Linking *in silico* and *in vitro* experiments to identify and evaluate a biomarker for enoxaparin activity

Abhishek Gulati

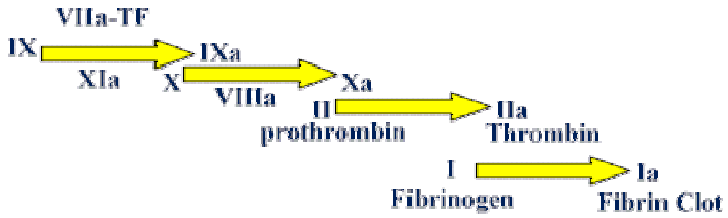
What is enoxaparin?

- Low molecular weight heparin anticoagulant
- Used to minimise the risk for thrombosis in patients with pulmonary embolism, deep vein thrombosis and acute coronary syndromes
- Increases the activity of a physiological inhibitor in blood called antithrombin ⟶ enhanced inactivation of Xa
- Inadequately controlled anticoagulation may result in bleeding or thrombosis ⟶ may be life threatening

Current test used to monitor enoxaparin therapy

Measurement of anti-Xa activity

- In Australasia
  - ✓ available only at few hospitals
  - ✓ takes several days for the results to become available
- No well accepted target value
- Focuses only on inhibitory activity directed against factor Xa



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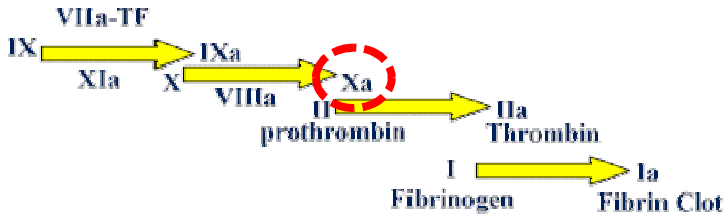

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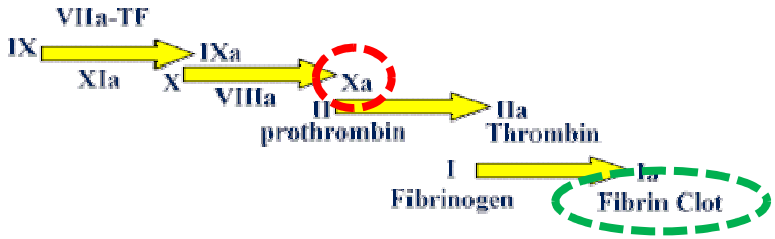


What we would like and what we have

- A test that assesses multiple stages of the clotting process and measures the endpoint ➔ FIBRIN CLOT
- Could be performed at any clinical haematology laboratory
- Clotting time tests currently exist that are used for
  - Unfractionated heparin: activated partial thromboplastin time (aPTT) test
  - Warfarin: prothrombin time (PT) test

BUT they do not produce significant dose-response changes with enoxaparin

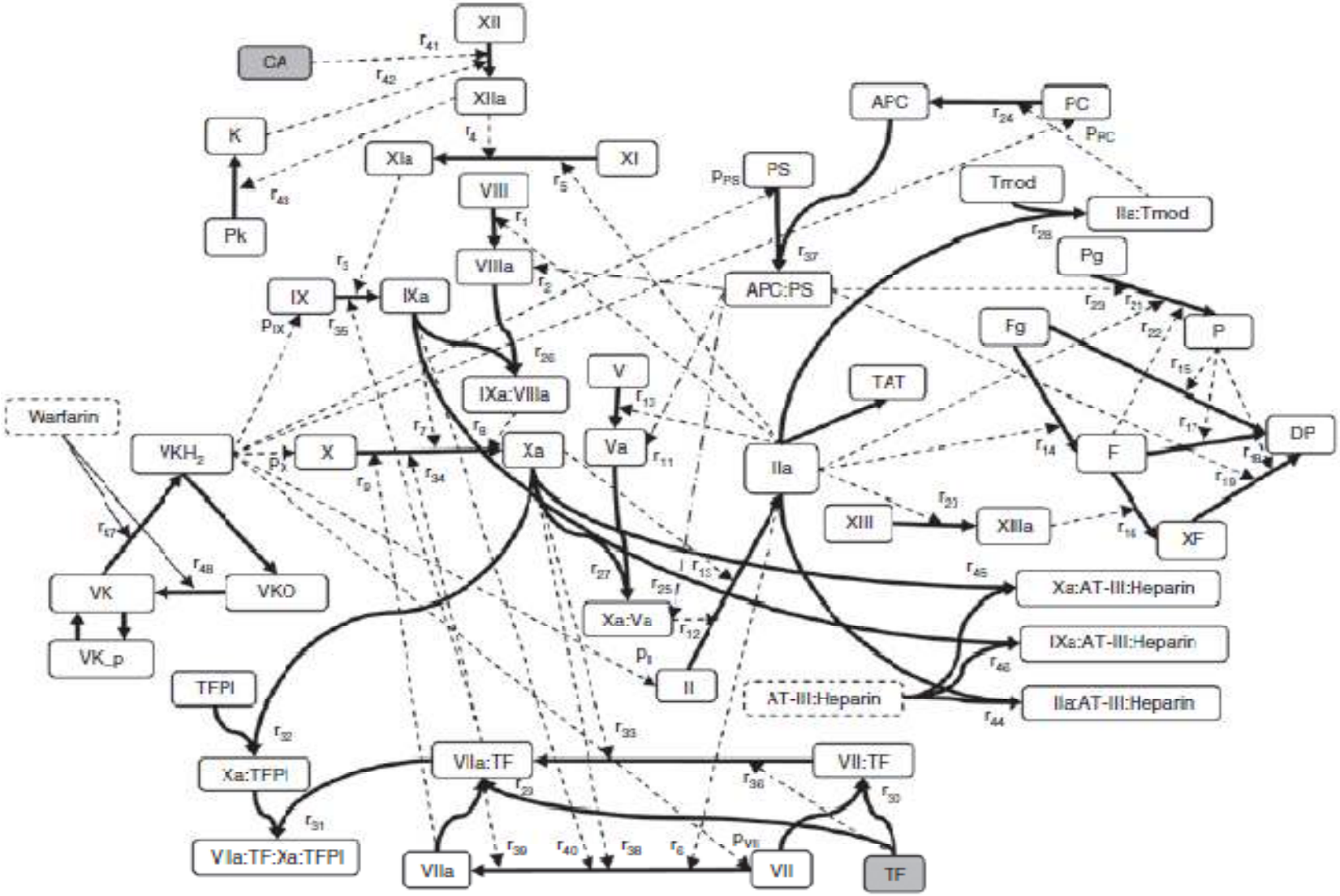
Aim and specific objectives

Aim: To identify and evaluate plausible activating agent(s) for a clotting time test to assess the anticoagulant effect of enoxaparin

