Global haemostasis assays: what will be the future?

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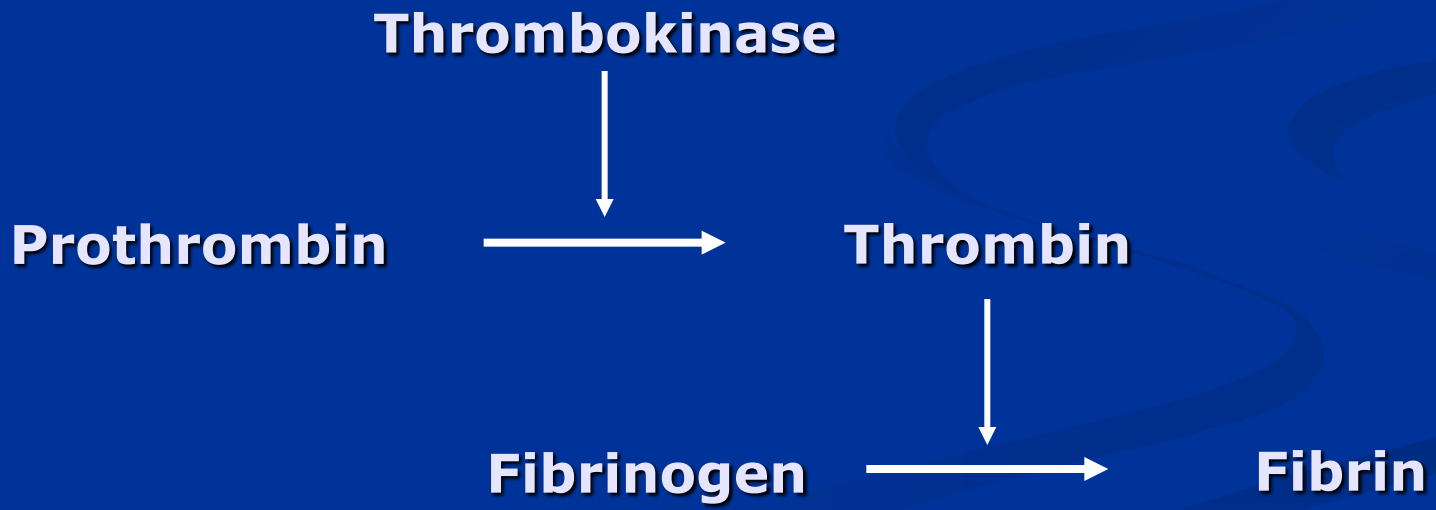
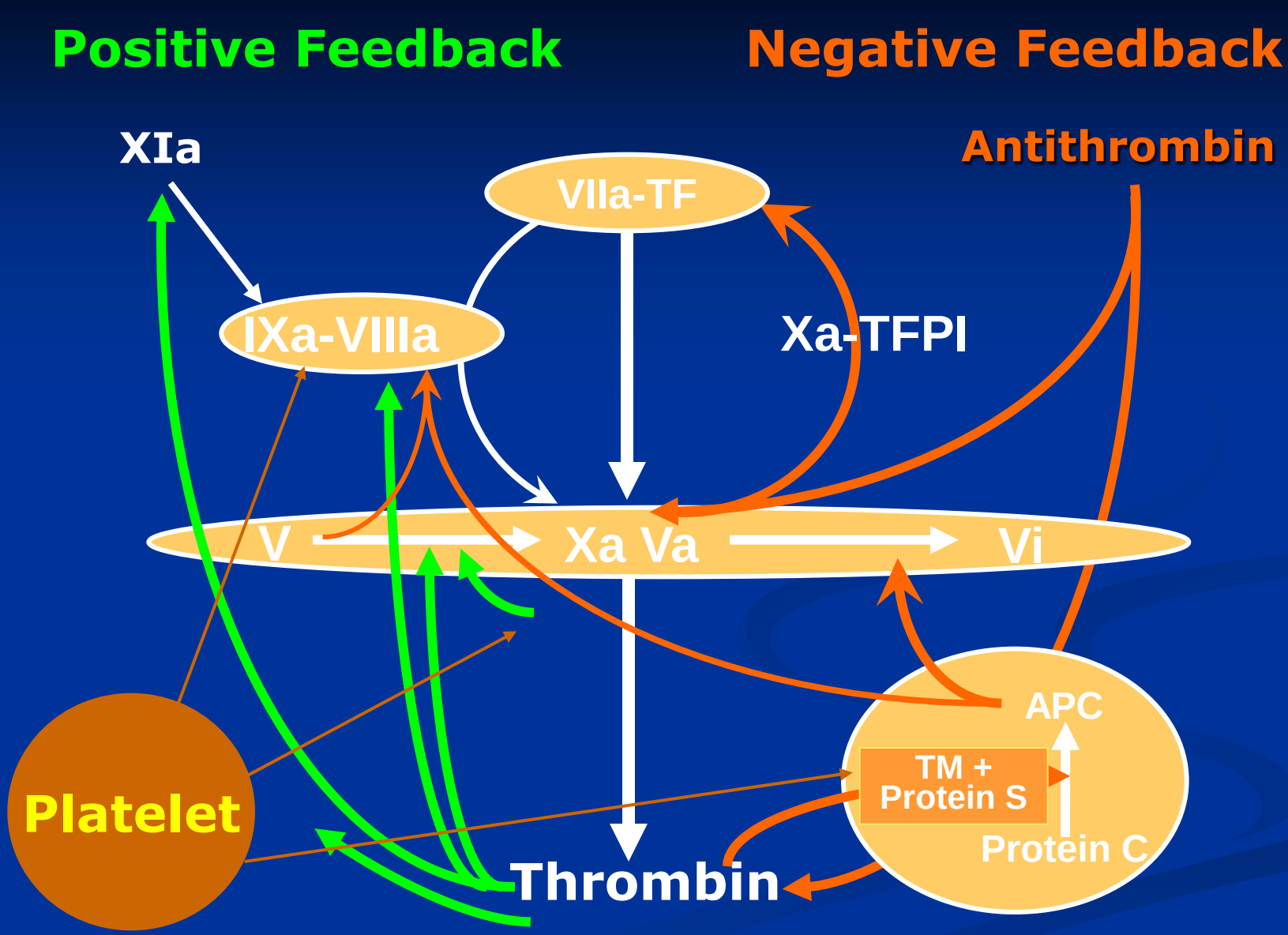
100 years ago clotting was simple

Morawitz 1905:



But then came biochemistry


Positive Feedback
Negative Feedback
XIa
Antithrombin
VIIa-TF
IXa-VIIIa
Xa-TFPI
V
Xa Va
Vi
APC
TM +
Protein S
Protein C
Platelet
Thrombin

Lots of sophisticated and special tests

- Clotting tests
- Coagulation Factors
- Anticoagulant proteins
- Activation markers (TAT, F1.2, D-dimer)
- Clot retraction
- Platelet aggregation
- VWF tests
- etc.
- etc.

General consensus

We have to develop global hemostasis assays to assess the risk of bleeding, thrombosis, and monitor treatment in a single assay.

Such a global assay should be affected by coagulation factors, anticoagulant proteins and platelets and by abnormalities in the haemostatic system.

