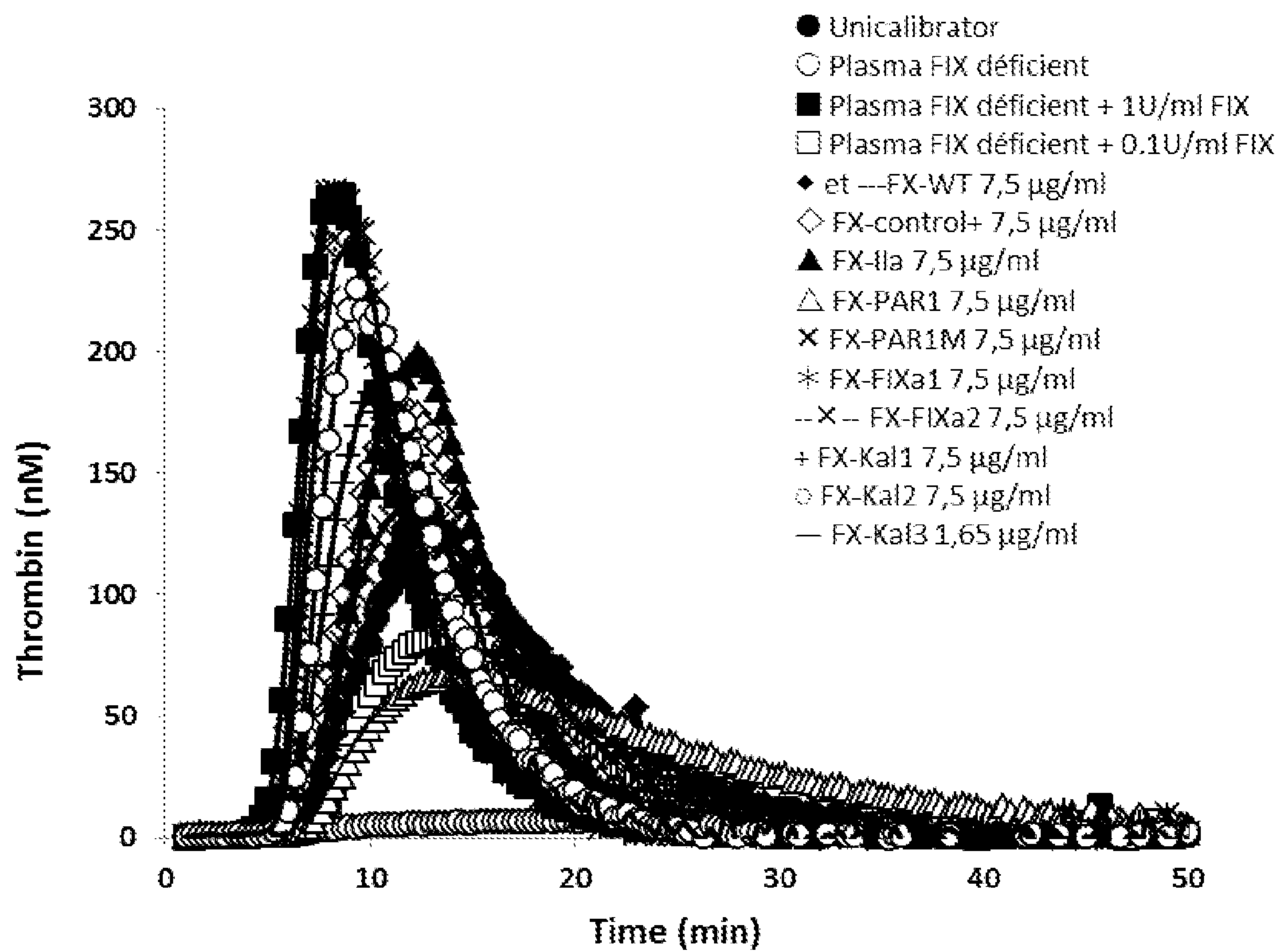


Figure 17