Specific objectives:

1. To learn *in silico* why enoxaparin does not prolong aPTT and PT
2. To identify new targets *in silico* for a clotting time test for enoxaparin
3. To confirm the *in silico* results using *in vitro* experiments
4. To assess whether the model supports the findings from the *in vitro* experiments

Mathematical model of the coagulation network



Wajima et al, *Clin Pharmacol Ther* (2009)

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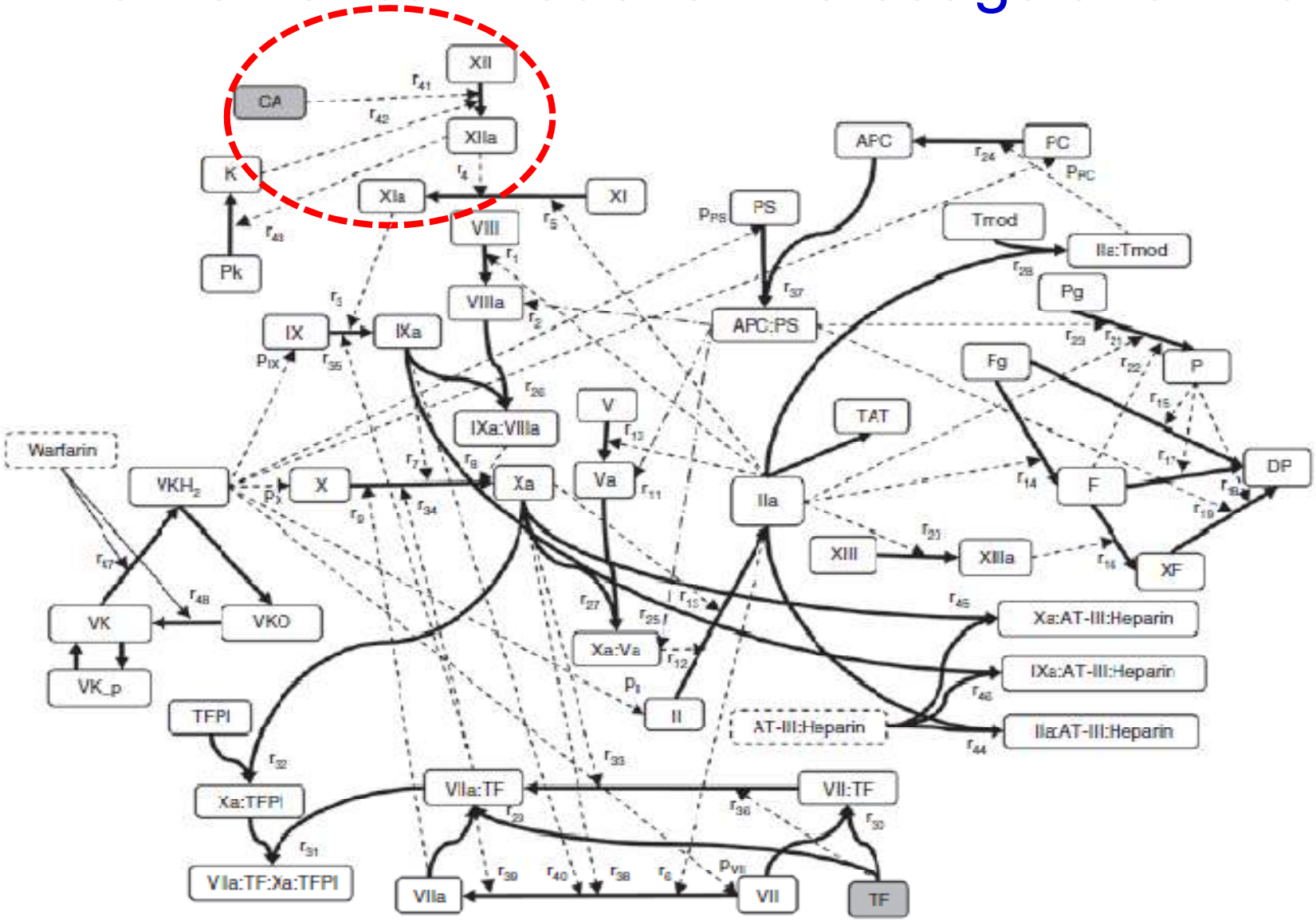

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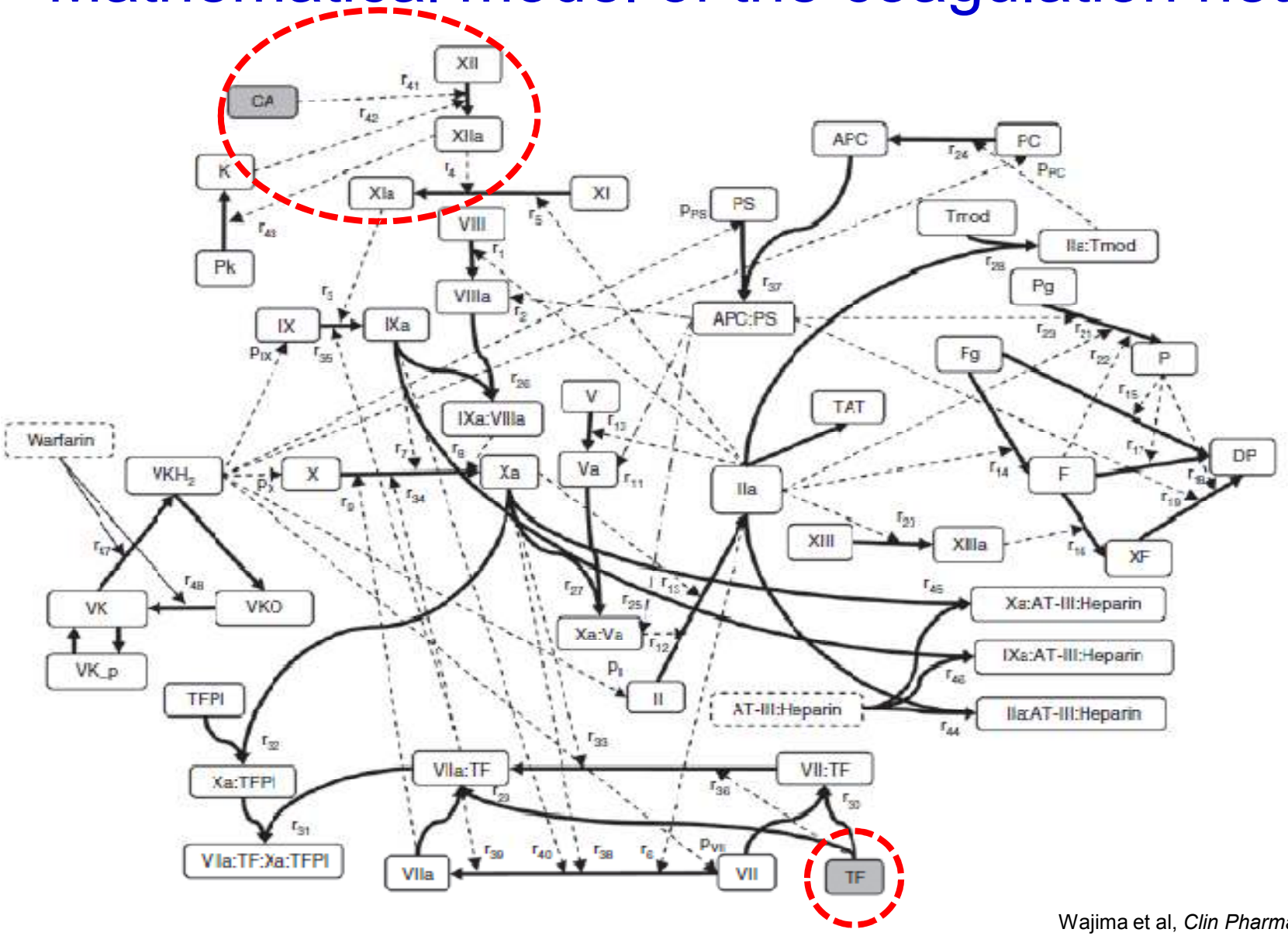

