

CONGRÈS  
d'FRANÇAIS  
d'HÉMOSTASE

10-12  
MAI  
2023



Palais des Congrès

SAINT-MALO

Le Grand Large



**SFTH**

SOCIÉTÉ FRANÇAISE  
DE THROMBOSE  
ET D'HÉMOSTASE

**HiTh**

INSERM UMR 1176

Hémostase

Inflammation - Thrombose

université  
PARIS-SACLAY

**Inserm**

La science pour la santé  
From science to health

# Agonistic nanobodies stimulating protein S function : sailing the unknown

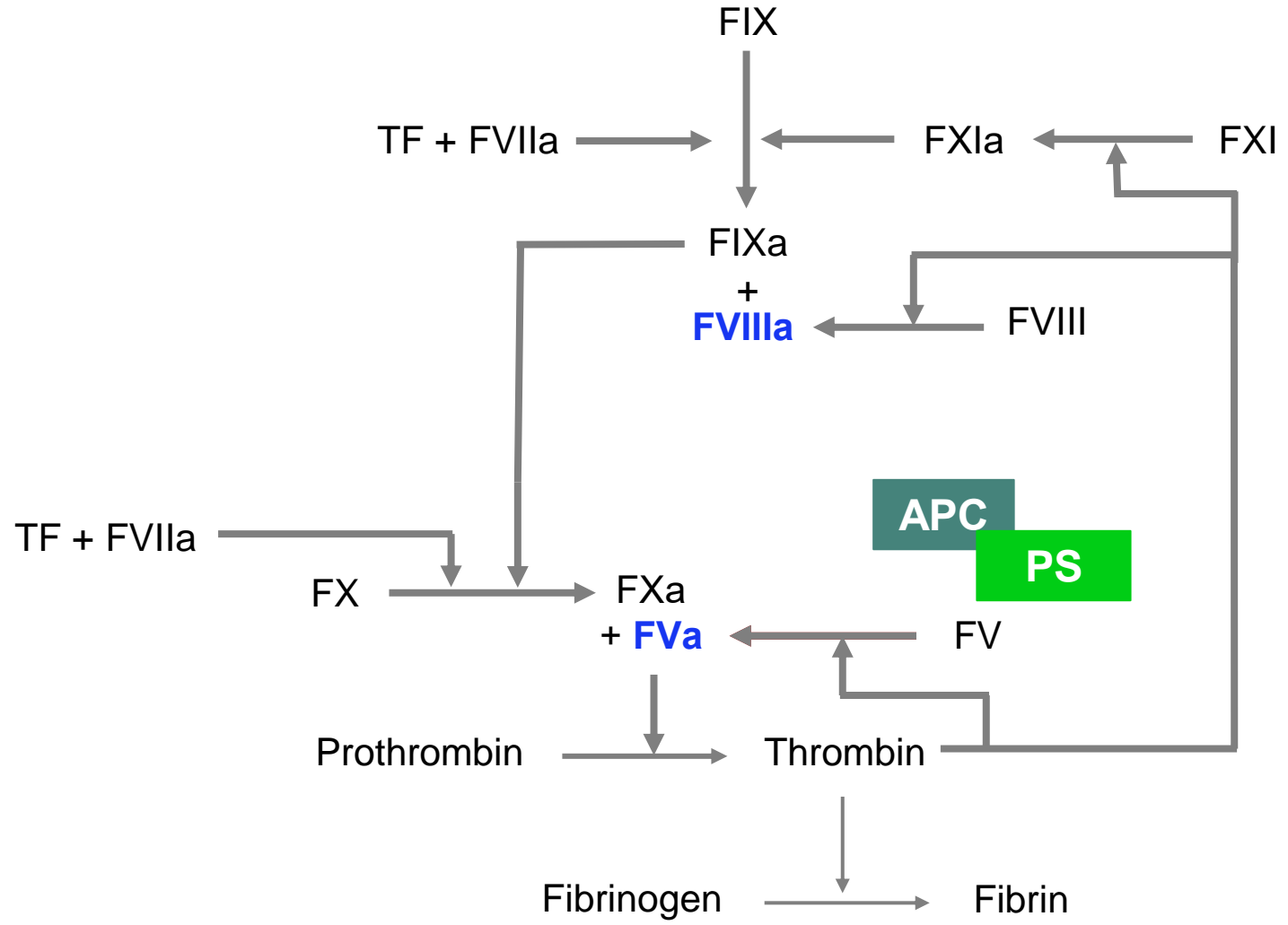
François Saller, PhD

# Disclosures for François Saller, PhD

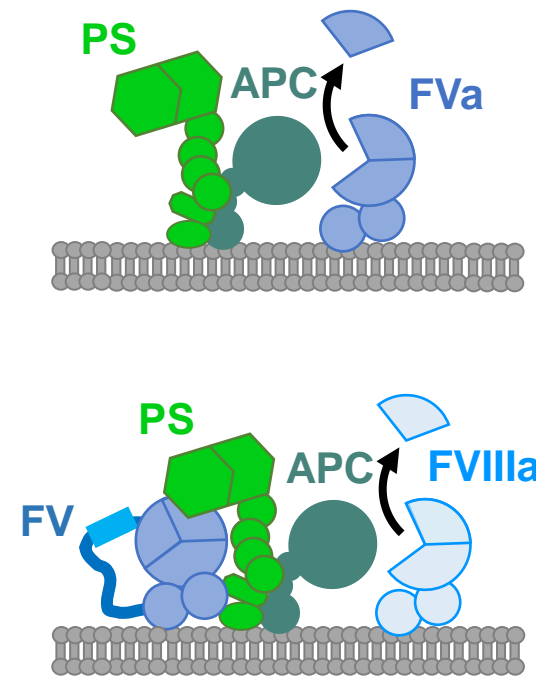
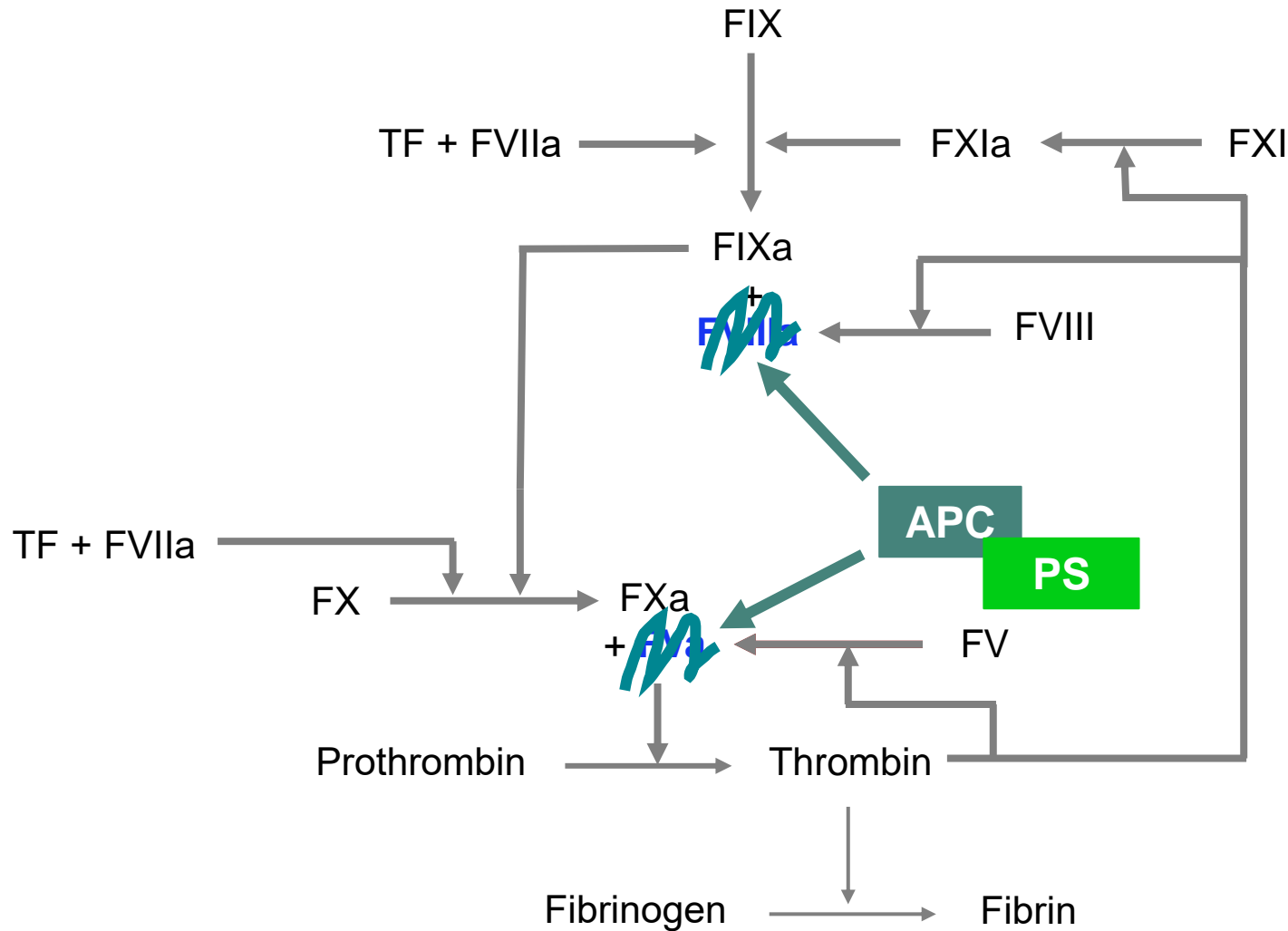
Research funded by the Agence Nationale de la Recherche (ANR)

Research funded by CSL Behring

# Anticoagulant functions of PS

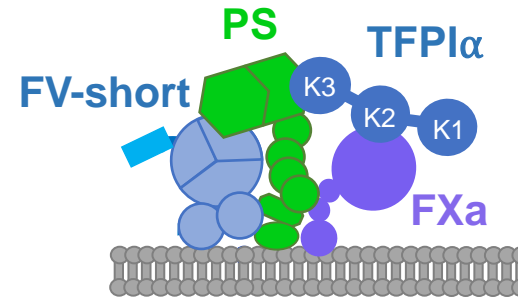
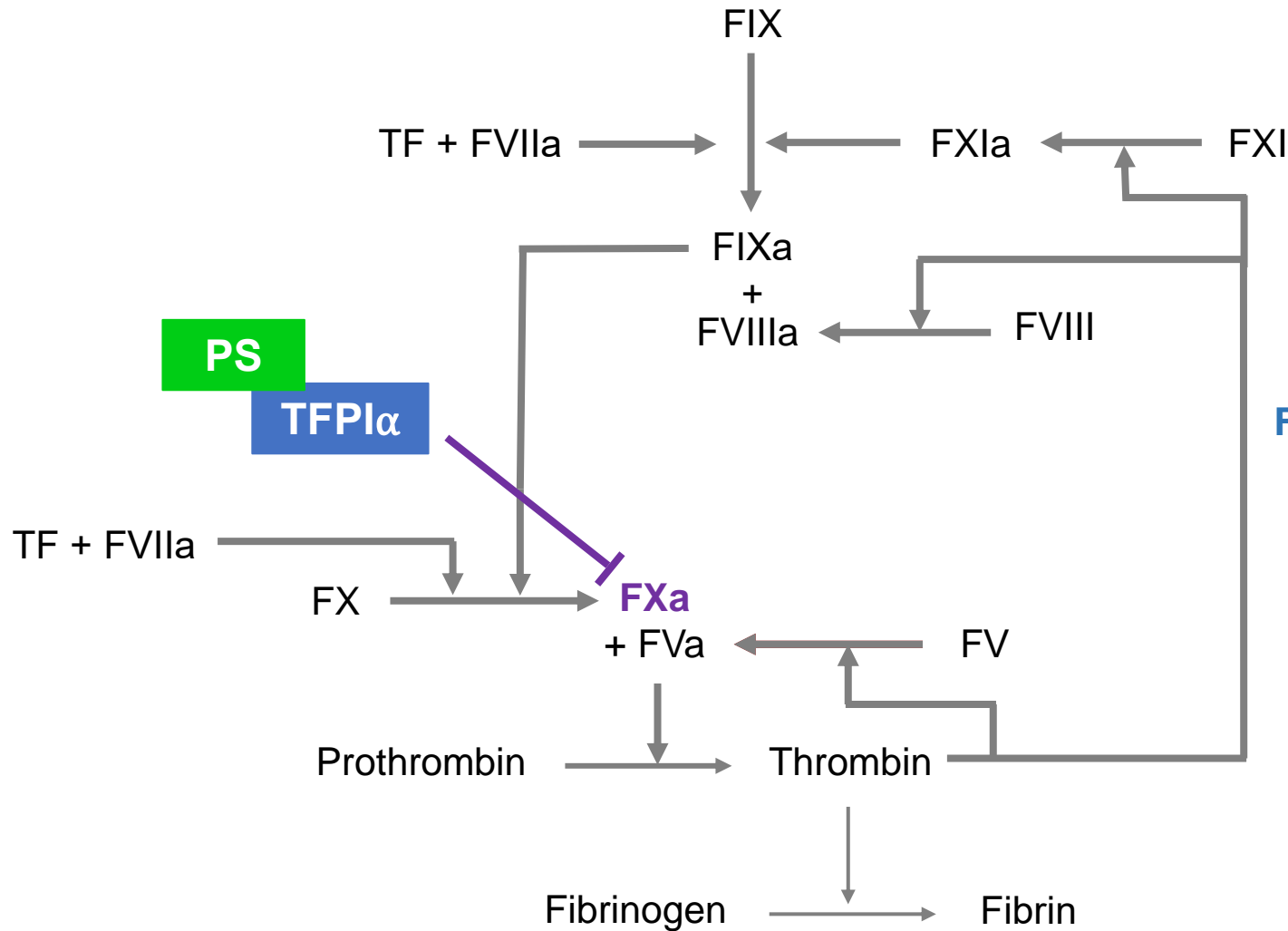


# Anticoagulant functions of PS



**APC-cofactor activity of PS**

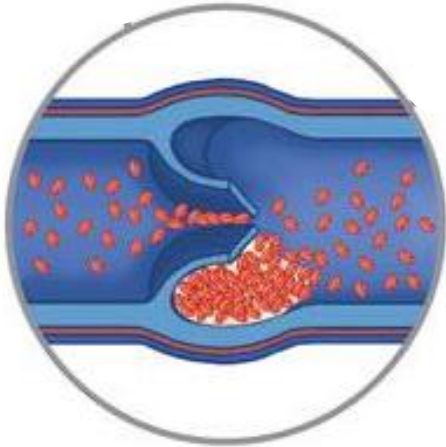
# Anticoagulant functions of PS



**TFPI $\alpha$ -cofactor activity of PS**

# Physiological importance of anticoagulant protein S (PS)

## Mild deficiency



Recurrent thrombo-embolic events

Deep Vein Thrombosis (DVT)

Pulmonary Embolism (PE)

## Severe deficiency

### Congenital



### Acquired



Microvascular thromboses

Disseminated Intravascular Coagulation (DIC)

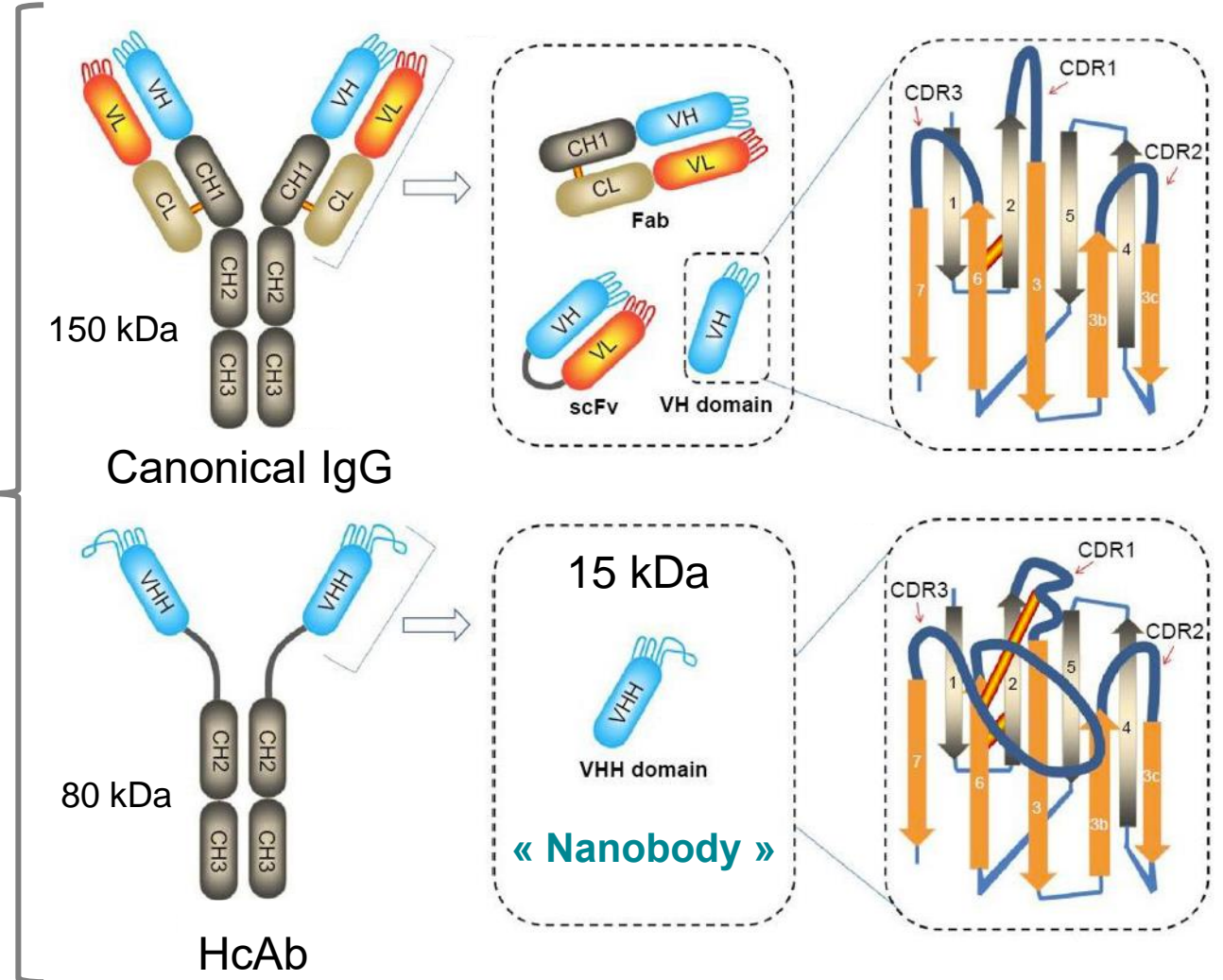
Purpura fulminans

Cutaneous necrosis

# « Nanobodies » or single-domain antibodies (sdAbs)



Llama (Camelids)



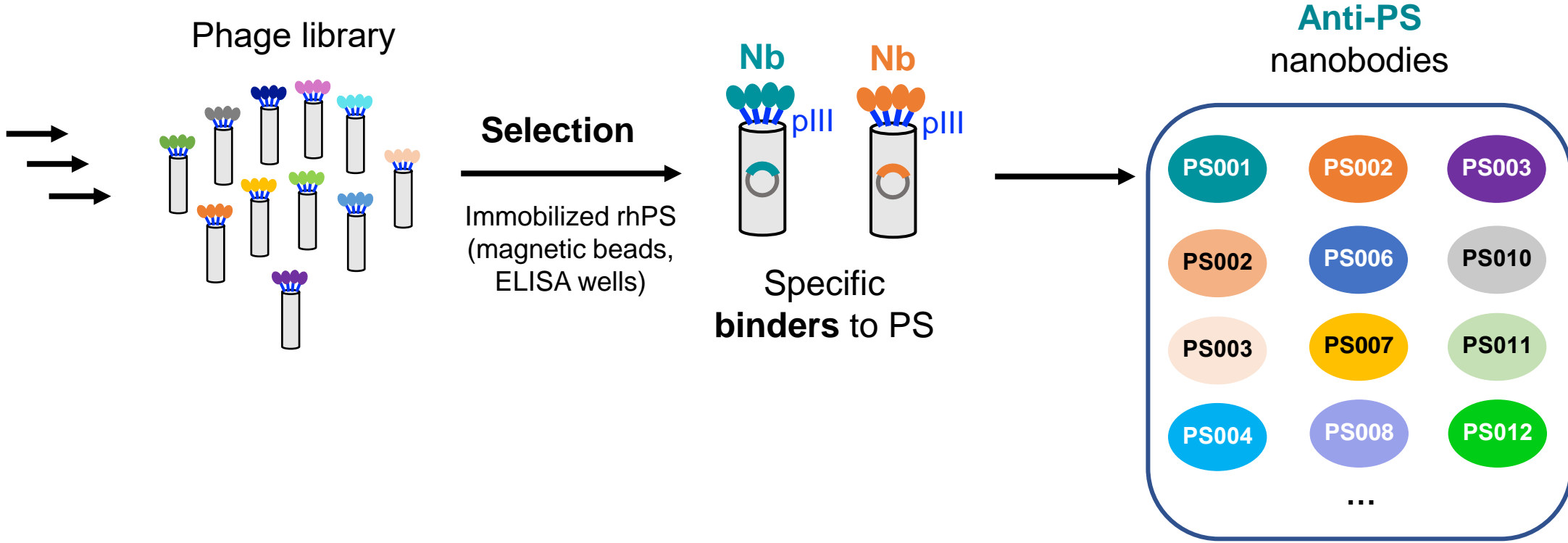
- Small, stable, soluble at high concentrations
- High tissue penetration
- Low immunogenicity *per se*
- Easy engineering and expression in *E. coli*
- Recognition of **cryptic & original epitopes**
- Can be identified by **phage-display**

