Mouse Models of Cancer-associated Thrombosis

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Outline

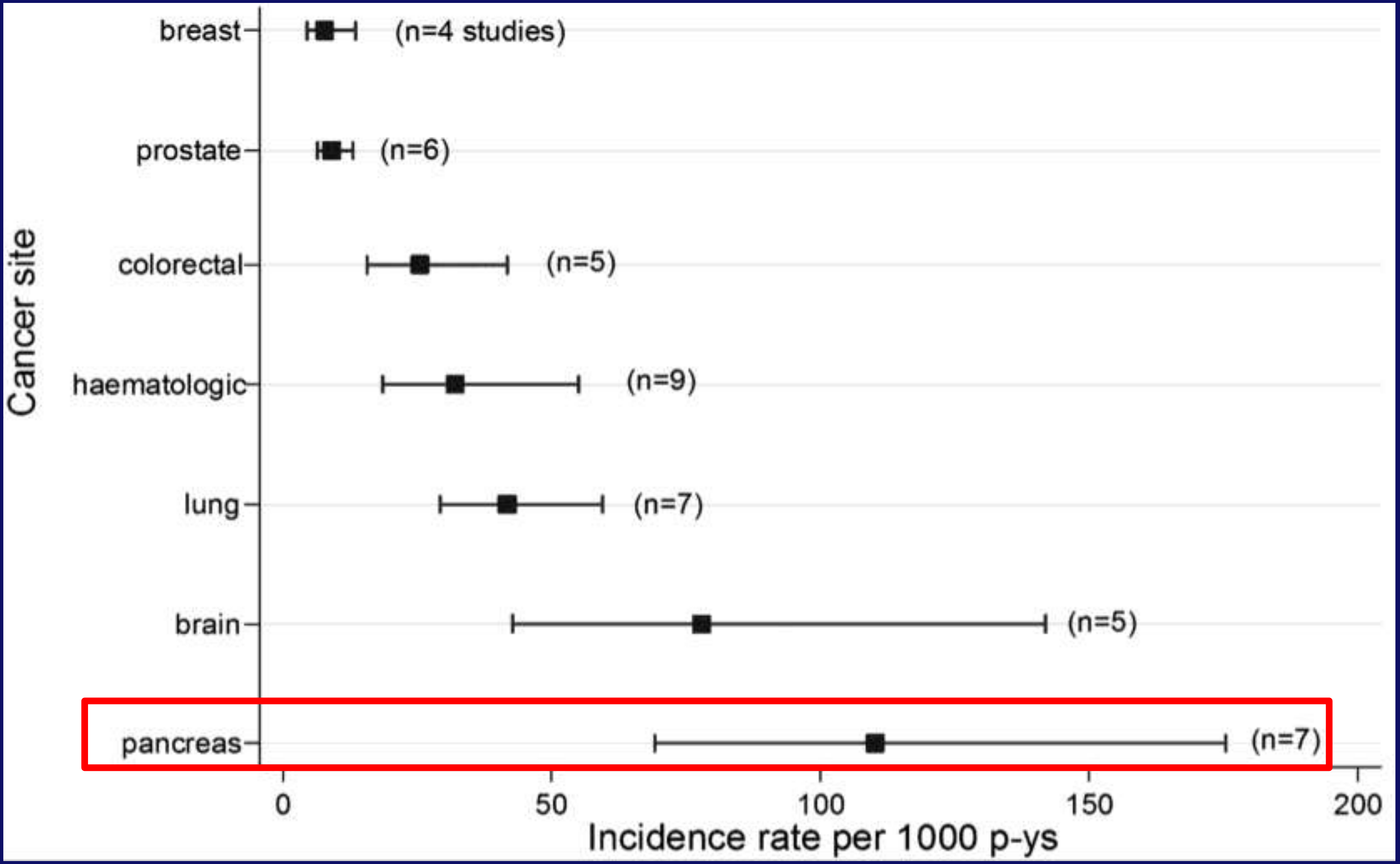
- Background
- Role of tumor-derived TF+ MVs in venous thrombosis in mice with pancreatic tumors
- Role of neutrophils and NETs in venous thrombosis in mice with pancreatic tumors

Cancer and Venous Thrombosis

Cancer patients have an a 4-7 fold increased risk for venous thrombosis – cancer-associated thrombosis (CAT)

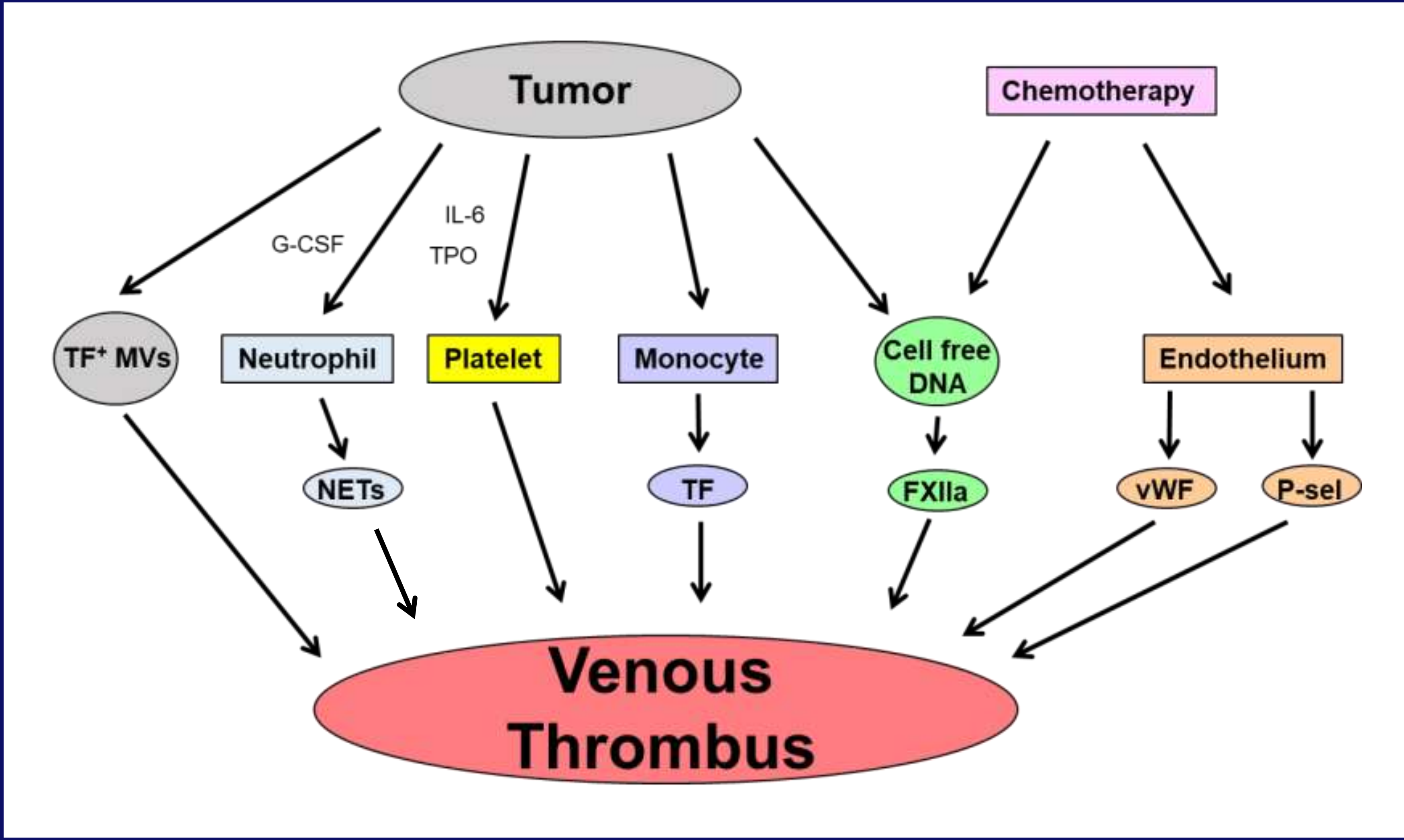
Blom JW et al. JTH 2006; Khorana AA et al. JTH 2007; Timp JF et al. Blood 2013

Incidence of Venous Thrombosis in Patients with Different Types of Cancer



Modiied from Timp JF et al. Blood 2013

Possible Pathways of Cancer-associated Thrombosis



Hisada Y et al JTH 2015

Mouse models are important tools to study the mechanisms of venous thrombosis in cancer patients

Mouse Models of Cancer-Associated Thrombosis

- Mouse strain- immunocompetent vs immunodeficient
- Thrombosis model
- Site of tumor growth- S.Q. vs orthotopic
- Tumor-type

Hisada Y and Mackman Thromb Res 2018

Mouse Models of Cancer-associated Thrombosis

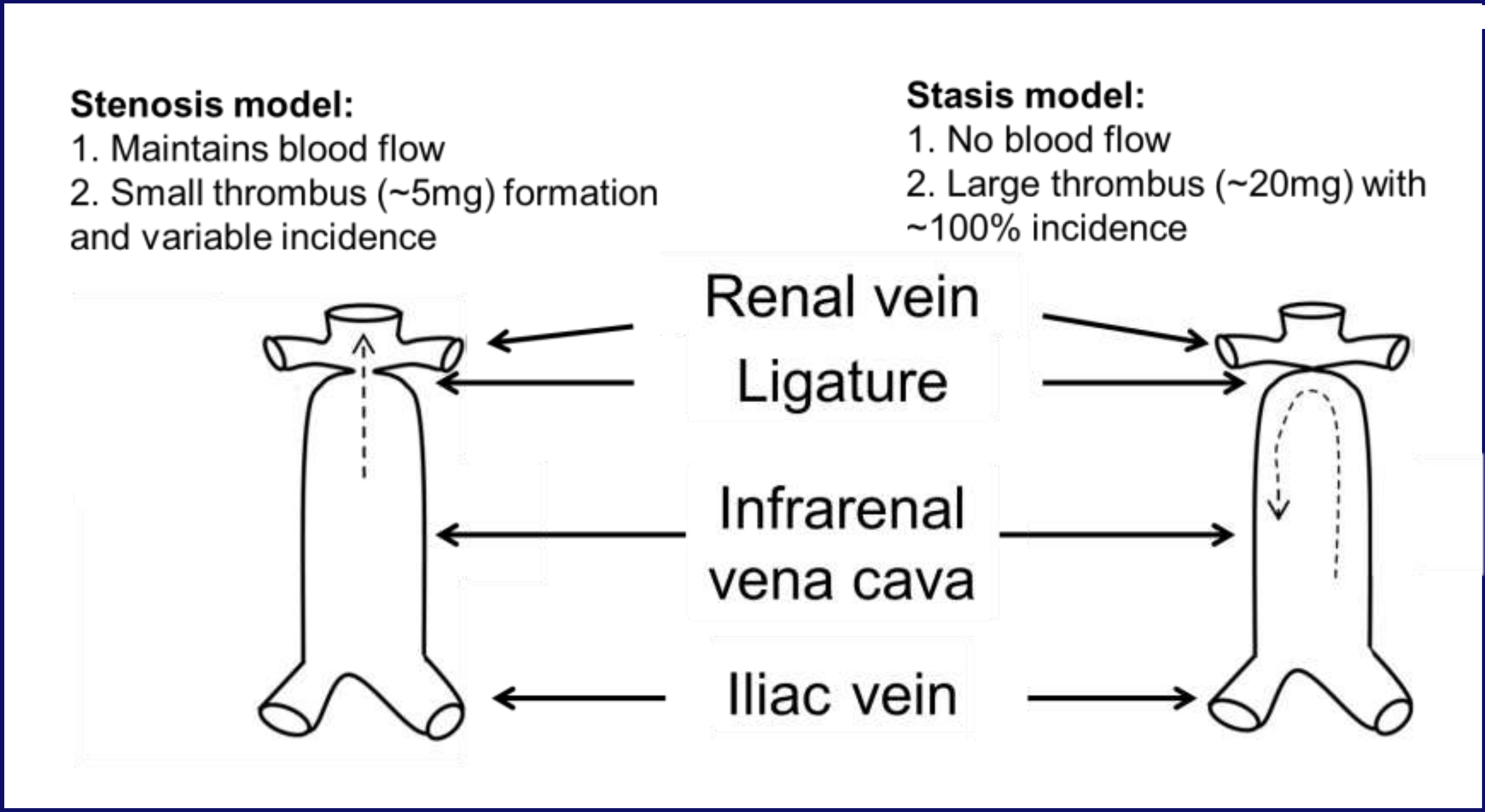
Immunocompetent mice

Can investigate the contribution of immune cells.
Can use different KO mice.
Limited number of murine cancer lines.

Immunodeficient mice

Can use human cancer cell lines and patient-derived xenografts.
Easy to image orthotopic tumors expressing reporters.
Mice lack various immune cells.

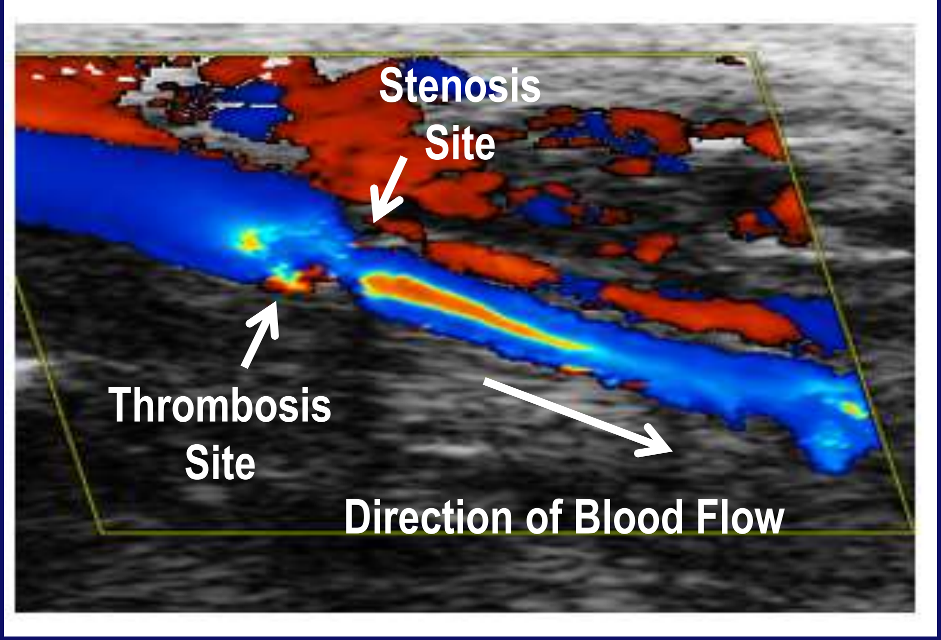
Mouse Venous Thrombosis Models: Infrarenal Vena Cava Stenosis and Stasis



Diaz J. et al. ATVB 2012

Kinetic Analysis of Thrombosis Formation

Color Doppler



Geddings J et al. JTH 2014

Summary of Allograft Mouse Models of Cancer-associated Thrombosis

Mouse strain	Cell line	Tumor site	Thrombosis model	Result	Ref
C57BL/6	LLC lung	S.Q.	$FeCl_3$/mesenteric	Occlusion time ↓	Thomas et al JEM 2009
C57BL/6	M27 lung	Orthotopic	$FeCl_3$/IVC	Clot weight ↑	Aghourian et al Blood 2016
BALB/c	4T1 breast	Orthotopic	Rose Bengal/Jagular	Occlusion time ↓	Leal et al Sci Rep 2016
C57BL/6	Pan02 pancreatic	S.Q.	$FeCl_3$/mesenteric	Occlusion time ↓	Thomas et al JEM 2009
C57BL/6	Pan02 pancreatic	S.Q.	Laser/cremaster	Clot ↑	Mesouar et al Int J Cancer 2015
C57BL/6	Pan02 pancreatic	S.Q.	IVC/stenosis	Clot incidence & weight ↑	Thomas et al JTH 2015

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Summary of Xenograft Mouse Models of Cancer-associated Thrombosis

Cell line	Thrombosis model	Result	Ref
A549 lung	IVC stenosis (3h)	Clot weight ↑	Chen et al Mol Cancer 2015
HPAF-II pancreatic	IVC stenosis (3h)	No change	Wang et al Blood 2009
BxPc-3 pancreatic	IVC stenosis (0-24h)	Clot incidence ↑	Geddings et al JTH 2015
BxPc-3 pancreatic	IVC stasis (0-48h)	Clot weight ↑	Hisada et al JTH 2017

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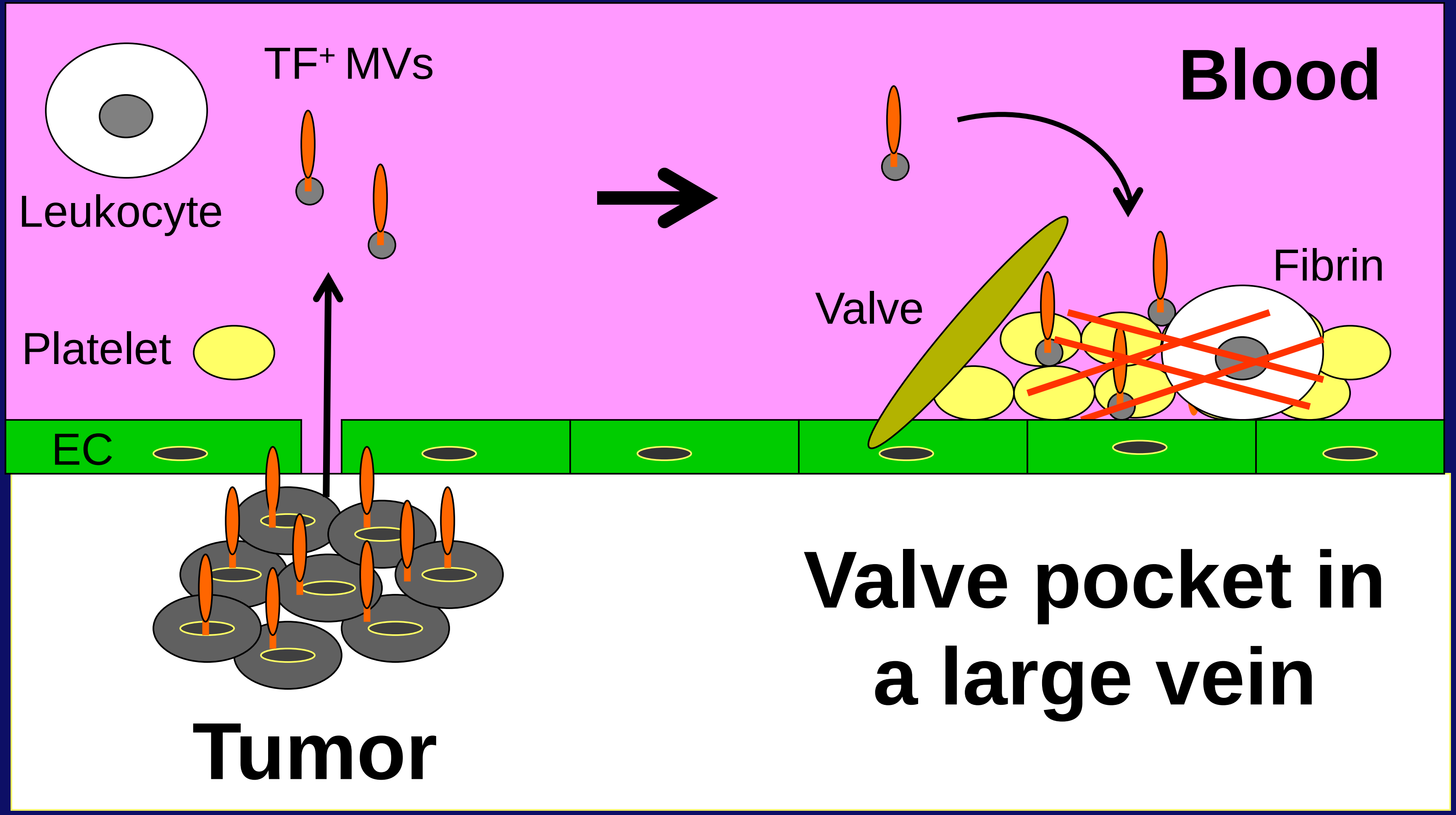
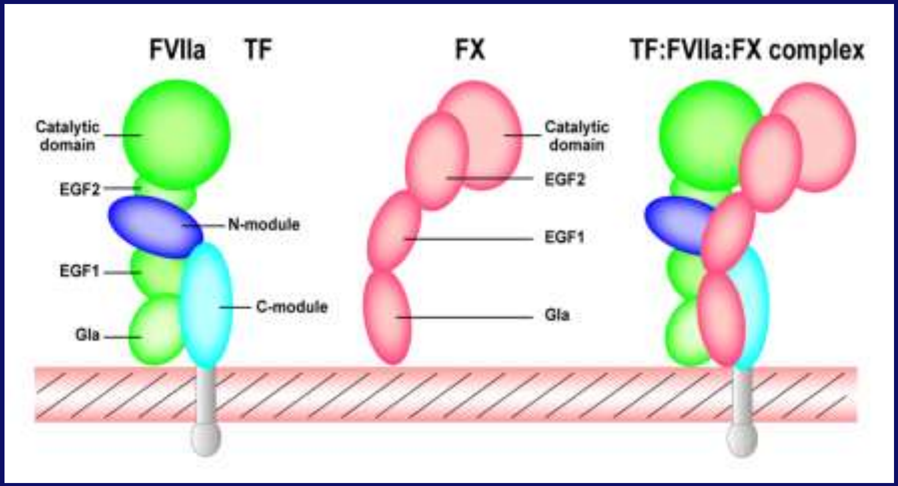
Hypothesis: Tumor-derived TF⁺ MVs Trigger Venous Thrombosis in Cancer Patients