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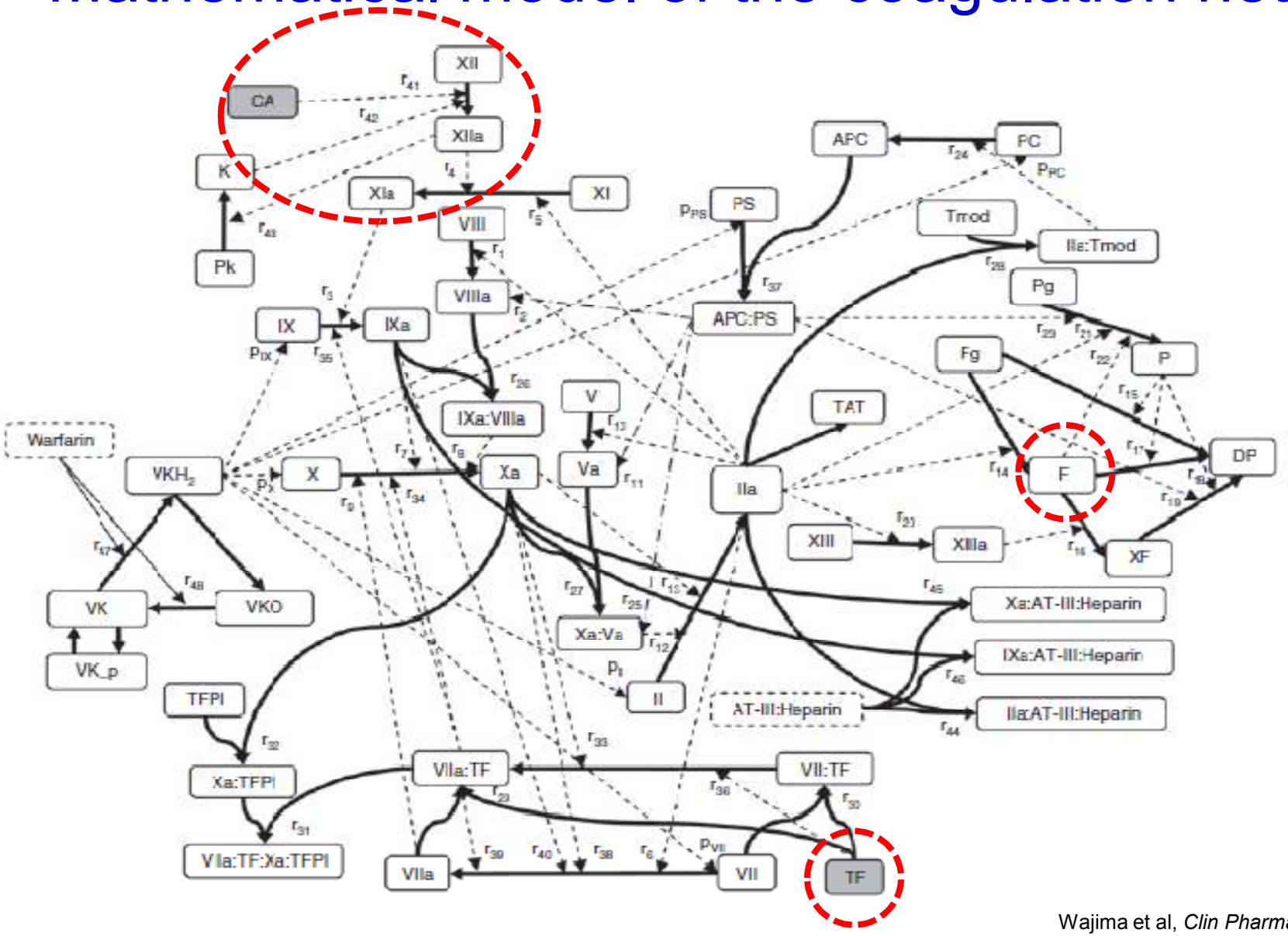