Wang *et al.* Int J Nanomed 2016

# Identification of anti-PS nanobodies by phage-display



Immunized  
with **rhPS**



Josepha Clara Sedzro



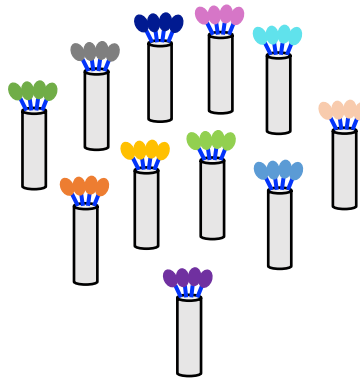


# Identification of anti-PS nanobodies by phage-display



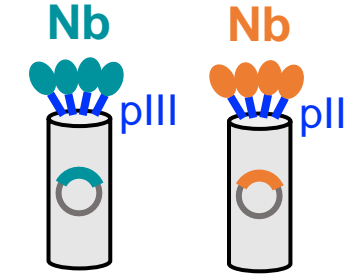
Immunized with rhPS

Phage library



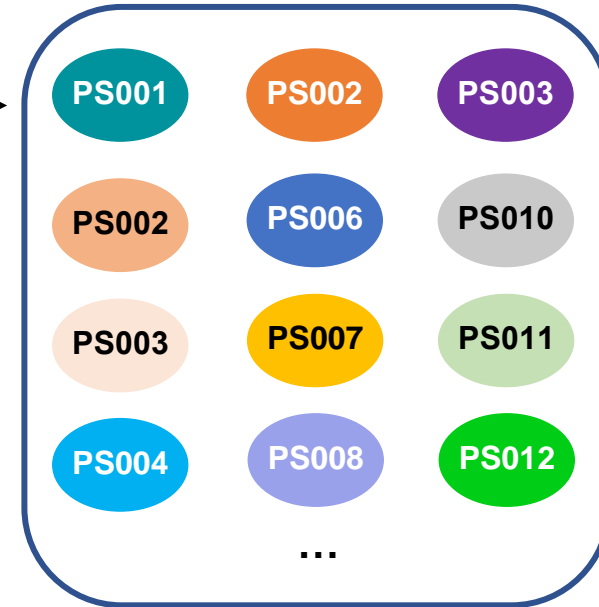
**Selection**

Immobilized rhPS  
(magnetic beads,  
ELISA wells)



Specific binders to PS

**Anti-PS nanobodies**

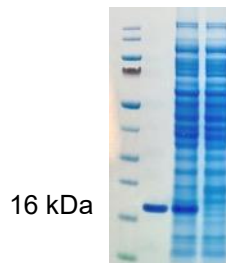


**Functional Screening**



APC-cofactor activity of PS

**Purification**



Lysis  
(Sonication)

**Expression**

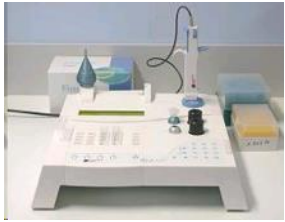


SHuffle *E. coli*

Josepha Clara Sedzro

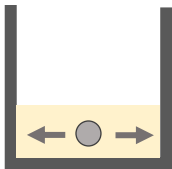


# Plasma-based APC-cofactor activity assay



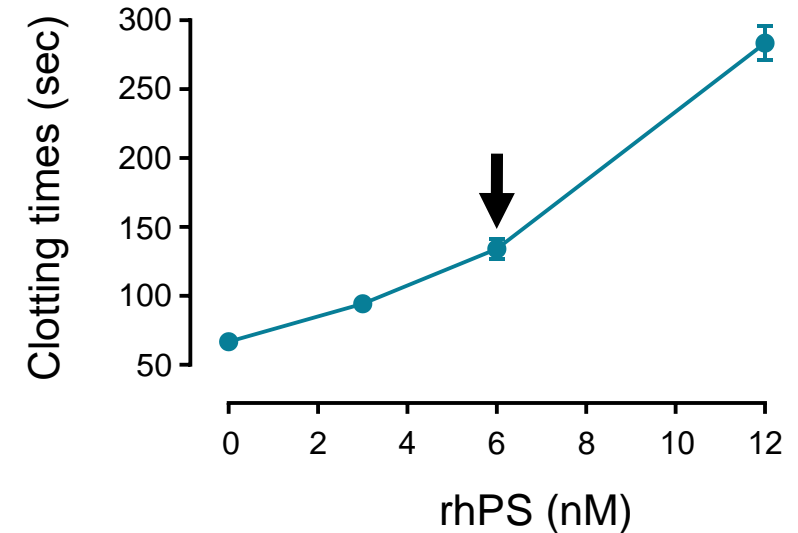
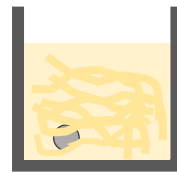
## APTT-based assay (STACLOT<sup>®</sup> PS)

PS-deficient  
plasma  
(25  $\mu$ L)



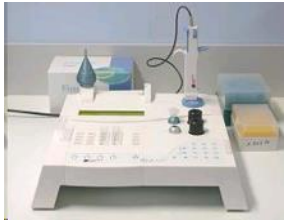
- 1) rhPS 25  $\mu$ L
  - 2) + Bovine FVa 25  $\mu$ L
  - 3) + APC 25  $\mu$ L
  - 4) + APTT Reagent + PL 25  $\mu$ L
- >
- 5) 2 min, 37°C
  - 6) + 25 mM CaCl<sub>2</sub> (25  $\mu$ L)

Coagulation



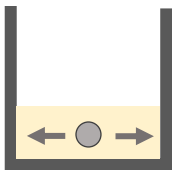
rhPS dose-dependently **prolongs clotting times**  
measured only **in the presence of APC**

# Functional screening of anti-PS nanobodies



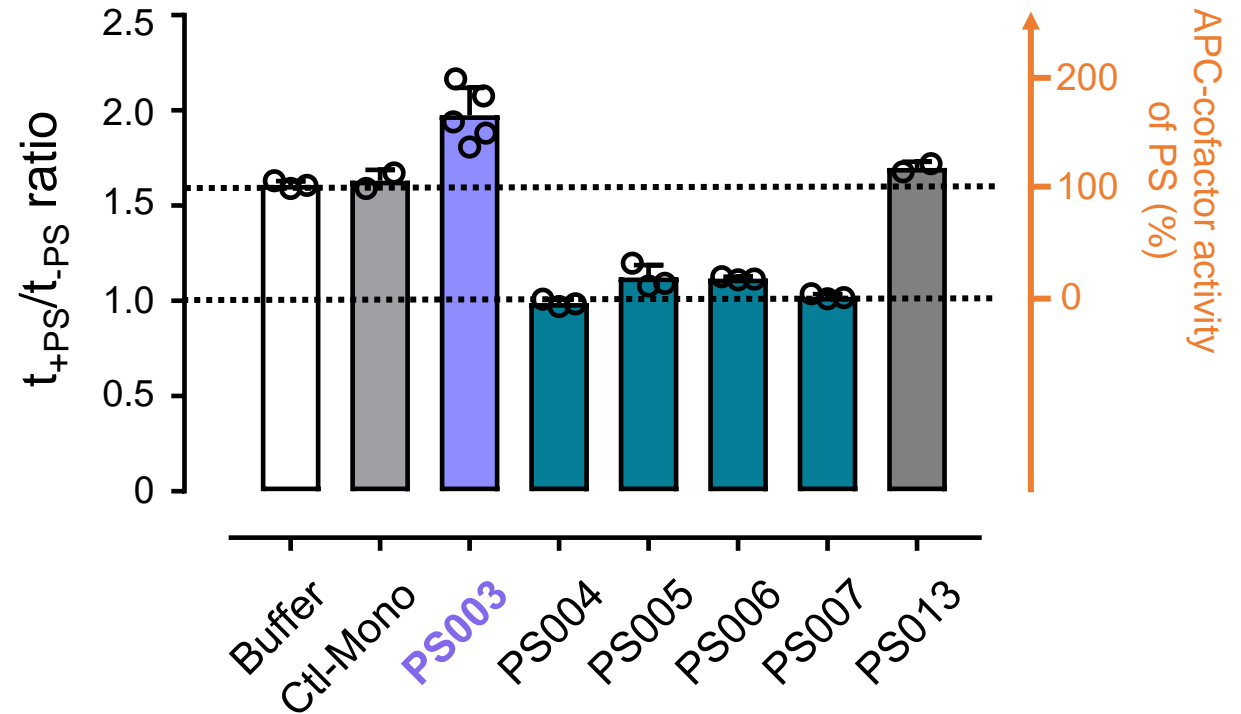
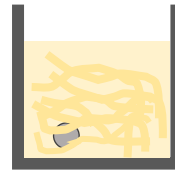
## APTT-based assay (STACLOT® PS)

PS-deficient plasma (25  $\mu$ L)



- 1) rhPS + sdAb 25  $\mu$ L
- 2) + Bovine FVa 25  $\mu$ L
- 3) + APC 25  $\mu$ L
- 4) + APTT Reagent + PL 25  $\mu$ L
- 5) 2 min, 37°C
- 6) + 25 mM CaCl<sub>2</sub> (25  $\mu$ L)

Coagulation

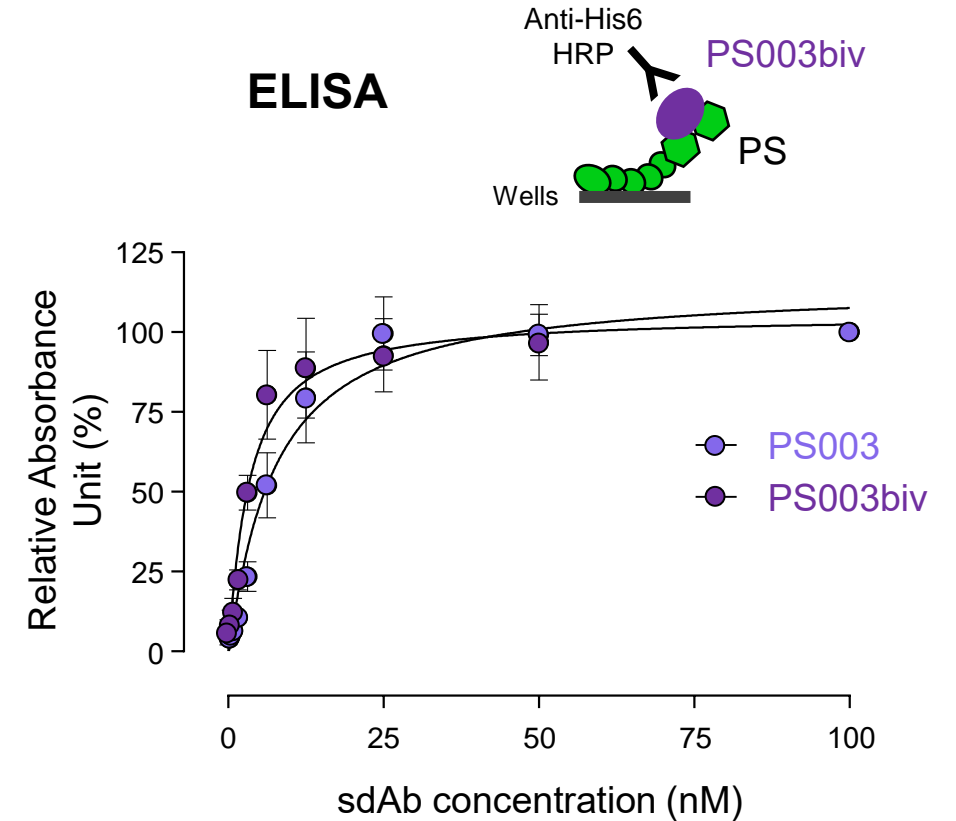
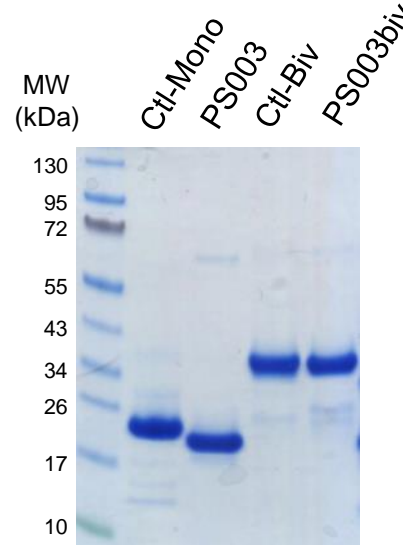
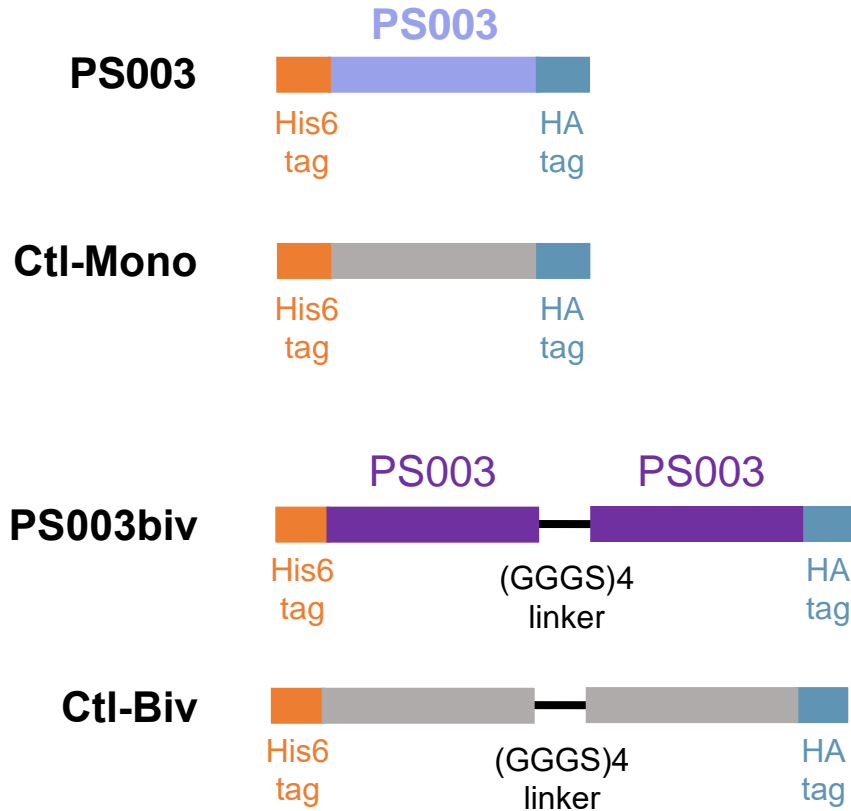


**PS003** appeared to **enhance the APC-cofactor activity of PS** in this assay !!



Josepha Clara Sedzro

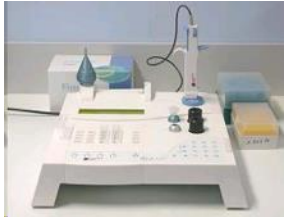
# Generation of monovalent and bivalent forms of PS003



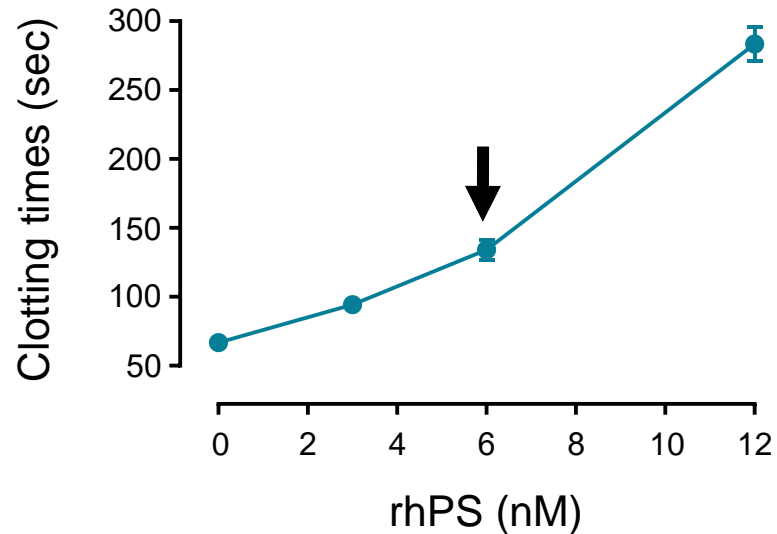
$K_{d\ app} = 6.7 \pm 1.2\ nM$  for PS003

$K_{d\ app} = 2.8 \pm 0.6\ nM$  for PS003biv

# Effects of PS003 in our APC-cofactor activity assay

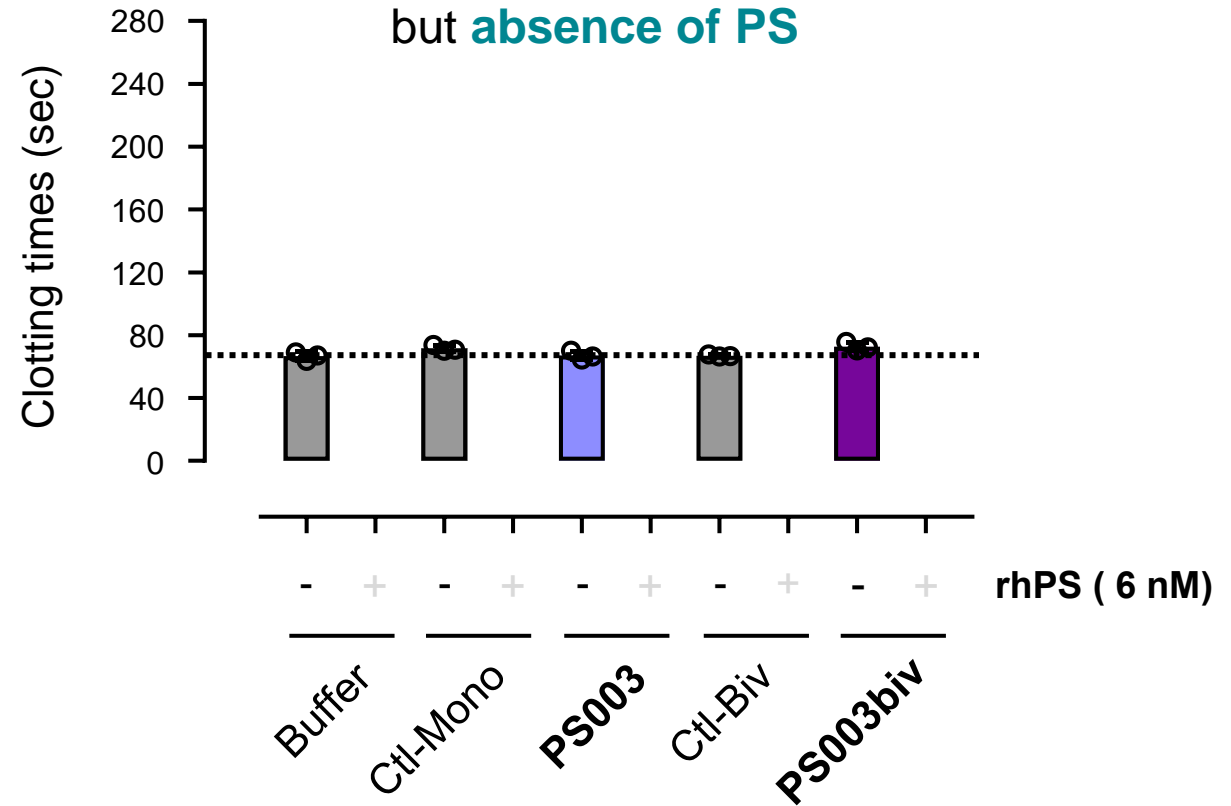


APTT-based assay  
 (STACLOT® PS)