Tissue Factor (TF): The Primary Activator of Blood Coagulation

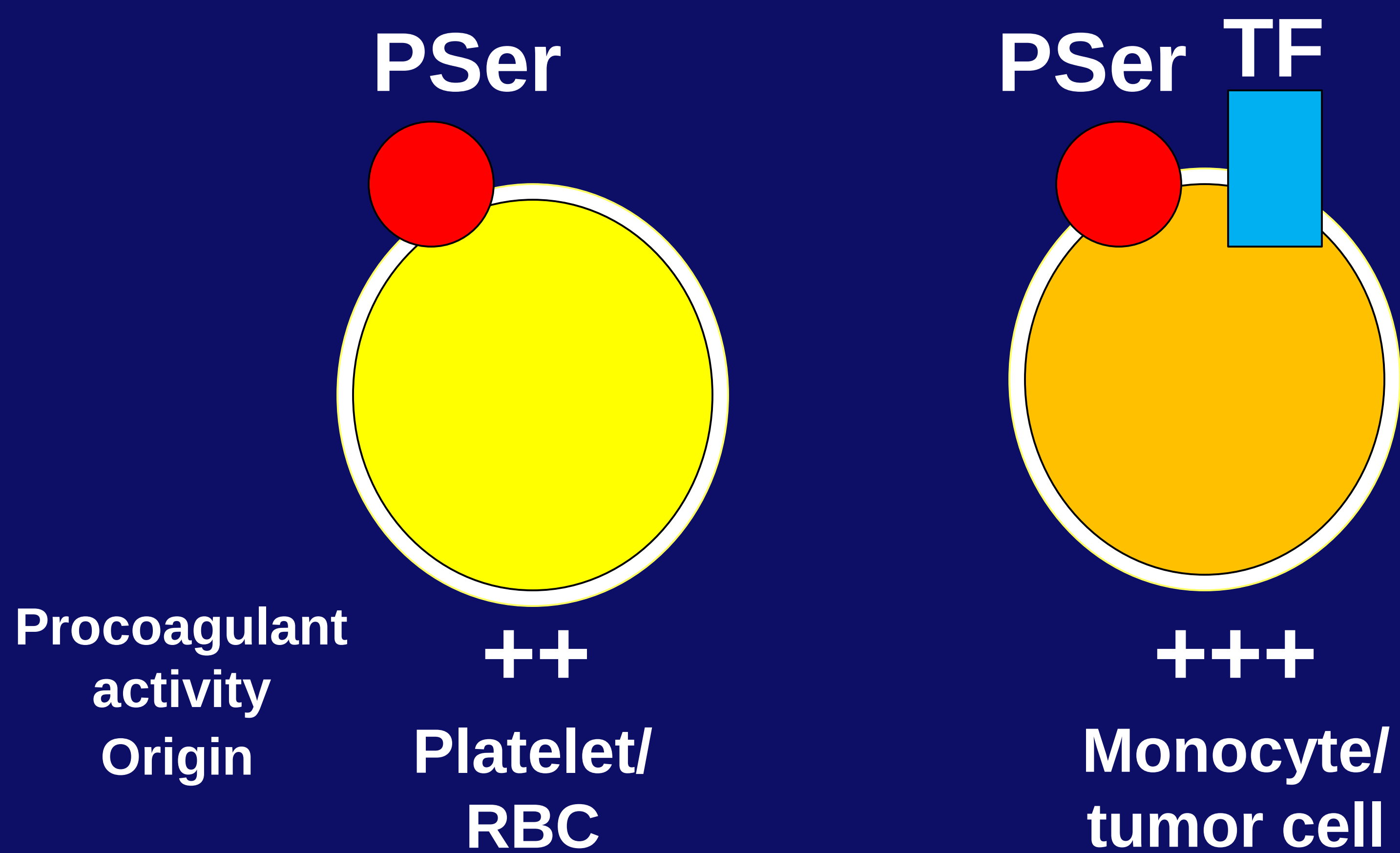


- 47 kDa transmembrane glycoprotein
- Receptor for FVII/FVIIa
- The TF:FVIIa complex activates both FIX and FX

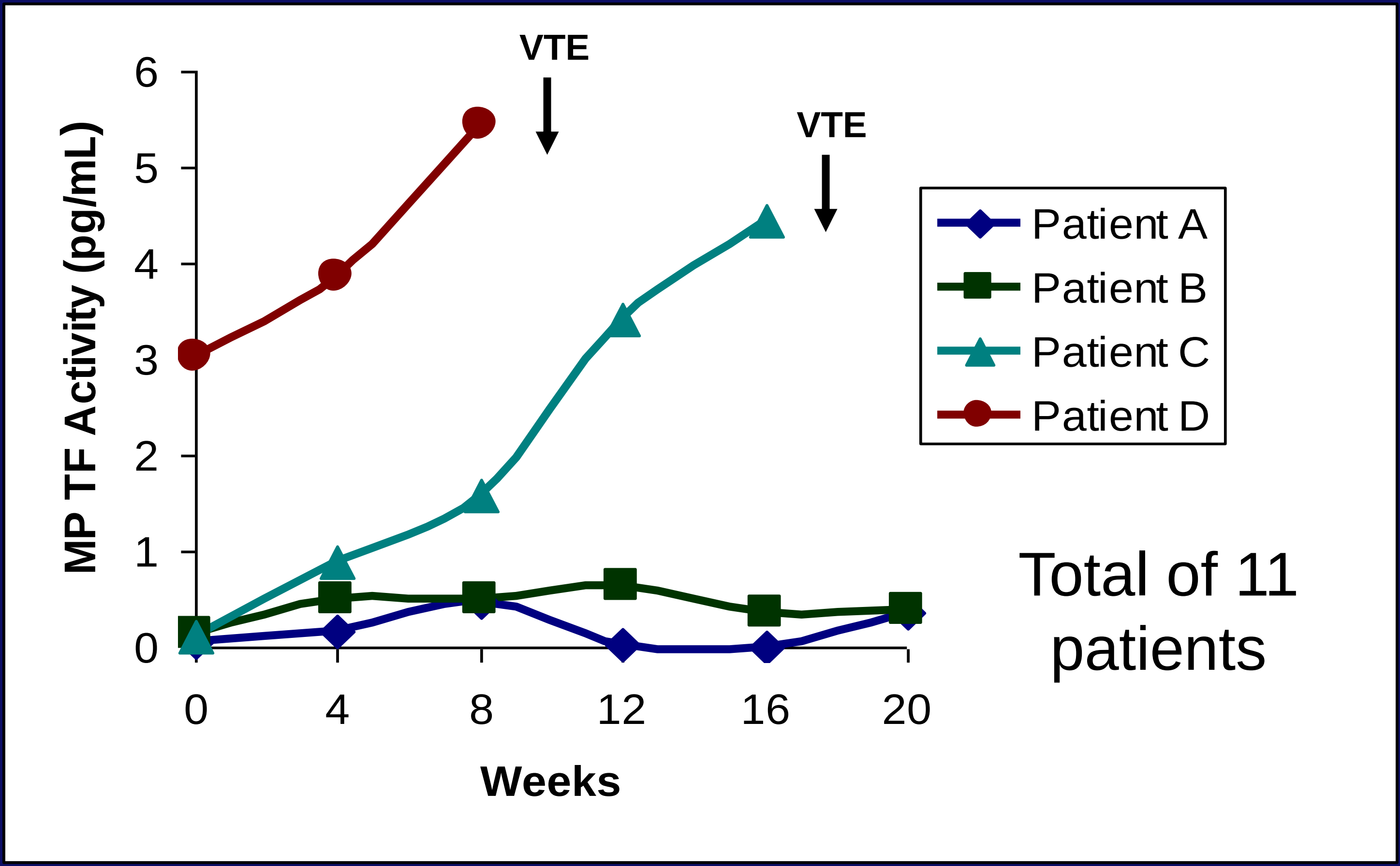
Mackman Thromb Haemost 2007

Types of Procoagulant MVs

Microvesicles (MVs) are defined as small (0.1-1 µm) membrane vesicles that are released from activated or apoptotic cells



Levels of MV TF Activity Increase in Pancreatic Cancer Patients before VTE

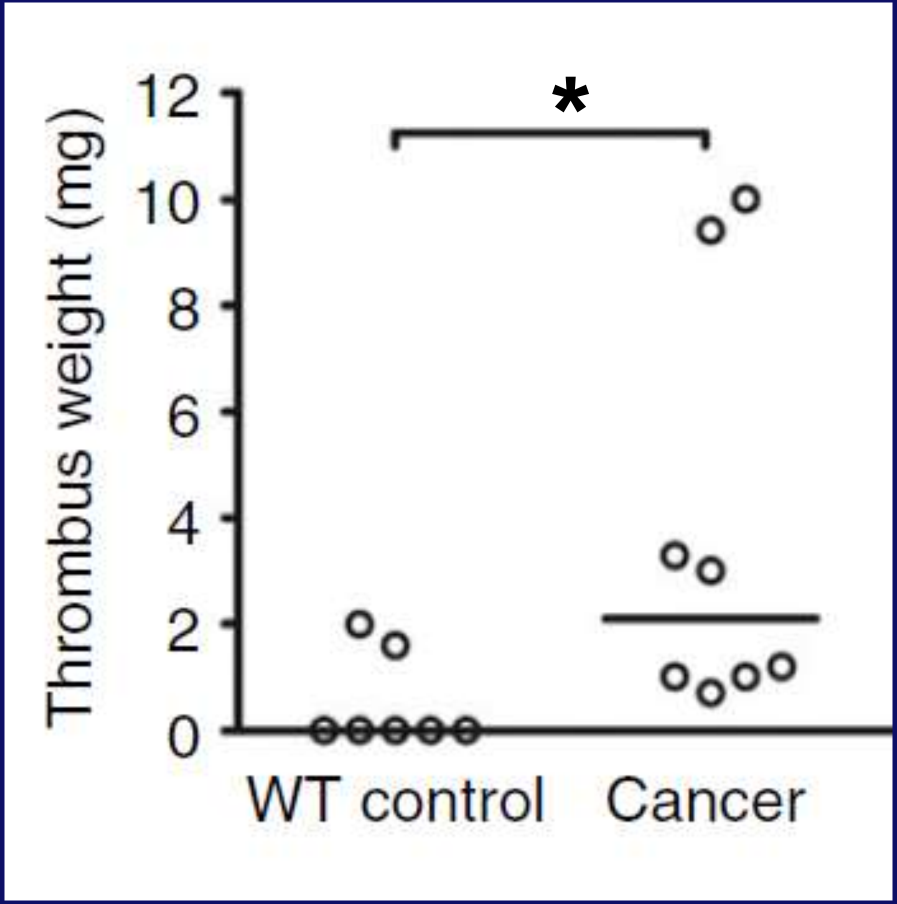


Khorana A et al JTH 2008

Association of MV TF Activity and VTE in Pancreatic Cancer Patients

Study	Patients	Time of Follow-up	Association between MV TF and VTE?
Khorana, 2008	2/9 patients developed VTE	5 months	Yes
Van Doormaal, 2012	3/13 patients developed VTE	6 months	Yes
Thaler, 2012	12/60 patients developed VTE	2 years	Weak
Bharthuar, 2013	*52/117 patients developed VTE	6 months	Yes
Van Es, Resubmitted	9/89 patients developed VTE	6 months	Weak
Khorana, In revision	7/39 patients developed VTE	3 months	Yes
Ilich, In preparation	4/22 patients developed VTE	6 months	Yes

Increased Thrombosis in C57BL/6 Mice bearing Murine Pancreatic Tumors



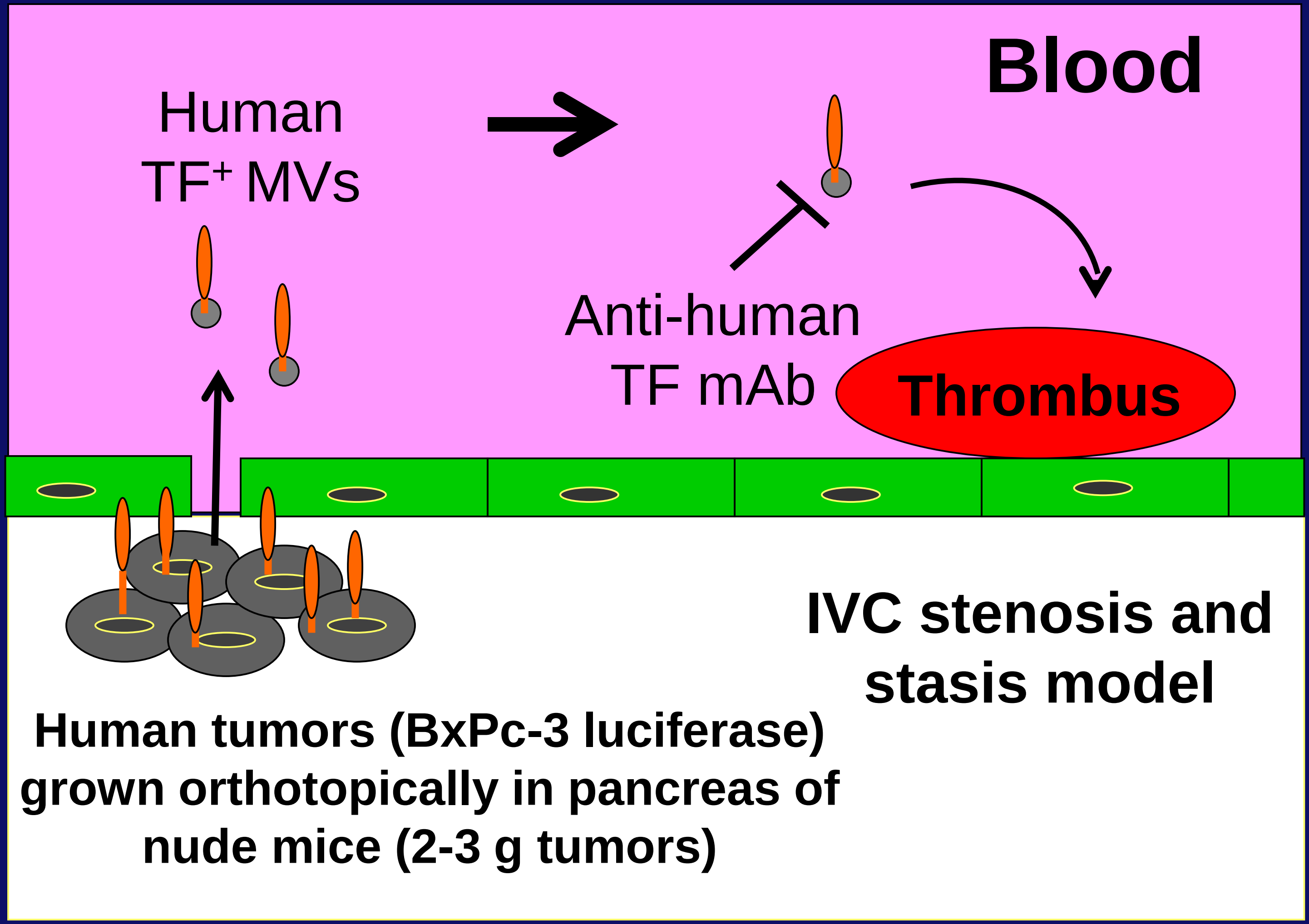
Clot measured at 3 h in the IVC stenosis model in mice bearing Panc02 tumors

Thomas G et al JTH 2015

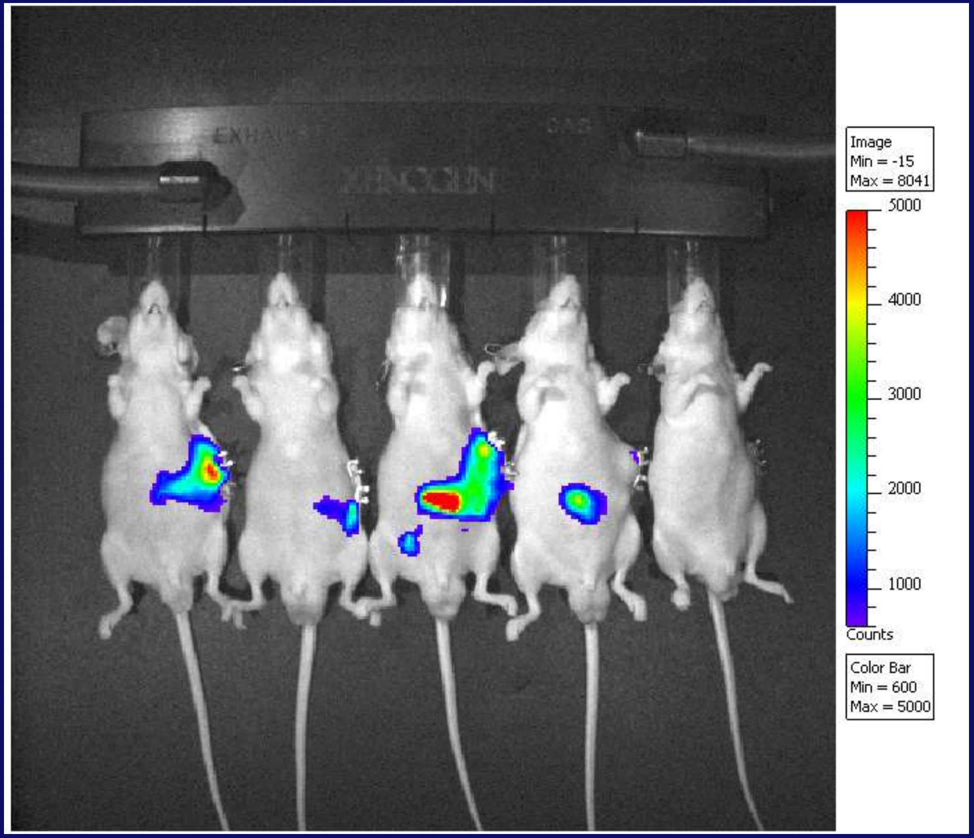
Model to Study the Role of Tumor-derived, TF⁺ MVs in Venous Thrombosis in Mice

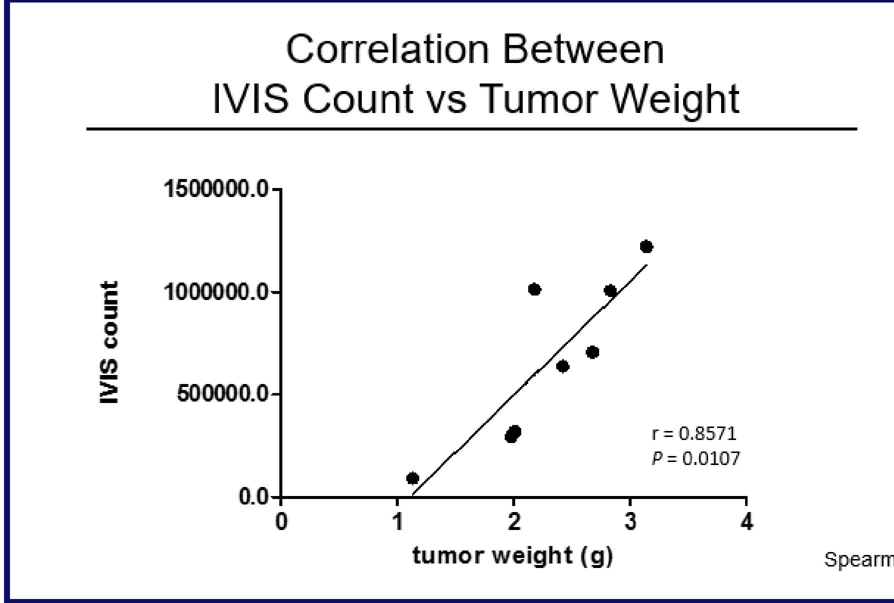
Julia Geddings

Yohei Hisada



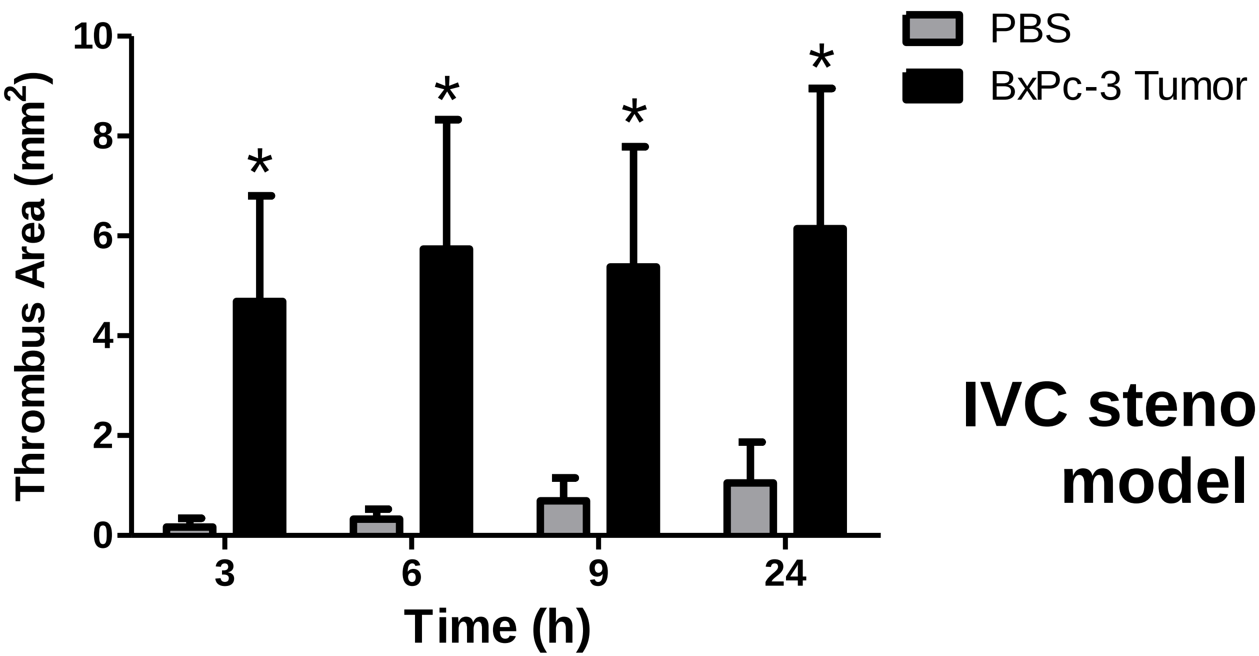
Imaging of BxPc-3-luciferase Tumors in the Pancreas of Mice