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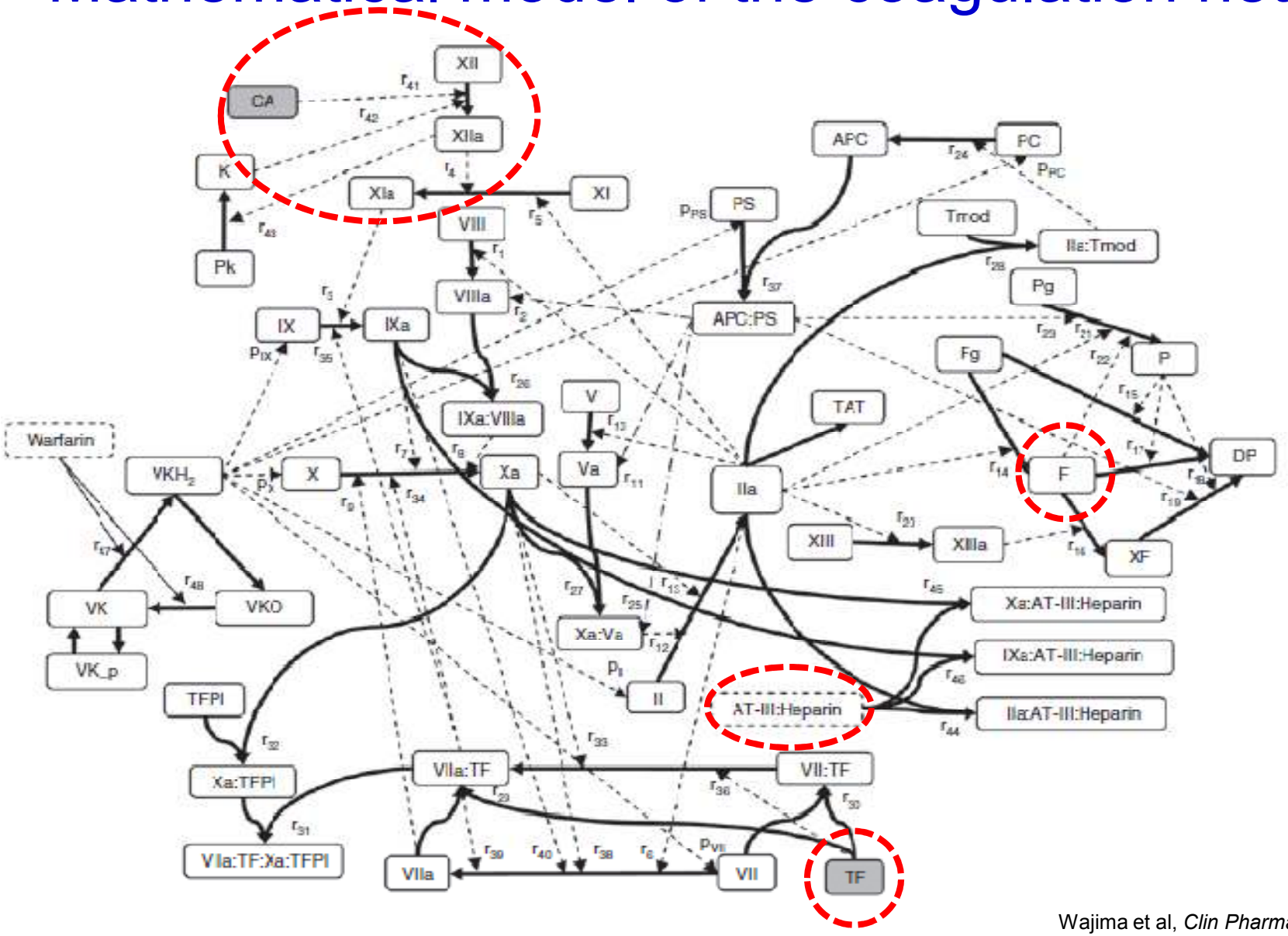

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Specific objective 1
"Learning" using *in silico* experiments

Why doesn't enoxaparin cause a significant (>2-fold) prolongation of current versions of aPTT and PT tests?

- ✓ Influences of various initial conditions was investigated
- ✓ Time courses of X and Xa in absence and presence of enoxaparin (0.5 IU/mL) were simulated

In silico assessment of activation of PT

[TF] (nM)	[Enox] (IU/mL)	PT (sec)	Fold increase in PT by enoxaparin	[Xa] at the time of clot formation	% reduction in [Xa] by enoxaparin
300	0	12		0.98 nM	

In silico assessment of activation of PT

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	0.5	14		0.37 nM	

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0.003	0	156		1.3 pM	

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300	0	12	1.2	0.98 nM	62%
	0.5	14		0.37 nM	
0.003	0	156	2.3	1.3 pM	96%
	0.5	354		0.05 pM	

In silico assessment of activation of aPTT

[XIa] (nM)	[Enox] (IU/mL)	aPTT (sec)	Fold increase in aPTT by enoxaparin	[Xa] at the time of clot formation	% reduction in [Xa] by enoxaparin
1.5	0	34	1.4	0.06 nM	85%
	0.5	49		0.0093 nM	
0.015	0	77	2.2	6.3 pM	94%
	0.5	167		0.4 pM	

What we learnt

- Concentration of the activating agents used in current versions of aPTT/PT tests results in formation of Xa that overwhelms the effect of enoxaparin

- However, the tests do work at low concentrations of the activating agents

Specific objective 2
Identifying new targets *in silico*

- To identify an activating agent in the form of a clotting factor that provides a reasonable clotting time (<60 seconds)
- And that was prolonged by at least 2-fold in the presence of enoxaparin (0.5 IU/mL)

↓

Clotting system was activated by a range of clotting factors or complexes:

IIa, Va, VIIa, TF, VIIa-TF, VIIIa, IXa, IXaVIIIa, Xa, XaVa, XIa, XIIa

Plausible activating agents

The model identified two plausible activating factors

- Factor Xa
  - ✓ more potent with clotting times of few seconds
- Tissue Factor
  - ✓ less potent with clotting times of few minutes

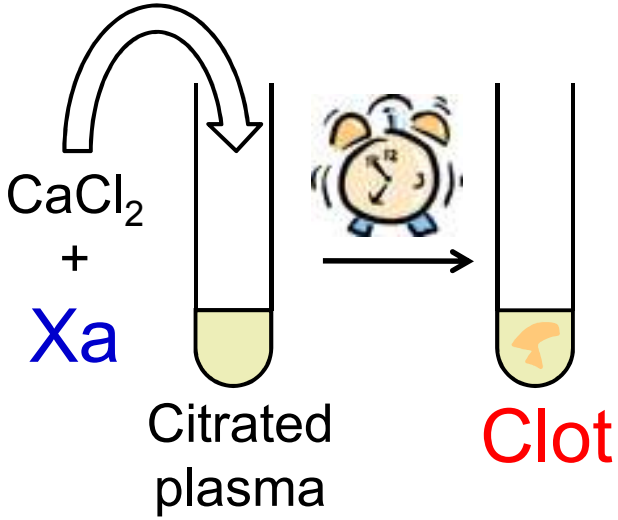
Xa was preferred because it produced shorter clotting times

Specific objective 3
"Confirming" through *in vitro* experiments

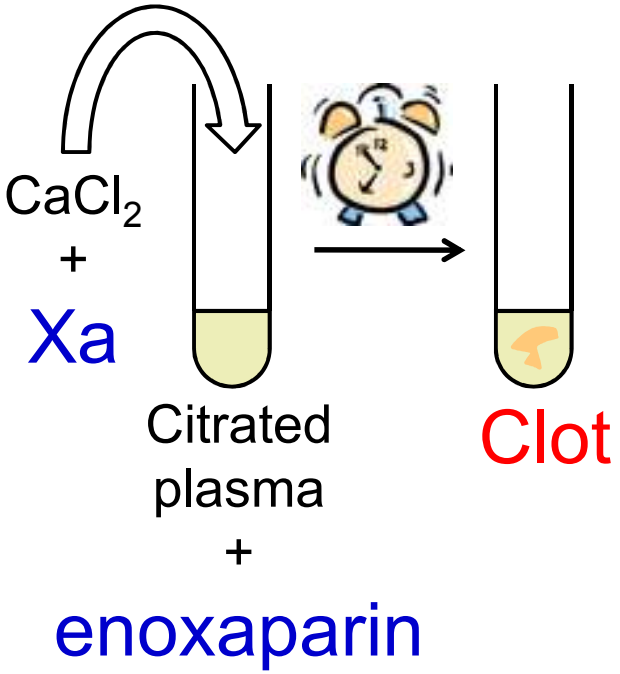
To demonstrate proof of mechanism of the clotting time test activated by Xa