Applications of global haemostasis tests

Diagnosis of thrombosis/bleeding disorder

D-Dimer, Duplex Ultrasonography, Contrast Venography

Cause of the thrombotic or bleeding disorder

Classical clotting tests/DNA analysis/ELISA

To monitor treatment

Risk of recurrence (prediction)

Risk of individuals with established risk factor (prediction)

Global haemostasis tests will be helpful

Risk of recurrence (prediction)

Monitor treatment

Risk of individuals with established risk factor (predictive value)

Why are classical coagulation tests not the ideal global assays?

- Thrombosis and bleeding are multifactorial diseases resulting from a complex interplay between genetic and acquired risk/protective factors.
- Global tests should reflect the overall effect of all risk/protective factors.
- Classical tests probe only a limited part of the haemostatic process

Thromboelastography

Thromboelastography measures the physical properties of clot formed in whole blood.
Routine application: Diagnosis of bleeding and monitoring the transfusion of blood products during surgery.

Multiplate analysis

The Multiplate analyzer measures the rate and extent of platelet aggregation in whole blood
Routine application: Diagnosis platelet abnormalities

Thrombin generation

Thrombin generation follows the formation and inhibition of thrombin in plasma or in whole blood
Routine Application: ??????????

Thrombin generation assays: what will be the future?

J. clin. Path. (1953), **6**, 3.

A THROMBIN GENERATION TEST

THE APPLICATION IN HAEMOPHILIA AND THROMBOCYTOPENIA

BY

R. G. MACFARLANE AND ROSEMARY BIGGS

From the Department of Clinical Pathology, Radcliffe Infirmary, Oxford

(RECEIVED FOR PUBLICATION OCTOBER 30, 1952)

Much recent research on blood clotting has dealt with prothrombin and factors which influence its activation. In the development of such investigations experimental procedures have become increasingly complex and artificial. They usually involve the use of anticoagulants, often multistage fractionation by precipitation, adsorption or filtration, and, almost invariably, the addition of tissue extracts to promote thrombin generation. These techniques have yielded information of great importance, but they must have a limited application, since they are far removed from the natural process of clotting.

Normal blood taken by a clean venepuncture into a glass vessel clots firmly in a few minutes without any stimulus other than surface contact. The following paper uses unaltered whole blood from which serial samples are simply transferred to fibrinogen, the clotting time of which indicates the thrombin concentration of the blood at the time of sampling. By plotting thrombin concentration against time, curves are obtained which show the speed of thrombin generation and destruction and thus may indicate the rate of production and the activity of blood thromboplastin.

The generation of thrombin in whole blood can only reflect thromboplastic activity if irrelevant variable factors are controlled as much as possible. Variations in prothrombin, antithrombin, and calcium concentration are likely to have a considerable effect on thrombin generation, but they were avoided by the use of normal blood or

Development of the thrombin generation assay

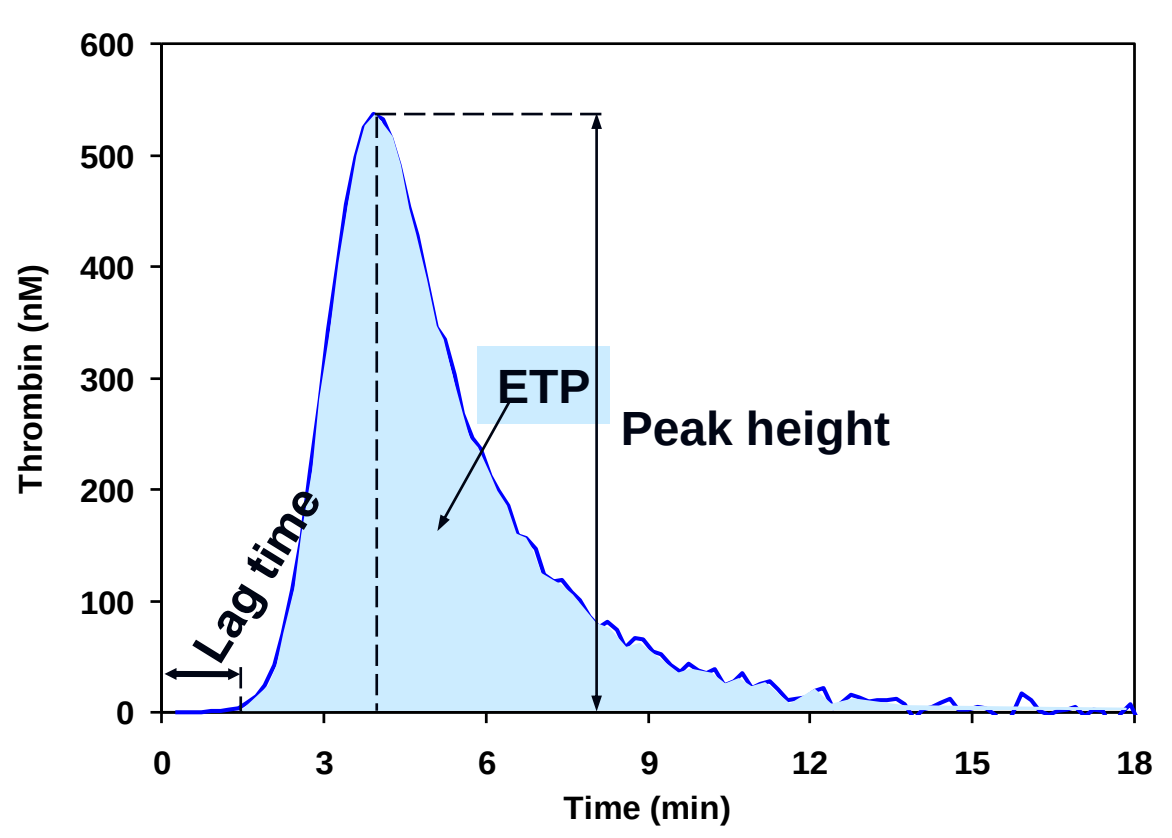
1953: Subsampling to fibrinogen and quantification of thrombin via a clotting assay (MacFarlane and Biggs)

1985: Subsampling to a cuvet with a thrombin-specific chromogenic substrate (Beguin and Hemker)

1993: Continuous registration with a weak thrombin-specific chromogenic substrate in defibrinated plasma (Hemker et al.)

2003: Continuous registration with a weak thrombin-specific fluorogenic substrate in full plasma (Hemker et al.)

Thrombin generation parameters



Lag time ~ clotting time

ETP ~ number of PT molecules activated ~ activity of thrombin inhibitors

Peak height ~ rates of prothrombin/FX activation and thrombin inhibition

Applications of Thrombin generation

Numerous papers appeared in literature with applications in:

1) Thrombophilia screening

2) Estimating bleeding risk of haemophilia patients

3) Monitoring treatment of patients with venous thrombosis

4) Drug development *e.g.* antithrombotics

5) Epidemiological studies *e.g.* pill thrombosis

etc. etc.

However 60 years after its introduction:

Thrombin generation is hardly applied in routine laboratories

Why?