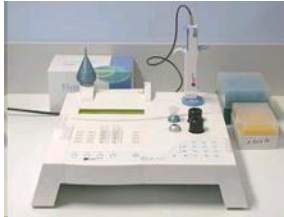


In the presence of APC

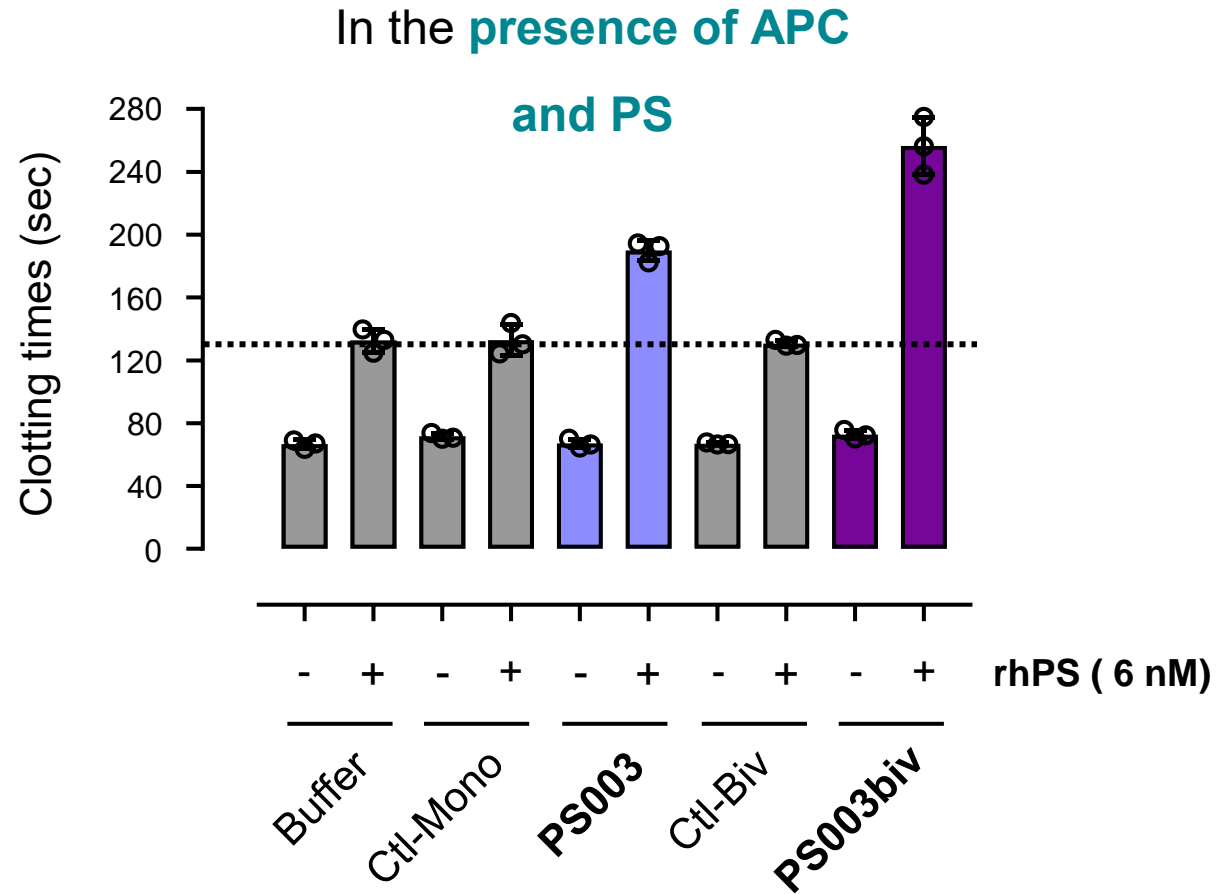
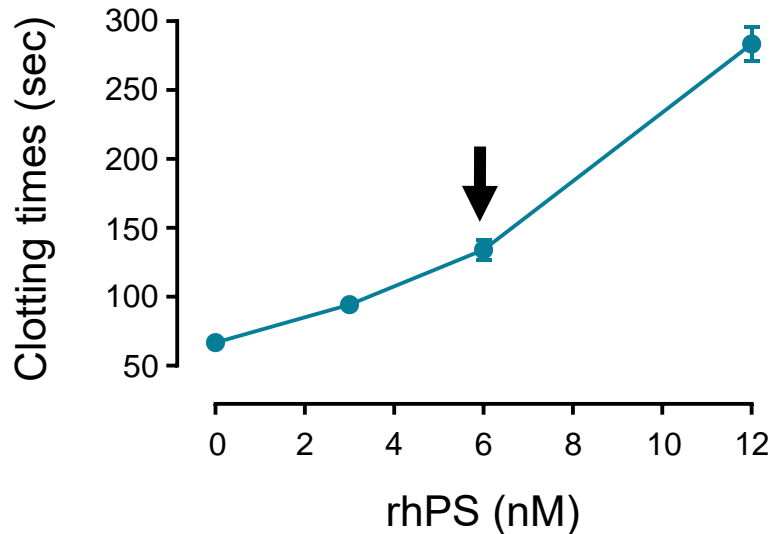
but absence of PS



# Effects of PS003 in our APC-cofactor activity assay



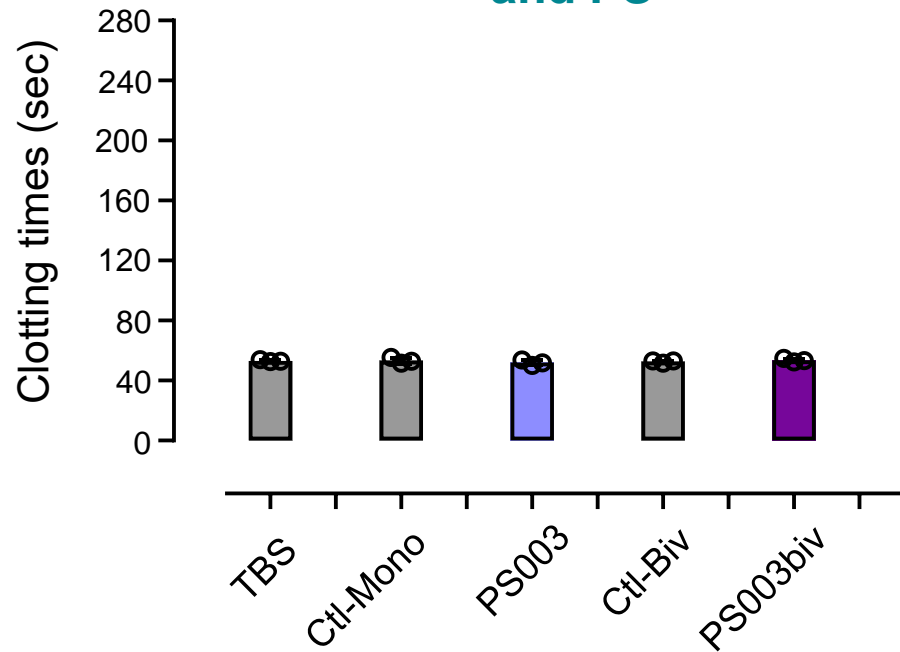
APTT-based assay (STACLOT® PS)



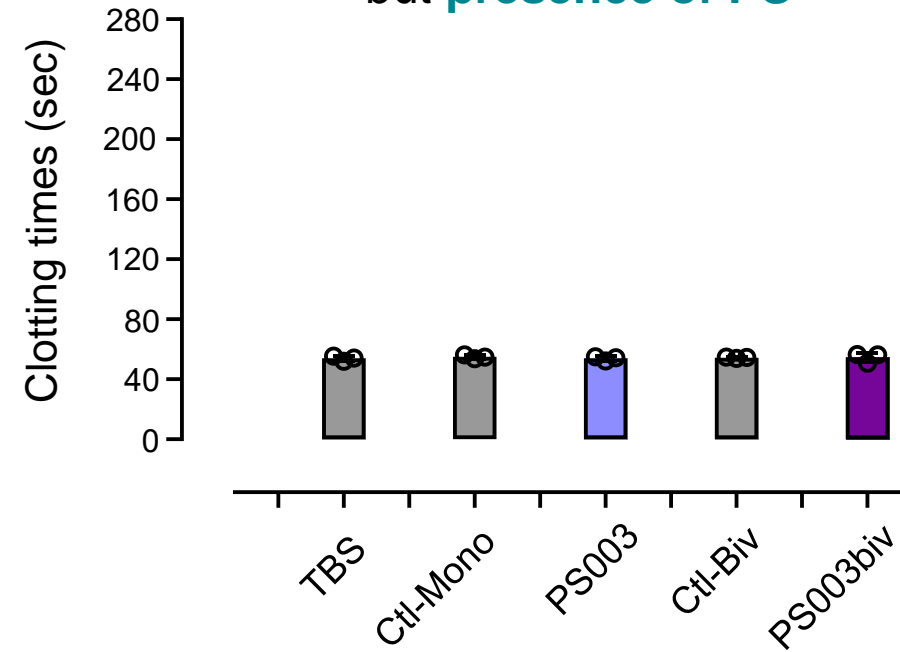
Bivalent PS003biv has a significantly **greater enhancing effect** than monovalent PS003

# Effects of PS003 in our APC-cofactor activity assay

In the **absence of APC**  
**and PS**



In the **absence of APC**  
but **presence of PS**



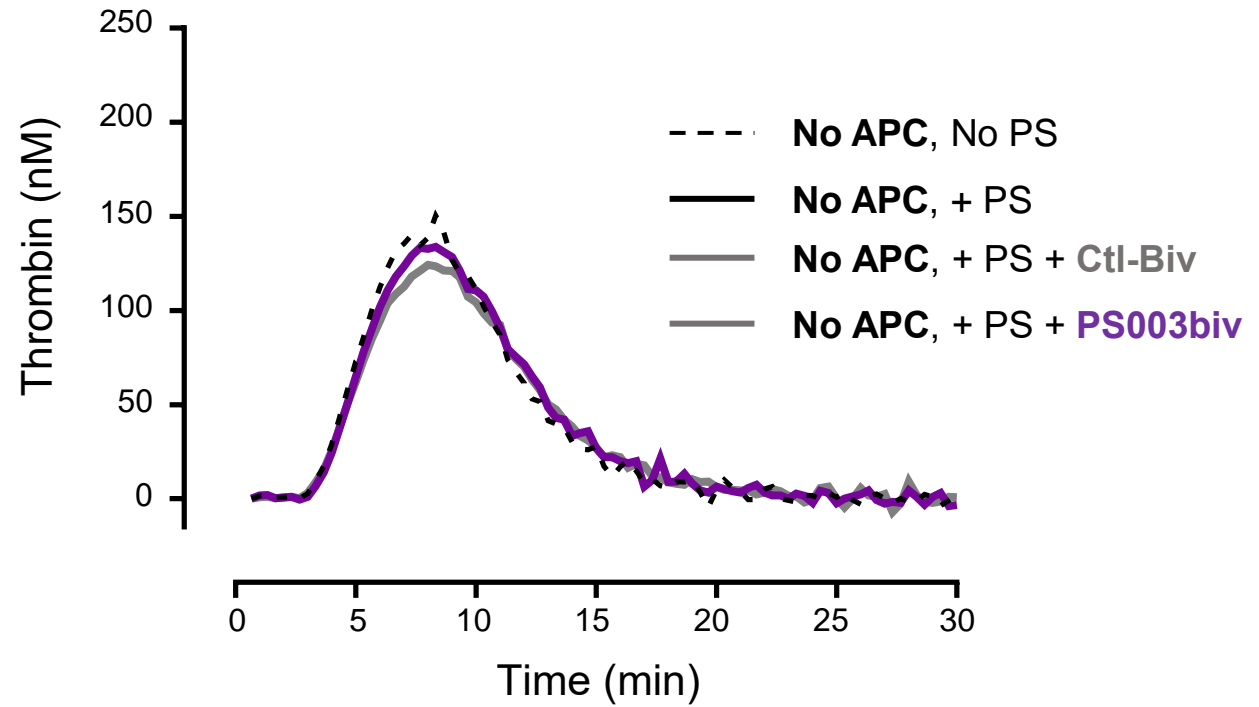
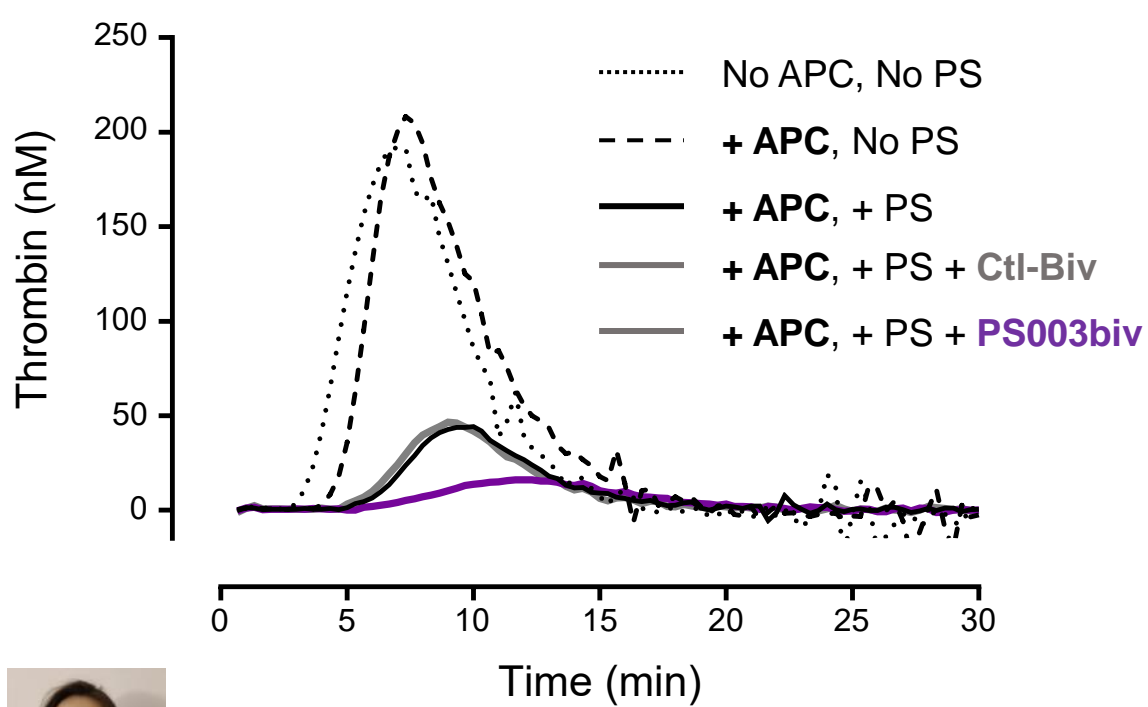
The functional effects of PS003 and PS003biv are **highly dependent on PS and APC**

No effects on **APC-independent** anticoagulant activities of PS

# Effects of PS003 in a thrombin generation assay (TGA)

Thrombin generation triggered by **1 pM tissue factor** (and 4  $\mu$ M PL) in the **presence of APC** (30 nM)

In a **PS-depleted plasma** supplemented with a **normal plasma** as a source of PS



Claire Auditeau  
 PhD student

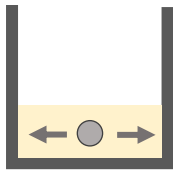


# Effects of PS003biv on FVa inactivation by APC/PS



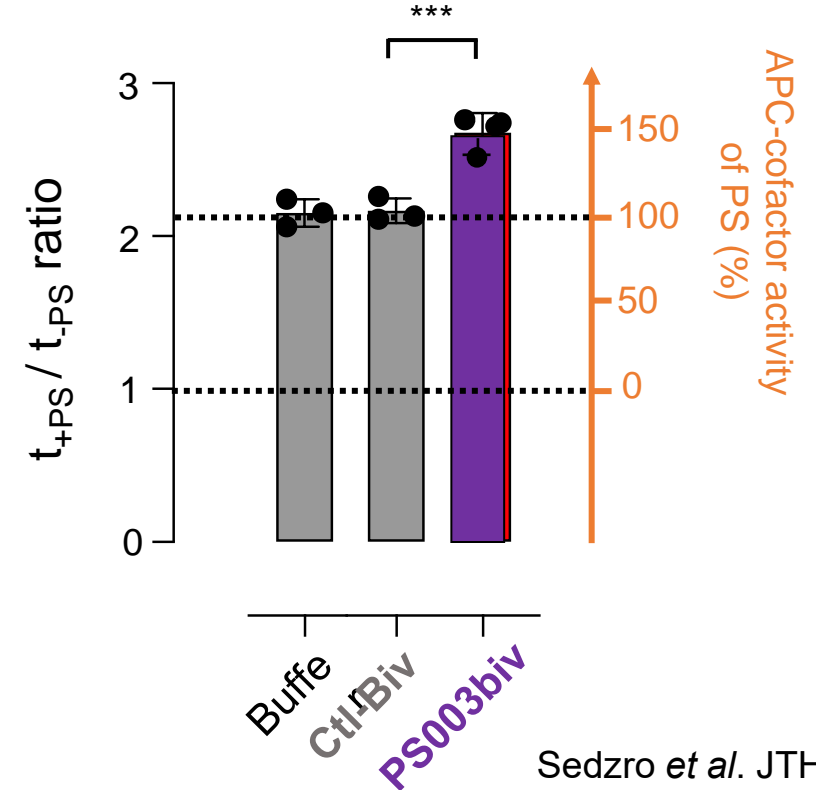
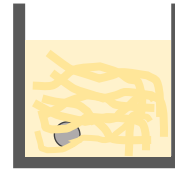
## FXa one-stage clotting assay

PS-deficient plasma (40  $\mu$ L)



- 1) + PL (10  $\mu$ M) 20  $\mu$ L
  - 2) + **RVV-X** (0.01 nM) 20  $\mu$ L
  - 3) + Purified APC (6.8 nM) 20  $\mu$ L
  - 4) + rhPS (50 nM)  $\pm$  sdAb (1.2  $\mu$ M) 20  $\mu$ L
- >
- 5) 2 min, 37°C
  - 6) + 25 mM CaCl<sub>2</sub> (50  $\mu$ L)

Coagulation



Sedzro *et al.* JTH 2022



Claire Auditeau  
 PhD student

PS003biv appears to **enhance FVa inactivation by APC**  
 in our plasma-based assay

# Effects of PS003biv on FVIIIa inactivation by APC/PS

Modified **plasma-based chromogenic FVIIIa activity** assay  
 (BIOPHEN™ FVIII:C kit, HYPHEN BioMed)

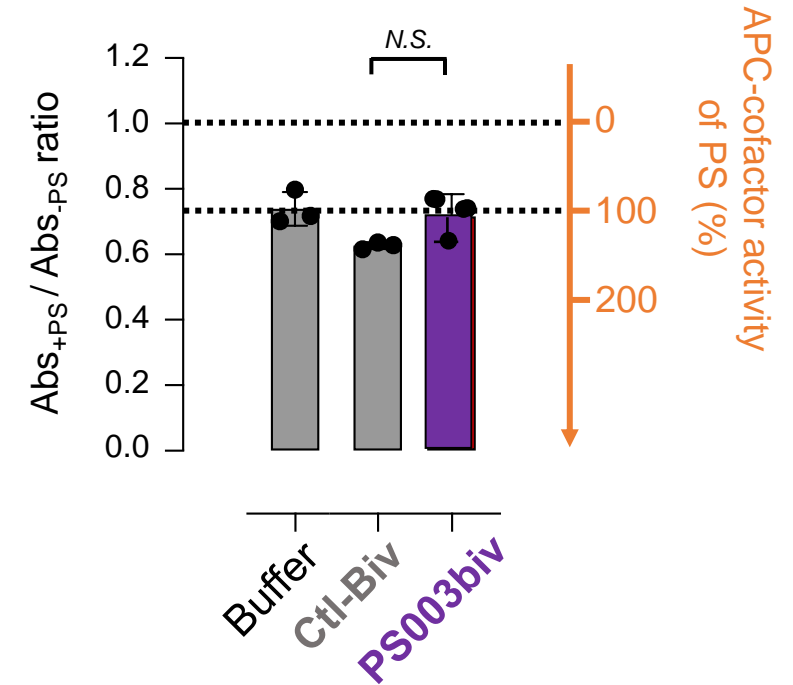
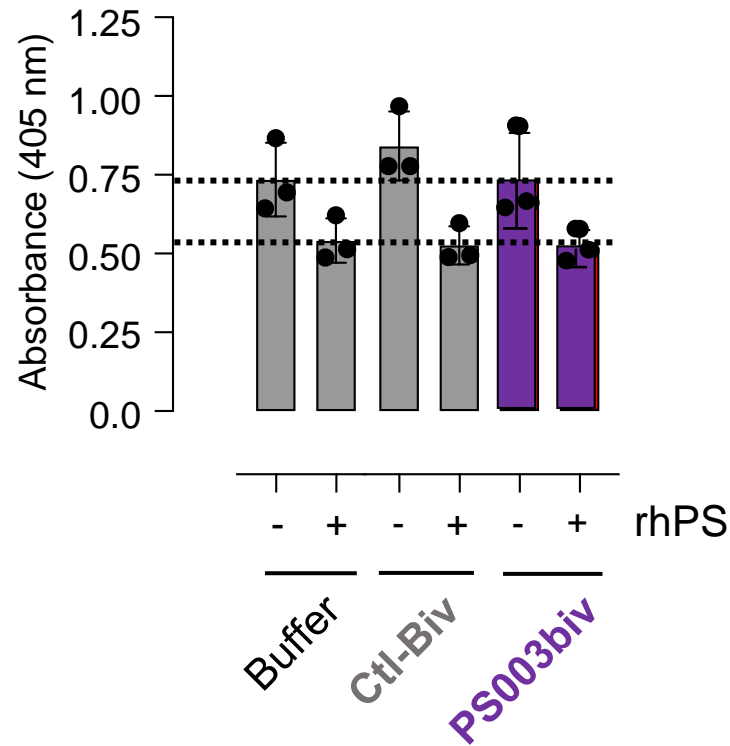