In vitro experiments with human plasma

Control



Enoxaparin treated

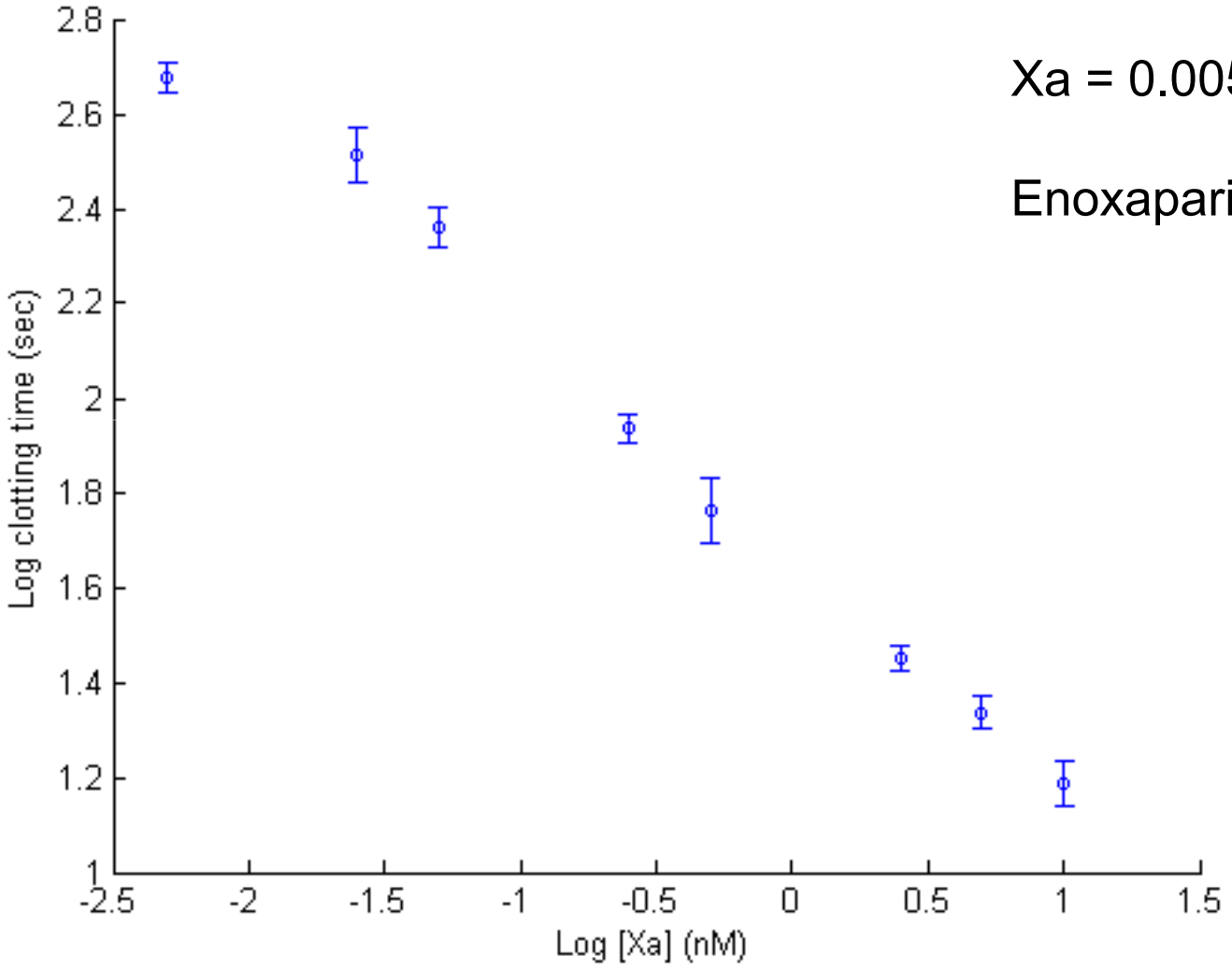


In vitro experiments

Clotting times were measured in three different sets of experiments

1.	Xa – varied	Enoxaparin – absent
2.	Xa – varied	Enoxaparin – constant