Lack of Standardisation

Pre-analytical variation *e.g* plasma preparation (citrate and centrifugation, Corn trypsin inhibitor)

Analytical variation *i.e.* test conditions: PFP, PPP or PRP Coagulation trigger, ± APC ± thrombomudulin ± CTI

Lack of ìnsight in background of the test

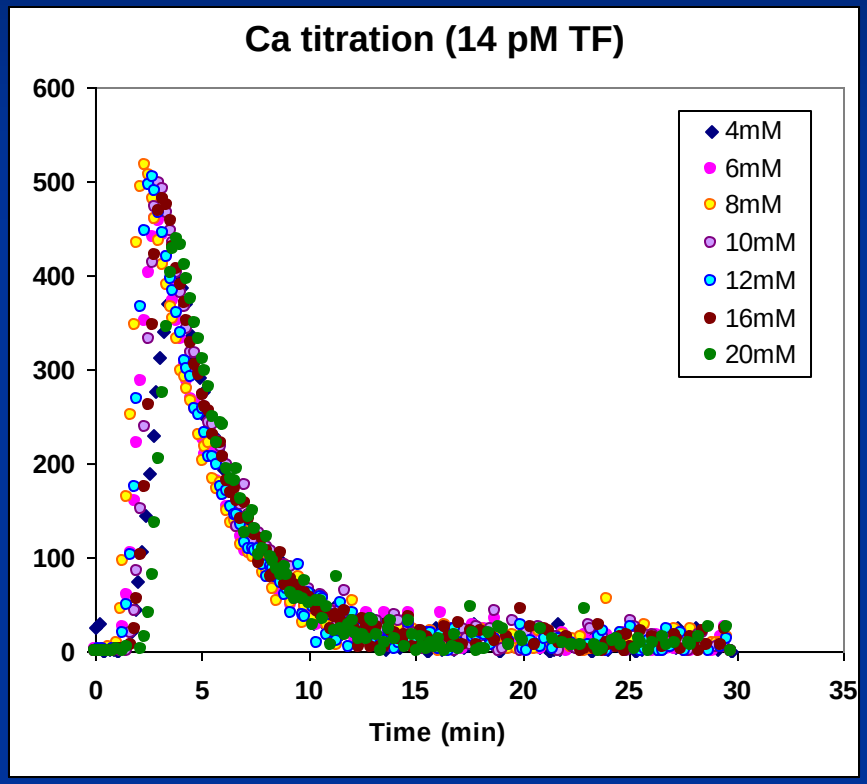
Insufficient knowledge of determinants of the various thrombin generation tests

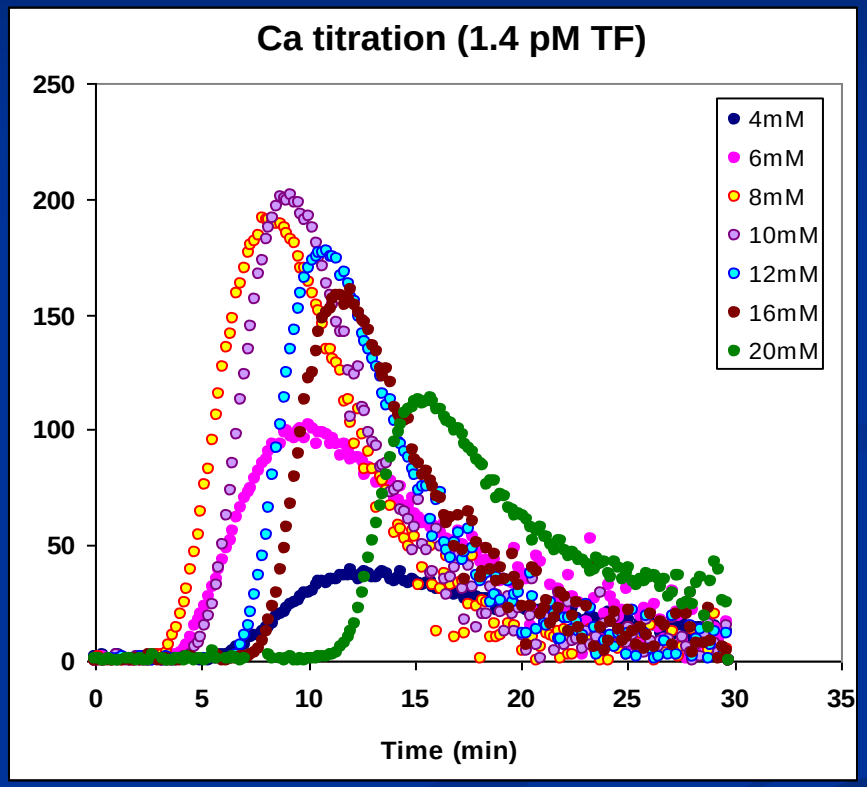
Lack of Clinical Validation

Limited clinical validation in small populations often with home-made tests

Thrombin generation in normal plasma

Effect of citrate/$CaCl_2$

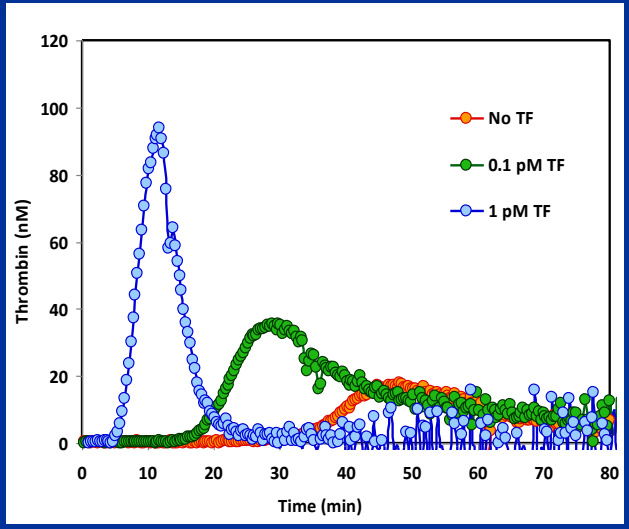




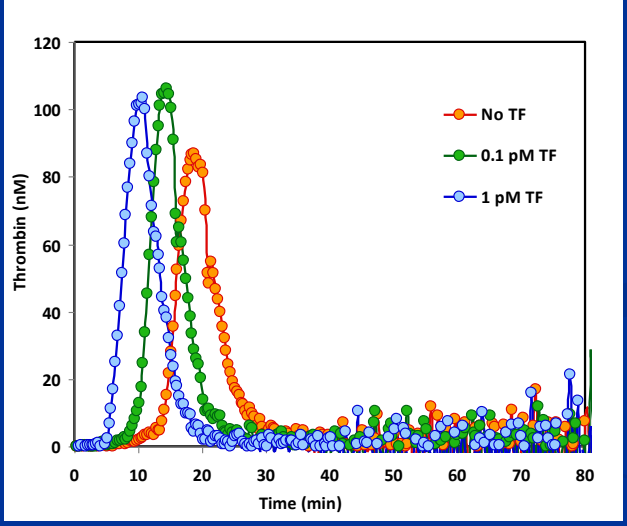
Effect of collection of blood in CTI

Thrombin generation in platelet-rich plasma

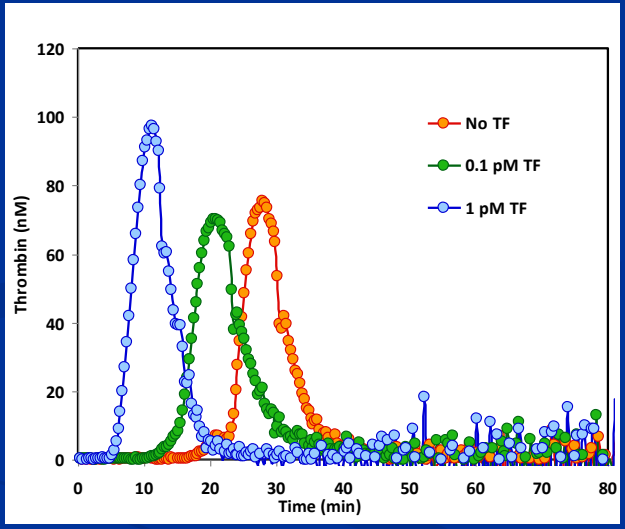
Collected in CTI



Not Collected in CTI



Not collected in CTI, CTI added later



Effect of analytical variables (test conditions) and determinants of tests

A determinant is a plasma variable that affects the outcome of a test.