PS-deficient plasma  
 + rhPS  
 + APC



Claire Auditeau  
 PhD student

Sedzro *et al.* JTH 2022

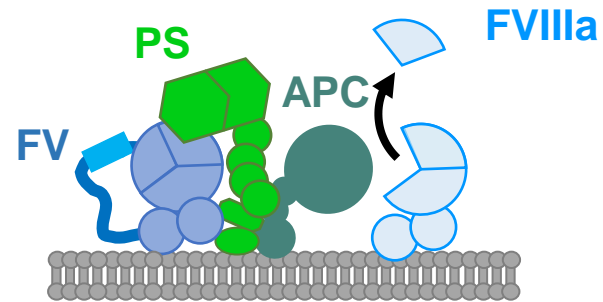
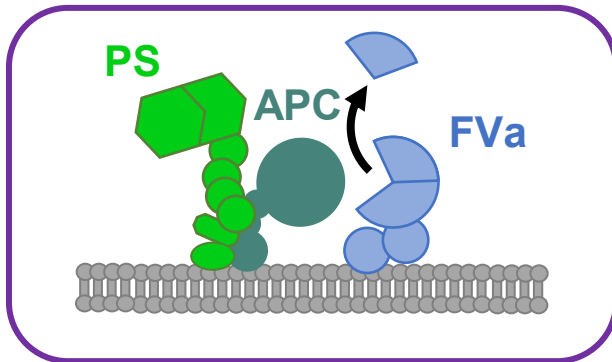


PS003biv did not **enhance FVIIIa inactivation by APC**  
 in this plasma-based assay

# Functional effects of PS003

## APC-cofactor activity

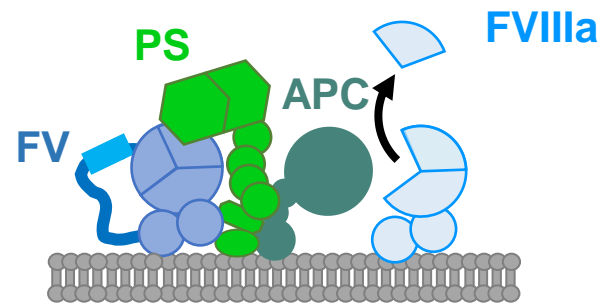
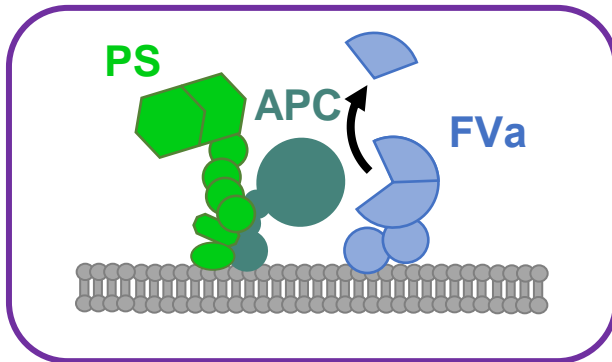
*Plasma-based assays*



# Functional effects of PS003

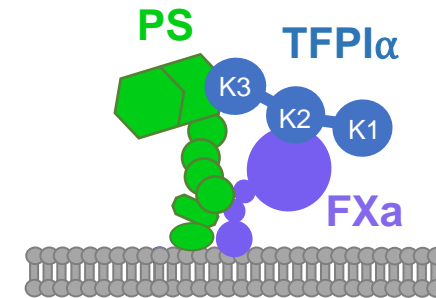
## APC-cofactor activity

*Plasma-based assays*



## TFPI $\alpha$ -cofactor activity

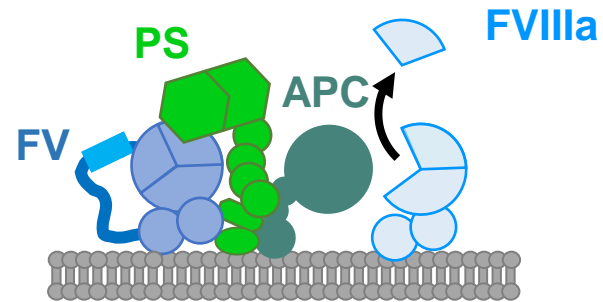
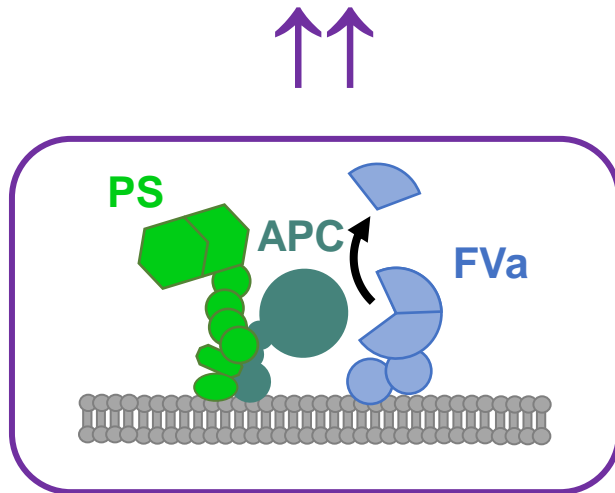
*Purified systems*



# Functional effects of PS003

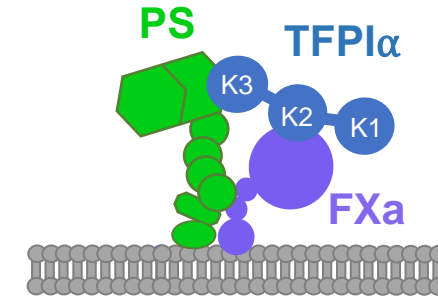
## APC-cofactor activity

*Plasma-based assays*



## TFPI $\alpha$ -cofactor activity

*Purified systems*

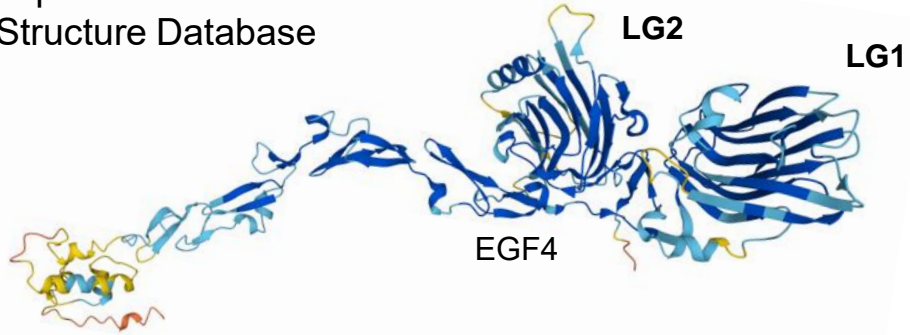


**Unexpected** and **mysterious** enhancing effect

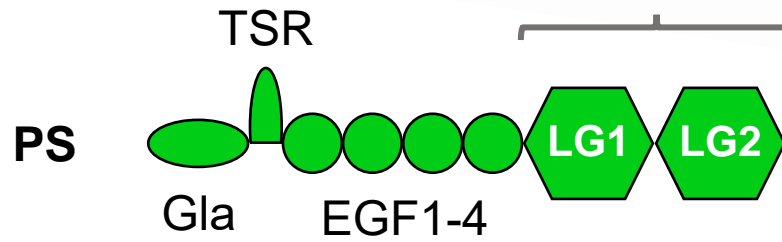
The mechanism of action is still **unknown** !!

# Epitope mapping of PS003

Alpha Fold Protein  
Structure Database

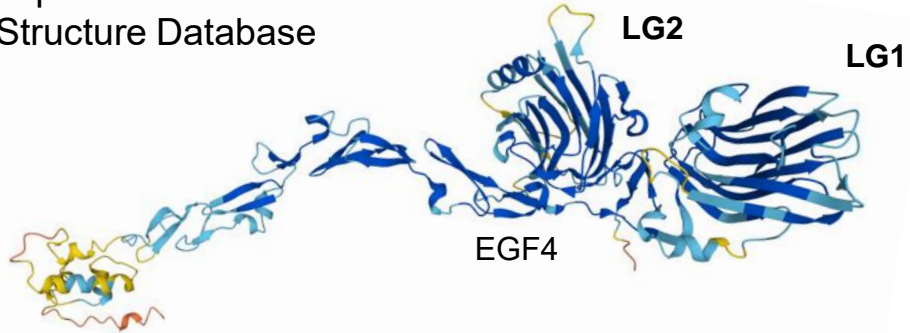


**SHBG domain**

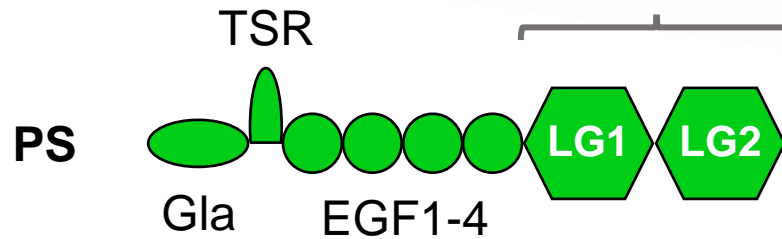


# Epitope mapping of PS003

Alpha Fold Protein  
Structure Database



**SHBG domain**

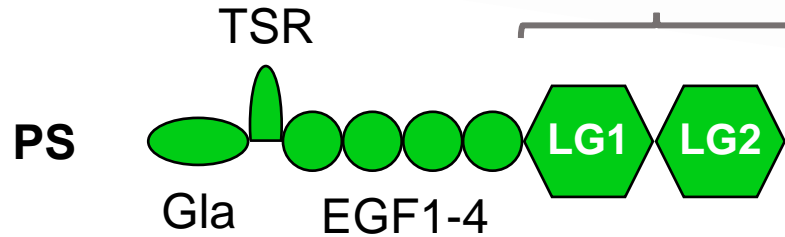
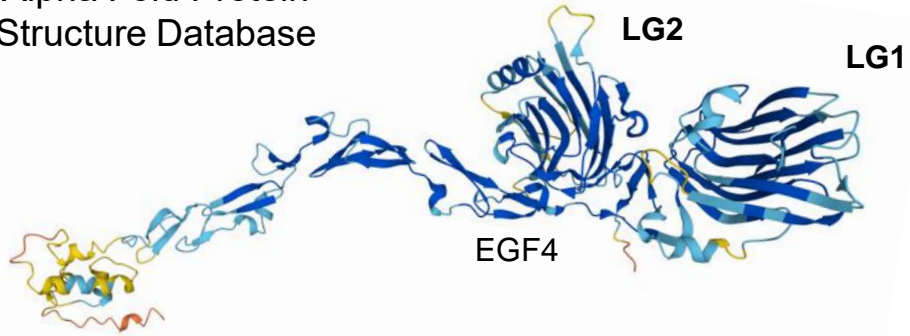


**rSHBG**

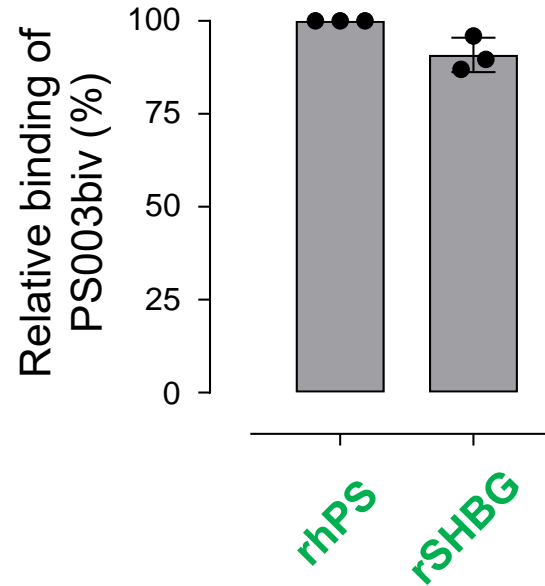


# Epitope mapping of PS003

Alpha Fold Protein Structure Database



rSHBG

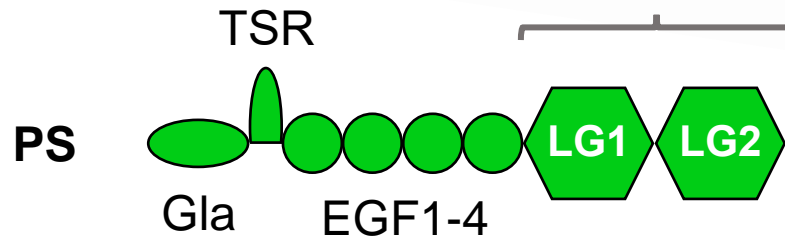
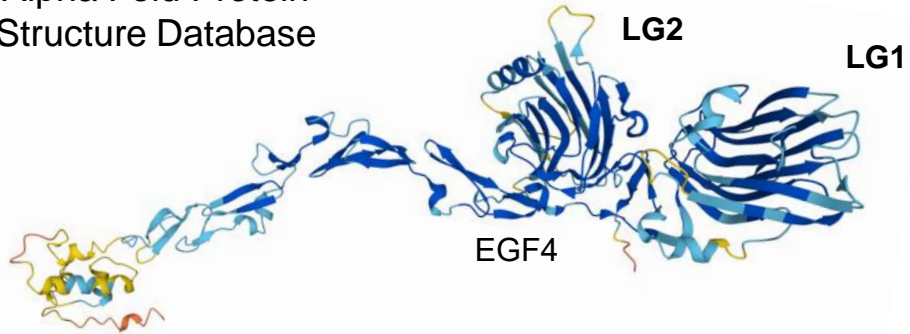


The epitope is localized within the **SHBG domain** of PS

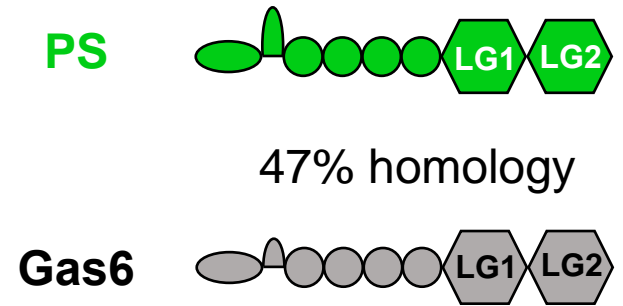
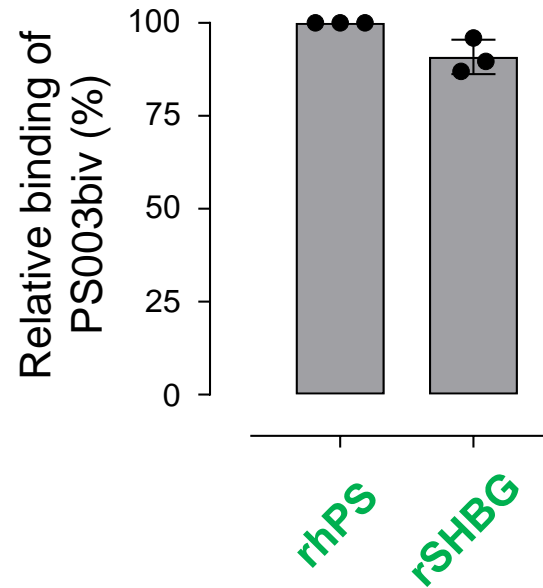


# Epitope mapping of PS003

Alpha Fold Protein Structure Database



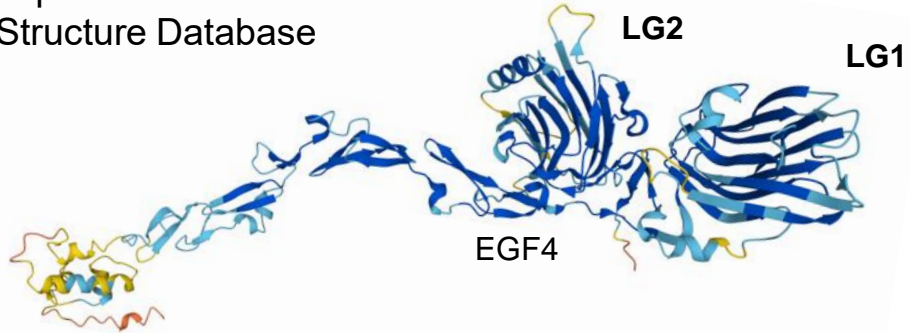
rSHBG



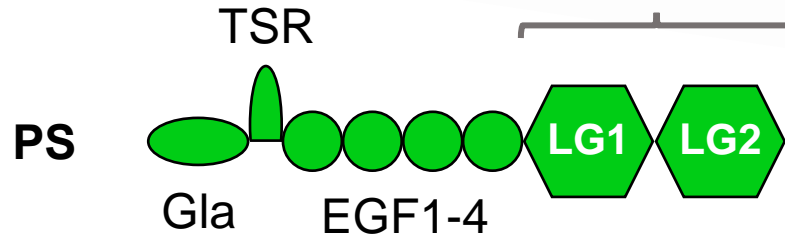
The epitope is localized within the **SHBG domain** of PS

# Epitope mapping of PS003

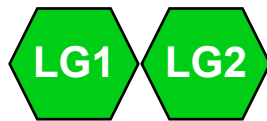
Alpha Fold Protein Structure Database



**SHBG domain**

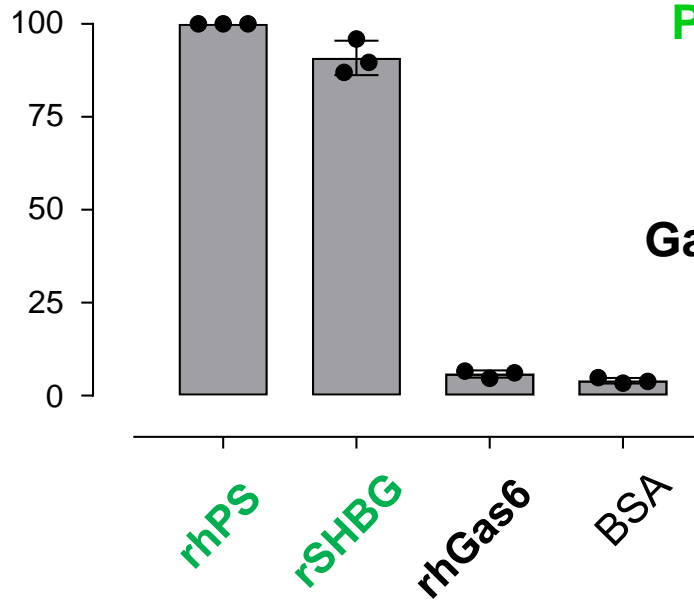


**rSHBG**

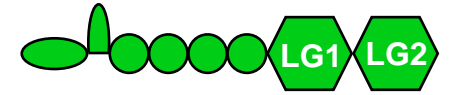


Saposnik *et al.* Biochem J 2003

Relative binding of PS003biv (%)