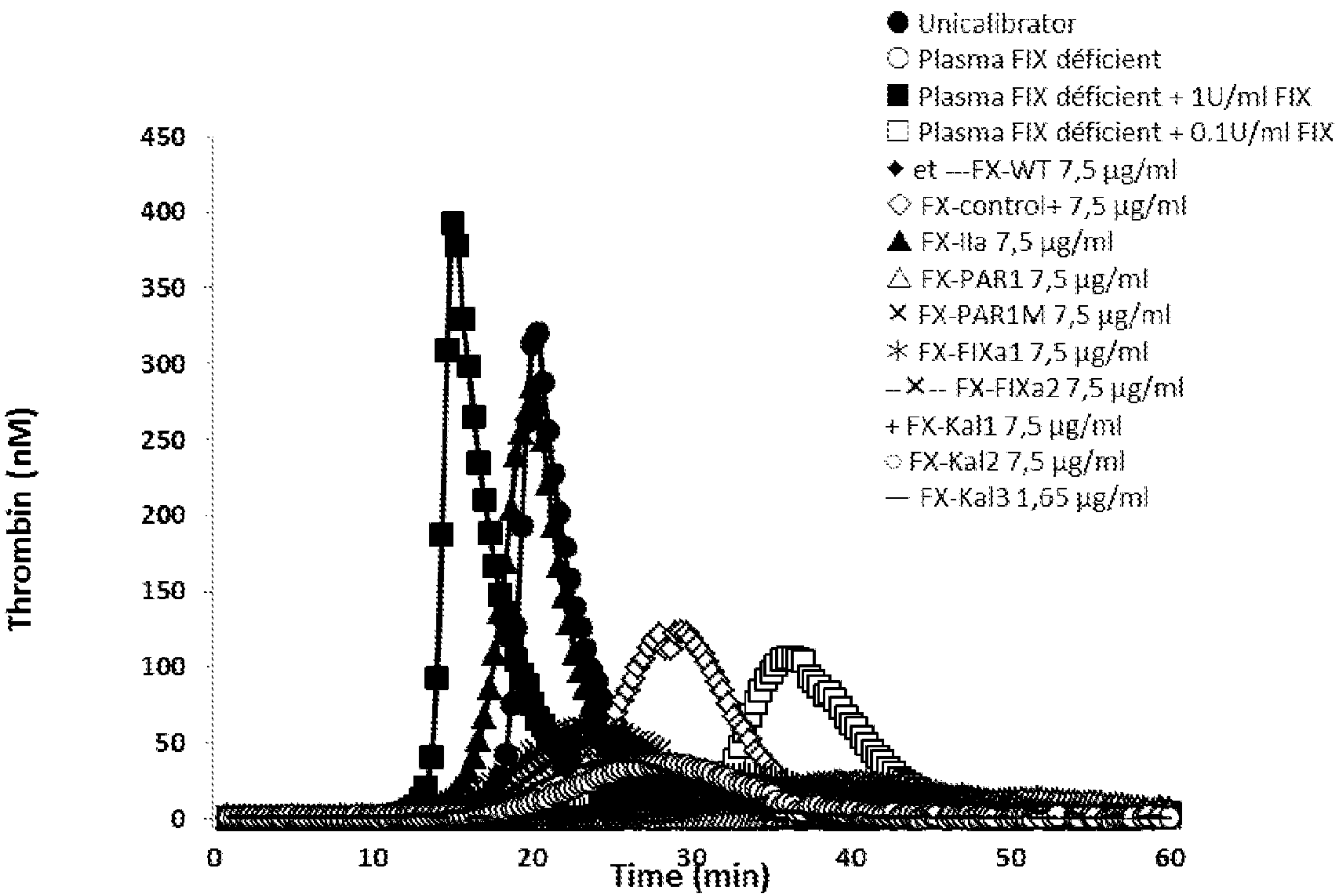


Figure 18