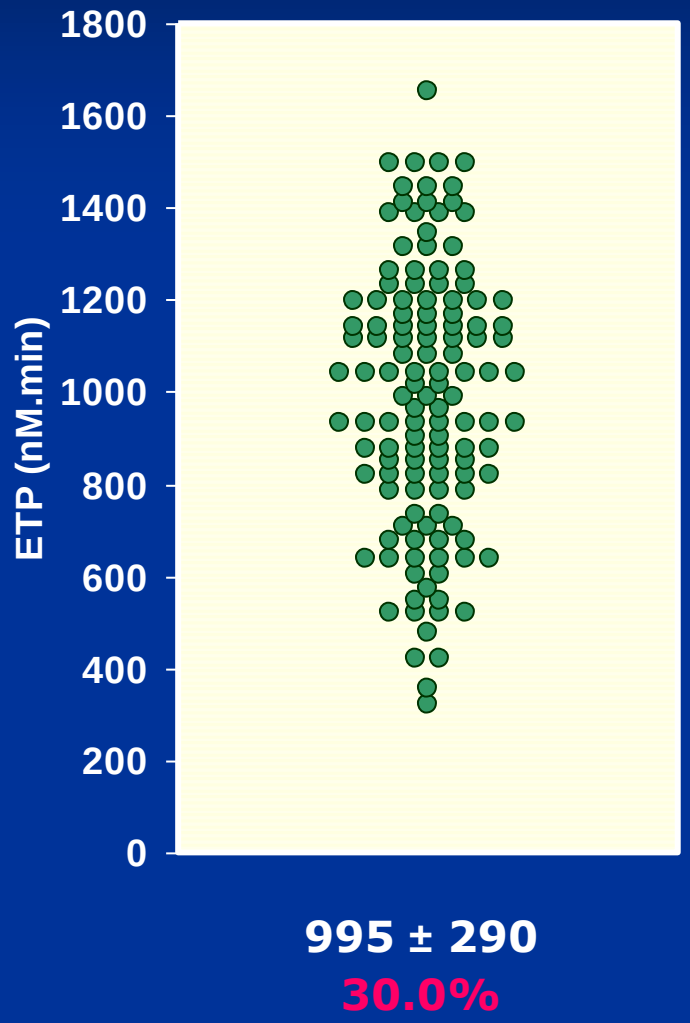
Approach:

Measure thrombin generation parameters in plasma samples of a large number of individuals.

Determine the plasma levels of coagulation factors and anticoagulant proteins

Perform a multi-regression analysis

Inter-individual variation (1 pM TF)



ETP (nM.min)		995 ± 290
	SD	Beta
Fibrinogen (g/L)	0.54	0.400
Prothrombin (U/dL)	18.2	n.s.
Factor V (U/dL)	18.6	n.s.
Factor X (U/dL)	15.5	n.s.
Factor XII (U/dL)	20.2	0.302
Antithrombin (U/dL)	10.1	-0.311
Protein C (U/dL)	19.8	n.s.
Free protein S (U/dL)	17.4	n.s.
Free TFPI (ng/mL)	3.05	-0.399

Based on: Dielis *et al*. J Thromb Haemost 2008

Assay determinants 1 pM TF
Lag time, thrombin peak, ETP

	Lag time	ETP	Peak height
Fibrinogen	0.197*	0.400*	0.368*
Prothrombin	0.063	-0.001	-0.181
FV	0.109	0.116	0.134
FVII	-0.232*	0.129	0.122
FX	0.090	0.125	0.097
FVIII	0.102	-0.013	0.119
Antithrombin	-0.092	-0.311*	-0.240*
Free protein S	0.295*	-0.101	0.012
Free TFPI	0.536*	-0.399*	-0.280*

FVIII????

Assay determinants
ETP

Assay conditions	Coagulation factors	Coagulation inhibitors
1 pM TF	Fibrinogen Factor XII	Antithrombin Free TFPI
14 pM TF	Fibrinogen Prothrombin Factor V	Antithrombin Free TFPI
14 pM TF +APC	Prothrombin Factor X (Factor V Leiden)	Free protein S Free TFPI

Thrombin generation routine?

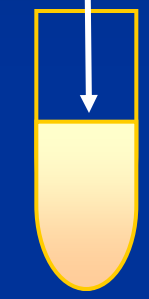
Not Yet!!

Other applications?