Hisada Y et al JTH 2017

Increased Thrombosis in Nude Mice bearing Human Pancreatic Tumors



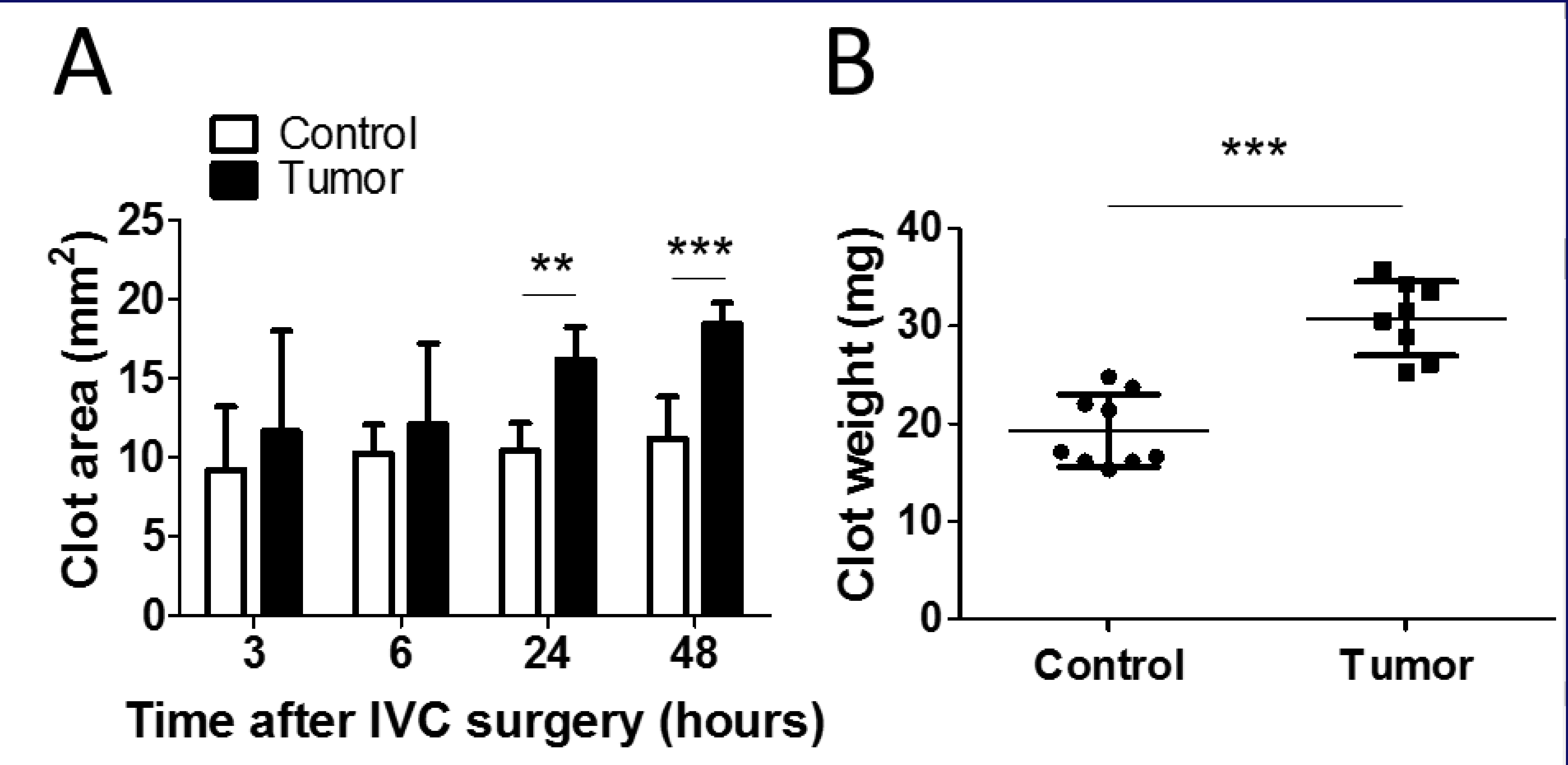
IVC stenosis model

Thrombus Incidence

Time (h)	3	6	9	24
PBS	17%	33%	33%	33%
BxPc-3 Tumor	50%	50%	50%	50%

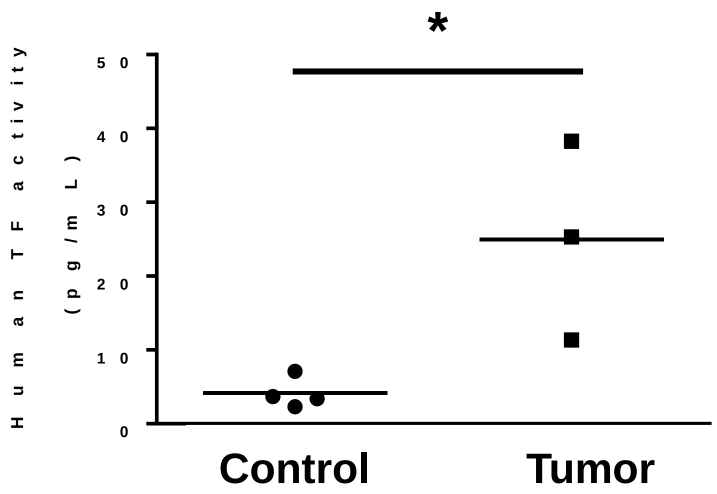
Geddings J et al. JTH 2015

Human Pancreatic Tumors increase Clot Size in Nude Mice



Hisada Y et al JTH 2017

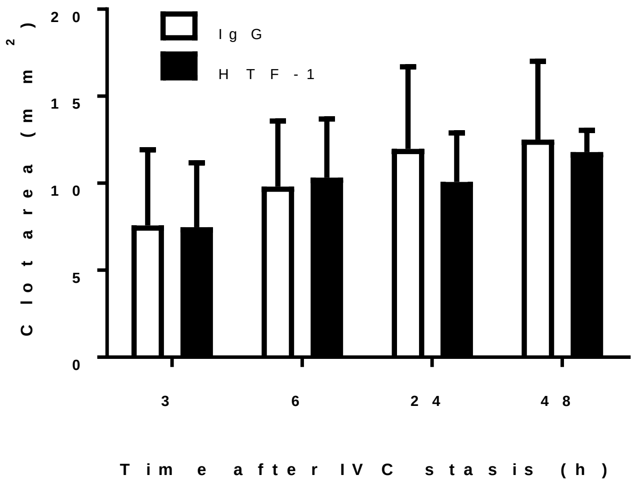
Clots from Tumor-bearing Mice Contain Human TF

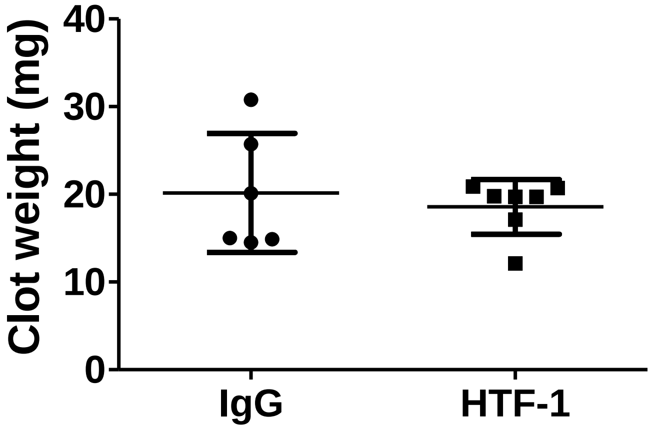


Hisada Y et al JTH 2017

Inhibition of Human TF Reduces Clot Size in Tumor Bearing Mice

Control



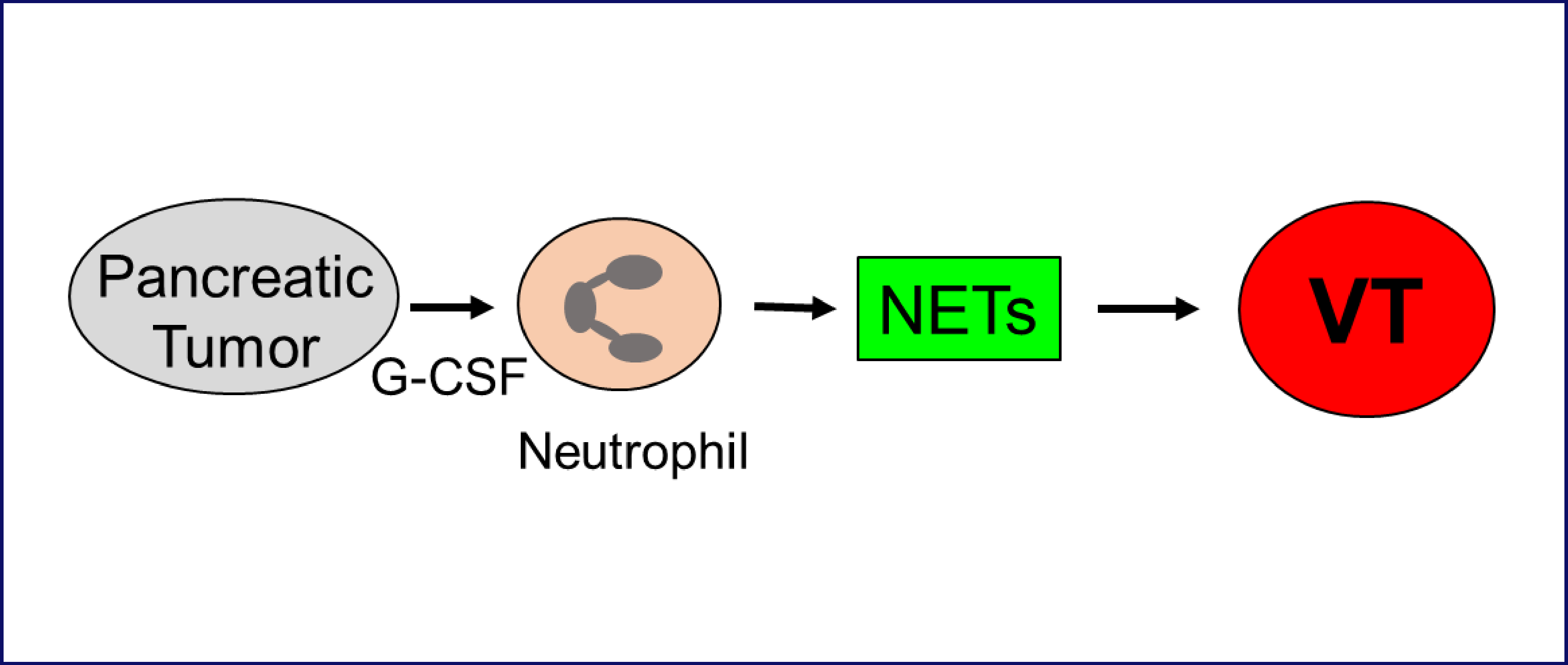


Hisada Y et al JTH 2017

Conclusion

Tumor-derived TF^{+} MVs enhance venous thrombosis in mice with pancreatic tumors

Hypothesis: Neutrophils and NETs Contribute Venous Thrombosis in Cancer Patients



Neutrophil Extracellular Traps- NETs

NETs are extracellular DNA fibers comprising of histones and neutrophil antimicrobial proteins- formed by a cell-death program called NETosis

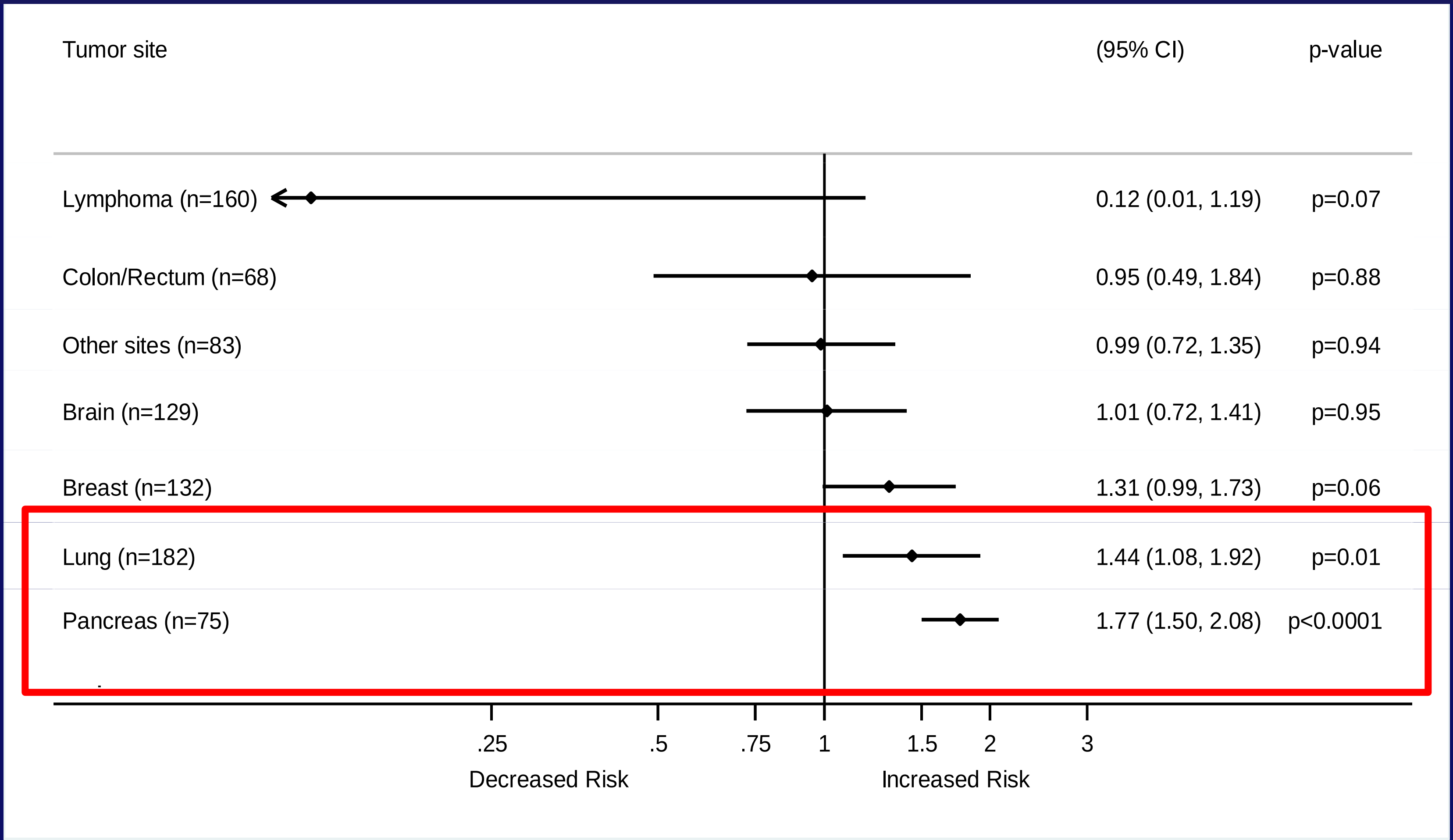
Brinkmann et al Science 2004 Neutrophils extracellular traps kill bacteria

NETs and Thrombosis

- **NETs are found in venous and arterial clots.** **Fuchs et al PNAS 2010; Longstaff et al JBC 2013; Mangold et al Circ Res 2015.**

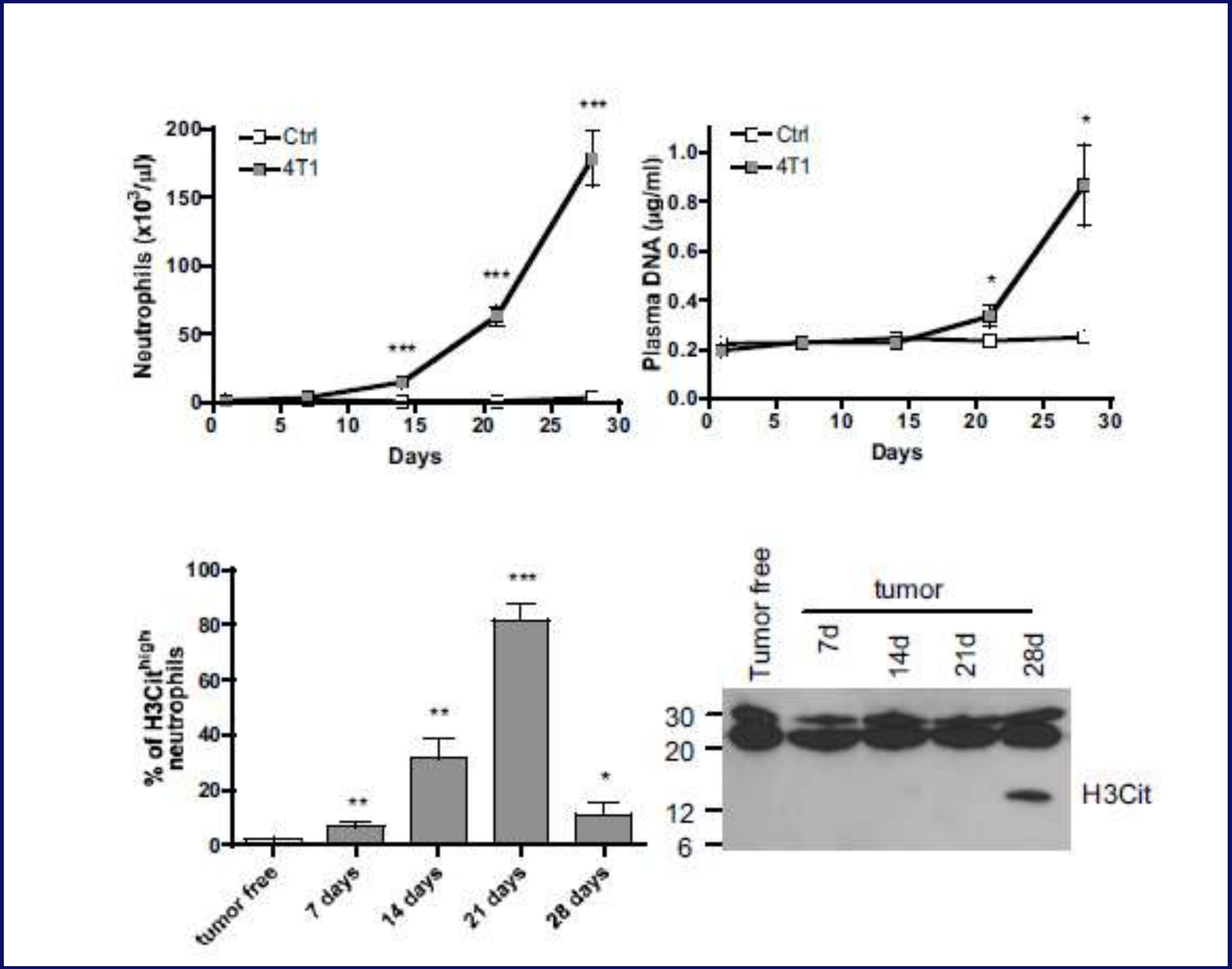
- **NETs contribute to venous thrombosis in mouse models.** **Brill et al JTH 2012; Martinod et al PNAS 2013.**