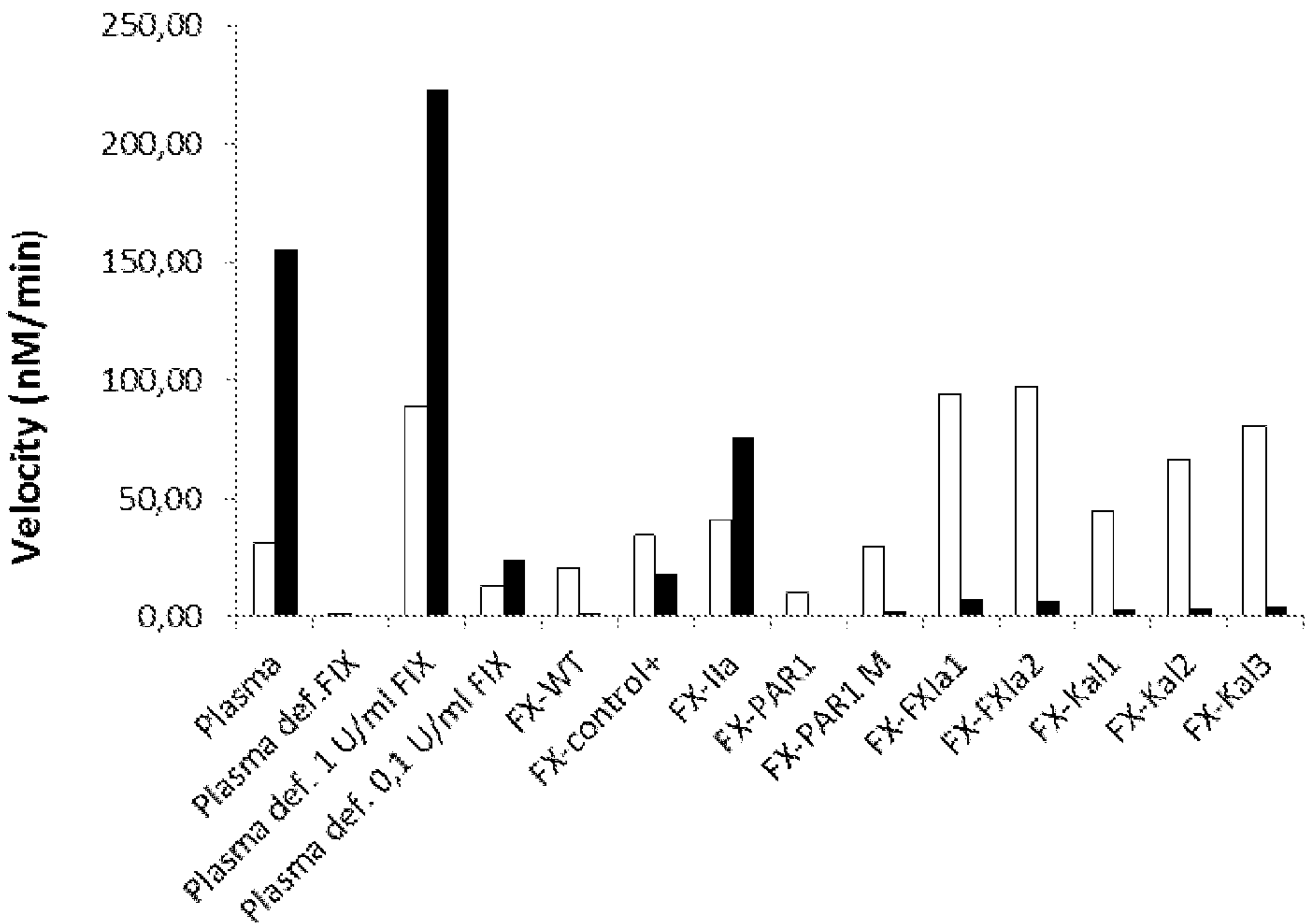


Figure 19