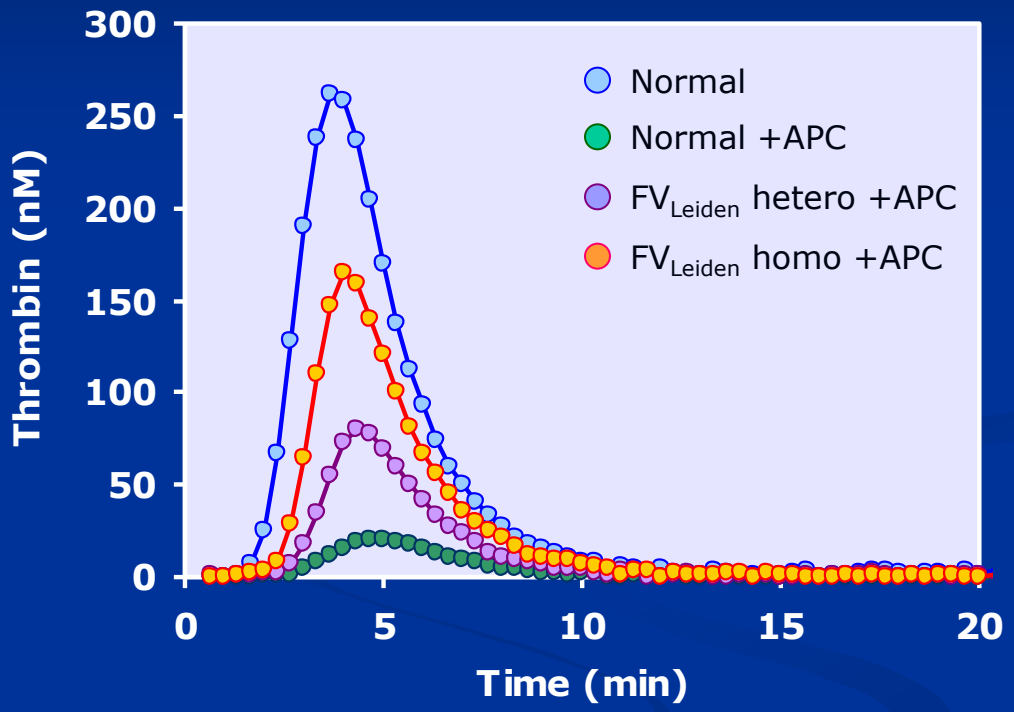
Application in clinical studies

Thrombin Generation-based APC Resistance Test

Tissue factor/$CaCl_2$
+/- APC

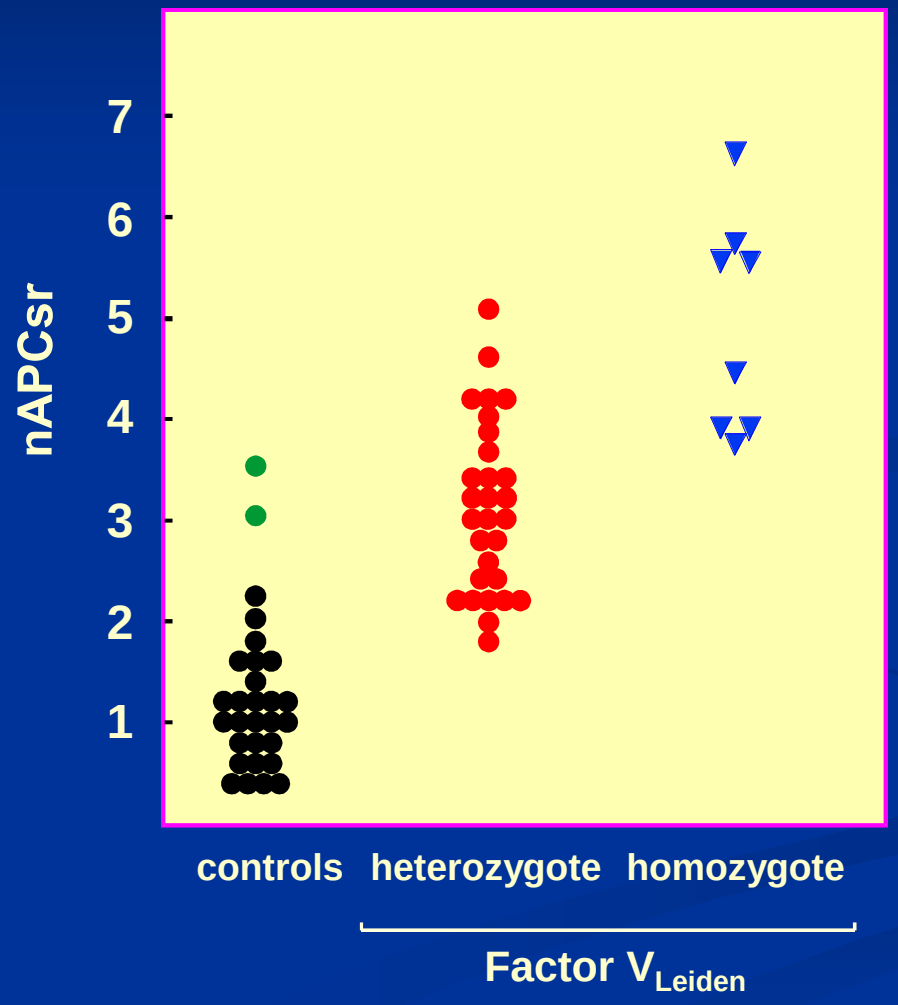


Plasma

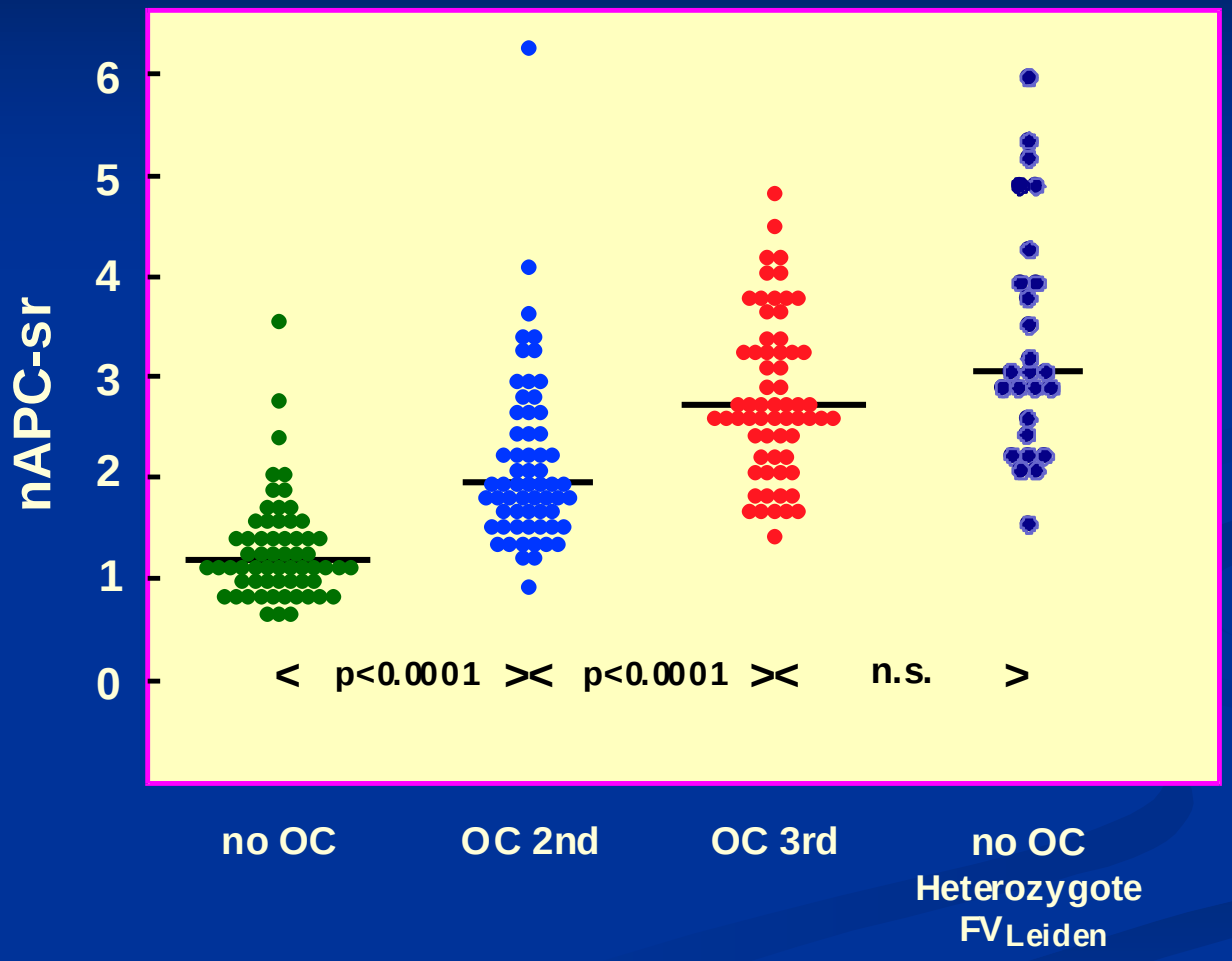


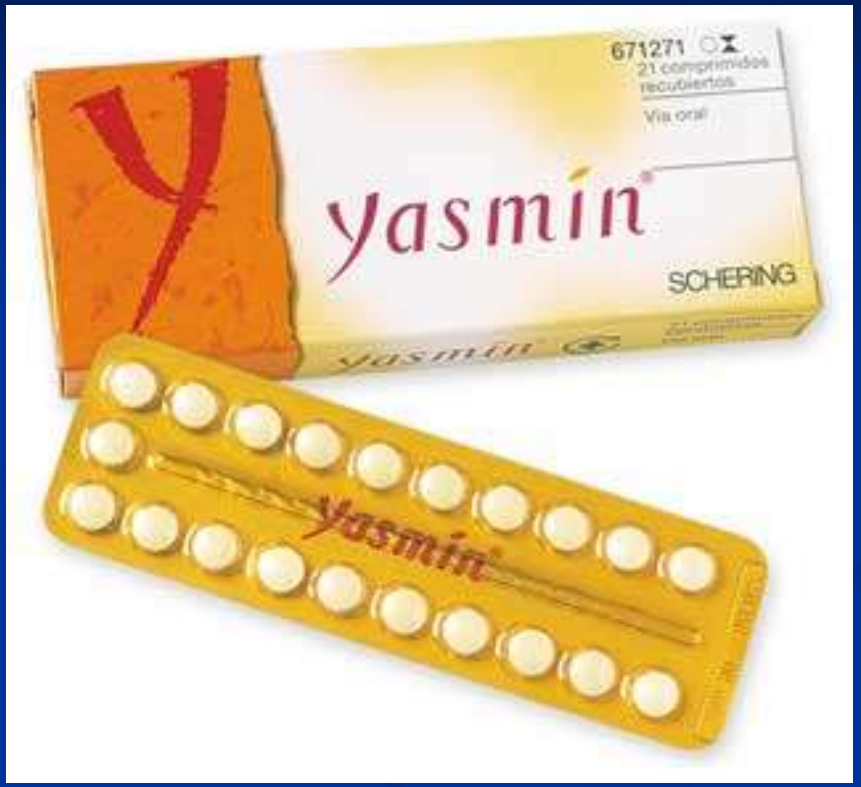
$$nAPCsr = \frac{(\%\ \text{rest thrombin formation}_{+APC})_{patient}}{(\%\ \text{rest thrombin formation}_{+APC})_{normal\ plasma}}$$

APC Sensitivity Ratio in Normal and Factor V_{Leiden} Plasma



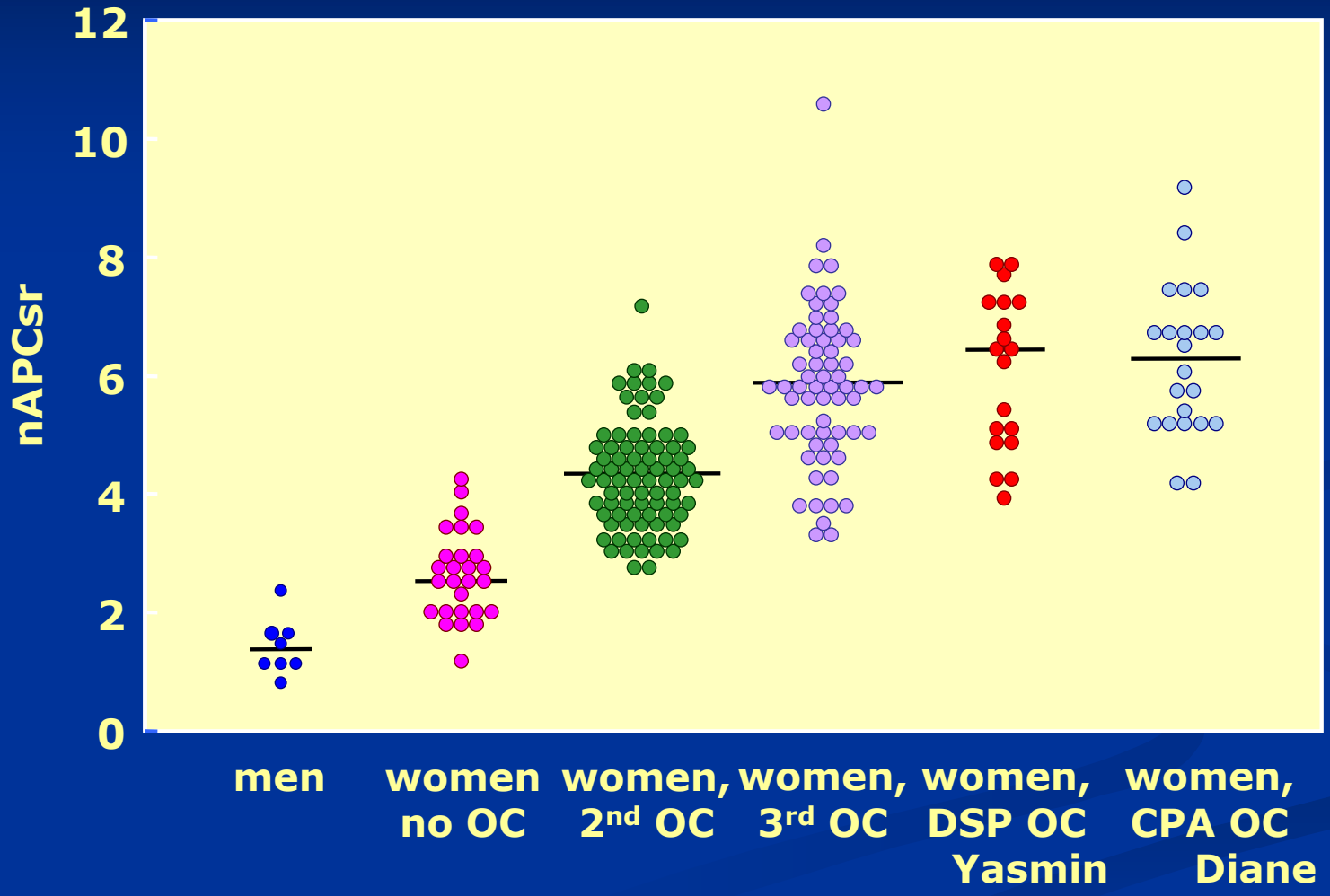
Acquired APC Resistance and Oral Contraceptives





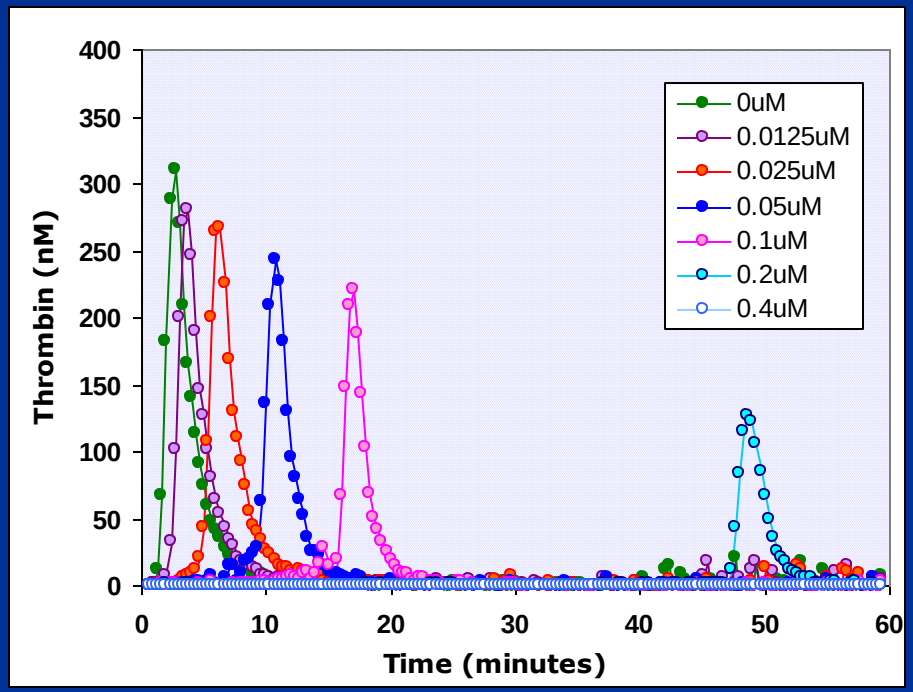
Diane-35: 35 μg ethinylestradiol 2 mg cyproterone acetate
Yasmin: 30 μg ethinylestradiol 3 mg drospirenone