**PS**



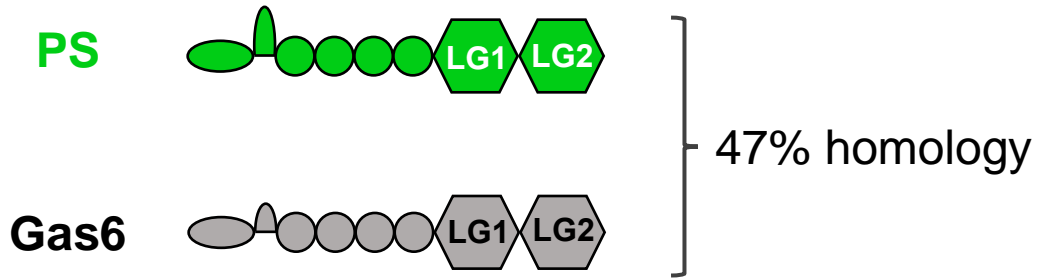
47% homology

**Gas6**

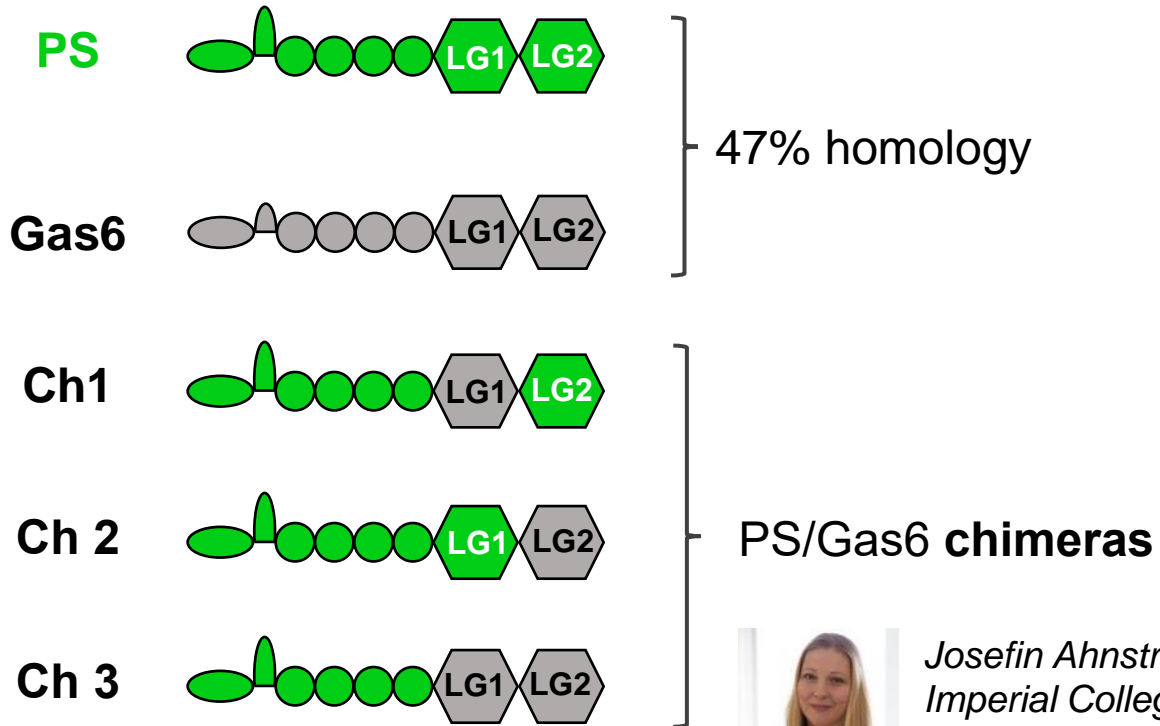


The epitope is localized within the **SHBG domain** of PS

# Epitope mapping of PS003



# Epitope mapping of PS003

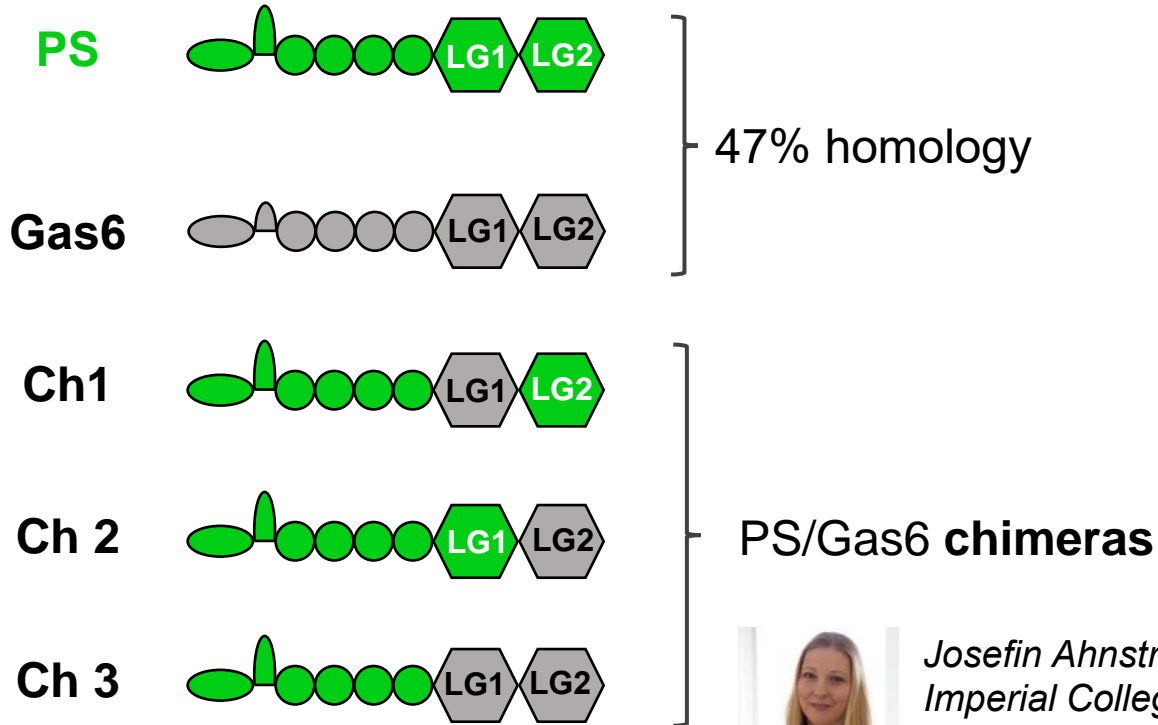


*Josefin Ahnström  
 Imperial College  
 London, UK*

Al Kafri *et al.* Biochem Biophys Rep 2022  
 Reglinska-Matveyev *et al.* Blood 2014  
 Evenäs P *et al.* Thromb Haemost. 2000  
 Nyberg P *et al.* FEBS Lett. 1998

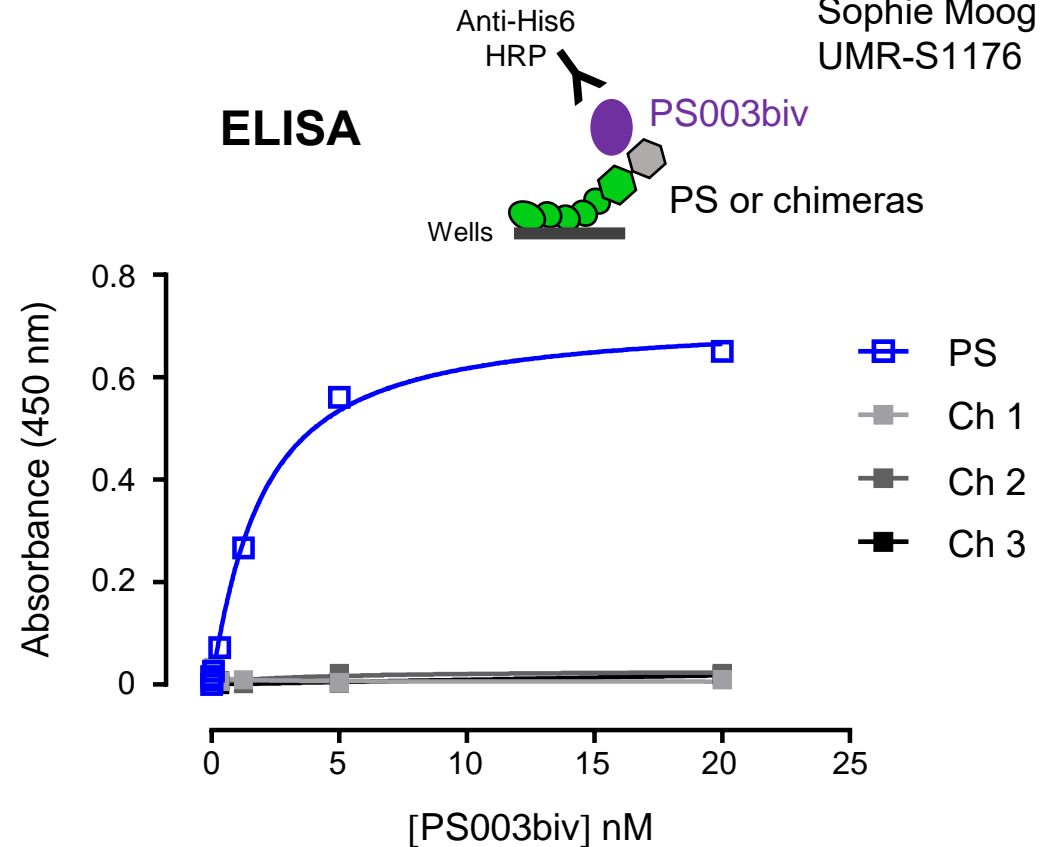
# Epitope mapping of PS003

Sophie Moog  
 UMR-S1176



Josefin Ahnström  
 Imperial College  
 London, UK

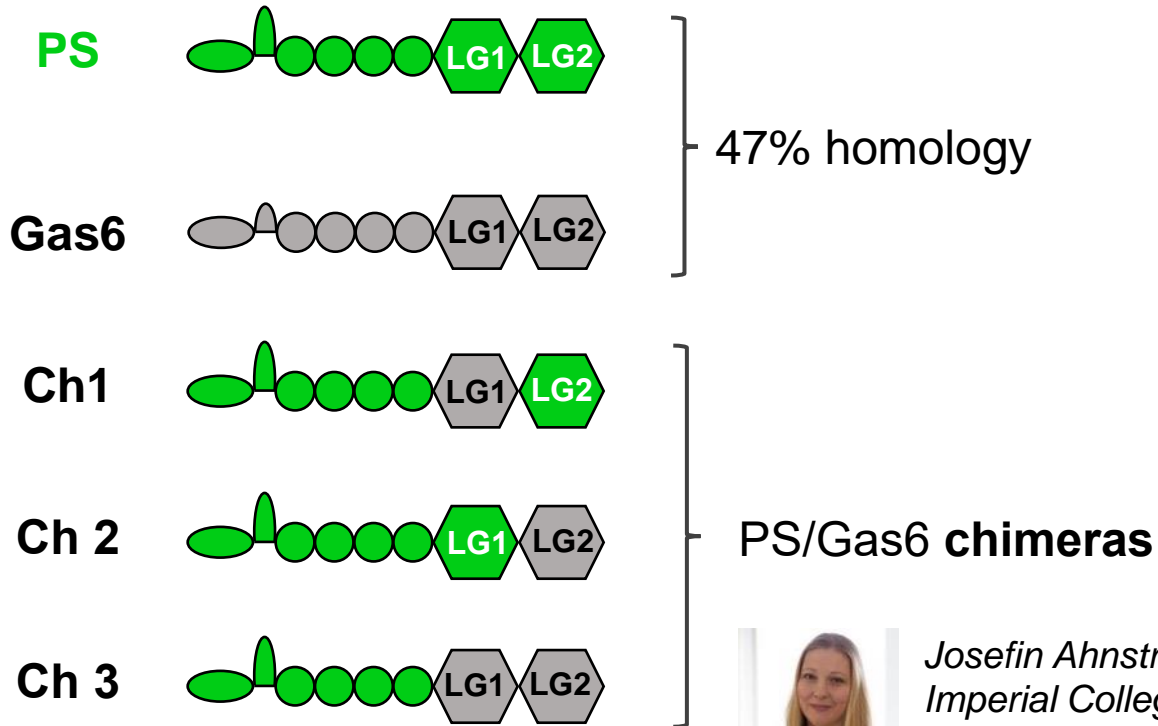
## ELISA



Al Kafri *et al.* Biochem Biophys Rep 2022  
 Reglinska-Matveyev *et al.* Blood 2014  
 Evenäs P *et al.* Thromb Haemost. 2000  
 Nyberg P *et al.* FEBS Lett. 1998

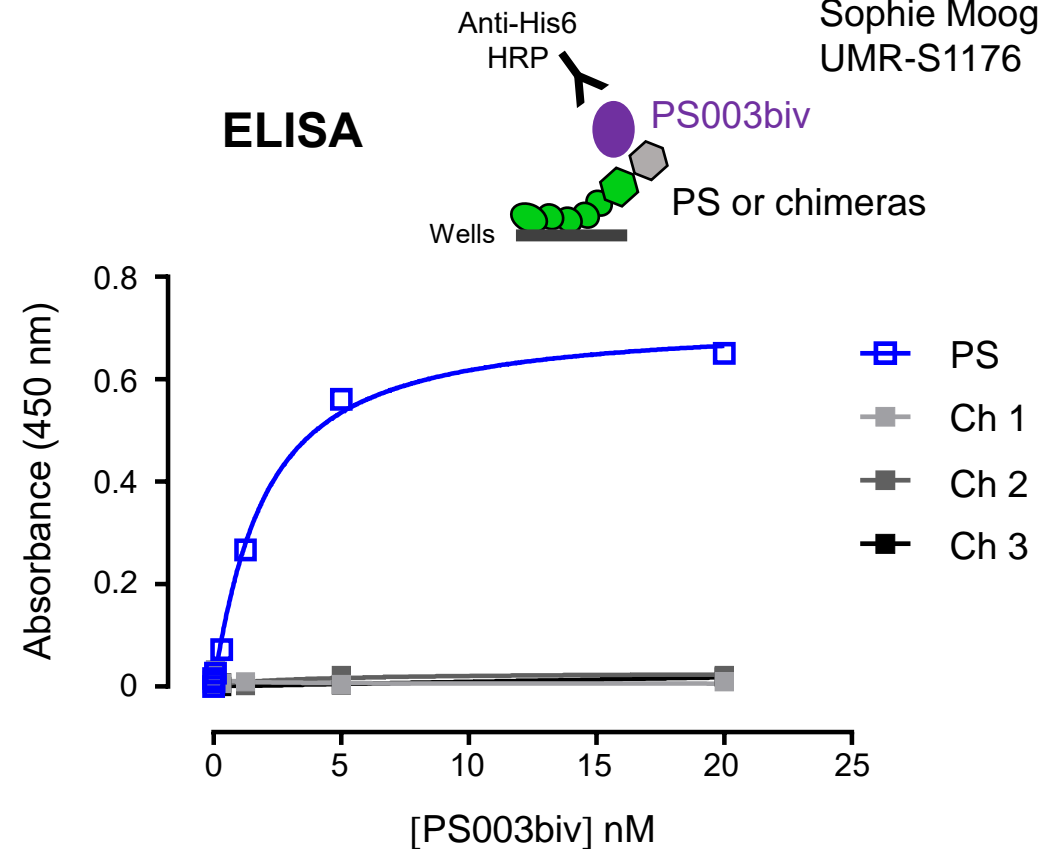
# Epitope mapping of PS003

Sophie Moog  
 UMR-S1176



Josefin Ahnström  
 Imperial College  
 London, UK

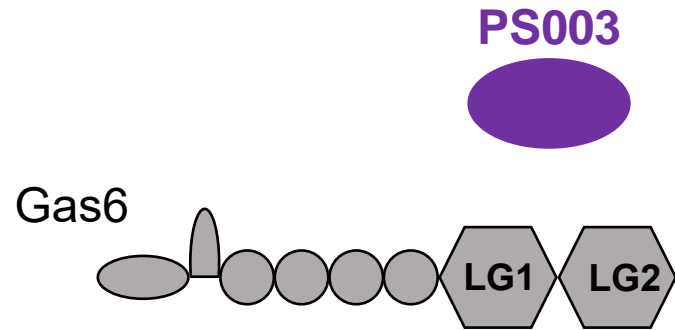
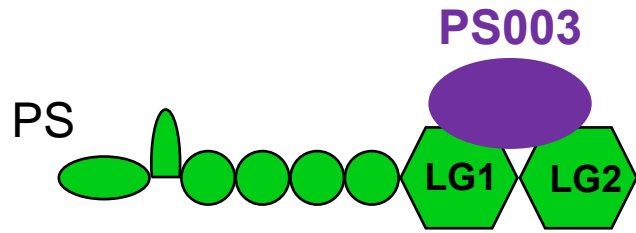
## ELISA



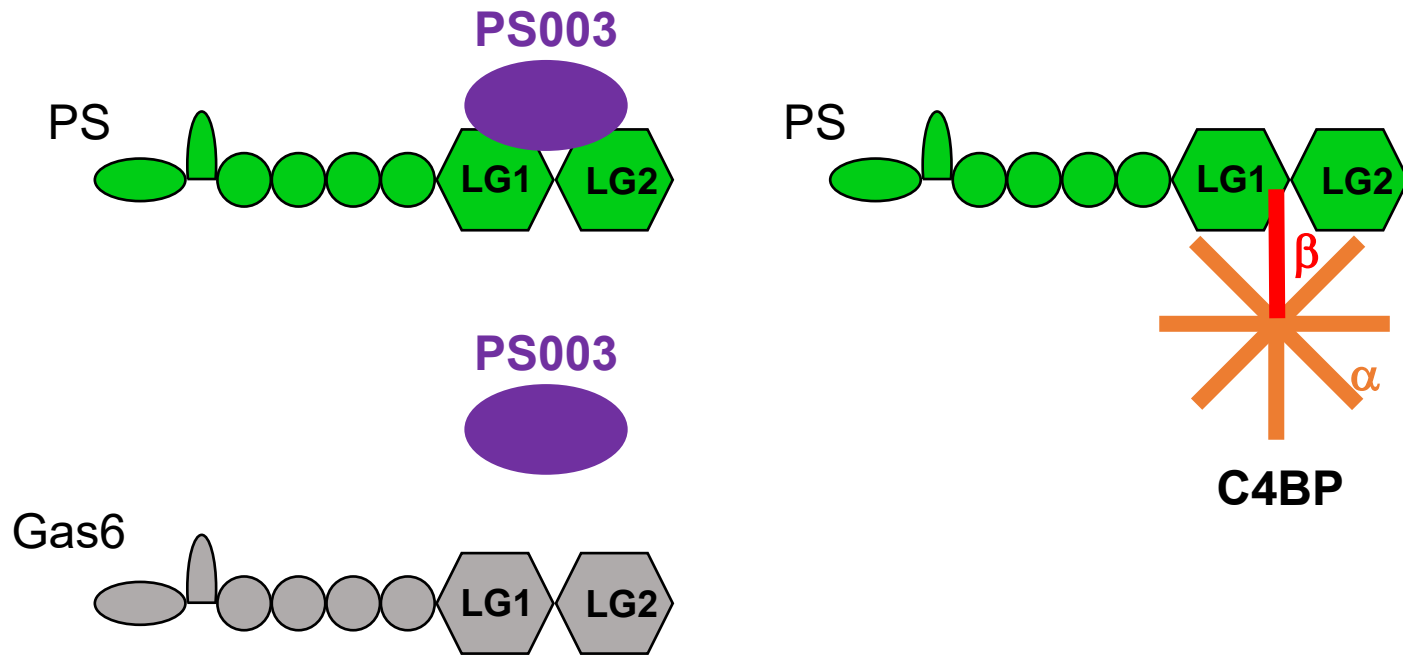
Al Kafri *et al.* Biochem Biophys Rep 2022  
 Reglinska-Matveyev *et al.* Blood 2014  
 Evenas P *et al.* Thromb Haemost. 2000  
 Nyberg P *et al.* FEBS Lett. 1998

Both **LG1 and LG2 domains** appear necessary  
 for the interaction between PS and PS003

# Binding of PS003 to the PS SHBG domain

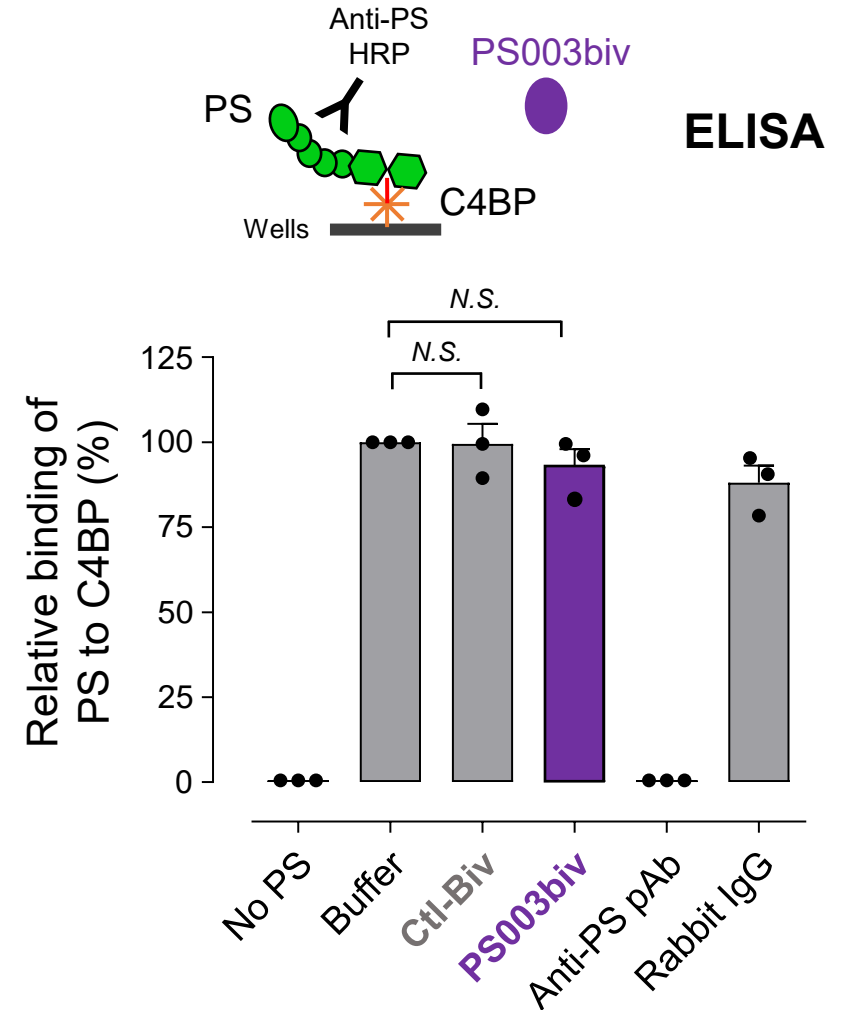
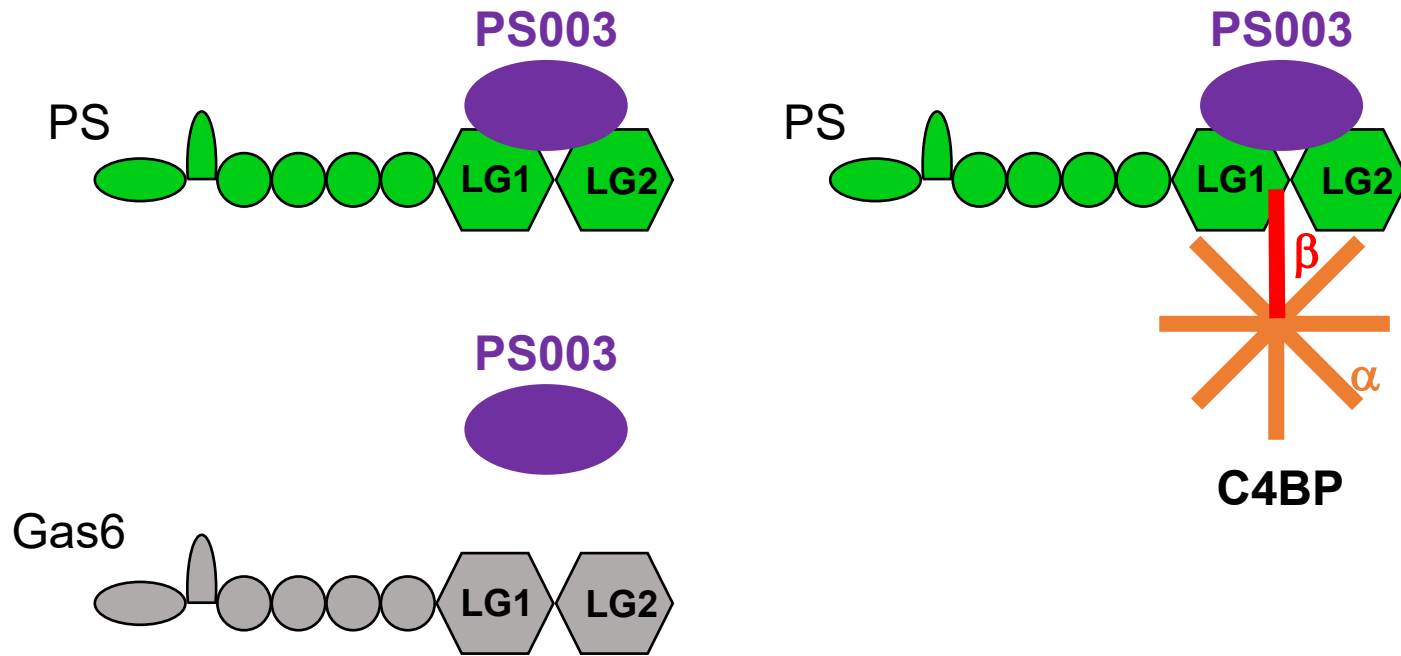