Levels of H3Cit are Associated with VTE Lung and Pancreatic Cancer



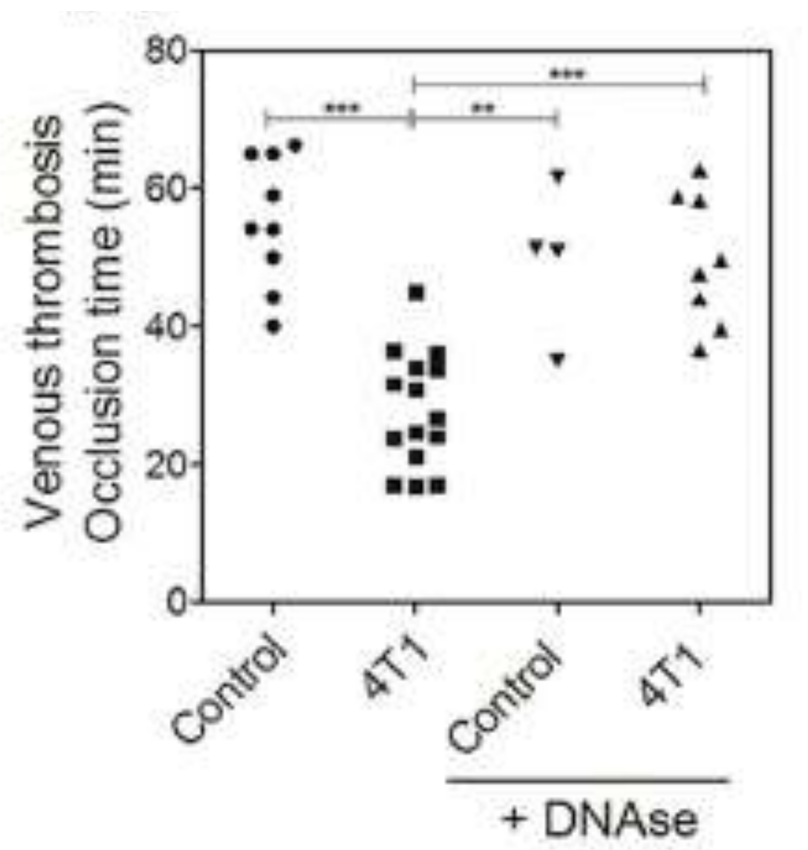
Modified from Mauracher LM et al. JTH 2018

Mice bearing 4T1 Breast Tumors have increased Neutrophils and NETs



Demers et al PNAS 2012

Mice bearing 4T1 Breast Tumors have increased Venous Thrombosis

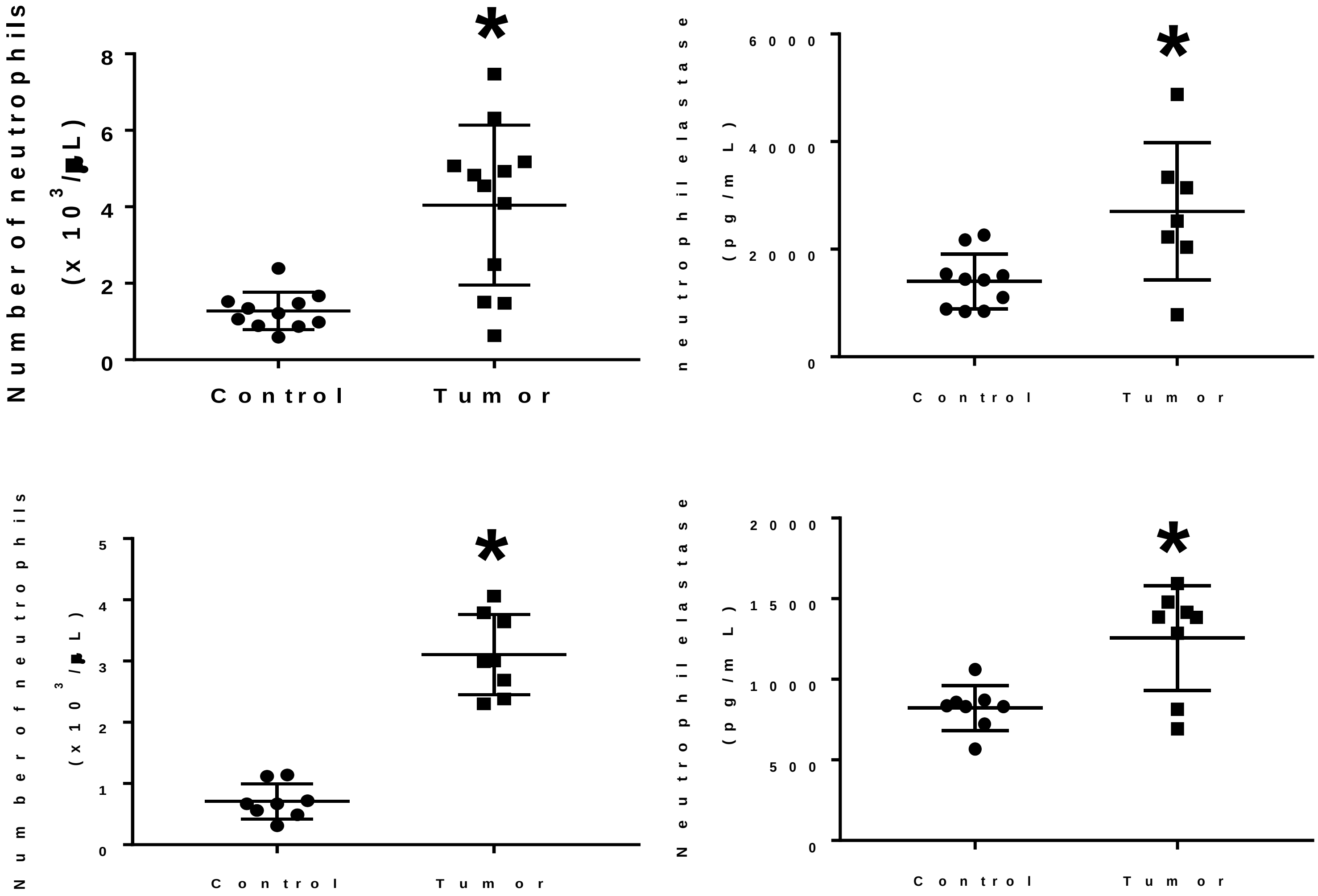


Leal et al Sci Rep 2017

Mice bearing Human and Murine Pancreatic Tumors have increased Neutrophils and Neutrophil Elastase

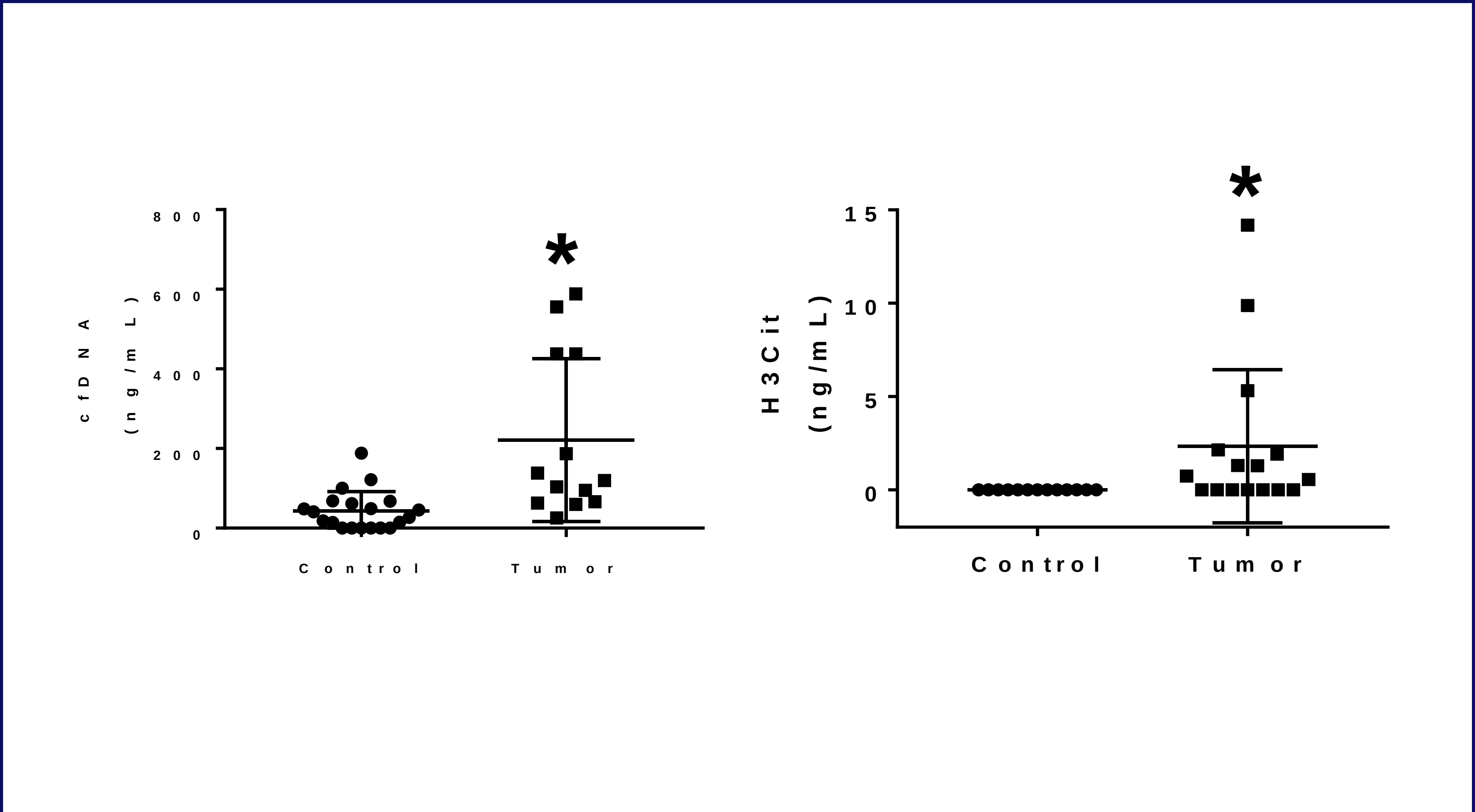
BxPc-3 (Human)

Pan02 (Mouse)



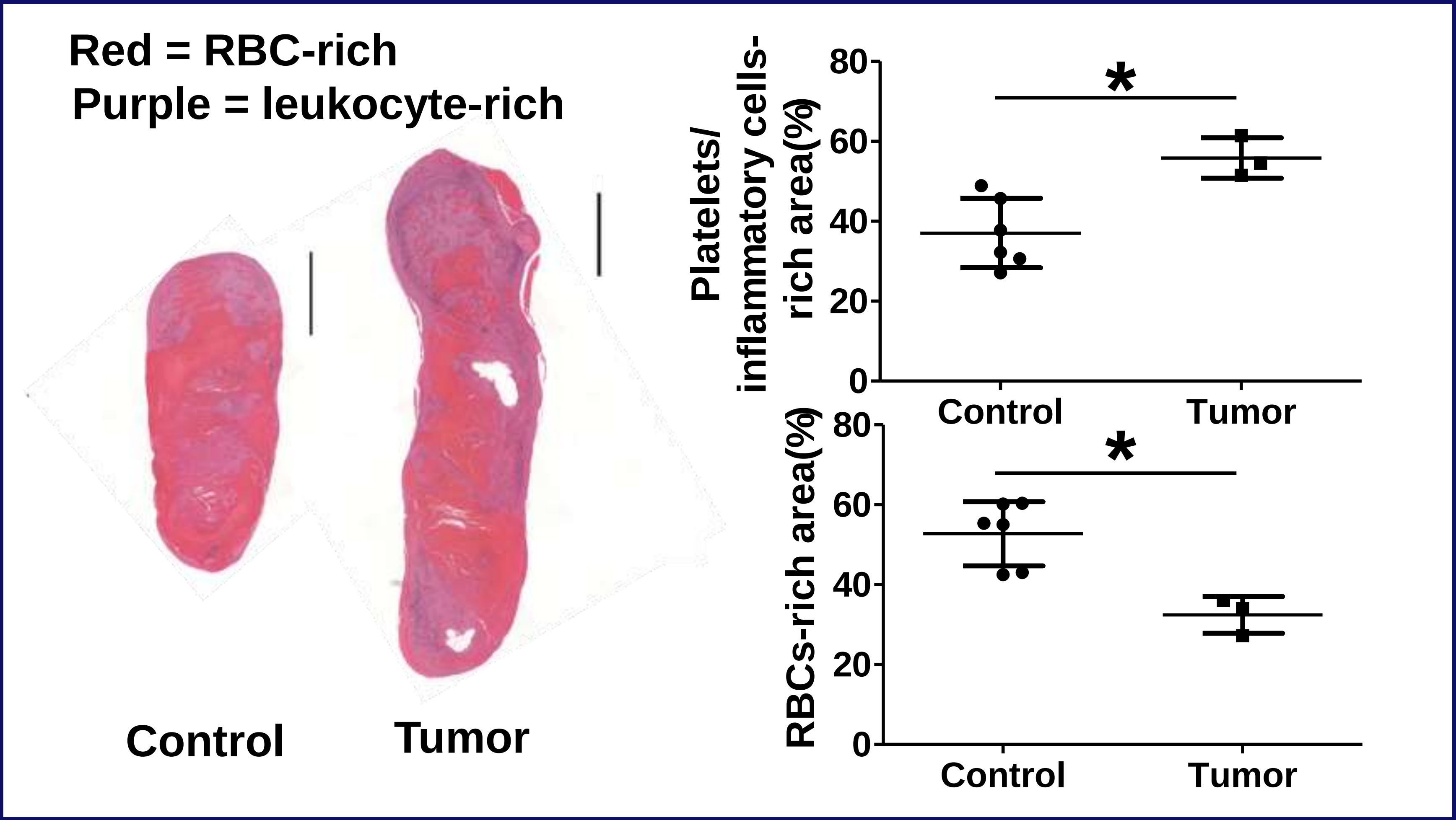
Hisada unpublished data

Mice bearing Human Pancreatic Tumors have increased Plasma cfDNA and NETs



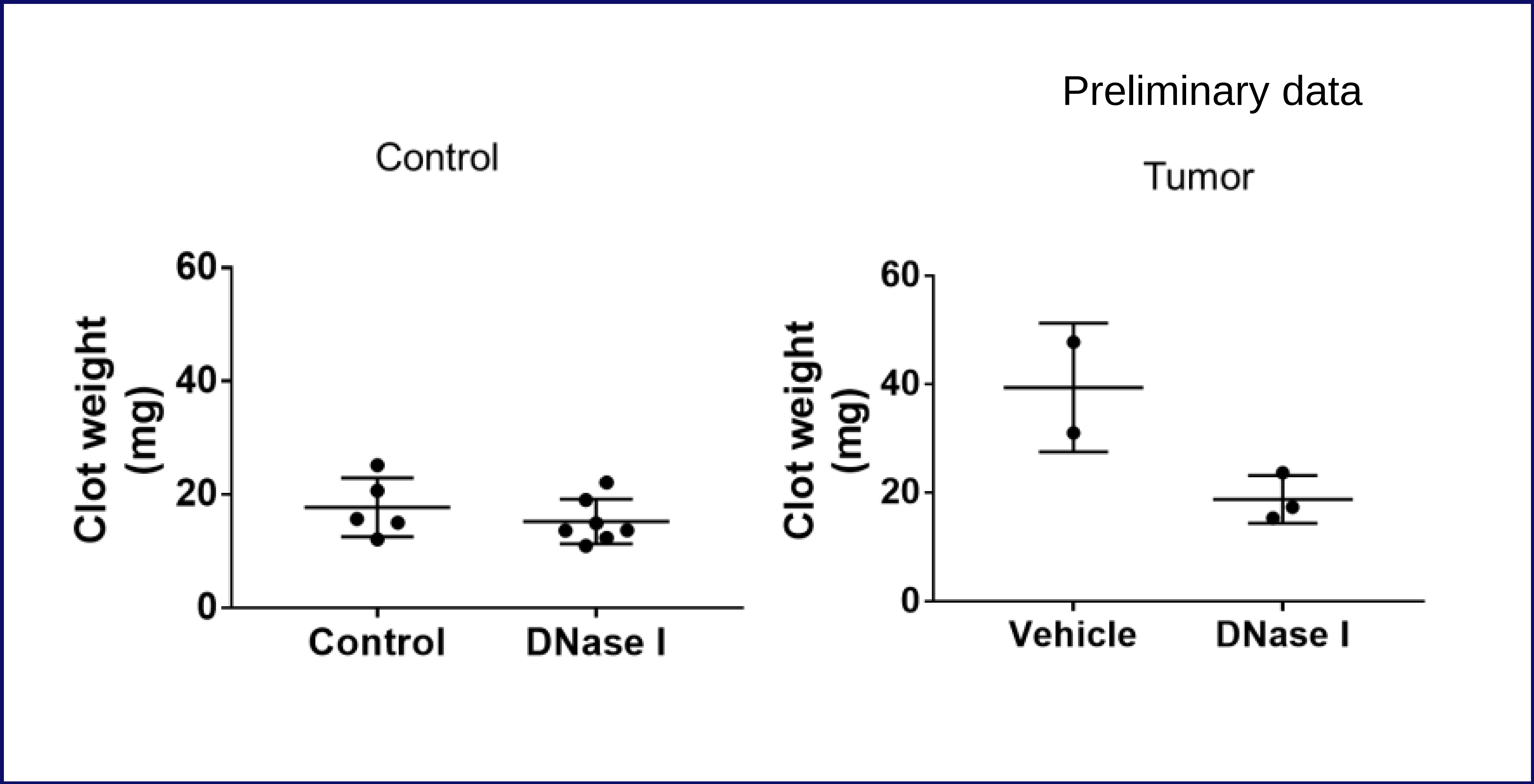
Hisada unpublished data

Clots from Mice bearing BxPc-3 Tumors have increased Leukocytes



Hisada et al JTH 2017

Effect of DNase I on Thrombus Size in an IVC Stasis Model in Control and Tumor bearing Mice



Hisada unpublished data

Conclusion

Neutrophils and NETs appear to contribute to venous thrombosis in mice with pancreatic tumors