Newer OC and APC Resistance

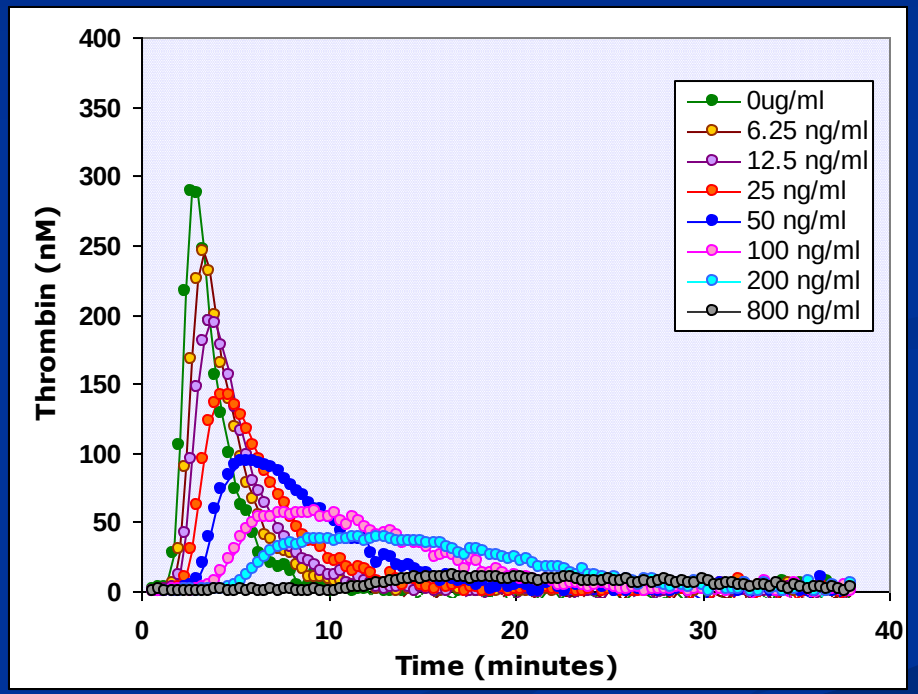


Application in clinical studies
Testing new anticoagulants

Thrombin generation + hirudin



Thrombin generation + rivaroxaban



Experiment: Kristin Winckers and Stella Thromassen

Application in clinical studies

Thrombin generation as a tool to discover new genetic risk factors for venous thrombosis (VTE)

VTE is a multifactorial disease with a strong genetic component

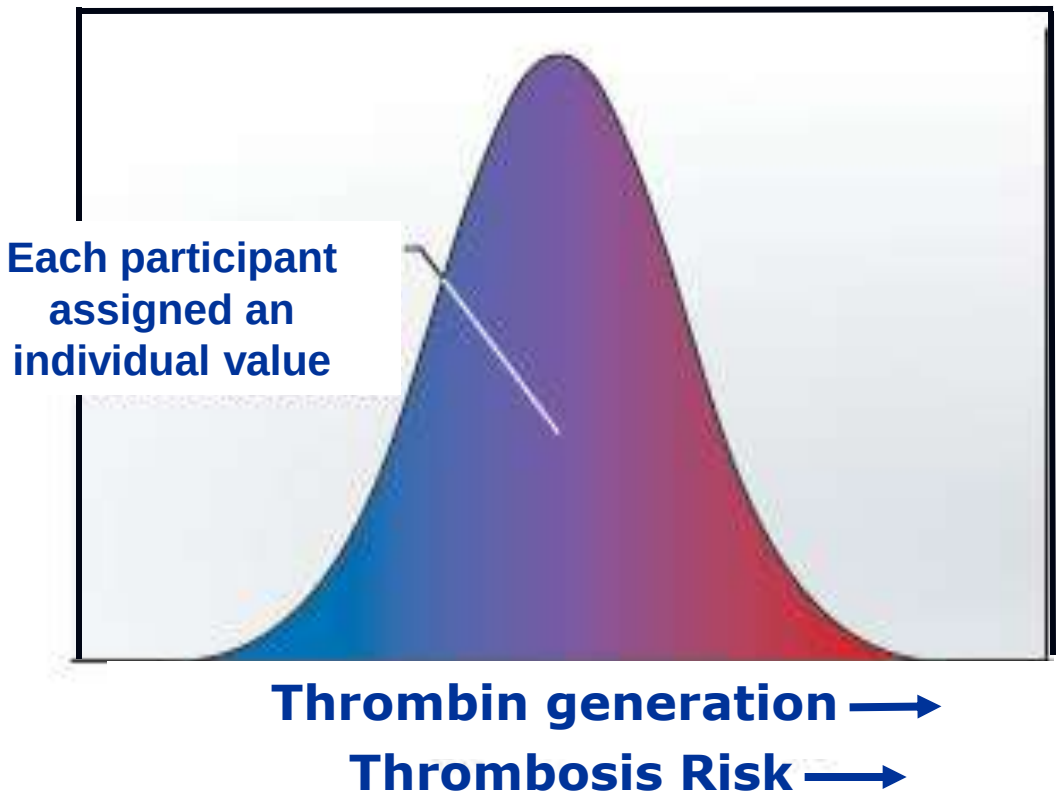
- Candidate gene and Genome-wide approach → Several new genetic risk factors for VTE

Problem: Difficult to establish (low risk, low prevalence)

Can thrombin generation as intermediate phenotype be of help to estimate thrombosis risk?

Intermediate phenotype

An intermediate phenotype is a clinical parameter the value of which correlates with the risk of a disease



Power Calculation

FV R2 haplotype

Associated with reduced FV levels and mild APC resistance

Does it increase the risk of VTE?