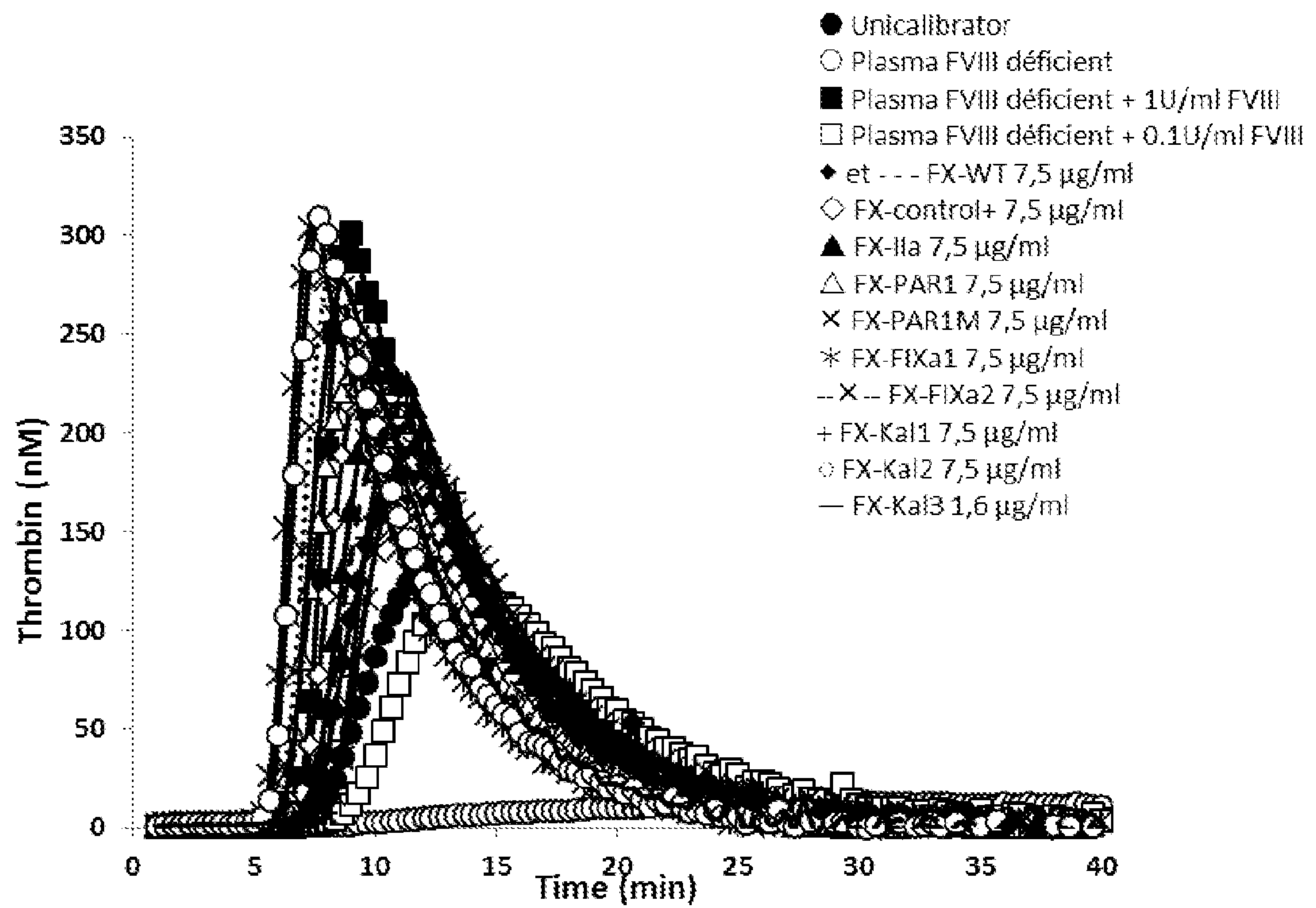


Figure 20

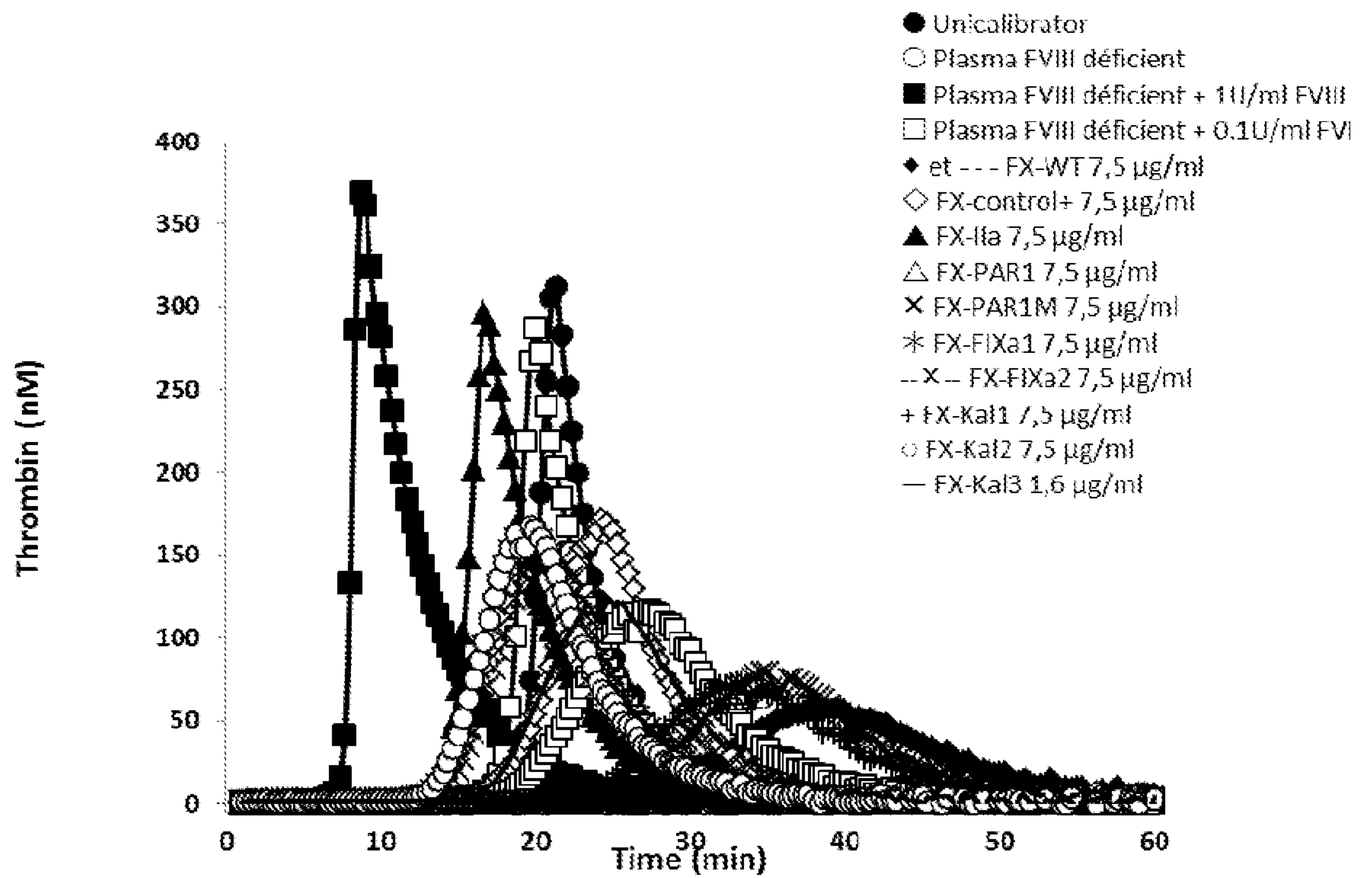