Acknowledgements

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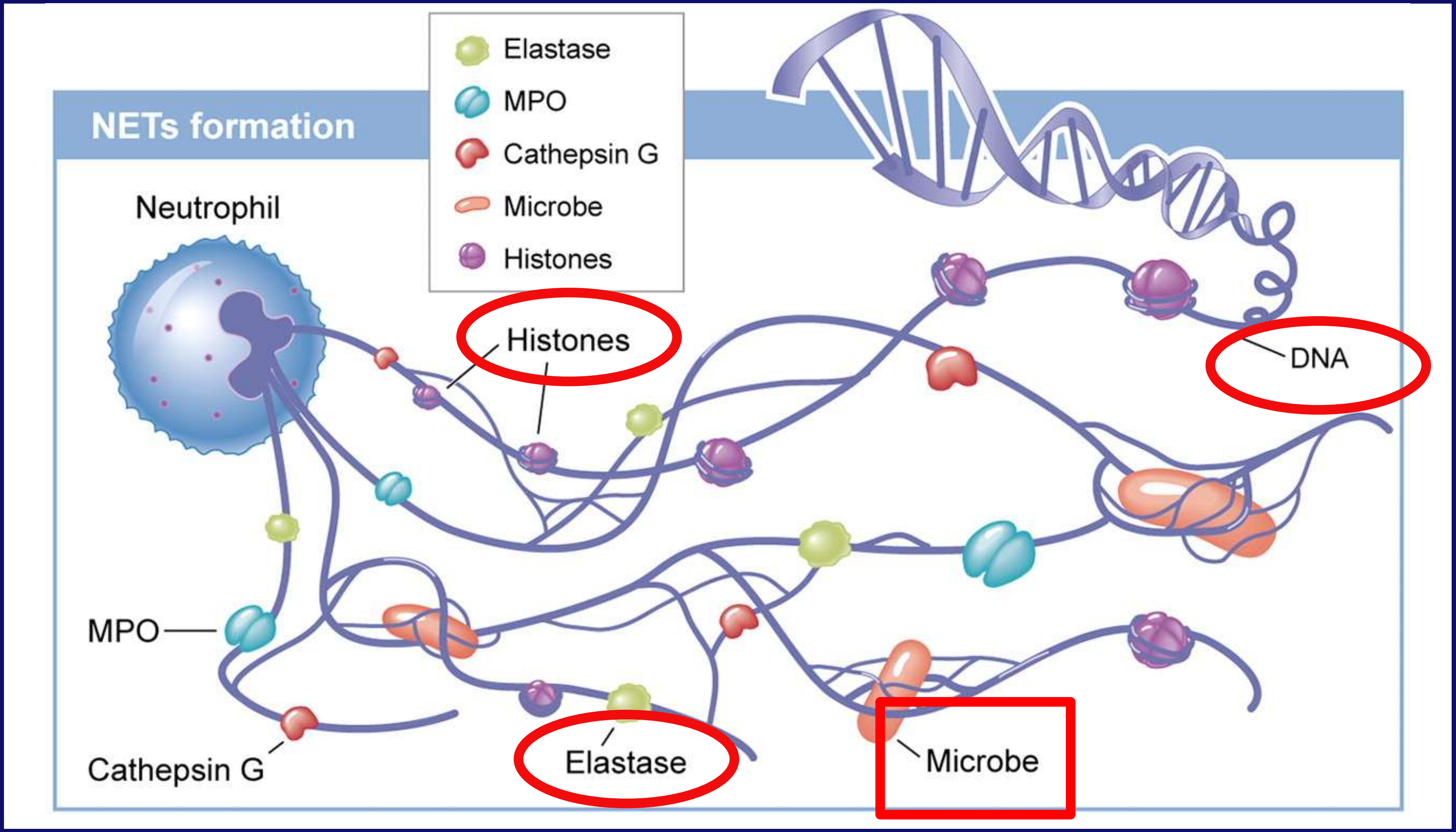
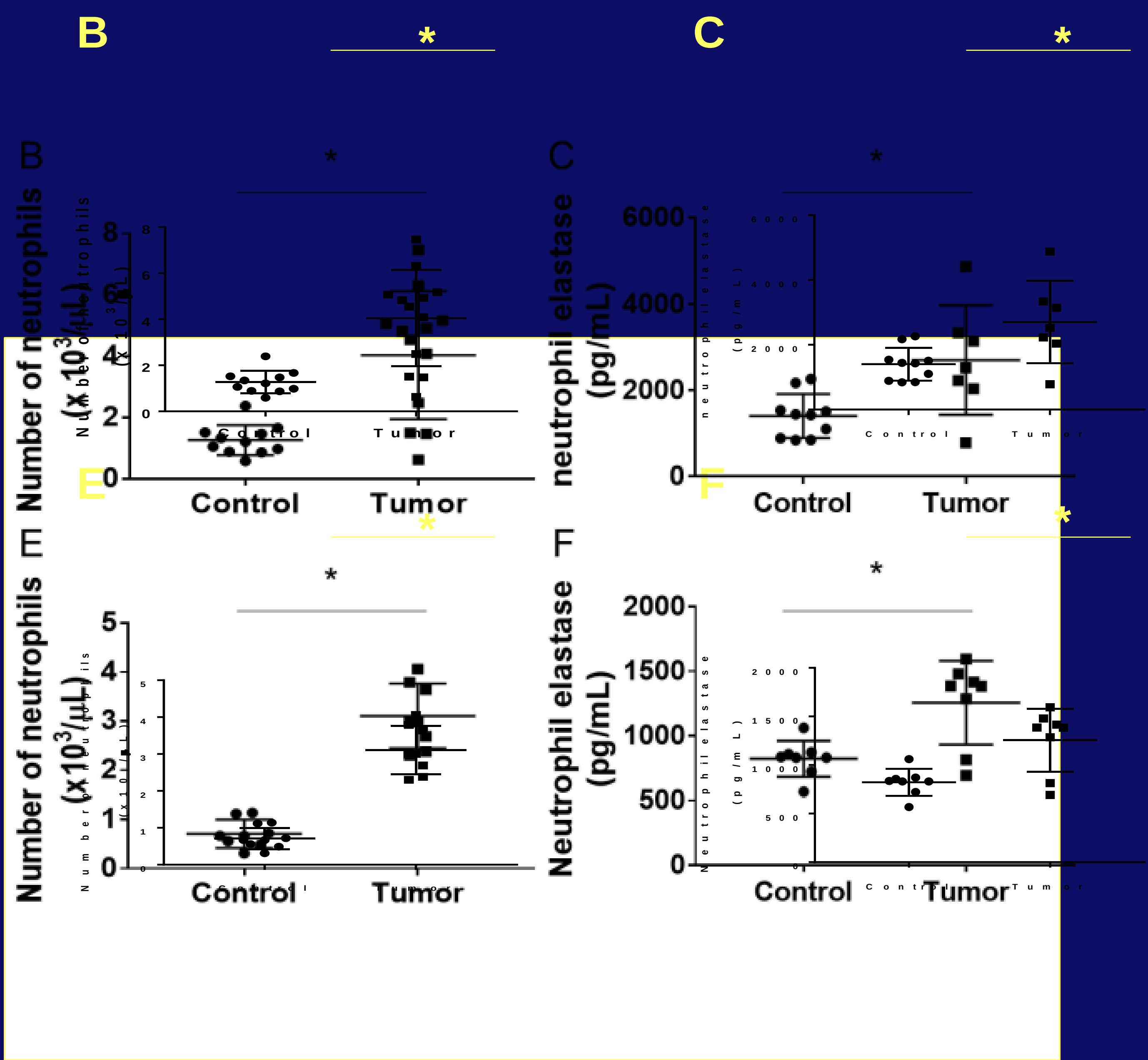
Neutrophil Extracelluar Traps (NETs)



Miyata T. and Fan X. Blood 2012

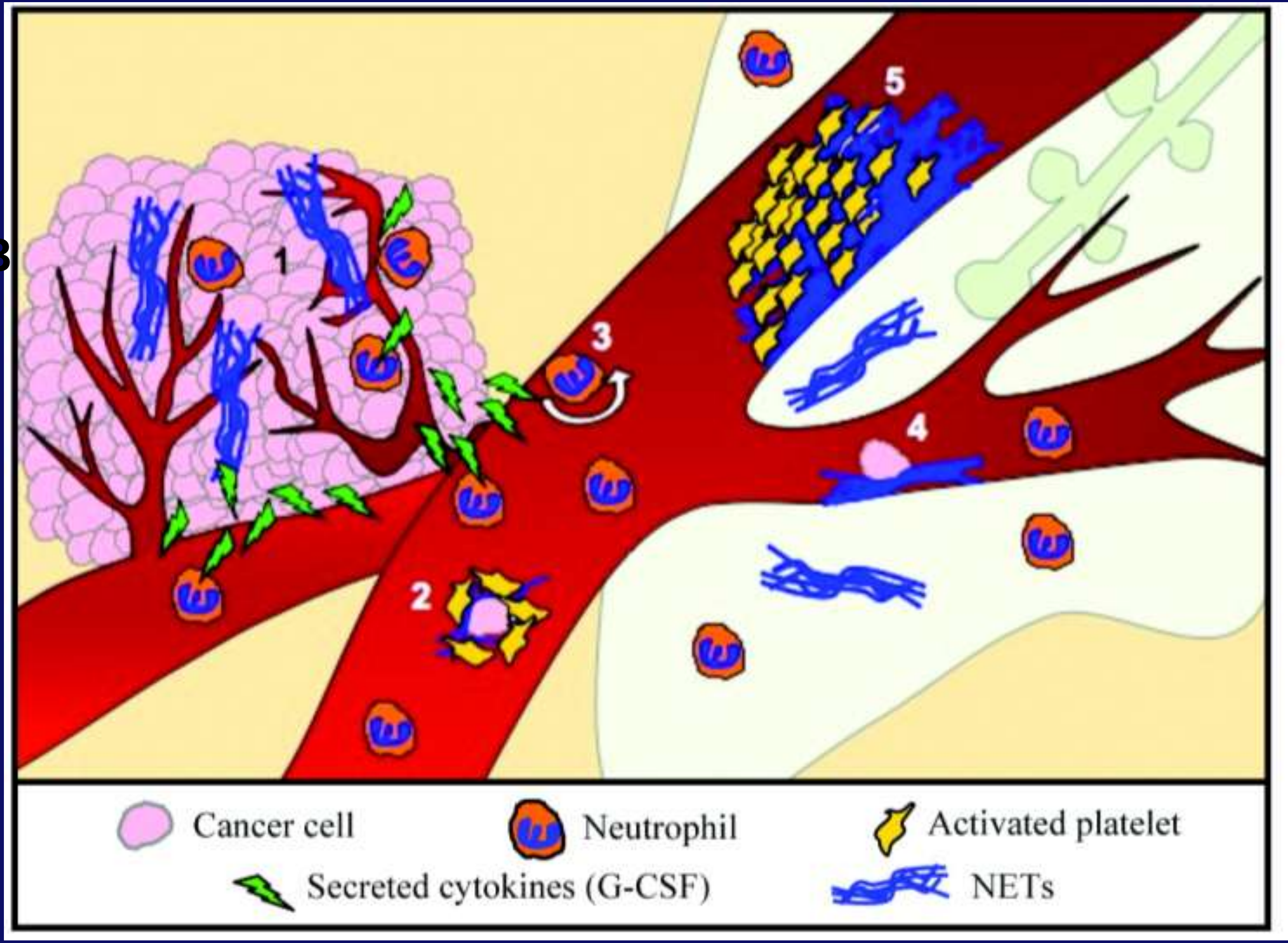
Conclusion

Tumor-derived TF^{+} MVs enhance venous thrombosis in mice with pancreatic tumors


B
*
C
*
Number of neutrophils (x 10^3/μL)
0
2
4
6
8
Control
Tumor
neutrophil elastase (pg/mL)
0
2000
4000
6000
E
*
F
Number of neutrophils (x10^3/μL)
0
1
2
3
4
5
Neutrophil elastase (pg/mL)
0
500
1000
1500
2000

NETs and Cancer-associated Thrombosis

BxPc-3



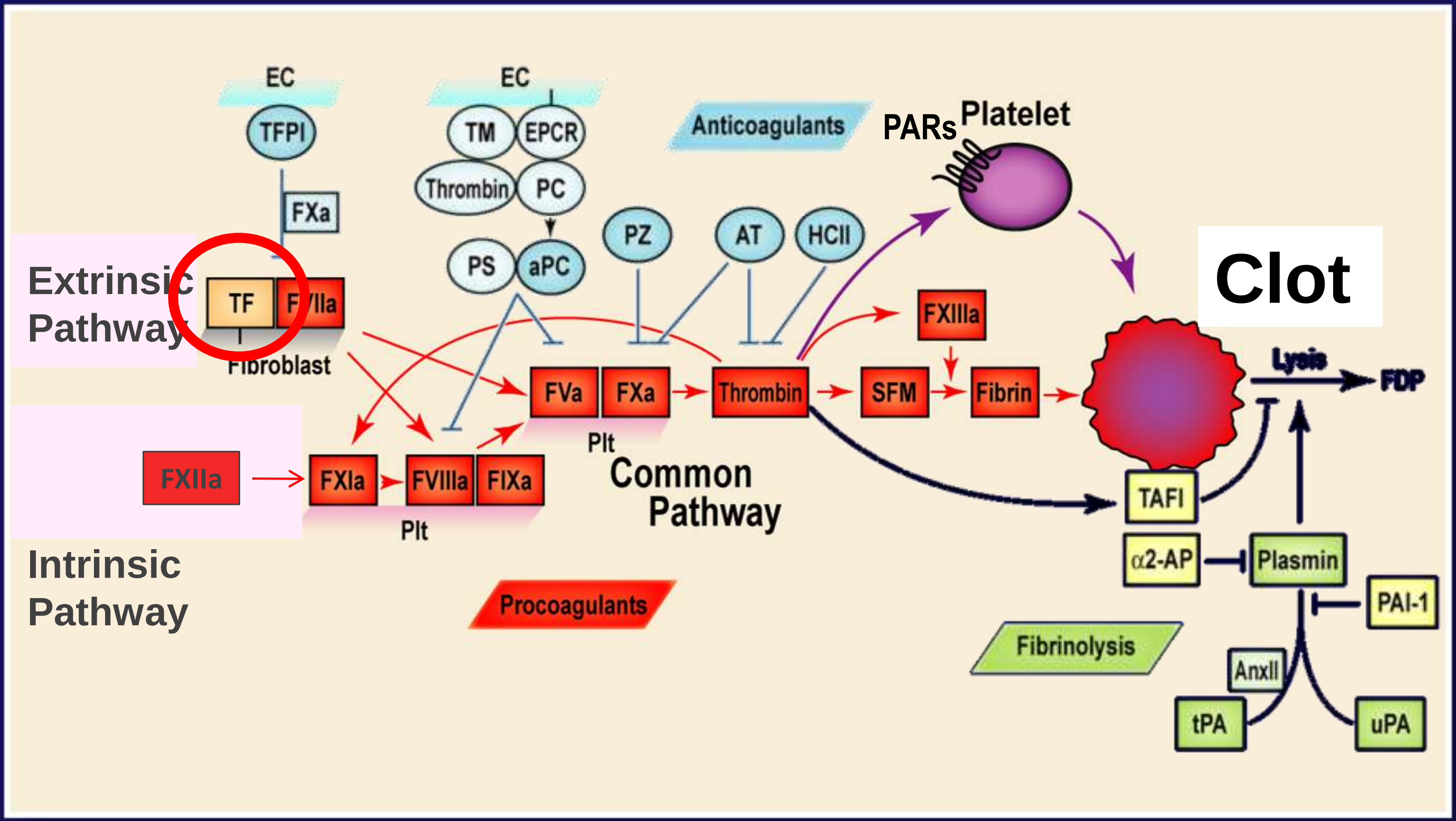
Demers M. and Wagner DD. Oncoimmunology 2013

Terminology

- Hemostasis - the arrest of bleeding
- Thrombosis - clotting within a blood vessel that may cause reduce blood flow and tissue oxygenation

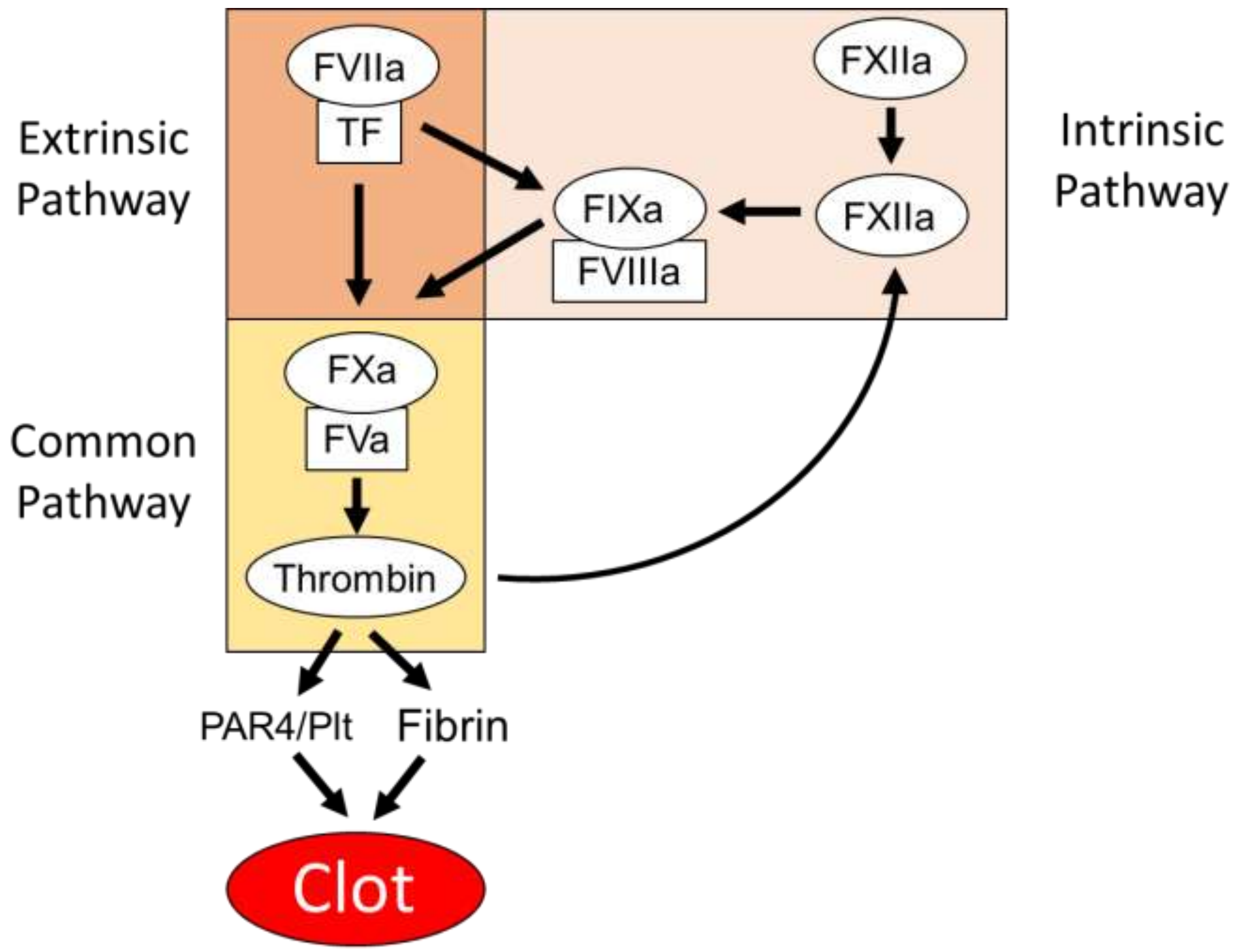
- Hemophilia - a disorder of the blood marked by a permanent tendency to hemorrhages
- Thrombophilia - a disorder of the blood in which there is a tendency to the occurrence of thrombosis

Formation and Lysis of a Clot



Mackman ATVB 2005

Simplified Blood Coagulation Cascade

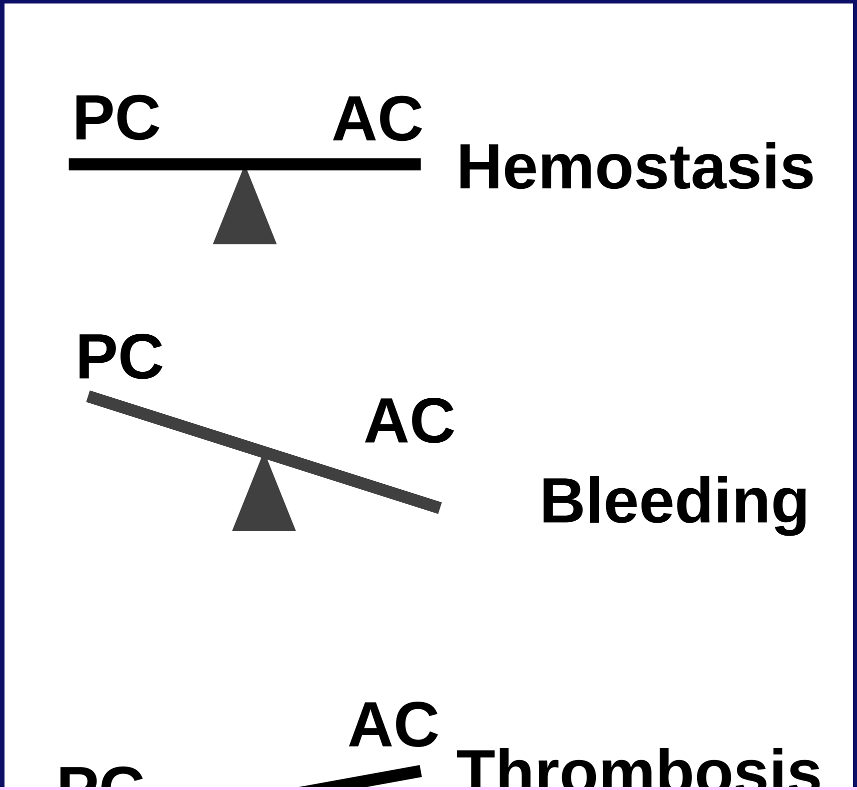


Balancing Clotting

Normal Hemostasis

Hemophilia
A (FVIII), B (IX) and C (XI)