# Binding of PS003 to the PS SHBG domain

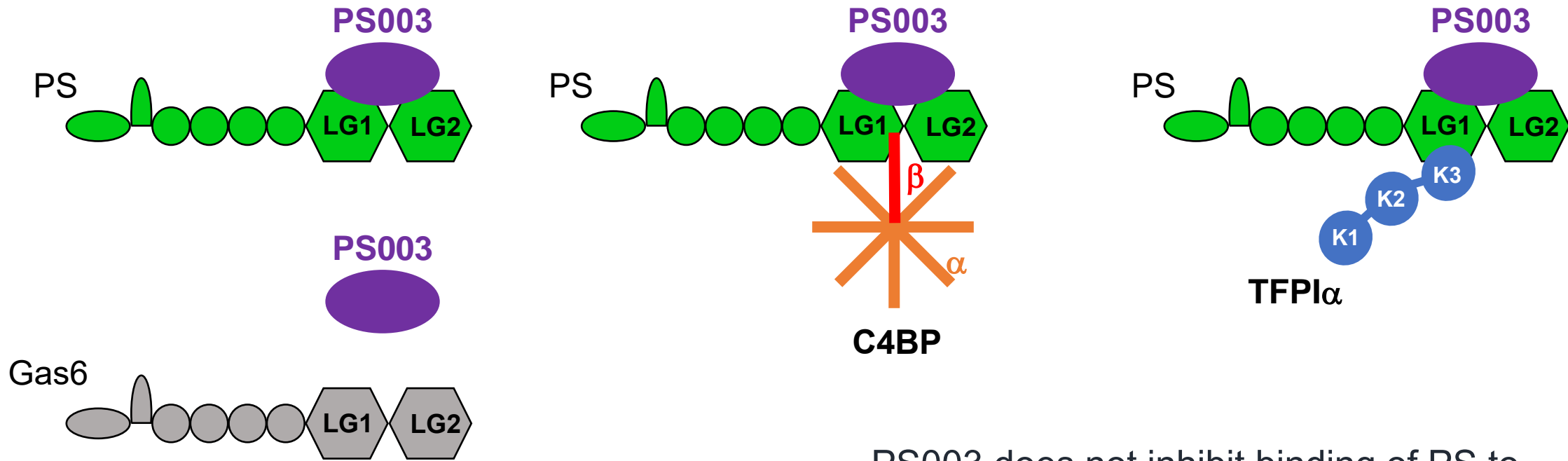




# Binding of PS003 to the PS SHBG domain

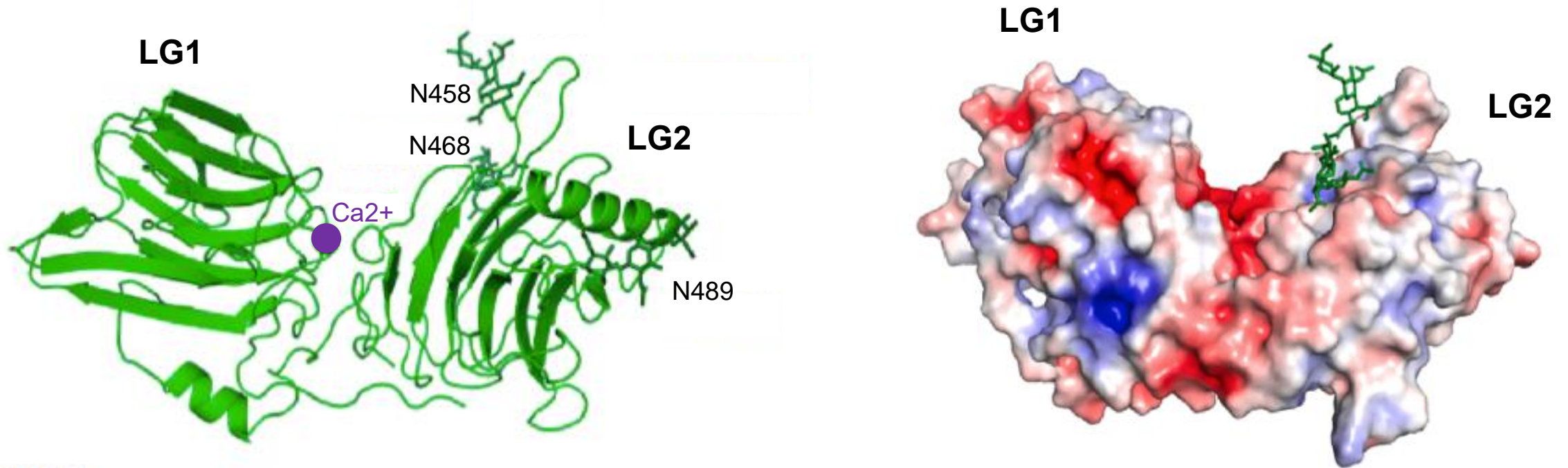


# Binding of PS003 to the PS SHBG domain



PS003 does not inhibit binding of PS to **C4b-binding protein (C4BP)** and **TFPI $\alpha$**

# Novel structural model of the PS SHBG domain

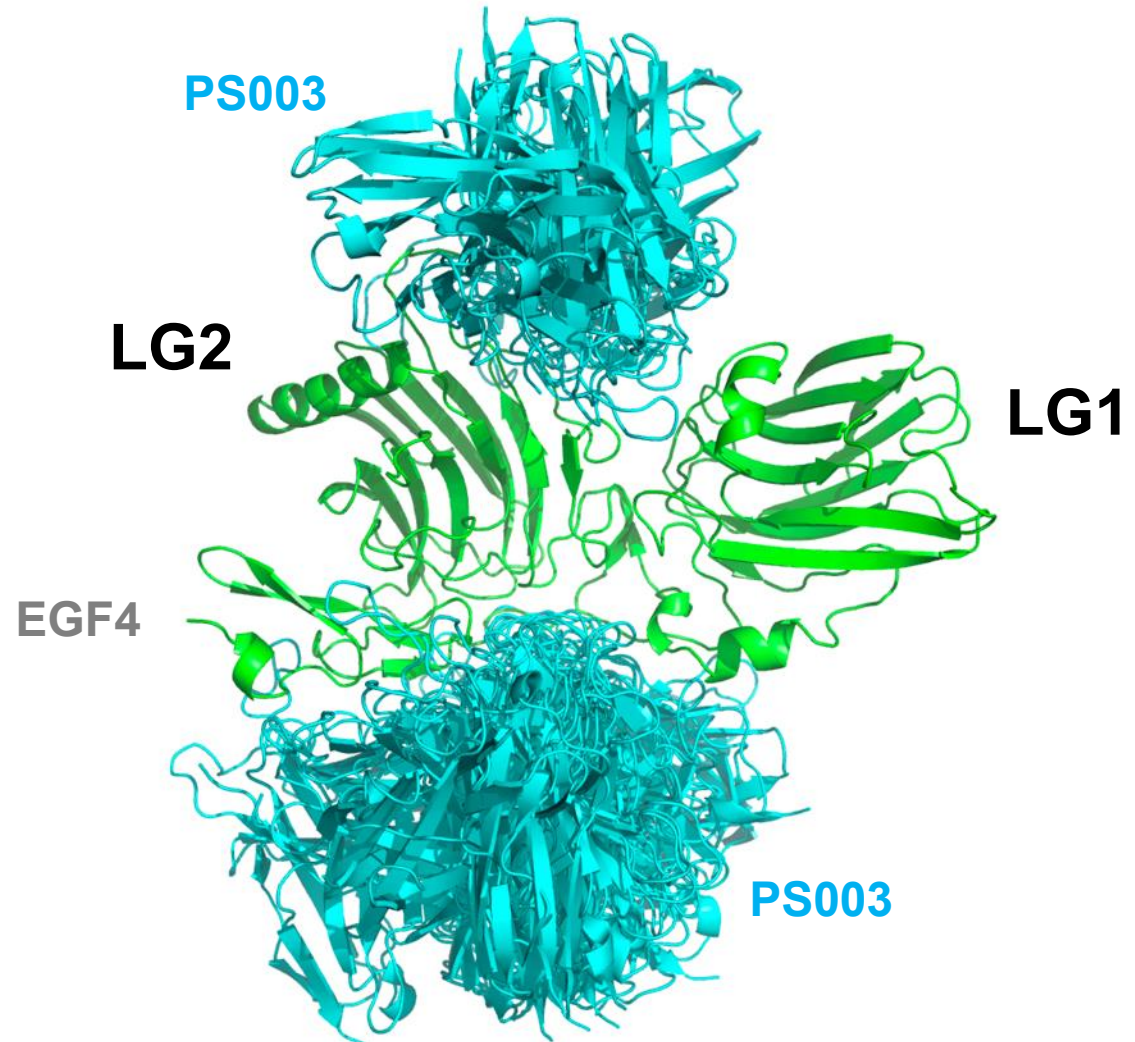
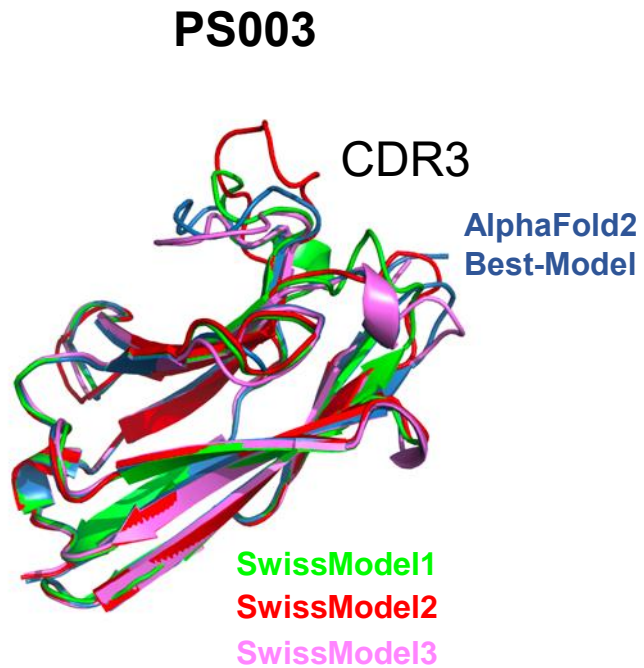


Al Kafri *et al.* Biochem Biophys Rep 2022



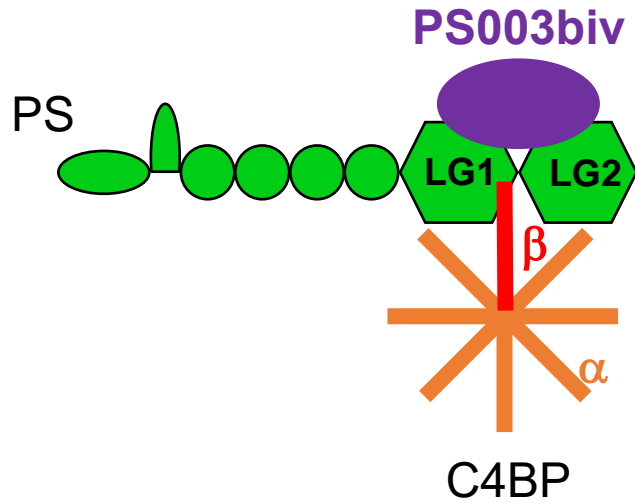
Bruno Villoutreix  
INSERM UMR 1141  
Paris, France

# Molecular docking of PS003 onto the PS SHBG domain

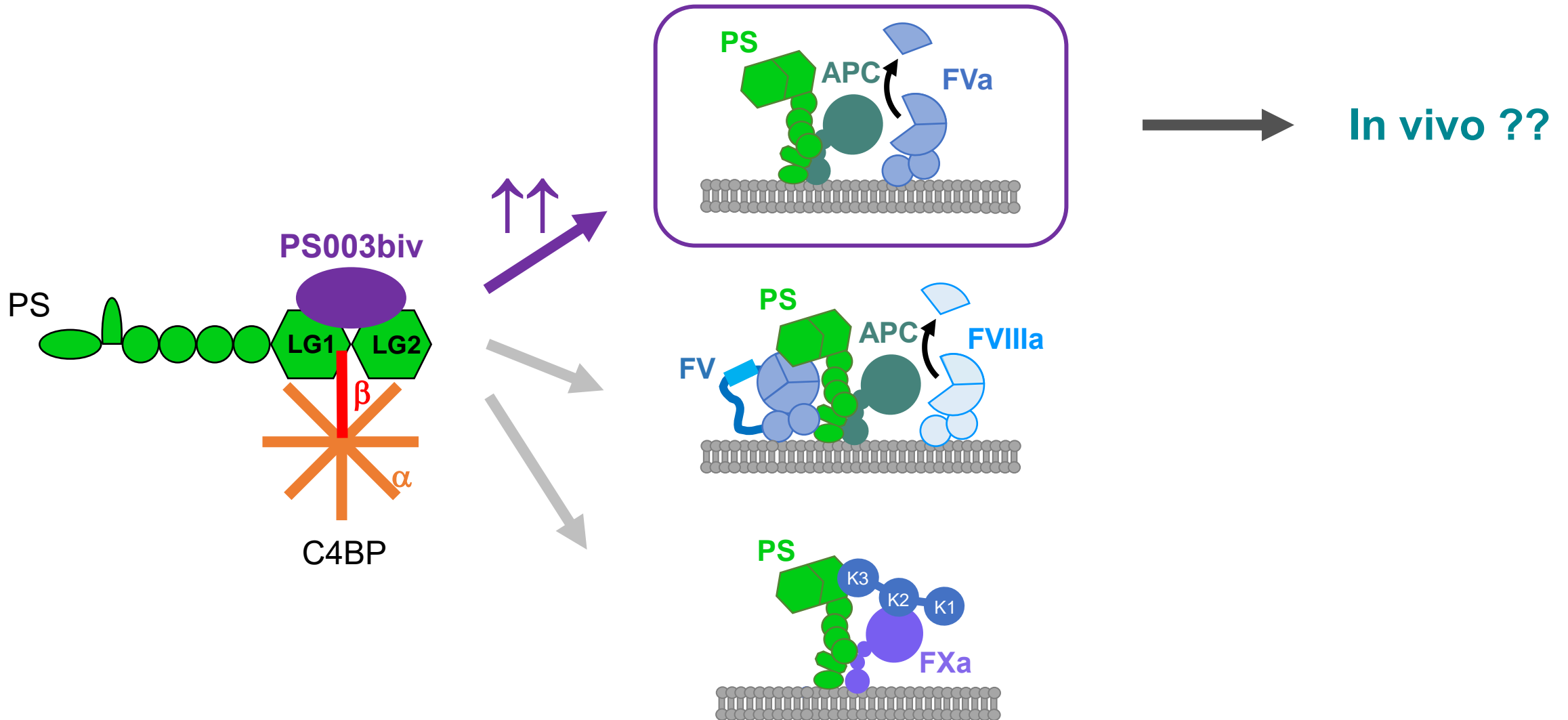


*Bruno Villoutreix*  
*INSERM UMR 1141*  
*Paris, France*

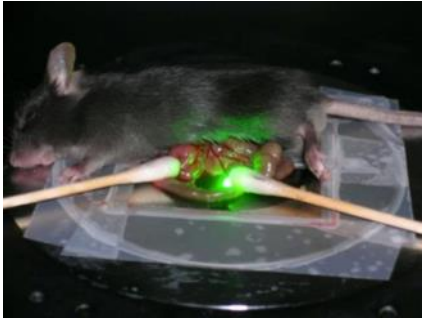
# A novel agent enhancing the APC-cofactor activity of PS



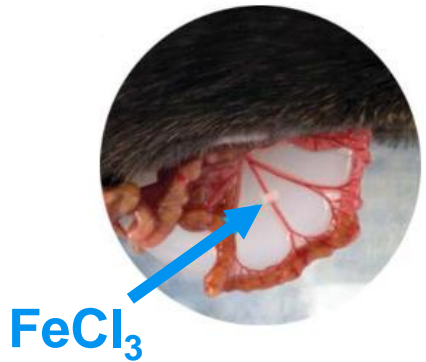
# A novel agent enhancing the APC-cofactor activity of PS



# In vivo antithrombotic effects of PS003biv

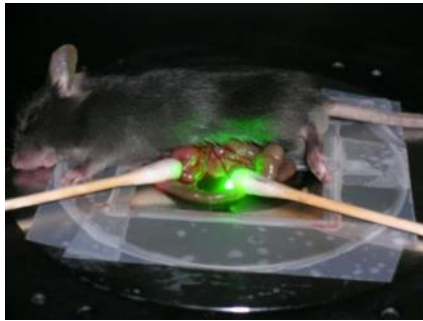


*Frédéric Adam (UMR-S1176)*



*Li et al. JoVE 2016*

# In vivo antithrombotic effects of PS003biv



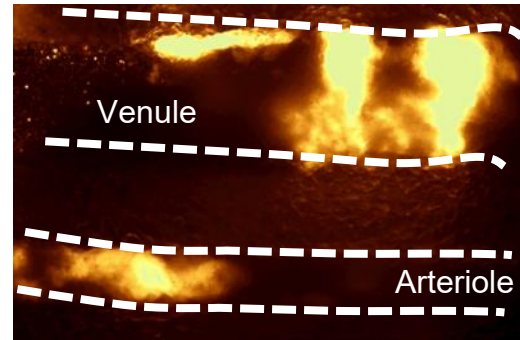
Frédéric Adam (UMR-S1176)