Epidemiological study

Carrier frequency 10% Odds Ratio 1.15

Power calculation: Significance p = 0.05 Power 90%

11470 Controls 11470 patients

Thrombin generation + APC

Carrier frequency 10% ETP_{+APC} 141 vs 236

Power calculation : Significance p = 0.05 Power 90%

130 individuals

Thrombin generation as an intermediate phenotype for venous thrombosis

A proof-of-concept study

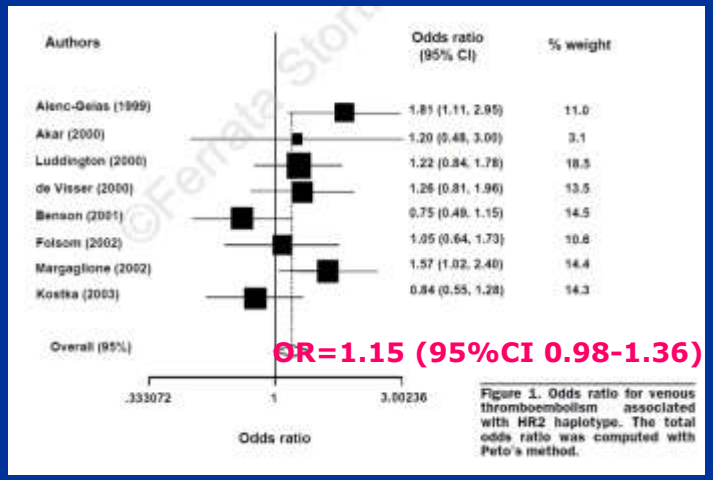
Olivier Segers[1]; René van Oerle[2]; Hugo ten Cate[2]; Jan Rosing[1]; Elisabetta Castoldi[1]

FV R2 haplotype

Does it increase the risk of VTE?

Meta-analysis

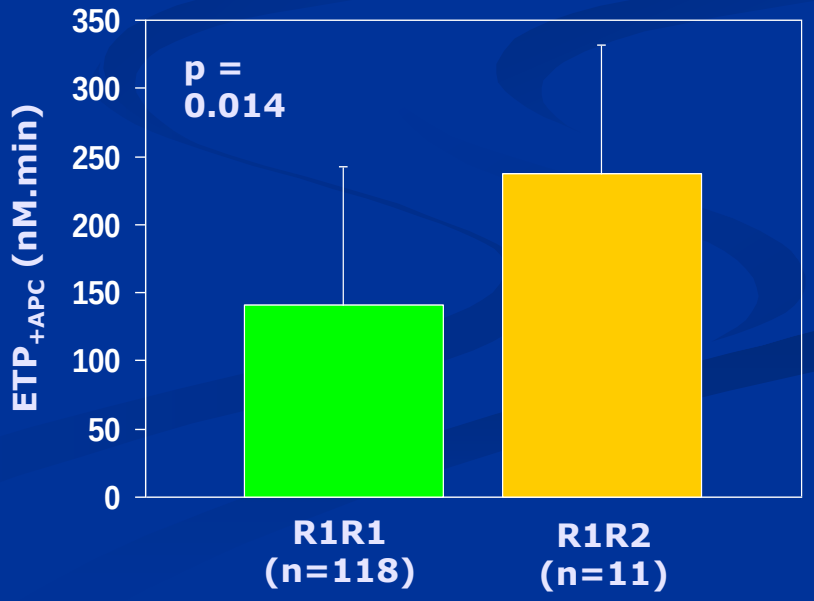
(2696 VTE cases & 7710 controls)



Castaman *et al.* 2003

Thrombin generation + APC

(129 healthy individuals)

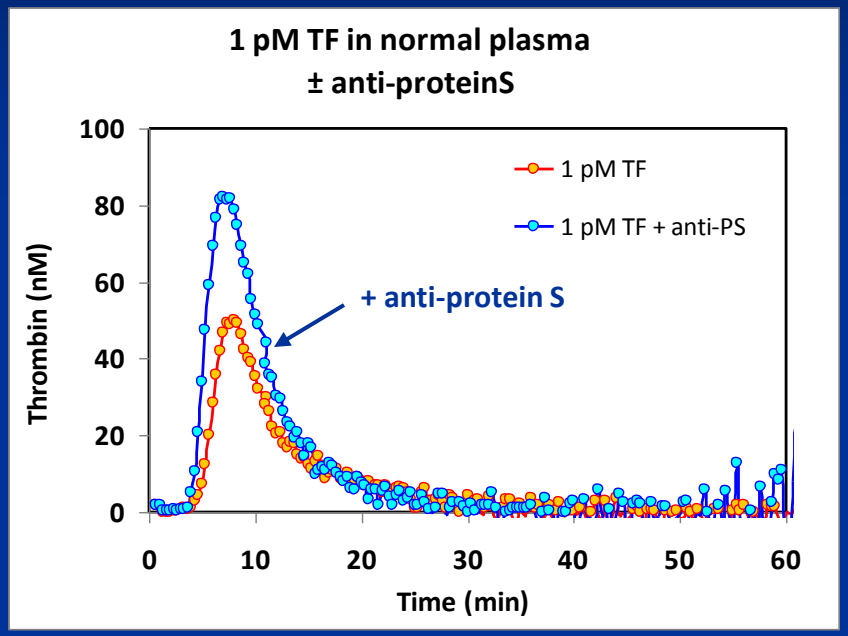


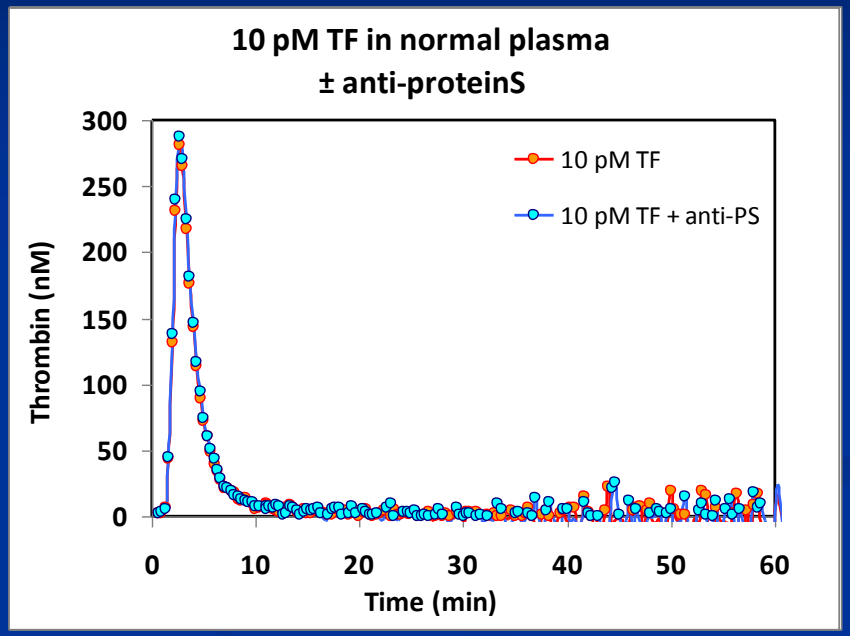
Application in research

Protein S

- Protein S is a cofactor of activated protein C (APC)
- Protein S exhibits anticoagulant activity in the absence of APC

Effect of protein S on thrombin generation





Protein S only expresses APC-independent anticoagulant activity in the presence of TFPI.

Conclusion: protein S acts as cofactor of TFPI

Conclusions

Future application of thrombin generation in routine laboratories requires:

- Rigourous standardisation of plasma preparation as well as test conditions.
- Clinical validation of standardised tests
- Knowledge of determinants at various test conditions

Thrombin generation is a powerful tool in:

- Clinical studies (testing drugs / establishing thrombosis risk factors
- Basic haemostasis research

Thank you for your attention!

Acknowledgements: Guido Tans, Stella Thomassen, Kristin Winckers, Coen Hemker, Tilman Hackeng et al.