3.	Xa – constant	Enoxaparin – varied

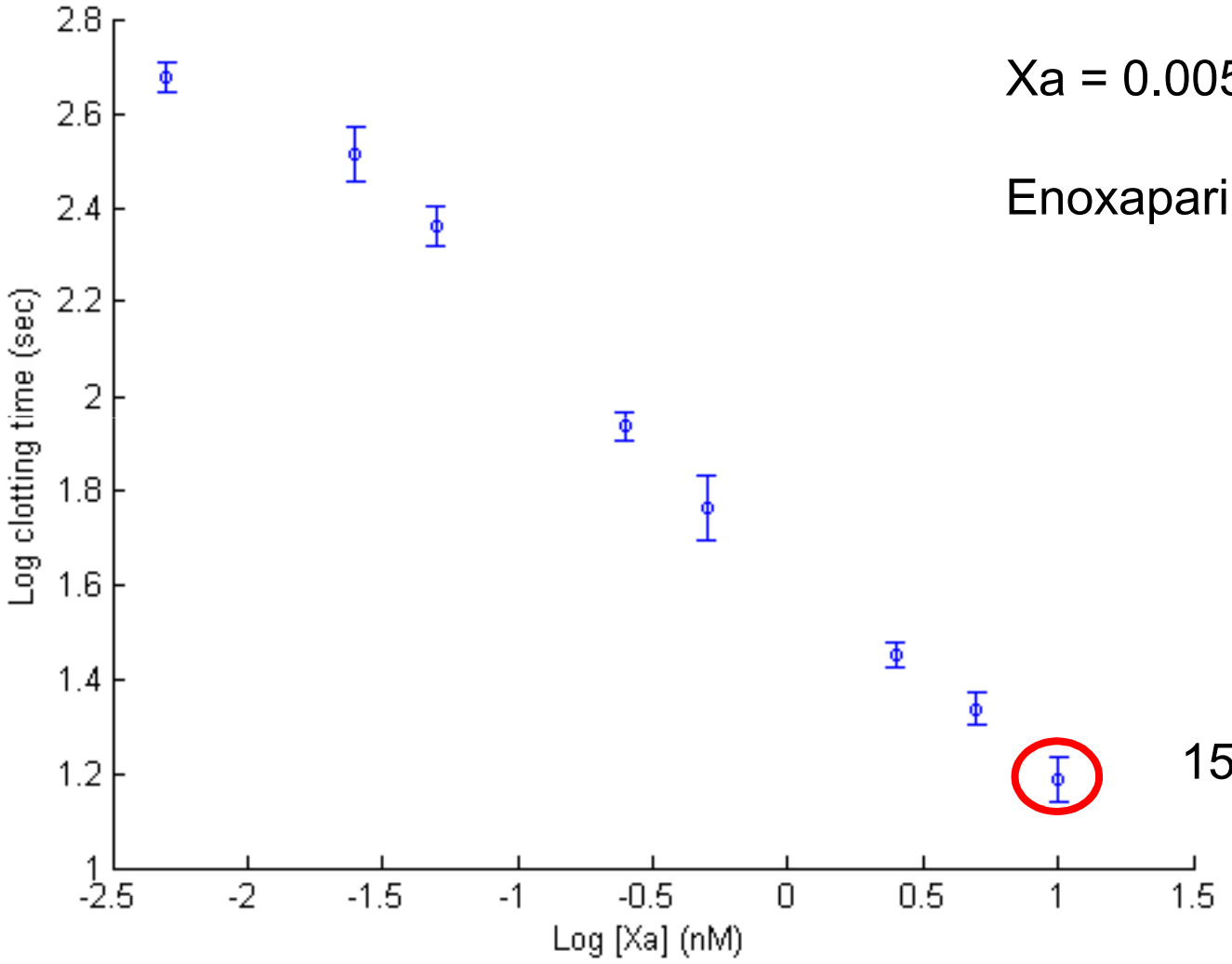
Xa varied in the absence of enoxaparin



Xa = 0.005 to 10 nM

Enoxaparin = 0

Xa varied in the absence of enoxaparin

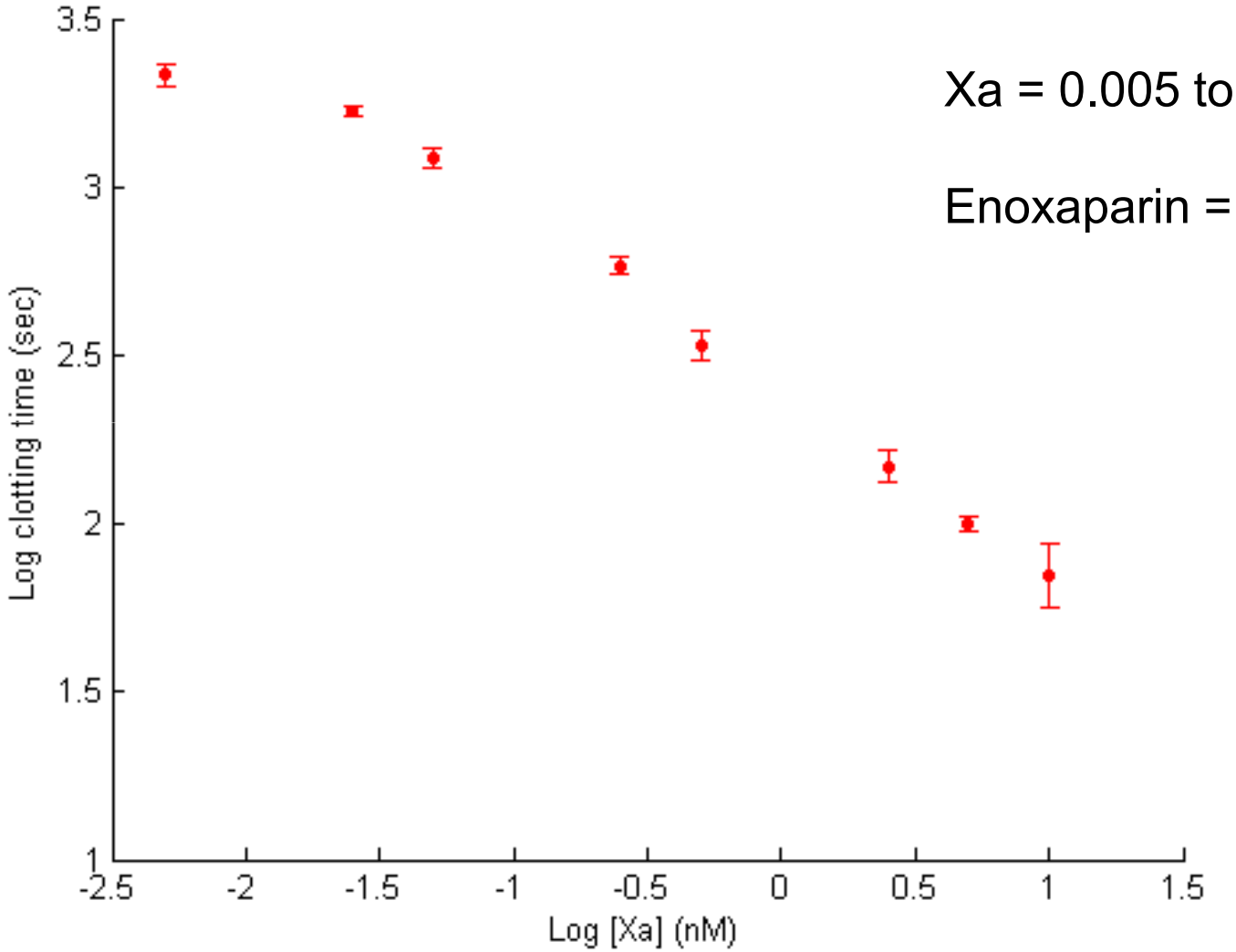


Xa = 0.005 to 10 nM

Enoxaparin = 0

15 sec