Figure 21

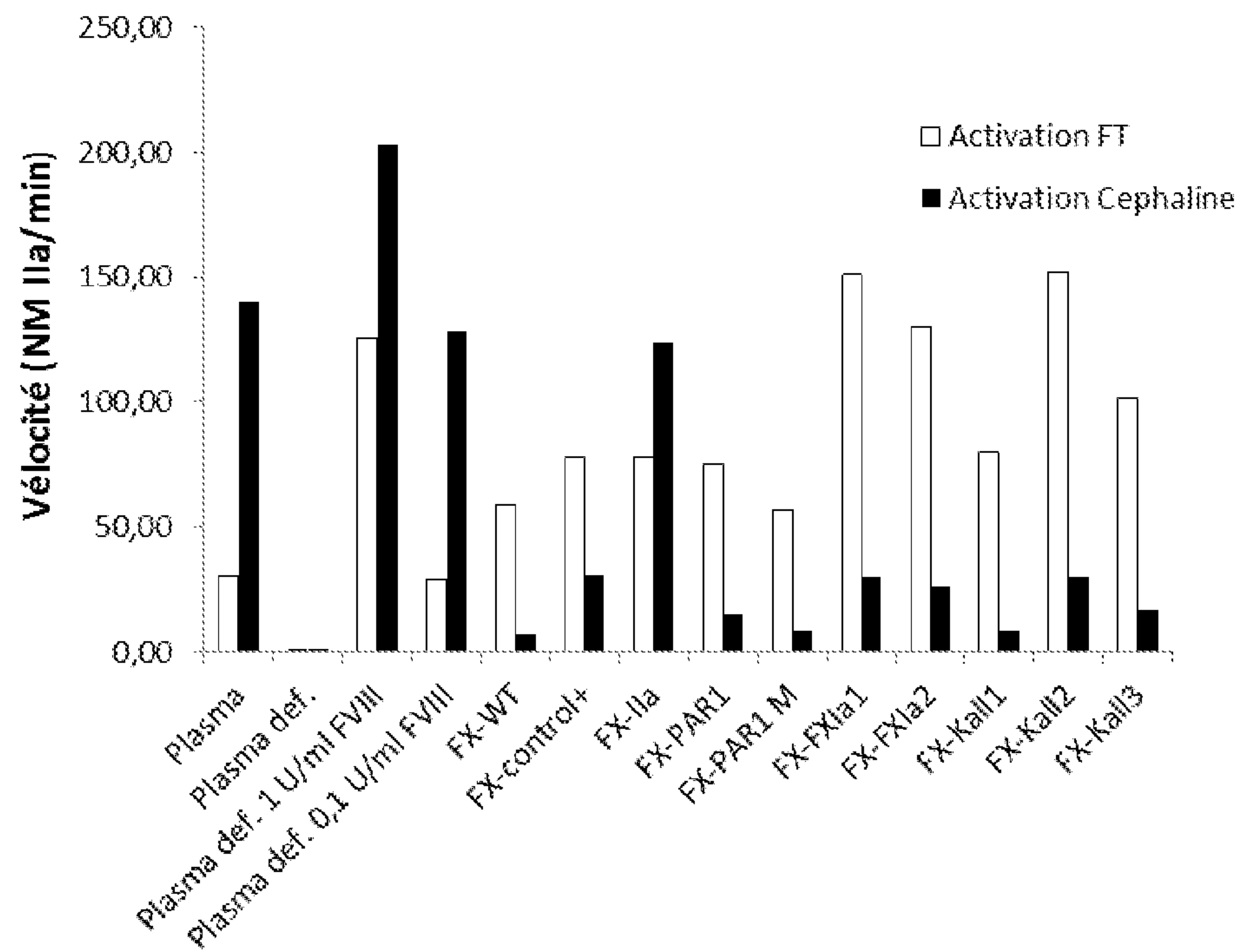