Thrombophilia

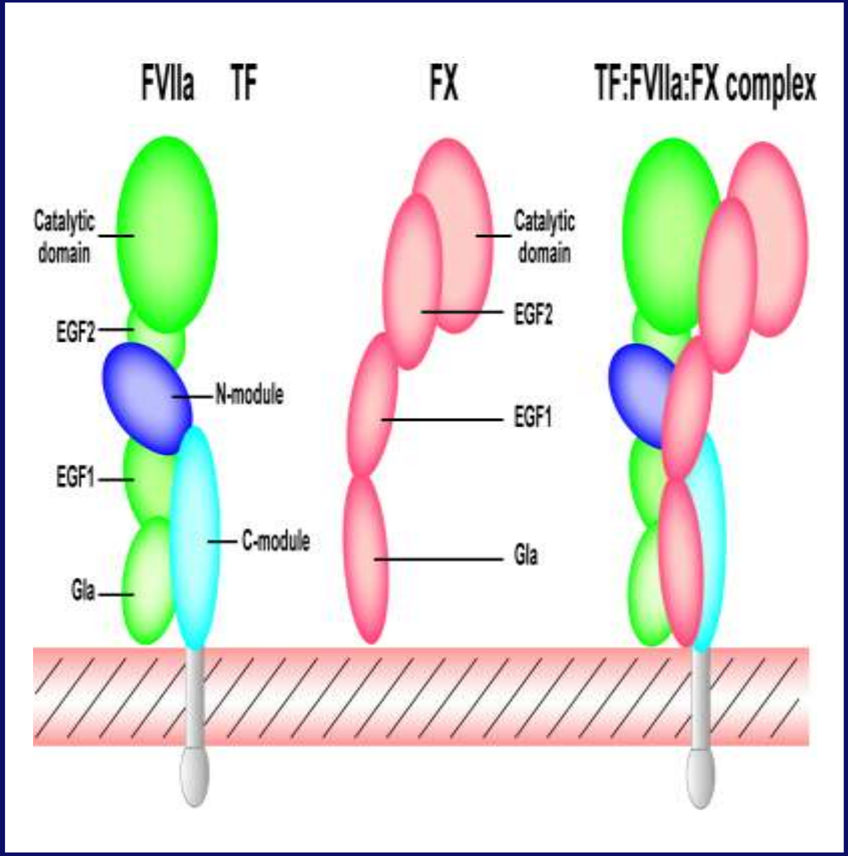


Restore hemostasis in hemophilia patients by decreasing levels of ACs

PC, procoagulant; AC, anticoagulant

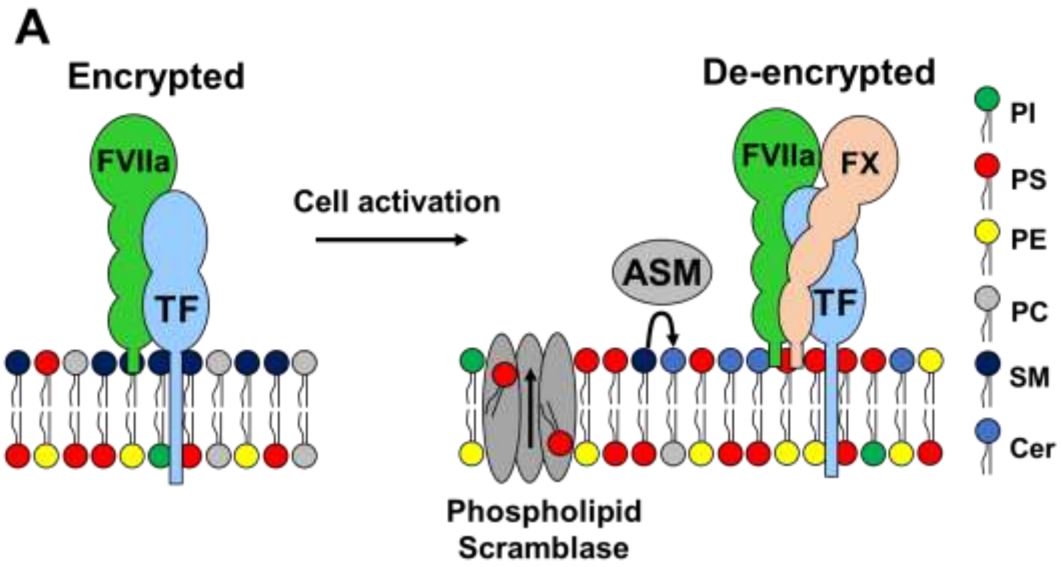
Tissue Factor (TF): The Primary Activator of Blood Coagulation

- 47 kDa transmembrane glycoprotein
- Receptor for FVII/FVIIa
- The TF:FVIIa complex activates both FIX and FX



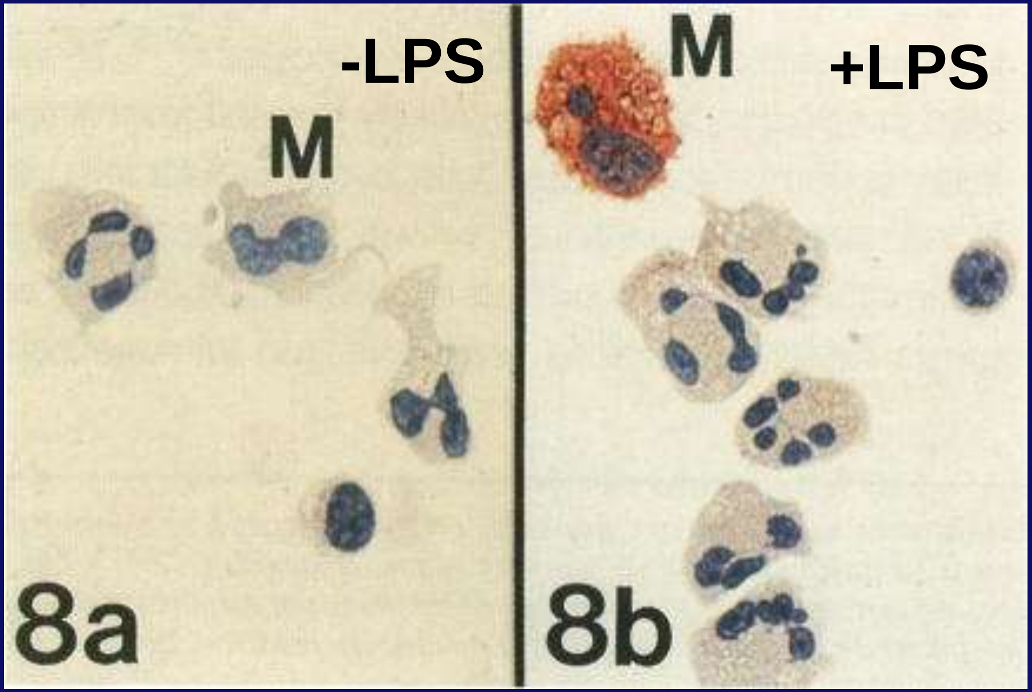
Mackman Thromb Haemost 2007

Mechanisms of TF De-encryption



Grover and Mackman ATVB in press

LPS Induction of TF Expression in Monocytes



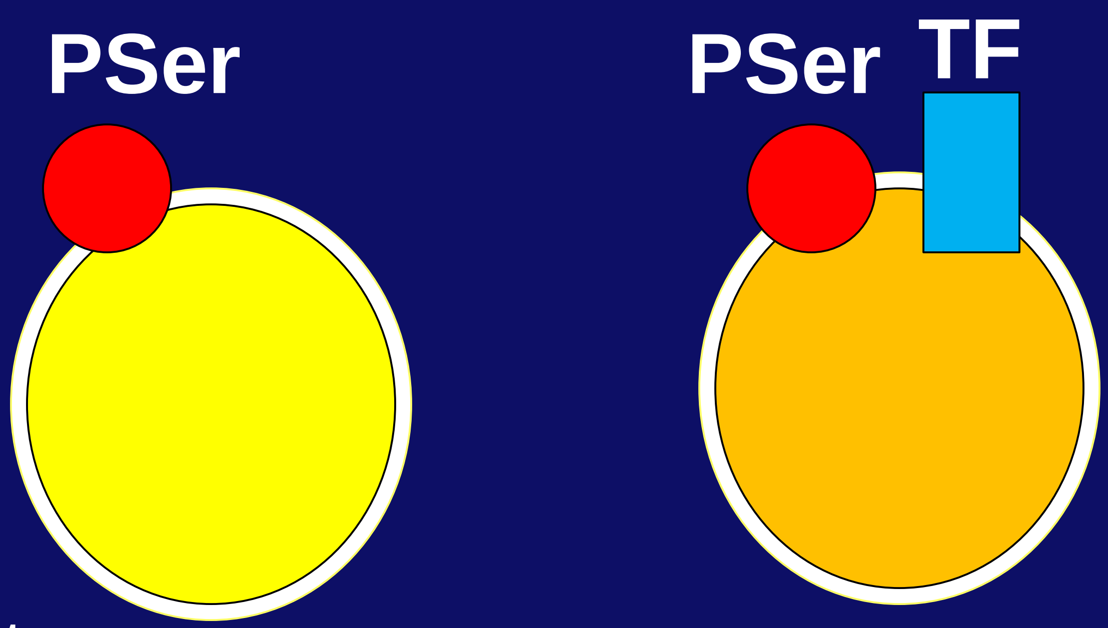
6 hrs

Host Defense

Drake et al AJP 1989

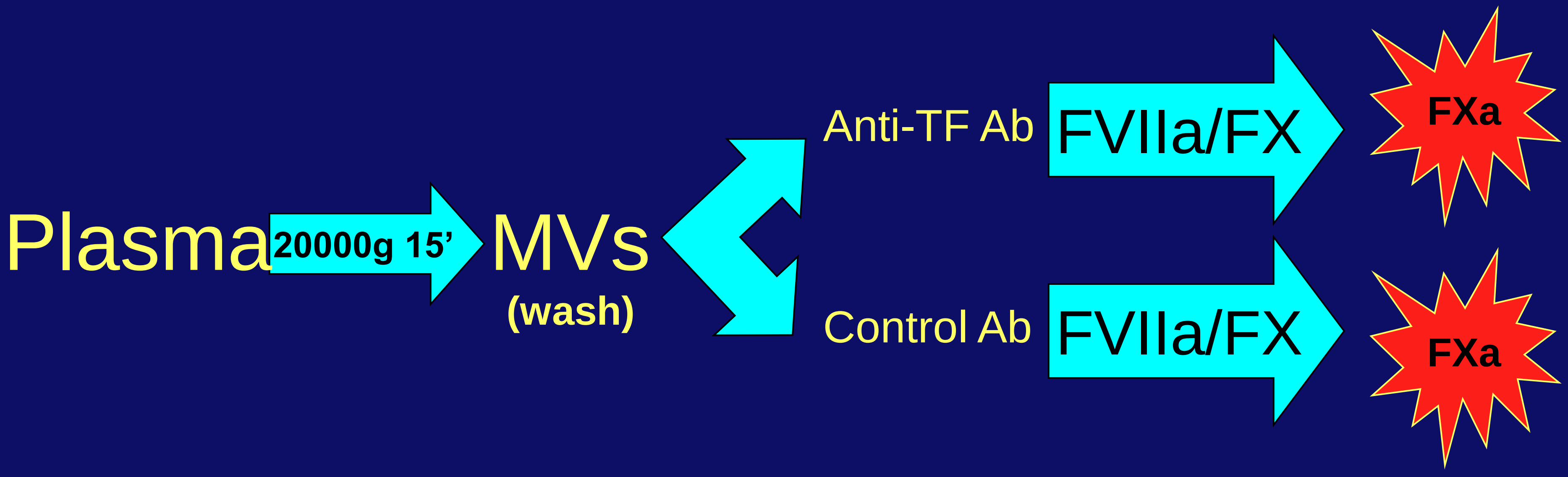
Types of Procoagulant MVs

Microvesicles (MVs) were originally defined as small (0.1-1 µm) membrane vesicles that are released from activated or apoptotic cells



Procoagulant activity	++	+++
Origin	Platelet/ RBC	Monocyte/ tumor cell

Development of a New Assay to Measure Levels of MV TF Activity in Plasma



TF-dependent factor Xa activity

Healthy individuals do not have detectable levels of TF^{+} MVs in the circulation

Diseases with Elevated Levels of Circulating TF$^+$ MVs

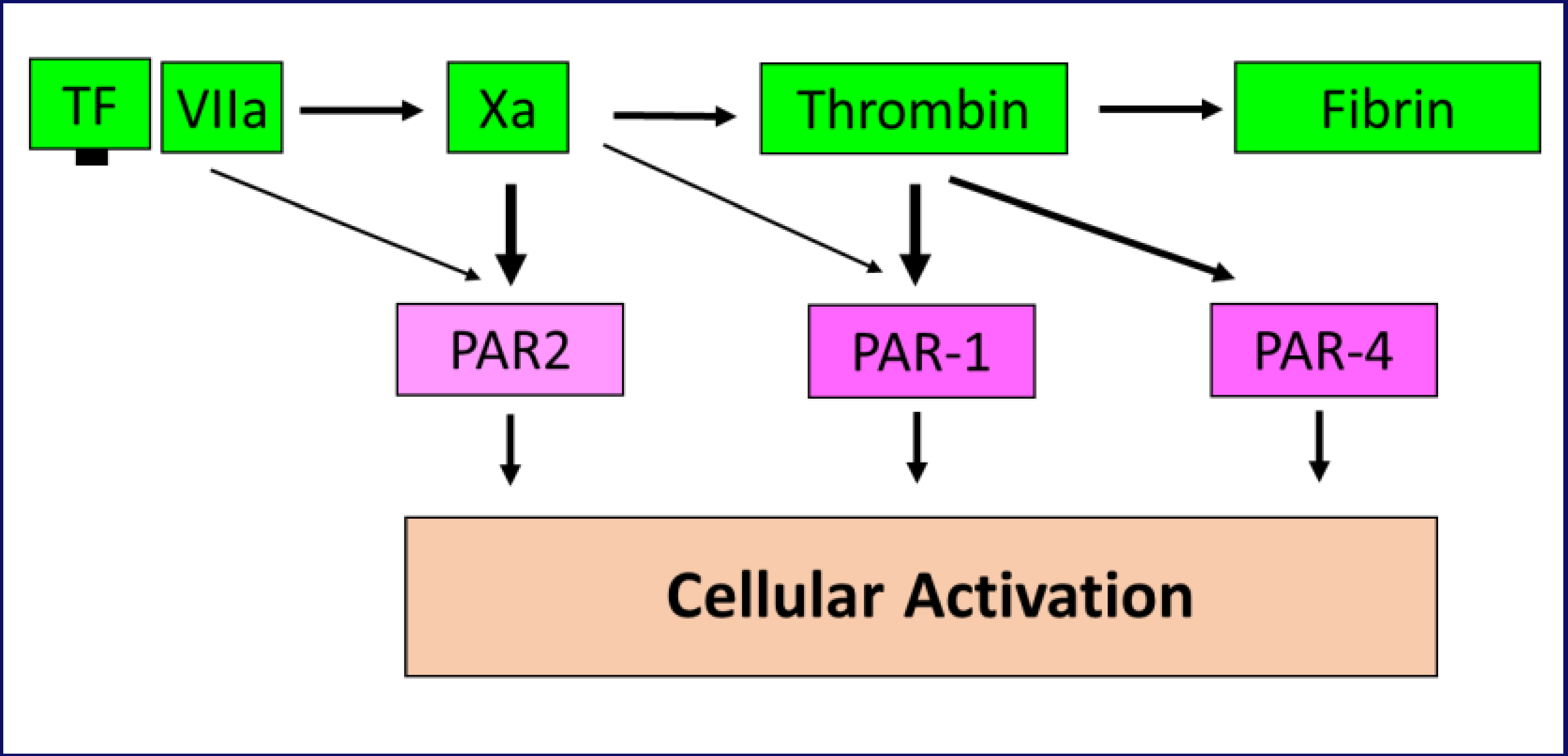
- Cancer
- Endotoxemia
- Sickle cell disease
- Liver injury

MV TF = biomarker of thrombotic risk

- Severe influenza A/H1N1 infection

Owens A and Mackman N Circ Res 2011;
Hisada Y et al Throm Res 2016

Coagulation Proteases Activate PARs



Mouse Models to Study Disease

TF/FVIIa → Coagulation Proteases

Fibrin
Plts
PARs

Coagulation Proteases → Hemostasis

Coagulation Proteases → Thrombosis

Coagulation Proteases → Cardiac Injury

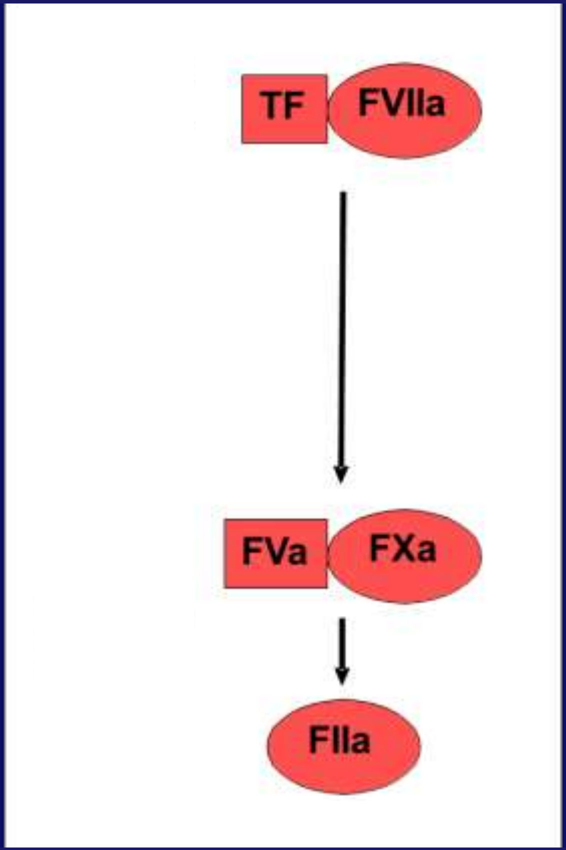
Coagulation Proteases → Infection

Hemostasis

- **Hemostasis** is a process which causes bleeding to stop. It is the first stage of wound healing. This involves coagulation, blood changing from a liquid to a gel (Wikipedia). Hemostasis also reduces the risk of infection.

Effect of Deletion of Different Procoagulant and Anticoagulant Proteins in Mice

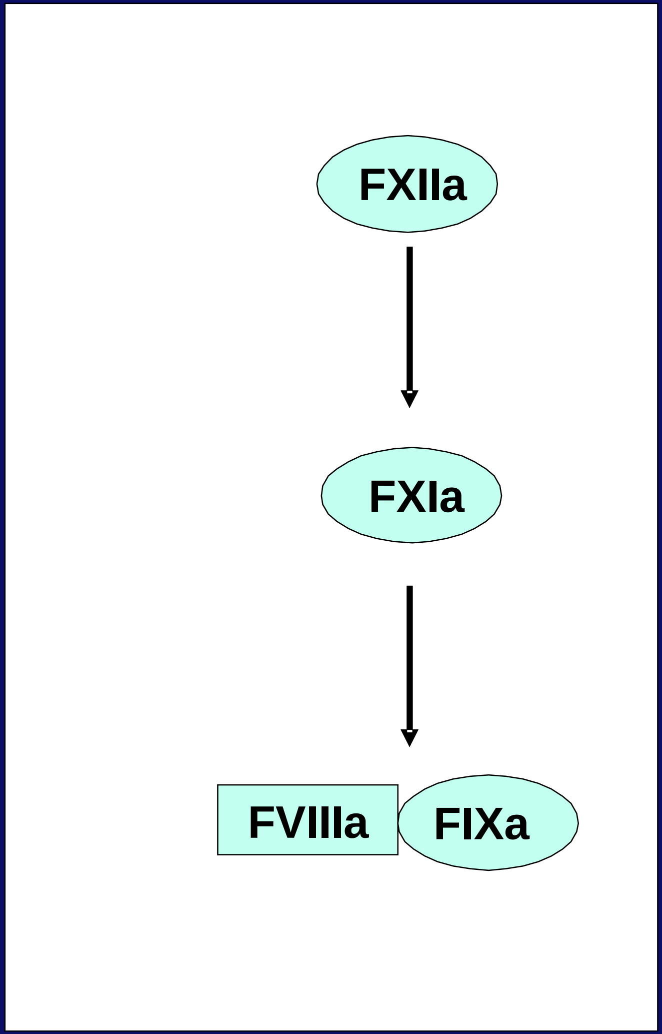
Effect of a Deficient of Extrinsic Pathway Factors on Survival in Mice



All die before wean

TF	Bugge PNAS 1996
	Carmeliet Nature 1996
	Toomey Blood 1996
FVII	Rosen Nature 1997
FX	Dewerchin T&H 2000
FV	Cui Nature 1996
FII	Sun PNAS 1998

Effect of a Deficiency of Contact and Intrinsic Pathway Factors on Survival in Mice



Mice are Viable

FXII	Pauer T&H 2004
FXI	Gailani B C & F 1997
FIX	Lin Blood 1997
FVIII	Bi Nat Genet 1995

Hemostatic Defects in Mice Lacking FVIII, FIX or FXI

Mouse	Increase in tail bleeding
$FXII^{-/-}$	**No**
$FXI^{-/-}$	**No**
$FIX^{-/-}$	**Yes**
$FVIII^{-/-}$	**Yes**

Broze G JTH 2001

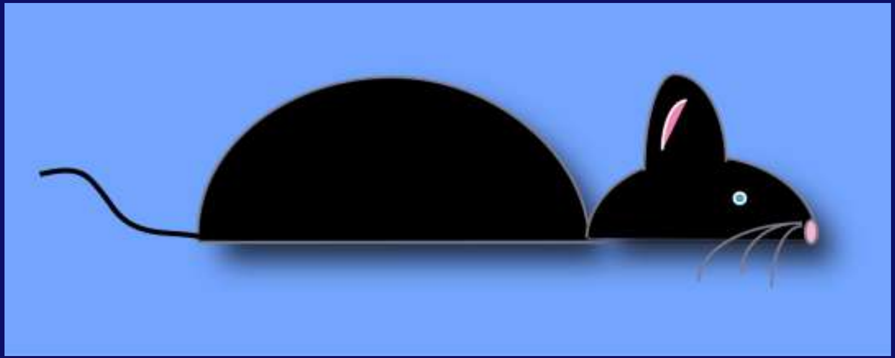
Effect of a Deficiency of Anticoagulant Proteins on Survival in Mice