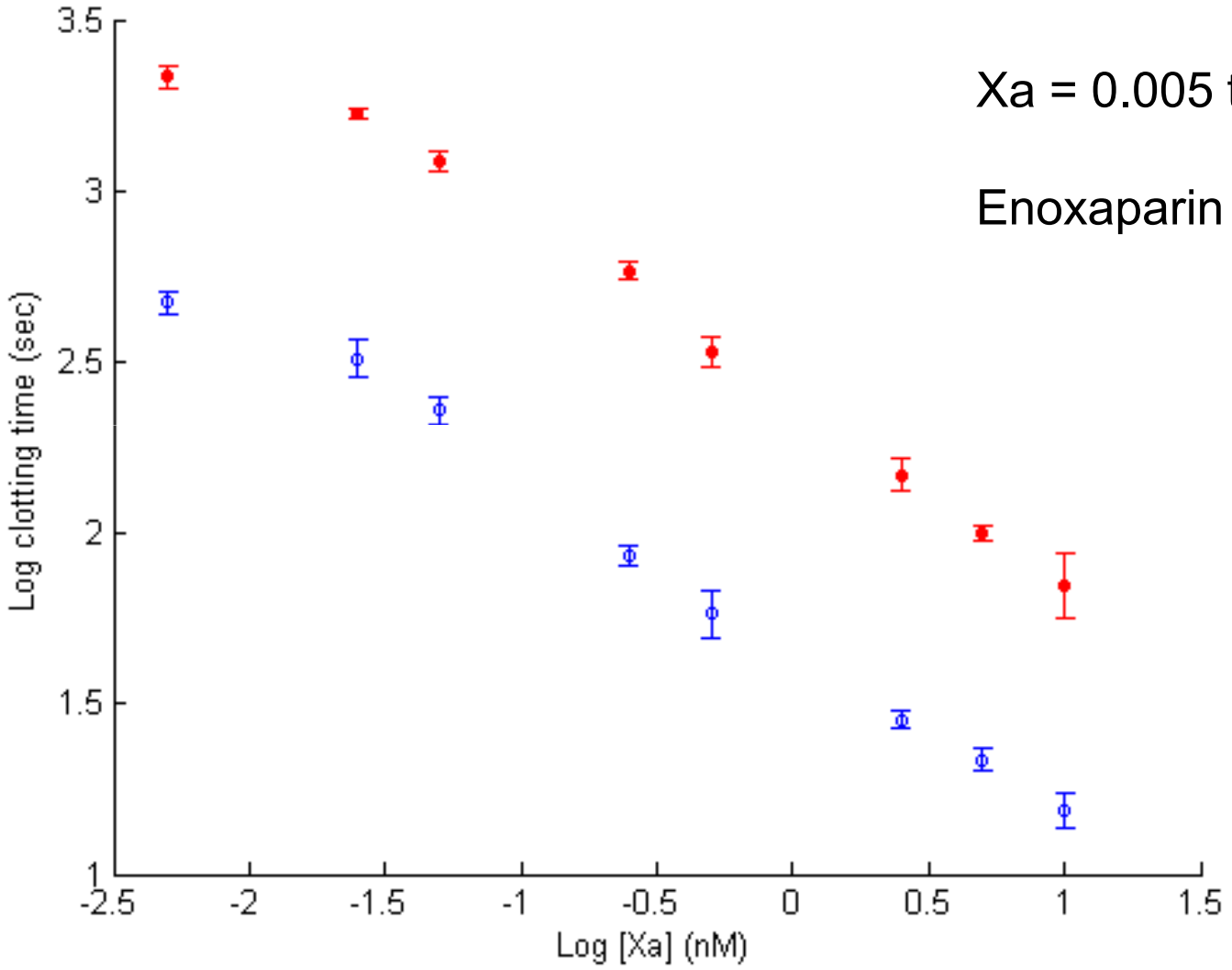
Xa varied in the presence of enoxaparin



Xa = 0.005 to 10 nM

Enoxaparin = 0.5 IU/mL

Xa varied in the presence of enoxaparin



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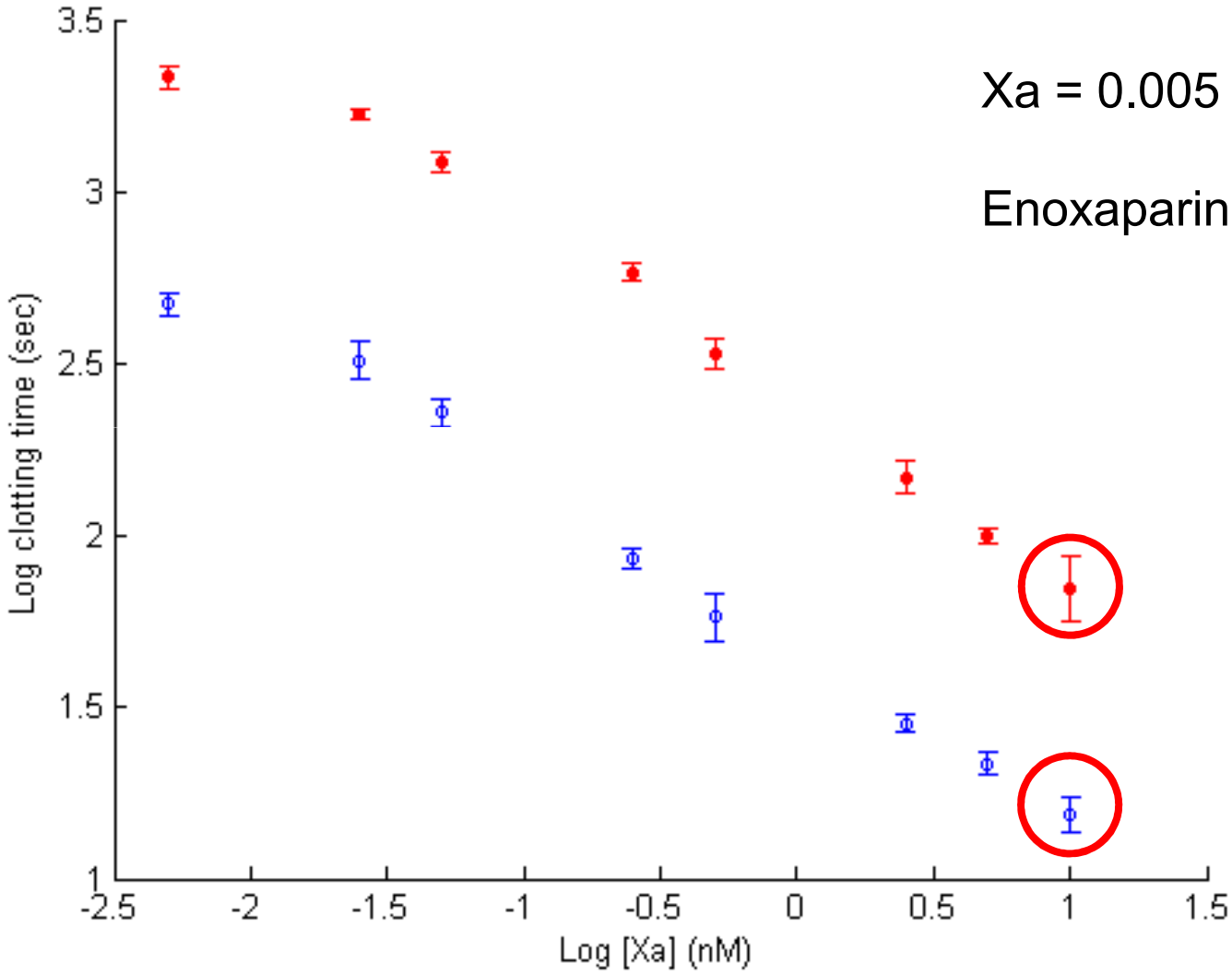
Xa varied in the presence of enoxaparin