Figure 22

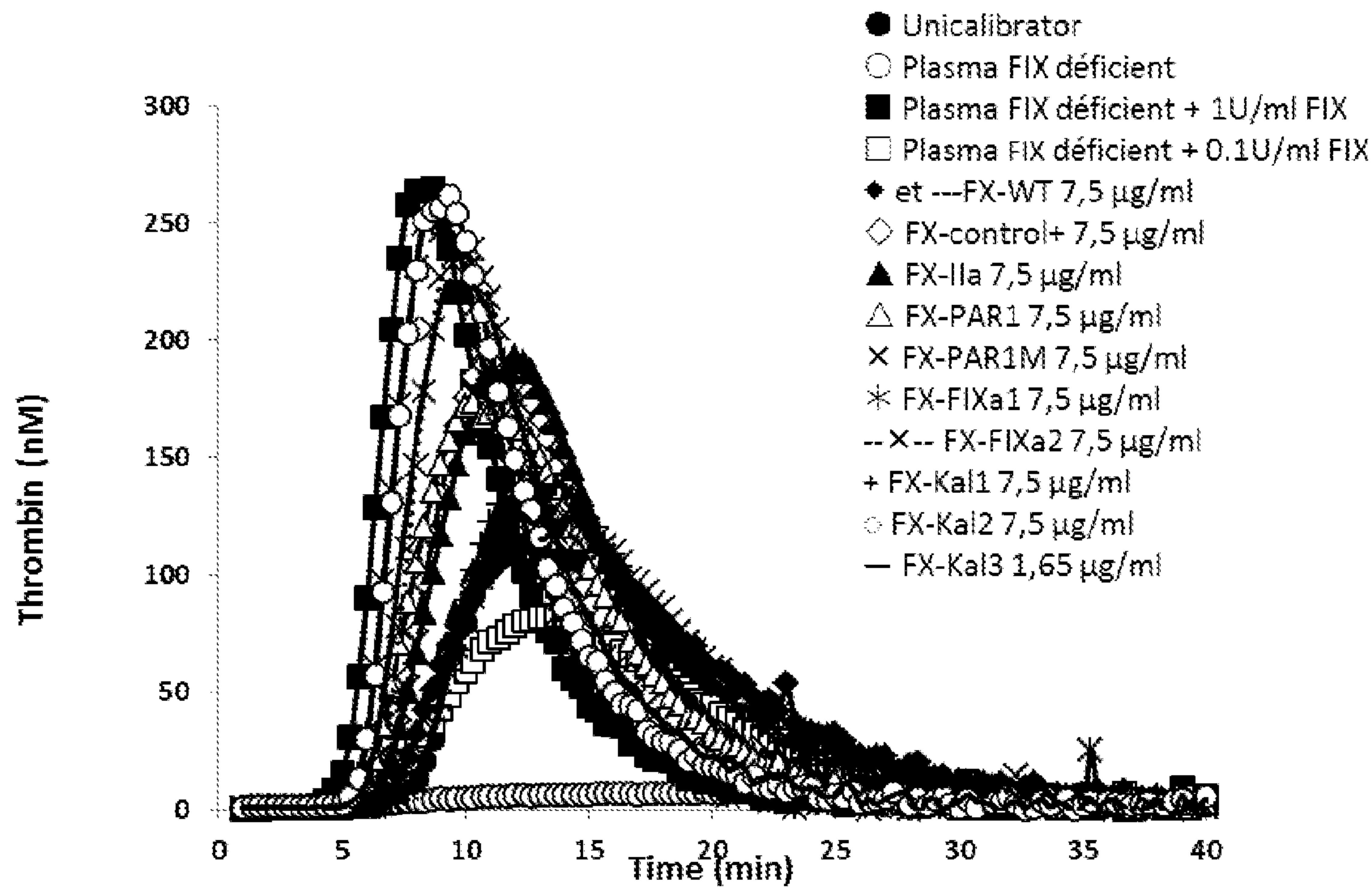