Gene	Survival at wean (%)	
TFPI	0	Huang Z et al Blood 1997
AT	0	Ishiguro K et al JCI 2000
TM	0	Healy A et al PNAS 1995
EPCR	0	Gu J et al JBC 2002
PC	0	Jalbert L et al JCI 1998
PS	0	Burstyn-Cohen et al JCI 2009

Each of the three anticoagulant pathways is required for regulation of the coagulation protease cascade

Rescue of $mTF^{-/-}$ Embryos with a Human TF (hTF) Transgene

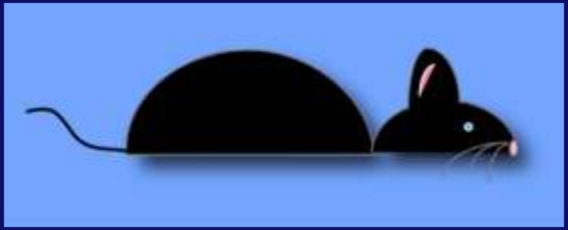
Cross = $mTF^{+/-}$,hTF^{+} x $mTF^{+/-}$,hTF^{+}



Low TF mice $mTF^{-/-}$,hTF^{+}

Low FVII mice Rosen E et al T&H 2005

Generation of Mice with Cell Type-specific Deletion of the TF Gene



TF$^{flox/flox}$ mice

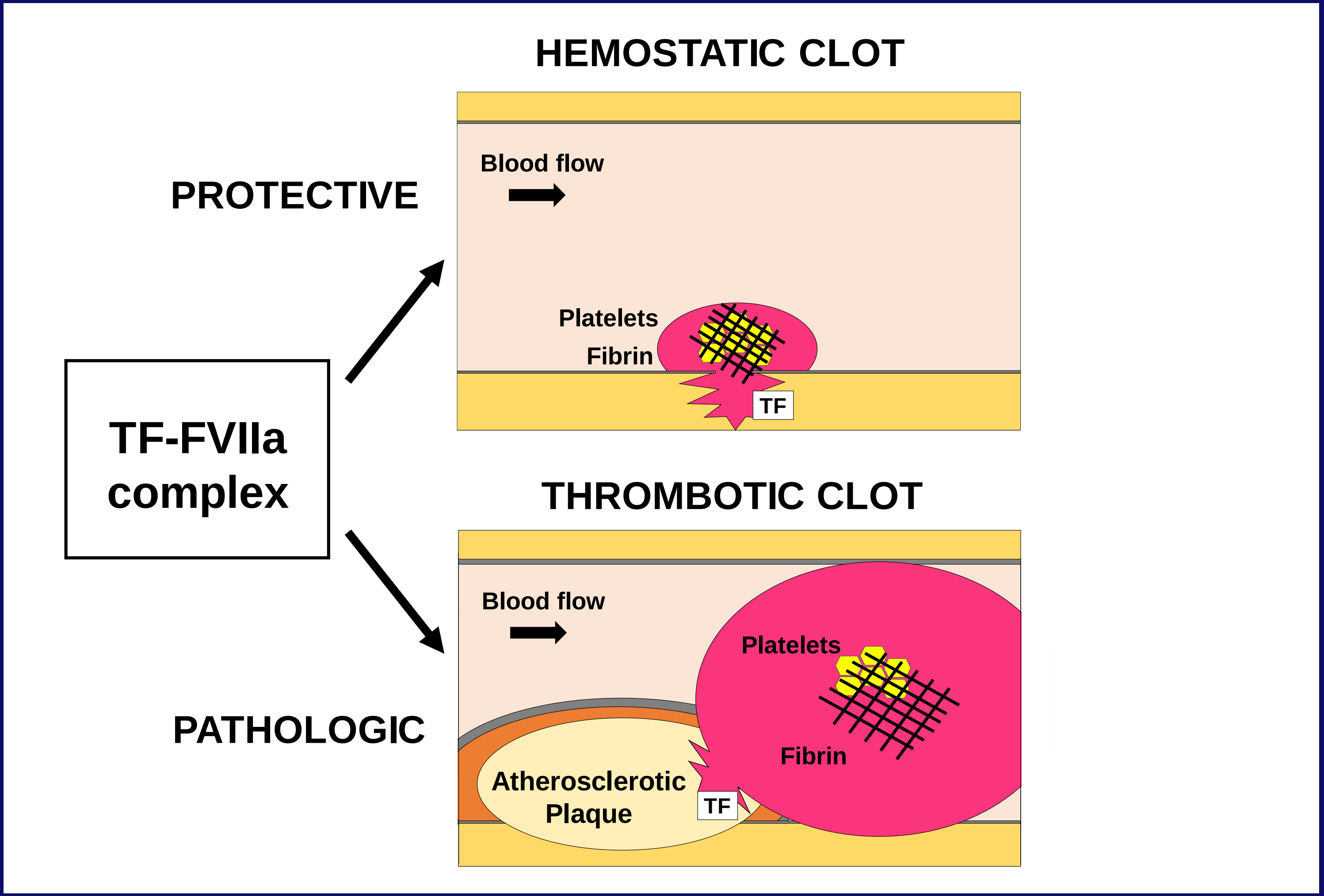
Cell type-specific deletion of the TF gene when crossed with different Cre^{+} lines

Pawlinski ..Mackman JTH 2007

Generation of Mice with Cell Type-Specific Deletion of the TF Gene

- **Mlc2v-Cre (cardiomyocytes).** Pawlinski et al, JTH 2007.
- **SM22-Cre (vascular smooth muscle cells).** Wang et al, Blood 2009.
- **LysM-Cre (myeloid cells). Pawlinski et al, Blood 2010.**
- **Tie2-Cre (EC and hemat cells).** Pawlinski et al, Blood 2010.
- **Alb-Cre (hepatocytes).** Sullivan et al, Blood 2013.
- **SPC-Cre (lung epithelial cells).** Shaver et al, AJRCMB 2015.
- **Nestin-Cre (CNS).** Wang et al JCI Insight 2016.

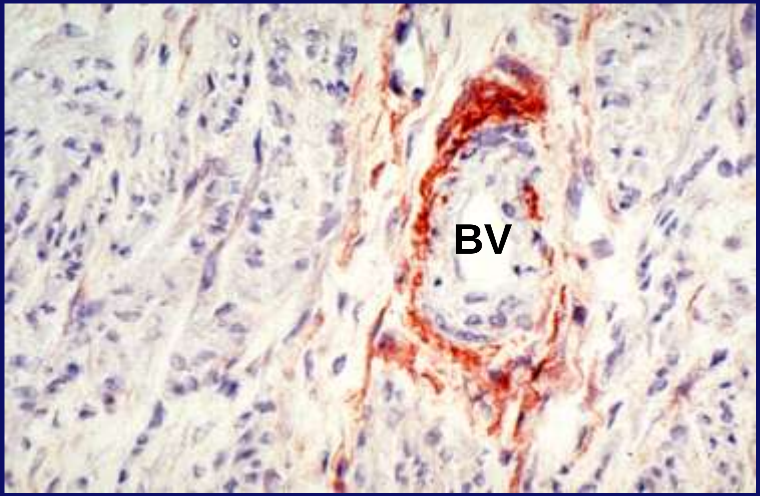
Roles of TF in Hemostasis and Thrombosis



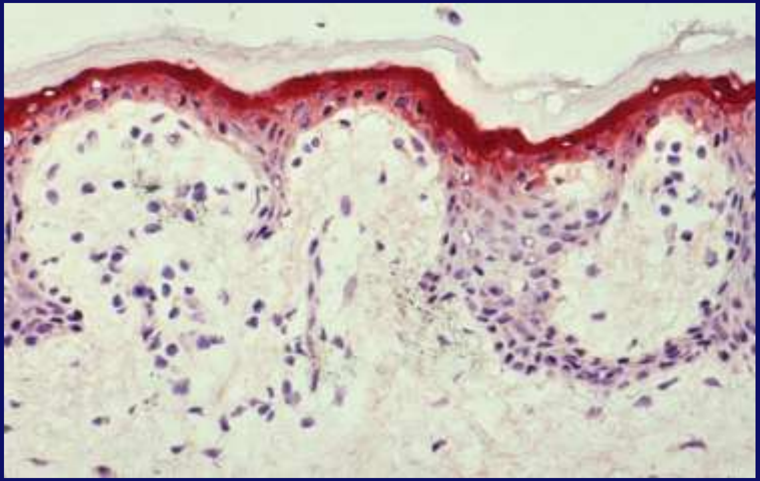
Grover and Mackman ATVB in press

Role of TF in Hemostasis

Blood Vessel



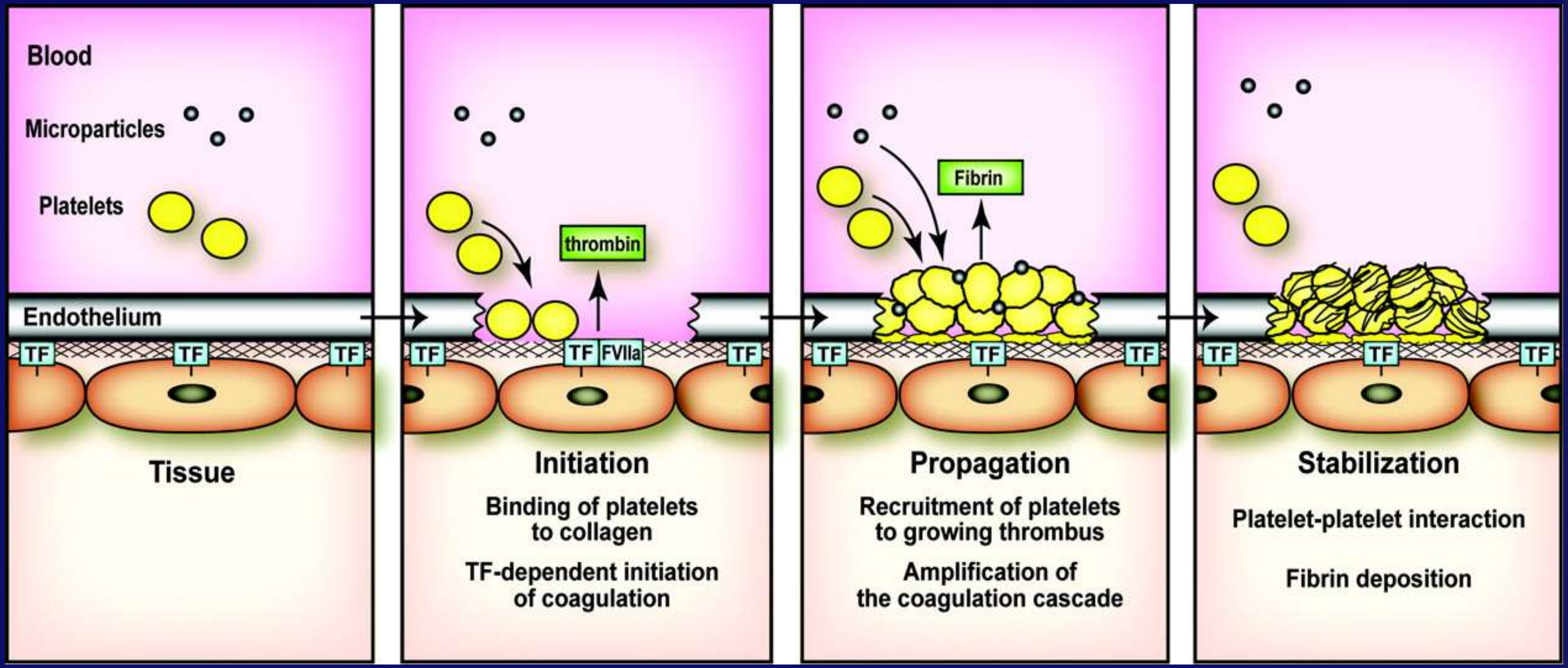
Skin



TF forms a "hemostatic envelope" around blood vessels and body surfaces

Drake et al AJP 1989

Perivascular TF Plays a Critical Role in Hemostasis After Vessel Injury



TF is the only clotting factor not present in blood

Mackman et al ATVB 2007

Expression of TF in Different Organs

High	Medium	Low
uterus	heart	skeletal muscle
skin	kidney	liver
brain	testis	spleen
lung		thymus
placenta		

Mackman ATVB 2005

Hypothesis:
TF provides additional hemostatic protection to select tissues

Analyze the hemostatic defects in Low TF mice

Spontaneous Hemostatic Defects in Low TF Mice

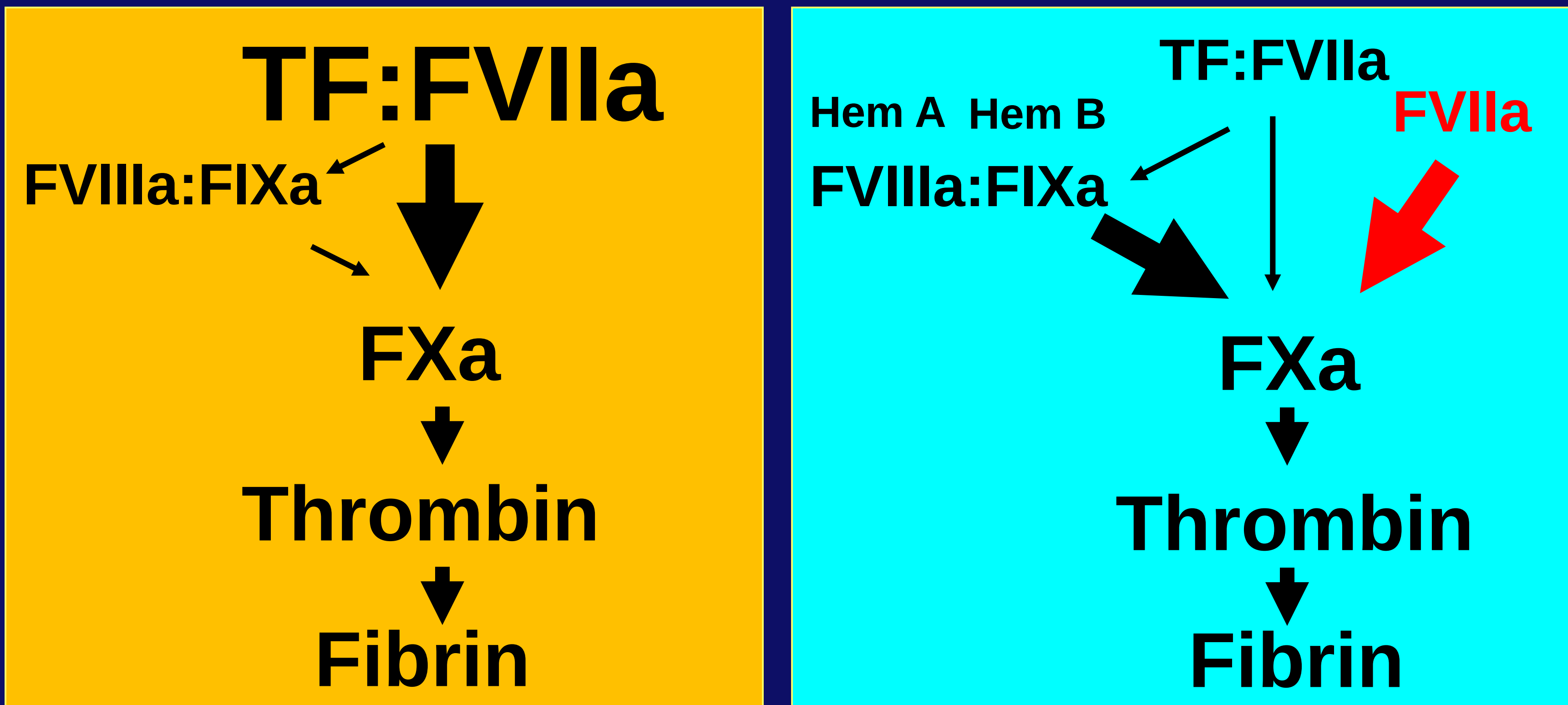
Uterus	Fatal hemorrhage post-partum	Erlich PNAS 1999
Placenta	Blood pools	Erlich PNAS 1999
Heart	Hemosiderin, fibrosis	Pawlinski PNAS 2002
Lung	Hemosiderin, fatal hemorrhages	Pedersen Blood 2005
Testis	Hemorrhage, calcification	Mackman JTH 2007
Brain	Occasional brain hemorrhage	Pawlinski T&H 2004

Blood vessels in the heart and lung may be more prone to mechanical damage

Tissue Specific Hemostasis

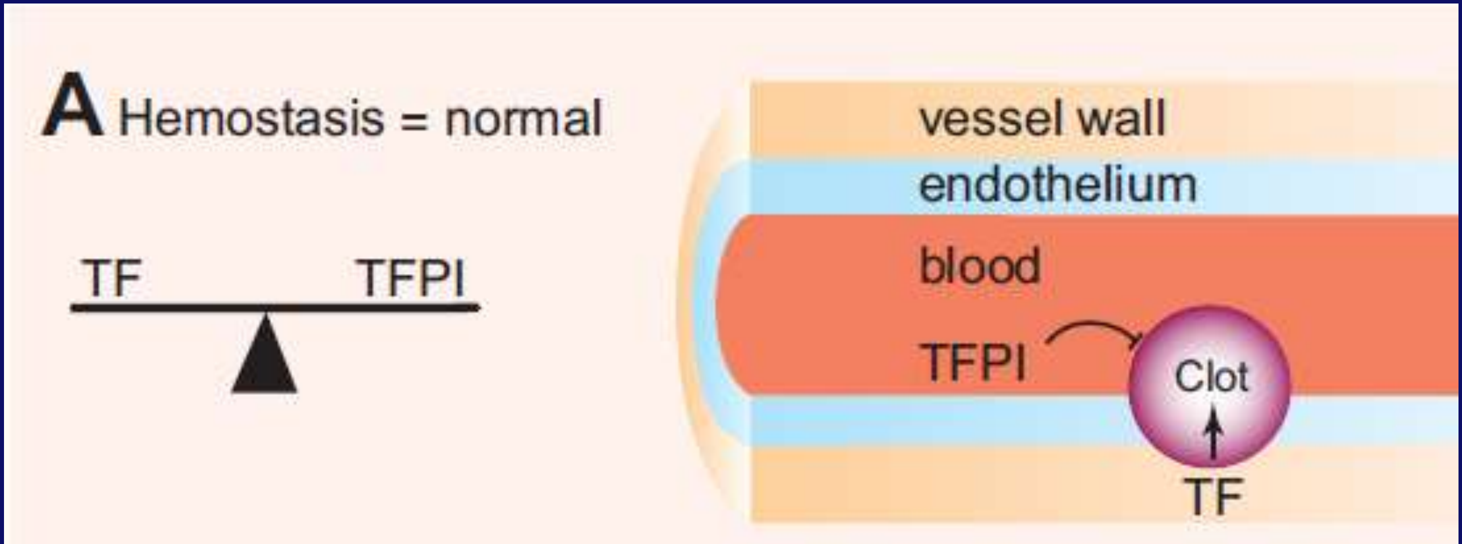
High TF: uterus, brain, heart, lung, placenta, testis

Low TF: skeletal muscle and joints



Mackman ATVB 2005

Balancing the TF Pathway



Mackman Blood 2010

Re-balancing the Clotting Cascade in Mice

Can we rescue the lethality of embryos lacking different anticoagulants with low levels of TF?

1/ TFPI$^{-/-}$ embryos are rescued by reducing the level of TF.
2/ Fatal bleeding in low TF mice is rescued by reducing the level of TFPI.