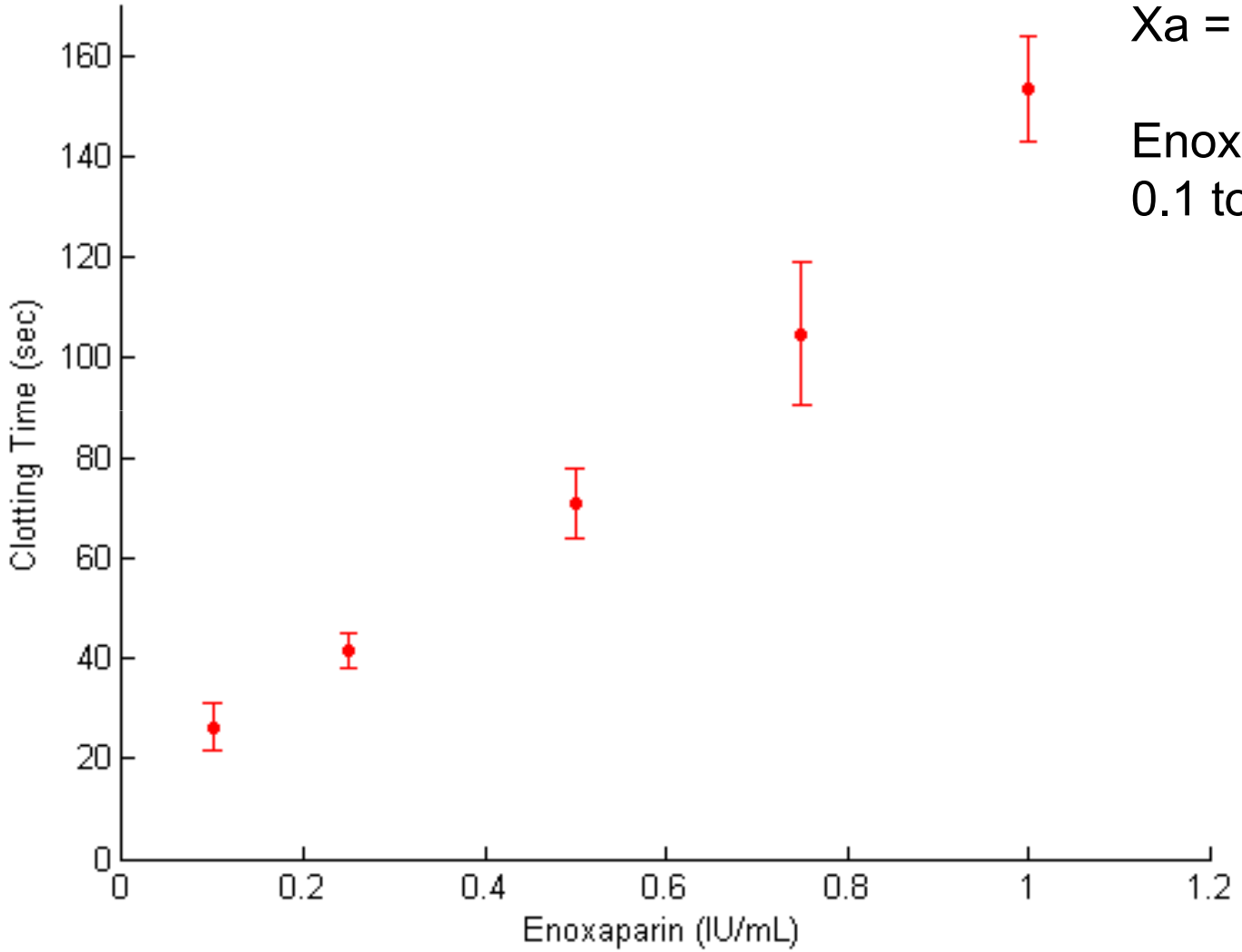
Xa = 0.005 to 10 nM

Enoxaparin = 0.5 IU/mL

71 sec

15 sec

Enoxaparin varied in the presence of specific Xa



Xa = 10 nM

Enoxaparin =
0.1 to 1.0 IU/mL

Enoxaparin varied in the presence of specific Xa

at Xa = 10 nM

Enoxaparin (IU/mL)	Clotting time (sec)	fold increase in clotting time by enoxaparin
0	15	0
0.1	26	1.7
0.25	42	2.8
0.5	71	4.7
0.75	105	7.0
1	153	10.2

Specific objective 4

In silico assessment of the new target

To assess whether the mathematical model supports the findings from the *in vitro* experiments

Addition to the model

Influence of variable antithrombin concentrations was accounted for:

$$f_{K_D} = 1 - \frac{[Enox]}{K_{D_E} + [Enox]}$$

$$\text{Concentration bound}(t) = B_{\max}(t) \times \frac{[Xa]}{\left(f_{K_D} \times K_{D_{Xa}}\right) + [Xa]}$$

$B_{\max}(t)$ = plasma concentration of $AT(t)$

$K_{D_{Xa}}$ and K_{D_E} = determined based on similarity of model predictions to *in vitro* results

In silico assessment of the new target

	In vitro results ([Xa] = 10nM)		*In silico* results ([Xa] = 0.1nM)	
[Enoxaparin] (IU/mL)	Clotting time (sec)	Fold increase in clotting time by enoxaparin	Clotting time (sec)	Fold increase in clotting time by enoxaparin
0	15	0	15	0
0.1	26	1.7	26	1.7
0.25	42	2.8	41	2.7
0.5	71	4.7	70	4.7
0.75	105	7.0	106	7.1
1	153	10.2	145	9.7

Conclusions

Using *in silico* simulations and *in vitro* experiments:

- learnt why enoxaparin does not prolong current versions of aPTT and PT tests
- identified Xa as a new target for a clotting time test for enoxaparin
- confirmed the *in silico* findings using *in vitro* experiments
- learnt that there was a difference between model predictions and *in vitro* results which could be due to the absence of calcium and phospholipids in the model

What next?

- Proof of concept (PoC) study for the Xa clotting time ("XaCT") test
- Successful PoC study would mean:
  - "XaCT Test" could be evaluated in patients receiving enoxaparin
- "XaCT Test" may provide a missing link for dose optimisation of drugs like enoxaparin
- The coagulation network model needs further development to describe coagulation pathways

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