Figure 23

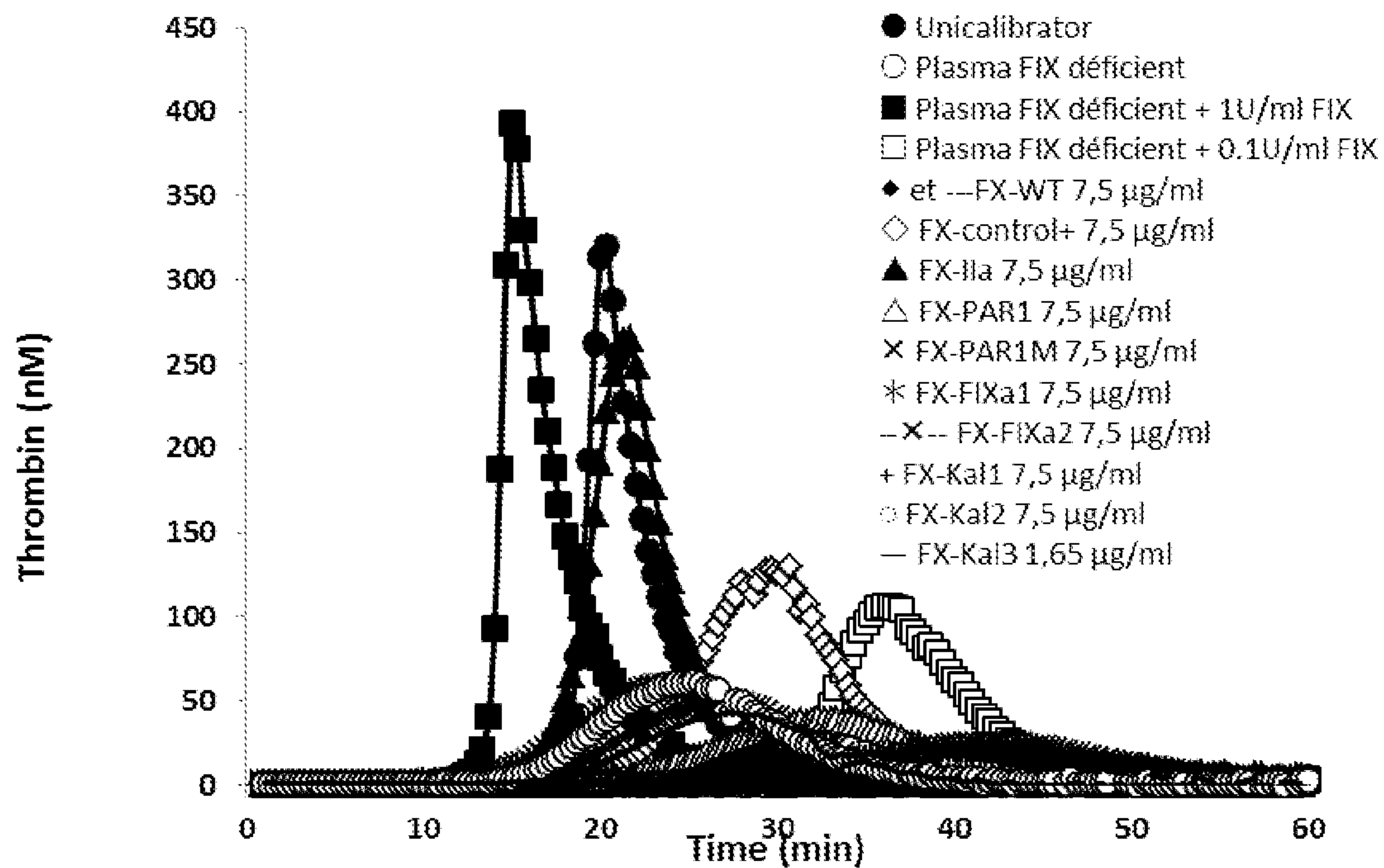