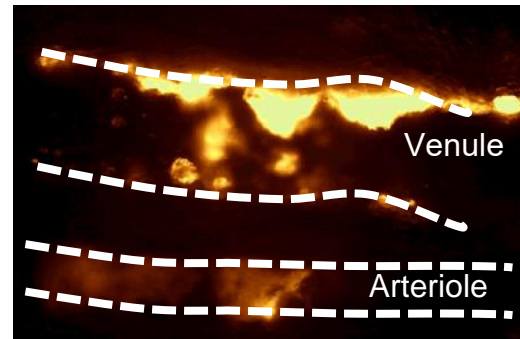
FeCl<sub>3</sub>

Li et al. JoVE 2016

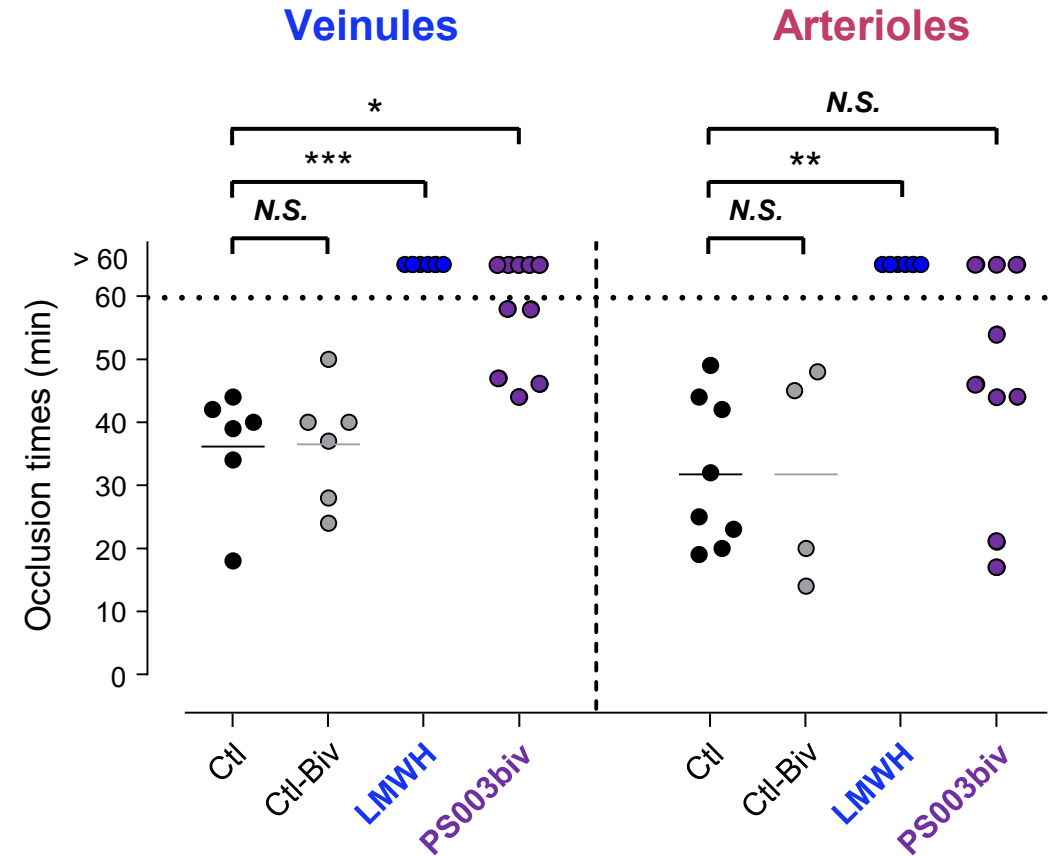
Ctl sdAb



PS003biv



+ High rate of embolization for PS003biv

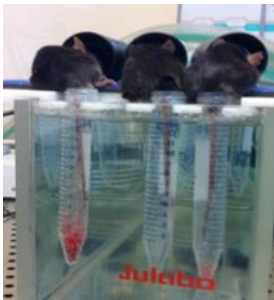
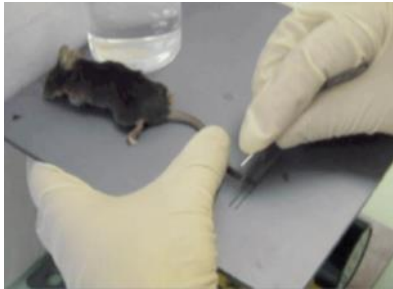


Administration of PS003biv results in **delayed occlusion times** associated with **thrombus instability** in mesenteric vessels

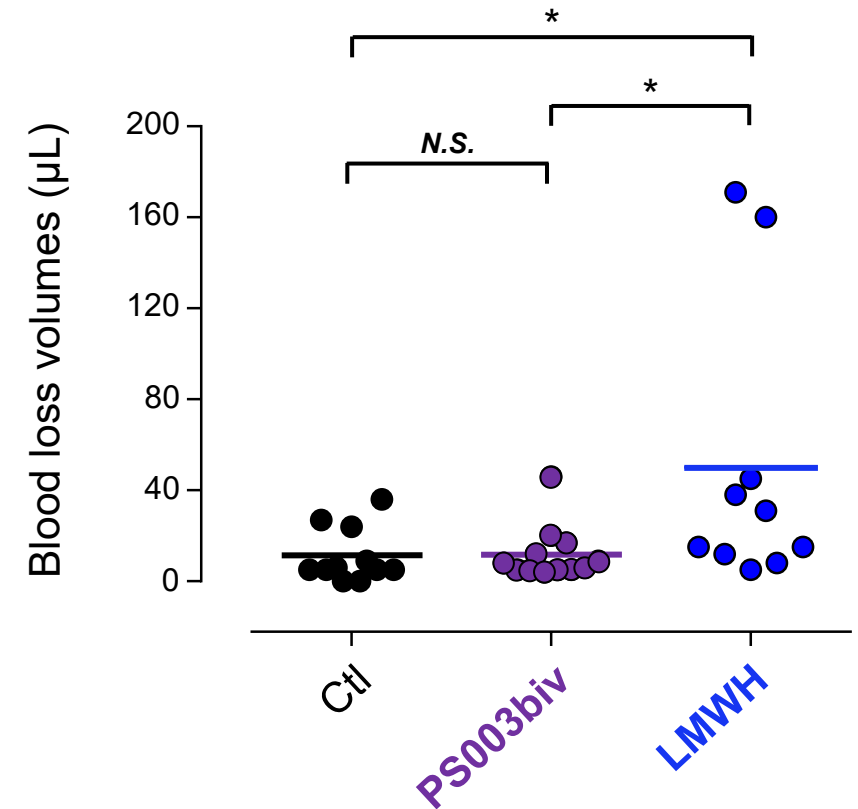
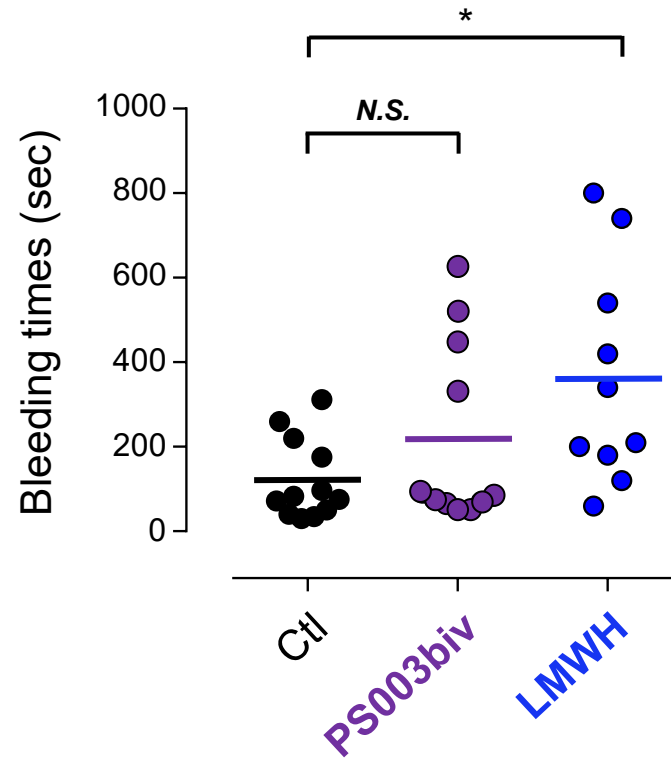


# Effects of PS003biv on physiological hemostasis

## Tail-clip **bleeding model**

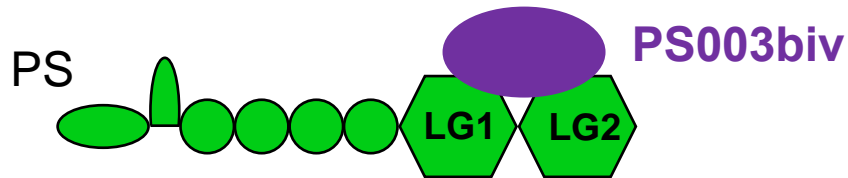
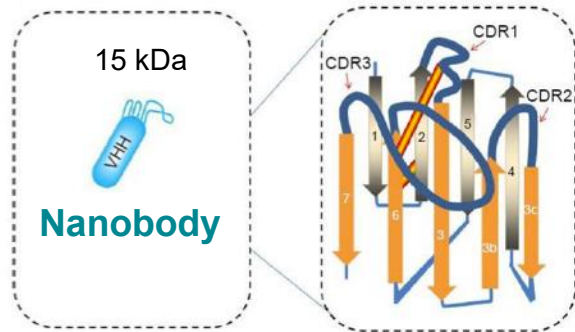


Cécile Denis, UMR-S1176



PS003biv has **no significant effects** on bleeding times and blood loss volumes

# PS003 might be an attractive antithrombotic strategy



Enhances the anticoagulant function of APC

By targeting PS

Exerts antithrombotic effects in mice

Sparing physiological hemostasis in mice

↓ **Therapeutic Potential**

In **(thrombotic) diseases** with :

- Defective protein C activation
- APC-resistance
- Acquired Protein S and/or Protein C deficiency

## PS levels are significantly reduced in SCD patients

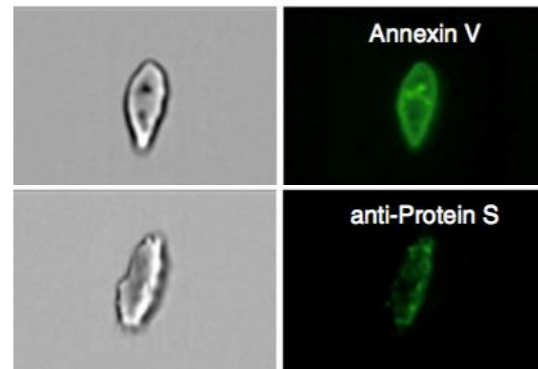
- Further reduction during vaso-occlusive crises (VOC)
- Binding to **PtdSer-exposing RBCs** and enhanced PS clearance ?
- Consumption after coagulation hyperactivation ?
- Hypoxia-induced reduction of PS ?
- Hepatic dysfunctions ?

Causes

**Table 1** Baseline blood and plasma characteristics in sickle cell disease (SCD) and control cohorts

	Control (n = 25)	SCD (n = 25)	P value
Baseline TAT (ng mL <sup>-1</sup> )	2.3 ± 1.1	10.2 ± 9.5	0.0004
phosphatidylserine+ RBCs (%)	0.26 ± 0.25	4.8 ± 4.0	<0.0001
Protein C (% activity)	126 ± 32	81 ± 21	0.0036
Protein S (% activity)	88 ± 18	53 ± 20	0.0005
Protein S (% free antigen)	81 ± 17	58 ± 18	0.0003
Factor V (% activity)	89 ± 21	97 ± 29	0.3027
Factor VIII (% activity)	112 ± 25	179 ± 45	0.0003
TFPI (% antigen)	86 ± 33	83 ± 24	0.6659

TAT, thrombin-antithrombin complexes; RBCs, red blood cells; TFPI, tissue factor pathway inhibitor.



Sickle RBCs

# PS deficiency and APC-resistance in Sickle Cell Disease

## PS levels are significantly reduced in SCD patients

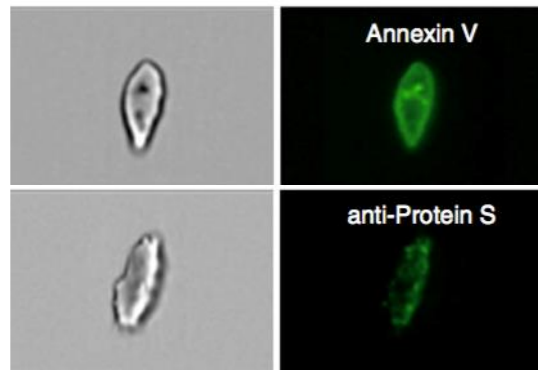
- Further reduction during VOC
- Binding to **PtdSer-exposing RBCs** and enhanced PS clearance ?
- Consumption after coagulation hyperactivation ?
- Hypoxia-induced reduction of PS ?
- Hepatic dysfunctions ?

Causes

**Table 1** Baseline blood and plasma characteristics in sickle cell disease (SCD) and control cohorts

	Control (n = 25)	SCD (n = 25)	P value
Baseline TAT (ng mL <sup>-1</sup> )	2.3 ± 1.1	10.2 ± 9.5	0.0004
phosphatidylserine+ RBCs (%)	0.26 ± 0.25	4.8 ± 4.0	<0.0001
Protein C (% activity)	126 ± 32	81 ± 21	0.0036
<b>Protein S (% activity)</b>	<b>88 ± 18</b>	<b>53 ± 20</b>	<b>0.0005</b>
<b>Protein S (% free antigen)</b>	<b>81 ± 17</b>	<b>58 ± 18</b>	<b>0.0003</b>
Factor V (% activity)	89 ± 21	97 ± 29	0.3027
Factor VIII (% activity)	112 ± 25	179 ± 45	0.0003
TFPI (% antigen)	86 ± 33	83 ± 24	0.6659

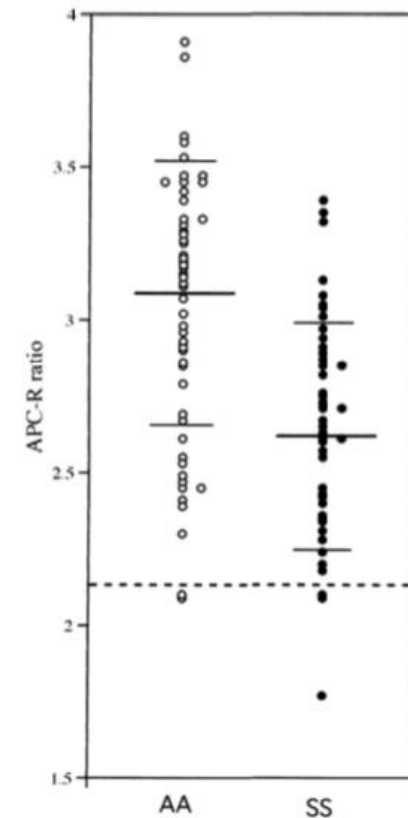
TAT, thrombin-antithrombin complexes; RBCs, red blood cells; TFPI, tissue factor pathway inhibitor.



Sickle RBCs

Whelihan *et al.* JTH 2016

## APC resistance in SCD patients



Wright *et al.* Br J Haematol 1997

# Coagulation activation and vaso-occlusive crises (VOC)

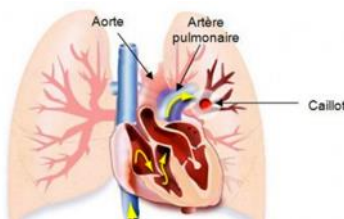
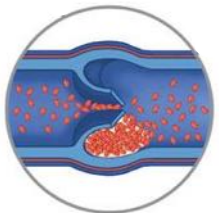
Impaired PC/PS system



Hypercoagulable  
State  
in SCD



High Risk  
of Venous  
Thromboembolism



# Coagulation activation and vaso-occlusive crises (VOC)

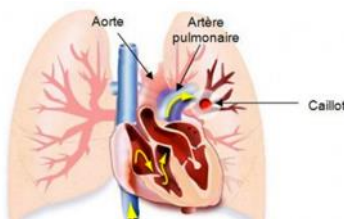
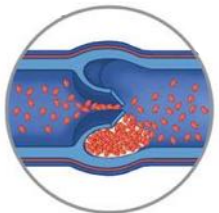
Impaired PC/PS system



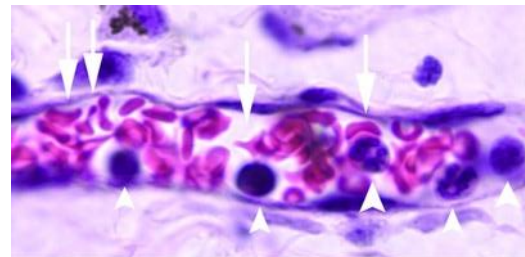
Hypercoagulable  
State  
in SCD



High Risk  
of Venous  
Thromboembolism



Vaso-Occlusive  
Crises



Kalemur *et al.* Am J Hematol 2004

# Coagulation activation and vaso-occlusive crises (VOC)

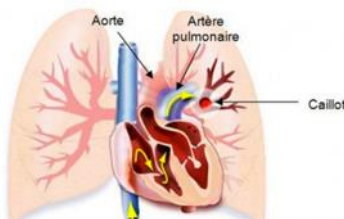
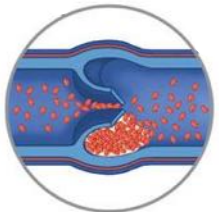
Impaired PC/PS system



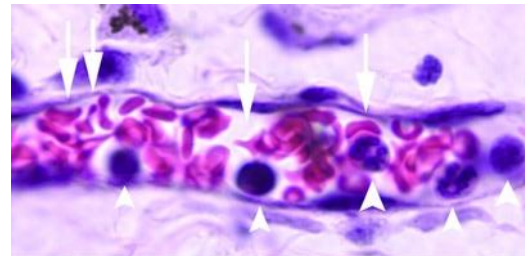
Hypercoagulable State in SCD



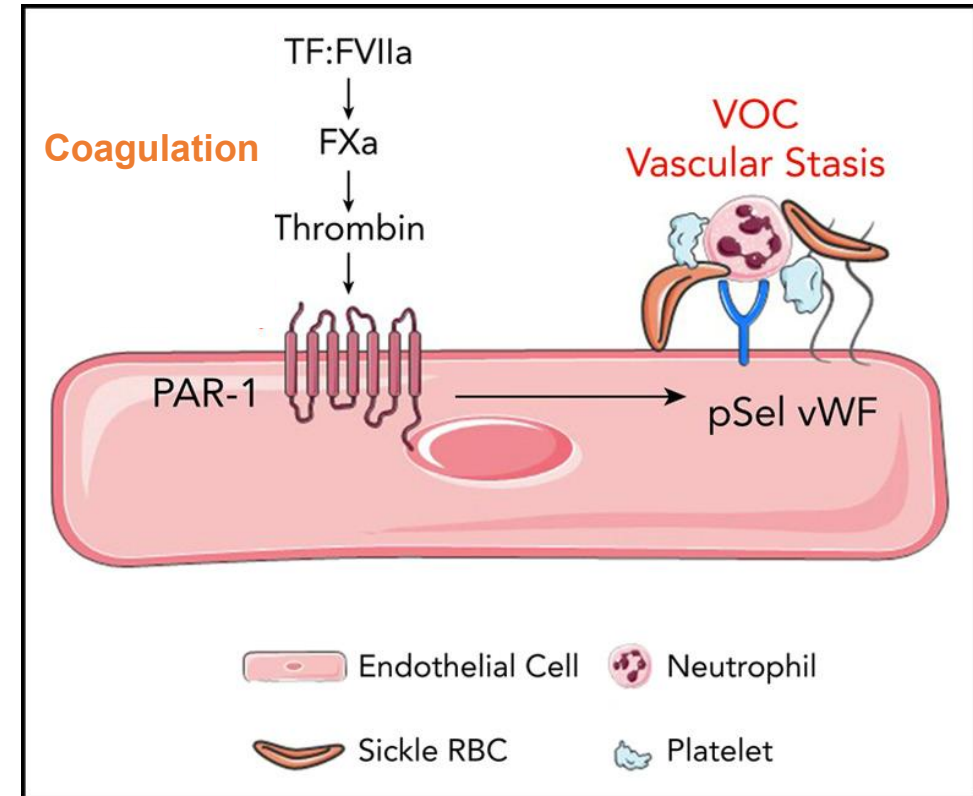
High Risk of Venous Thromboembolism



Vaso-Occlusive Crises



Kalemur *et al.* Am J Hematol 2004



Sparkenbaugh *et al.* Blood 2020

# Coagulation activation and vaso-occlusive crises (VOC)

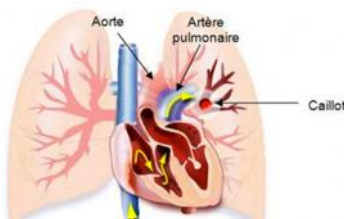
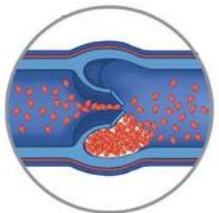
Impaired PC/PS system