Figure 24

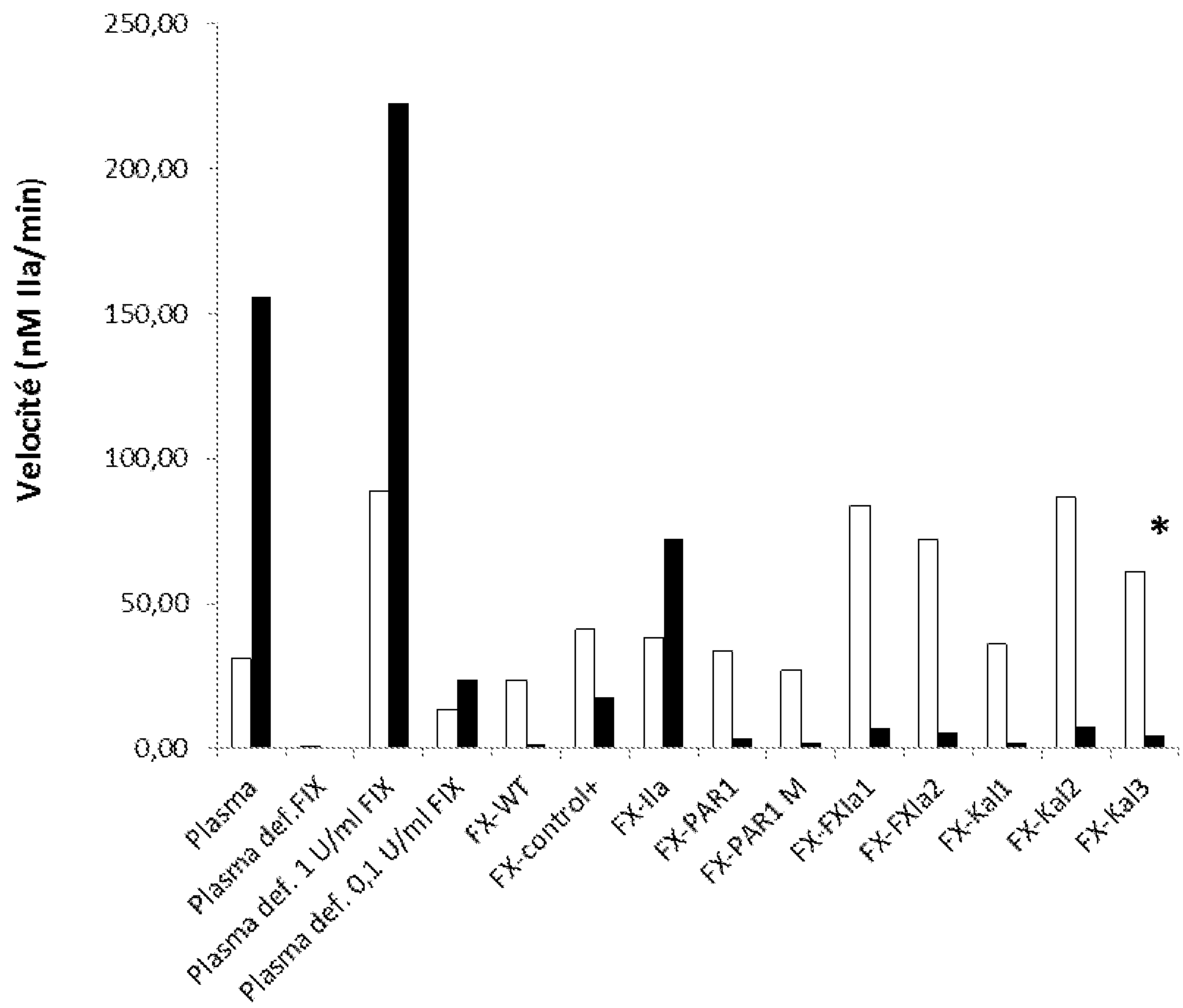


Figure 25