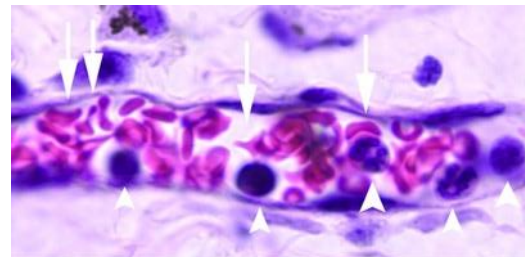
Hypercoagulable State in SCD



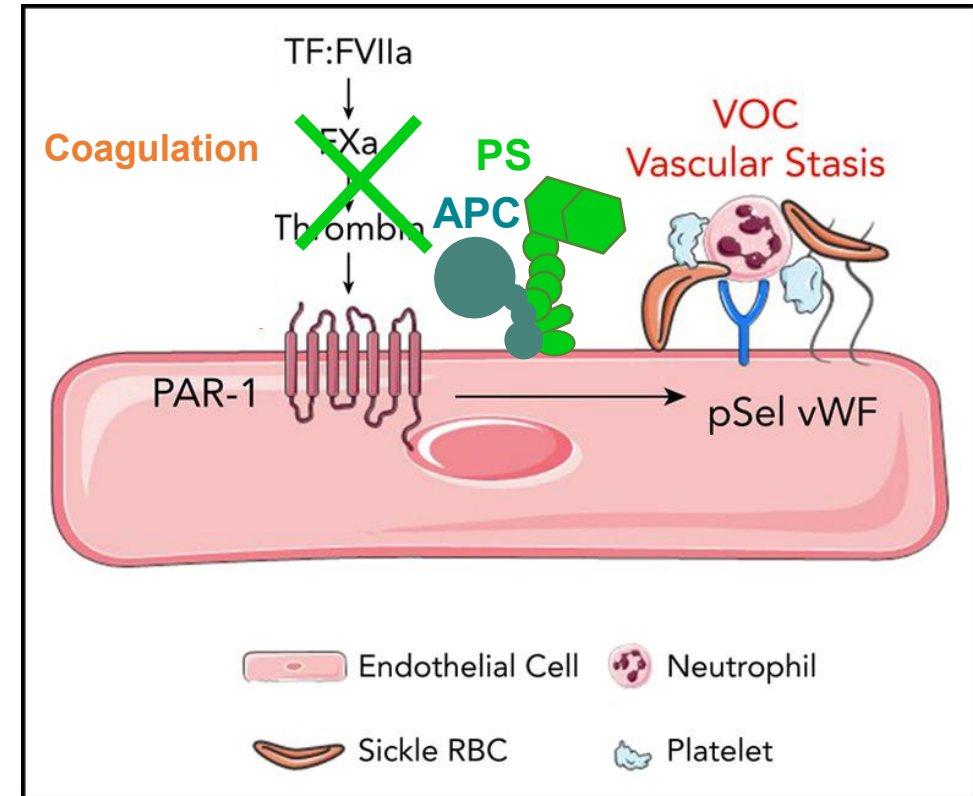
High Risk of Venous Thromboembolism



Vaso-Occlusive Crises



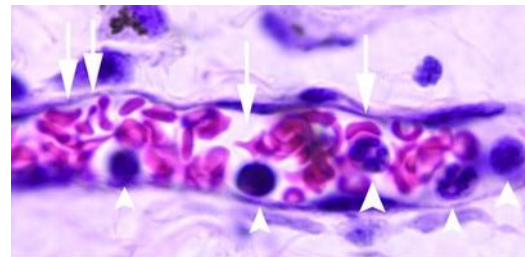
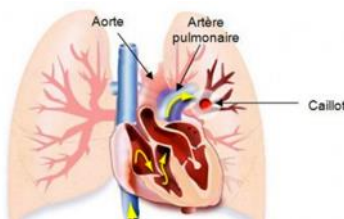
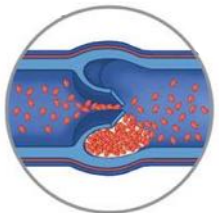
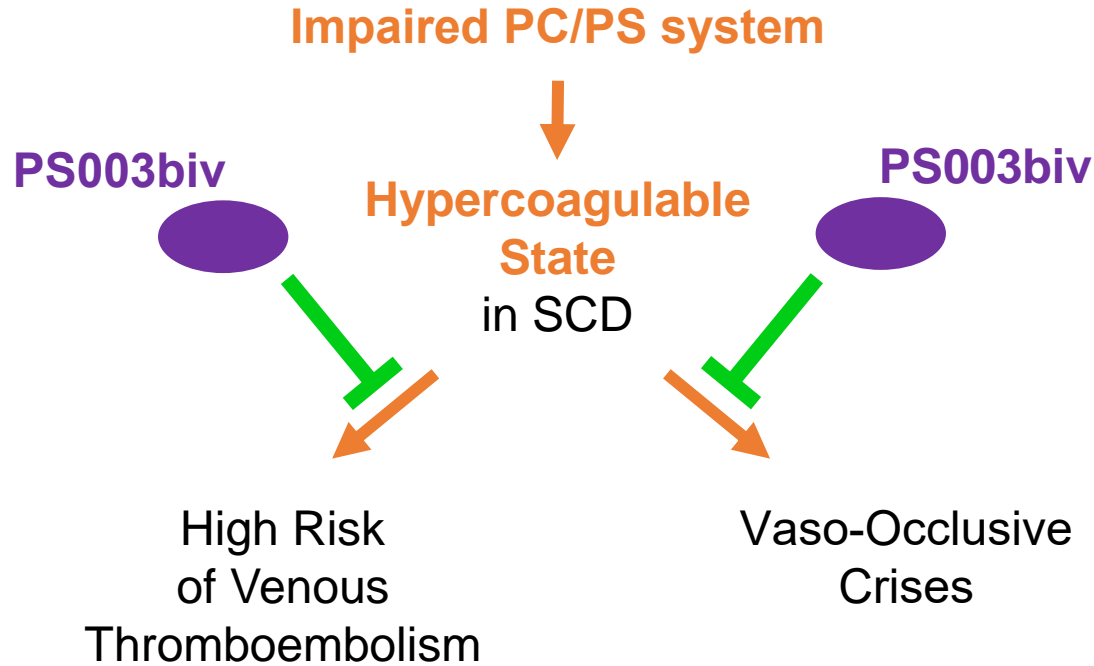
Kalemur *et al.* Am J Hematol 2004



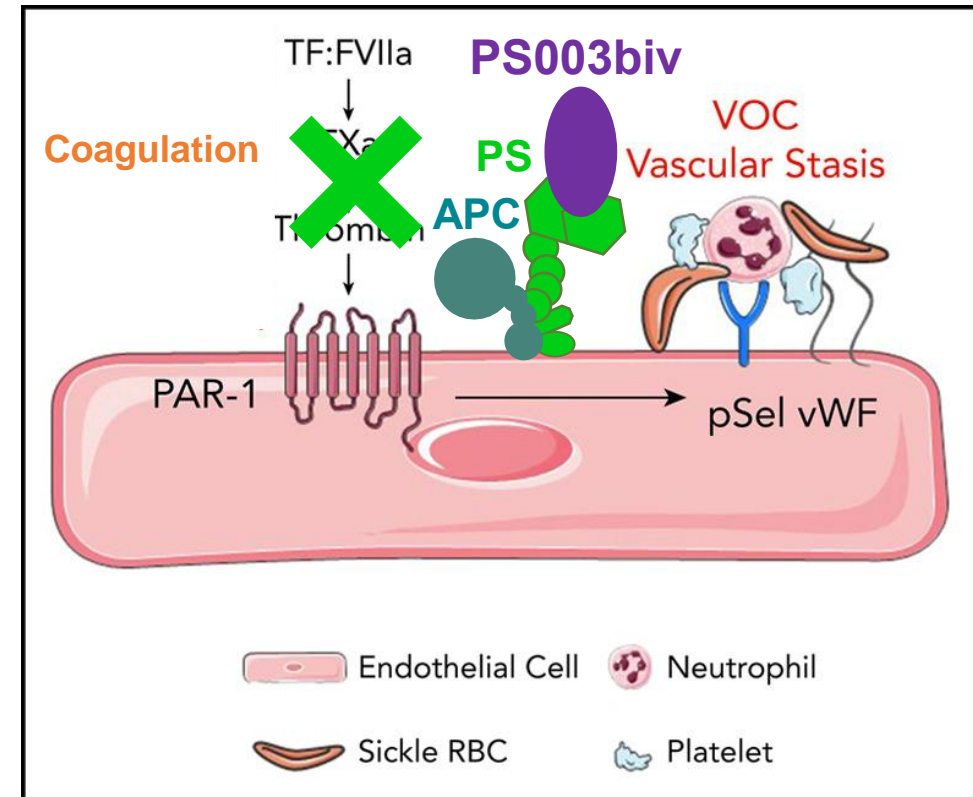
Sparkenbaugh *et al.* Blood 2020



# Coagulation activation and vaso-occlusive crises (VOC)



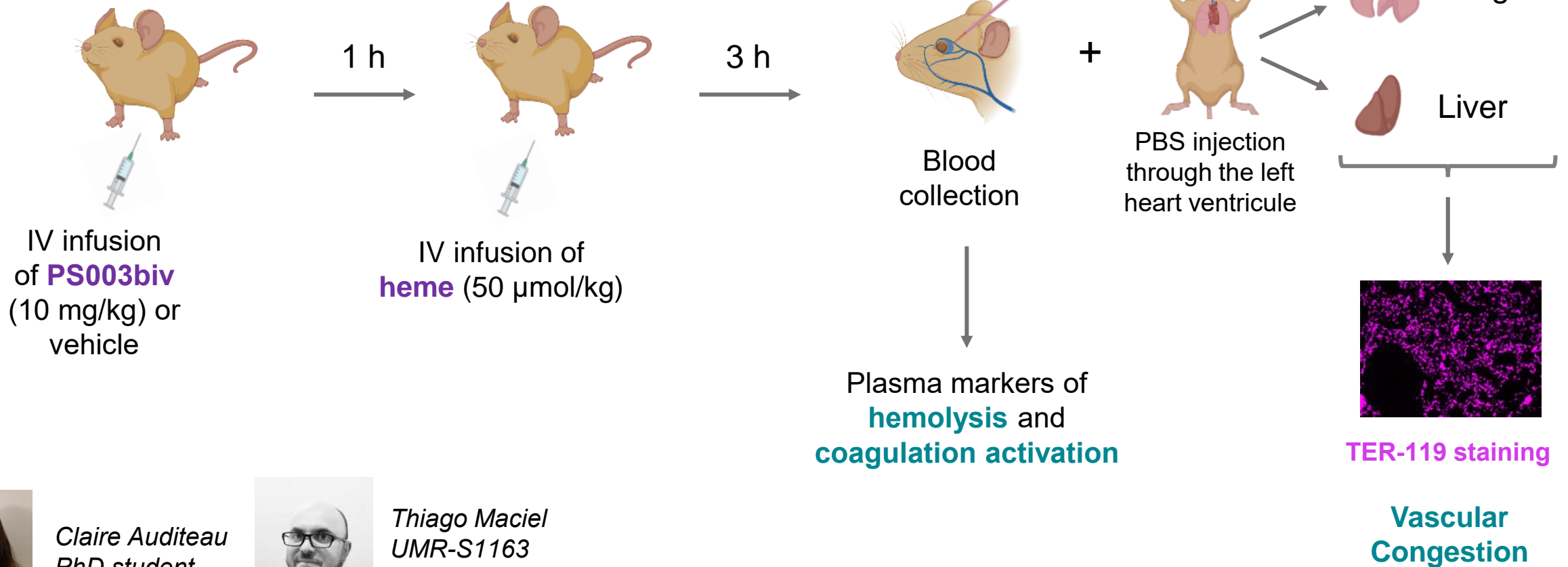
Kalemur *et al.* Am J Hematol 2004



Sparkenbaugh *et al.* Blood 2020

# Effects of PS003biv in a mouse model of VOC

## SCD mice (HbSS Townnes)



*Claire Auditeau*  
PhD student



*Thiago Maciel*  
UMR-S1163  
Institut Imagine, Paris

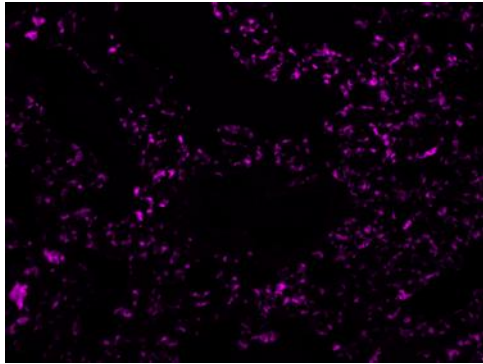
# Effects of PS003biv in a mouse model of VOC

Lungs

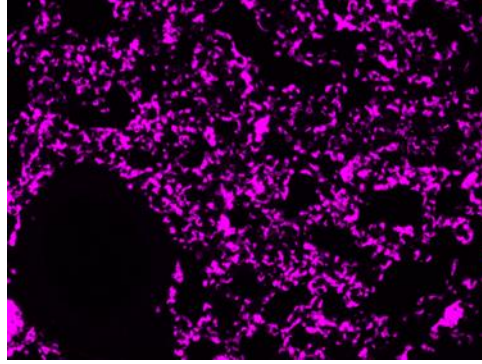


TER-119 staining

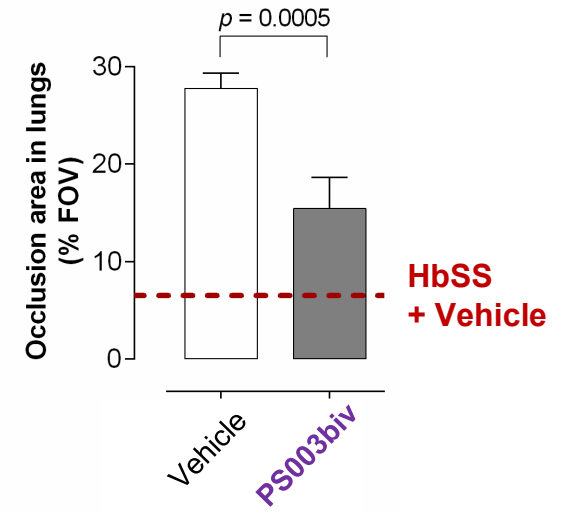
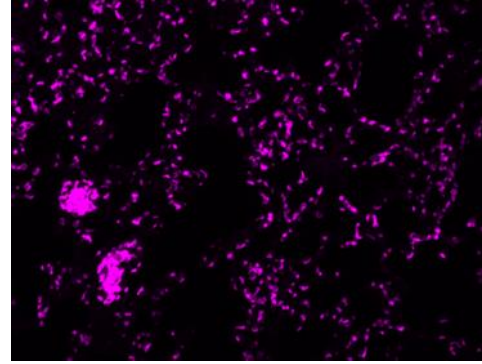
HbSS + Vehicle



HbSS + Vehicle  
Heme



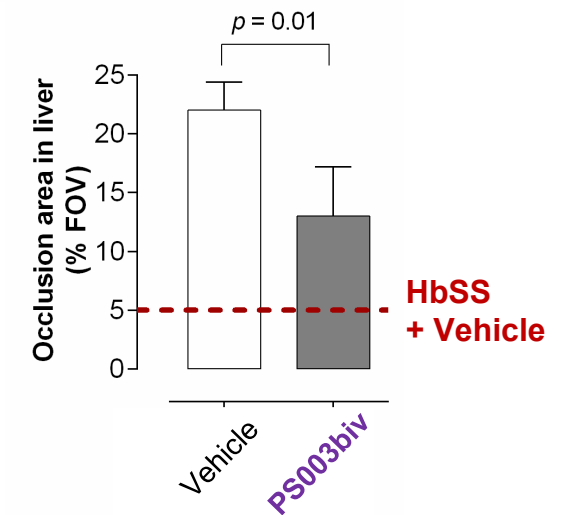
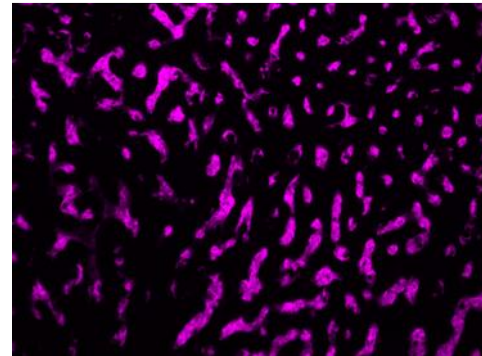
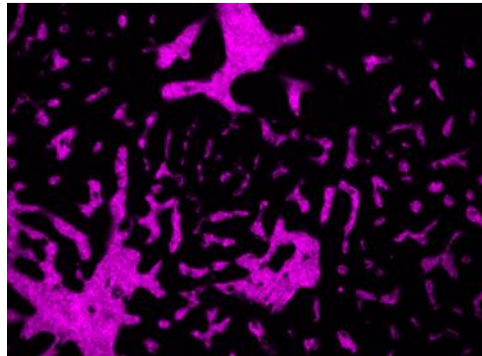
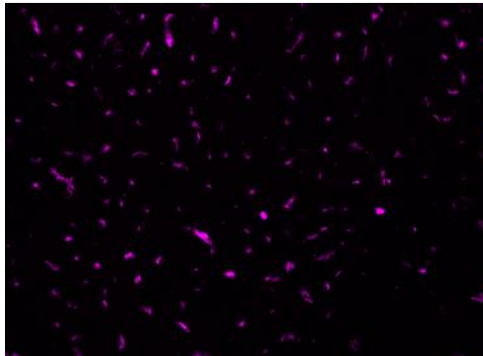
HbSS + PS003biv  
Heme



Liver

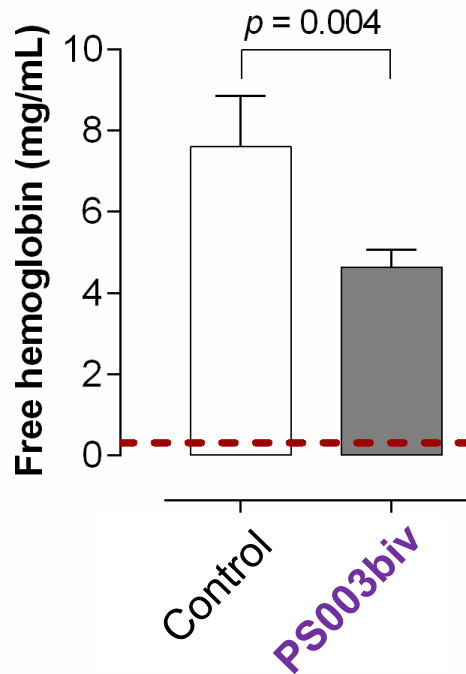


TER-119 staining



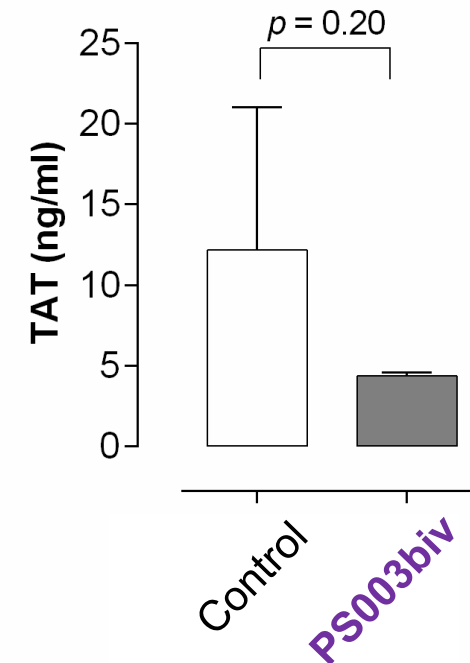
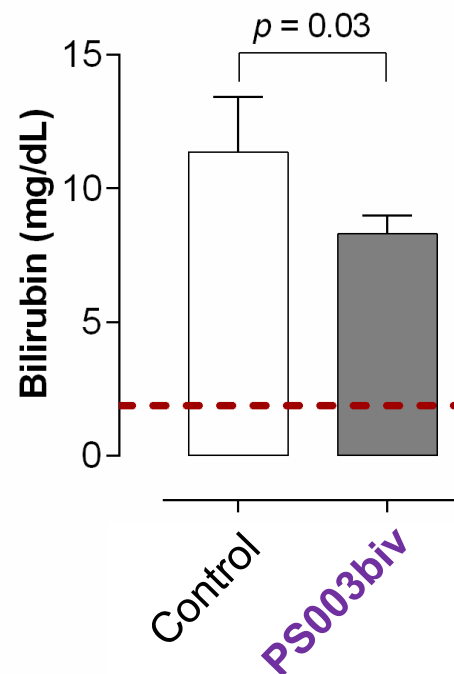
# Effects of PS003biv in a mouse model of VOC

## Hemolysis










----- HbSS - Vehicle

## Coagulation activation



# Conclusions

-  A nanobody **enhancing the function** of a physiological inhibitor of coagulation can be identified
-  **Originality** comes with **complexity** !
-  **Not yet elucidated** mechanism(s) of action
-  **Therapeutic potential** of enhancing the APC-cofactor activity of PS
-  **Protective** in models of VOC in SCD mice
-  Optimization of **pharmacological properties** needed (e.g. half-life, humanization)
-  This nanobody **stimulates new research projects** (PROSICK project, funded by the ANR)

# Acknowledgments



Delphine Borgel  
Josepha Clara Sedzro  
Claire Auditeau  
Ivan Peyron  
Gabriel Aymé  
Allan De Carvalho  
Sadyo Daramé  
Frédéric Adam  
Elsa Bianchini  
Sophie Moog  
Olivier Christophe  
Peter Lenting  
Cécile Denis

## Collaborators



Tilman Hackeng  
Stella Thomassen



Josefin Ahnström



Sophie Gandrille



Bruno Villoutreix



Thiago Maciel

## Research Fundings



PROSICK  
Protein S in Sickle Cell Disease  
ANR-22-CE14-0058