Pedersen B et al Blood 2005

Re-balancing the Clotting Cascade in Mice

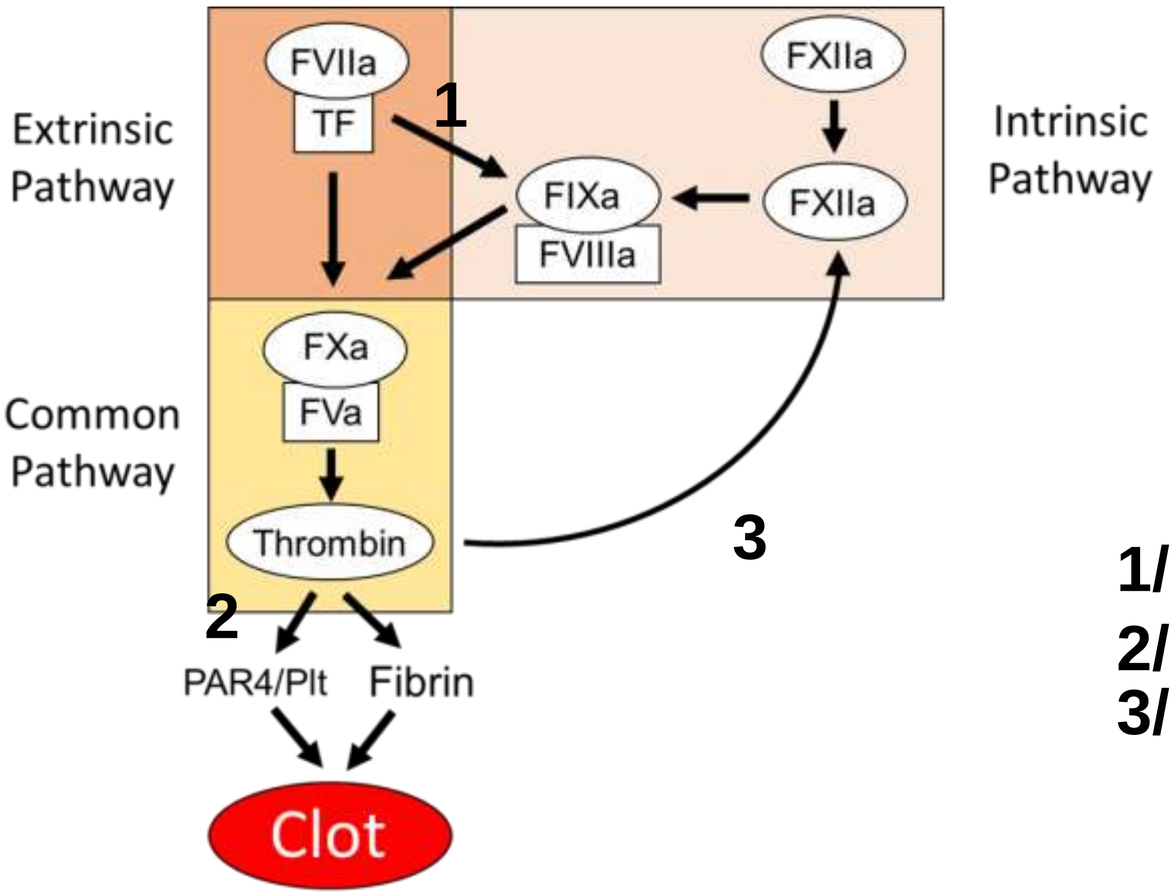
EPCR$^{-/-}$ x Low TF- rescue

Li W et al Blood 2005

AT$^{-/-}$ x Low TF- rescue

Hayashi M et al Blood 2005

Analysis of Different Pathways using a Genetic Approach



1/ TF:FVIIa – FIX

2/ Thrombin – PAR

3/ Thrombin – FXI

Effect of Combining Low Levels of TF with FIX Deficiency

Breeding Male $mTF^{-/-}$, $hTF^{+/+}$, $FIX^{+/y}$ x female $mTF^{+/-}$, $hTF^{-/-}$, $FIX^{+/-}$

Sex	mTF	hTF	F9	Expected (%)	E14.5 (n)	E14.5 Observed (%)
F	+/-	+	+/+	12.5	7	18.4
F	+/-	+	+/-	12.5	7	18.4
M	+/-	+	+/y	12.5	1	2.6
M	+/-	+	-/y	12.5	4	10.5
F	-/-	+	+/+	12.5	5	13.2
F	-/-	+	+/-	12.5	5	13.2
M	-/-	+	+/y	12.5	5	13.2
M	-/-	+	-/y	12.5	4	10.5
		Total		100	38	100

*2 dead

wean

Grover unpublished

Low TF, $FIX^{-/-}$ P1 Pups Die after Birth

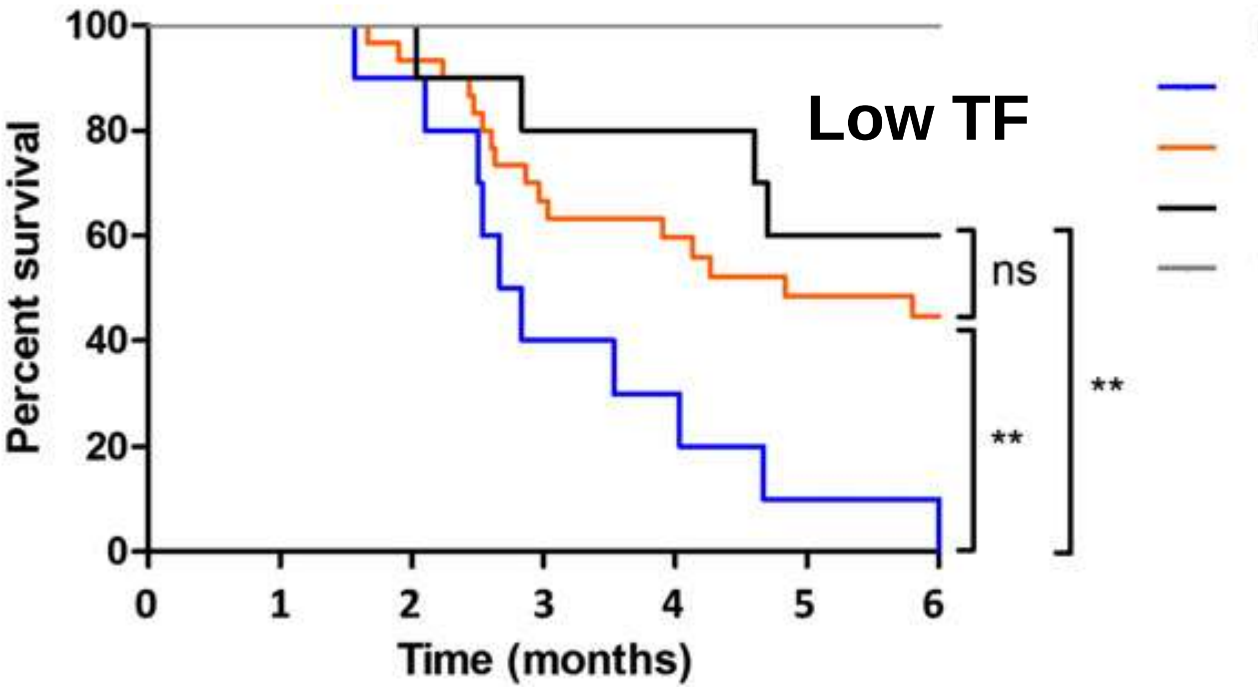
mTF+/-, hTF+, FIX+/-

mTF-/-, hTF+, FIX-/-

Grover unpublished

Effect of Combining Low Levels of TF with PAR4 Deficiency

Expected numbers of low TF, PAR4 deficient mice at wean



Bode M and Mackman N Thromb Res 2016

Effect of Combining Low Levels of TF with FXI Deficiency

Breeding- male $mTF^{+/-}$, $hTF^{+/-}$, $FXI^{+/-}$ x female $mTF^{+/-}$, $hTF^{+/-}$, $FXI^{+/-}$

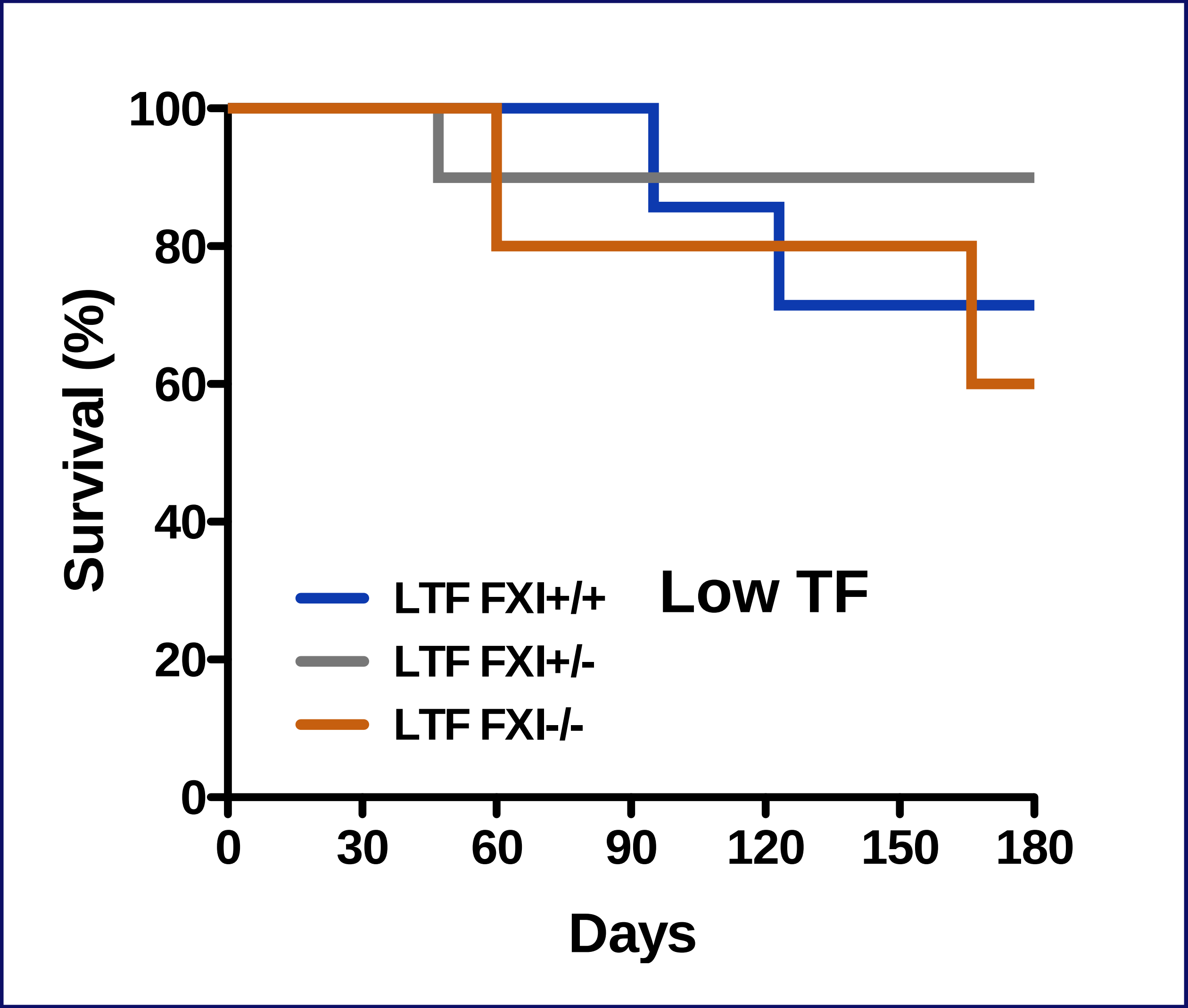
mTF	hTF+	FXI	Observed (N)	Observed (%)	Expected (%)
+/+	-	+/+	4	4.55	1.56
+/+	-	+/-	3	3.41	3.13
+/+	-	-/-	3	3.41	1.56
+/+	+	+/+	5	5.68	4.69
+/+	+	+/-	4	4.55	9.38
+/+	+	-/-	2	2.27	4.69
+/-	-	+/+	2	2.27	3.13
+/-	-	+/-	7	7.95	6.25
+/-	-	-/-	1	1.14	3.13
+/-	+	+/+	11	12.50	9.38
+/-	+	+/-	23	26.14	18.75
+/-	+	-/-	9	10.23	9.38
-/-	-	+/+	0	0.00	0
-/-	-	+/-	0	0.00	0
-/-	-	-/-	0	0.00	0

Low TF, $FXI^{-/-}$ mice are generated at the expected frequency

hTF+ = hTF+/- and hTF+/+

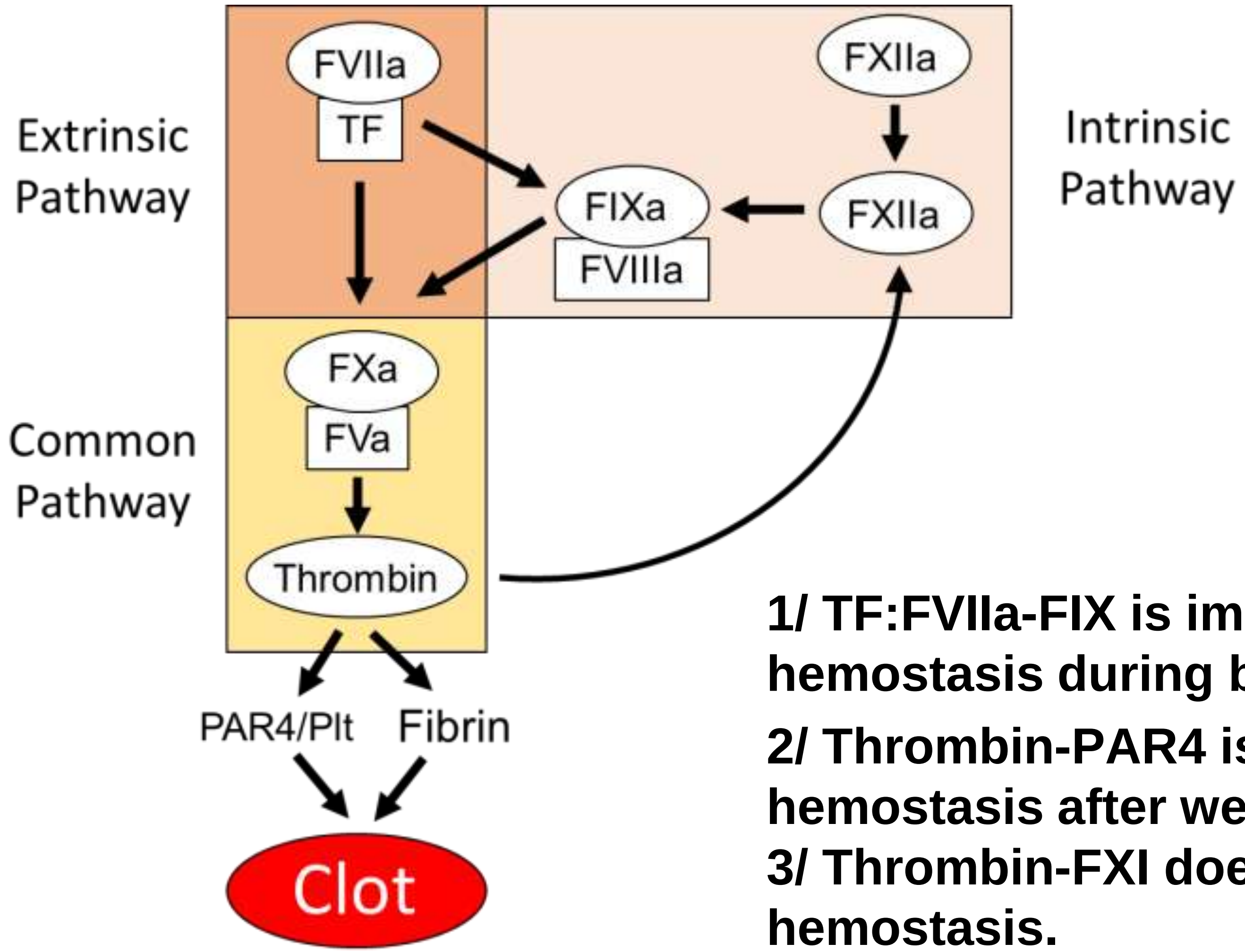
Grover unpublished

Survival of Low TF, FXI$^{-/-}$ Mice



Grover unpublished

Conclusions



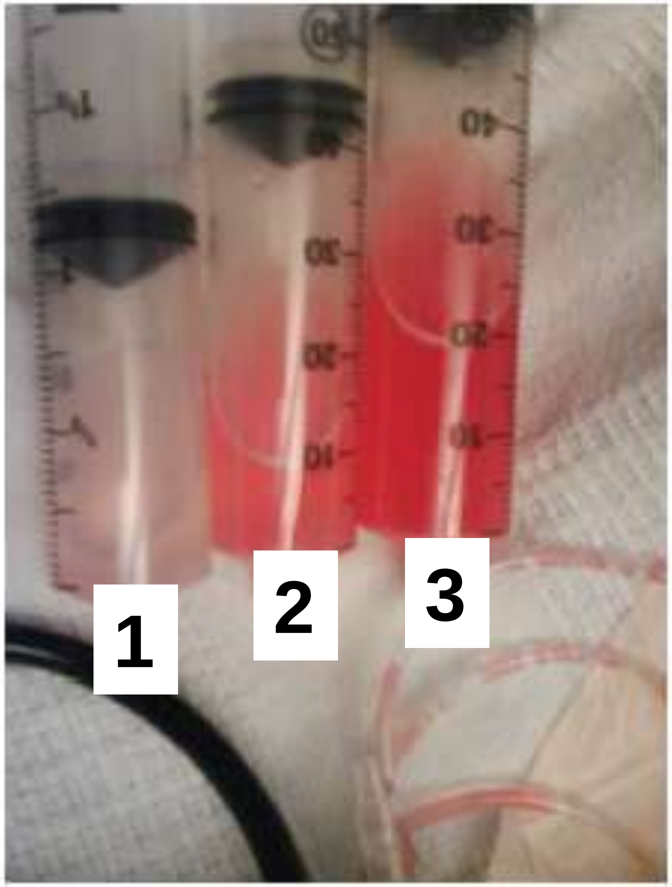
1/ TF:FVIIa-FIX is important for hemostasis during birth.

2/ Thrombin-PAR4 is important for hemostasis after wean.

3/ Thrombin-FXI does not affect hemostasis.

Alveolar Hemorrhage in Patients with Severe Influenza A Infection

Bronchiolar lavage fluid from a patient infected with influenza A



Gilbert et al Respit Care 2010

Role of Lung Epithelial Cell TF in Influenza A Infection

Hypothesis:
A deficiency of TF in the lung will increased alveolar hemorrhage after influenza A infection

Silvio Antoniak

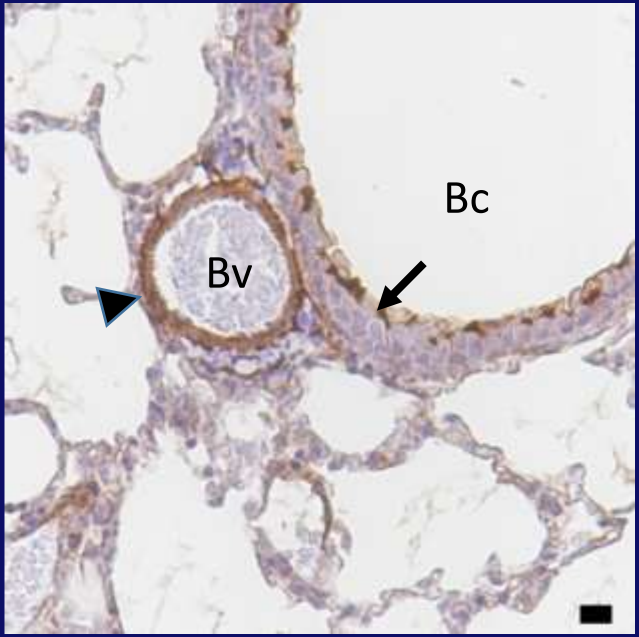
We used a genetic approach:
($TF^{fl/fl}$,SPC-Cre mice)

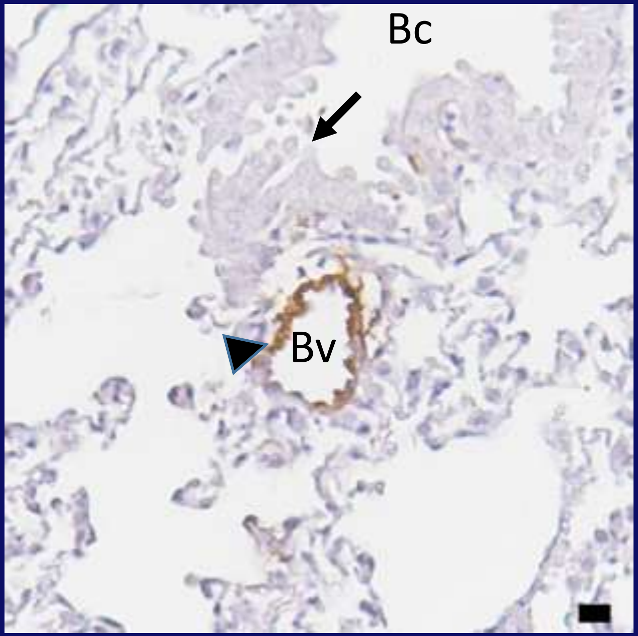
SPC = surfactant protein C

Characterization of $TF^{f//fl}$,SPC-Cre Mice

$TF^{fl/fl}$

$TF^{fl/fl}$,SPC-Cre^{+}





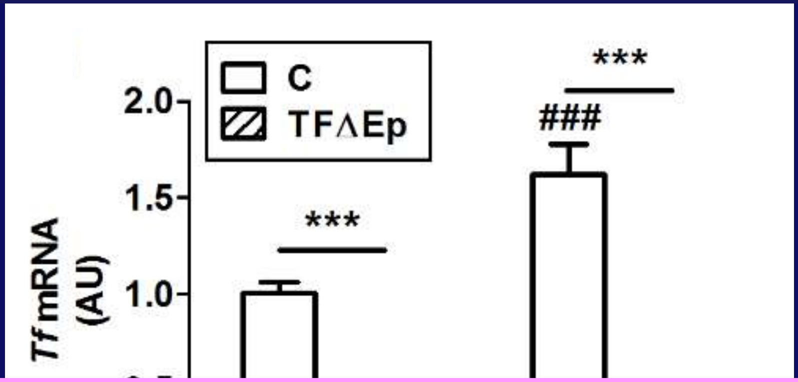
$TF^{fl/fl}$,SPC-Cre^{+} have the TF gene deleted in all epithelial cells- bronchiolar and alveolar

Mouse Model of Influenza A Infection (IAV)

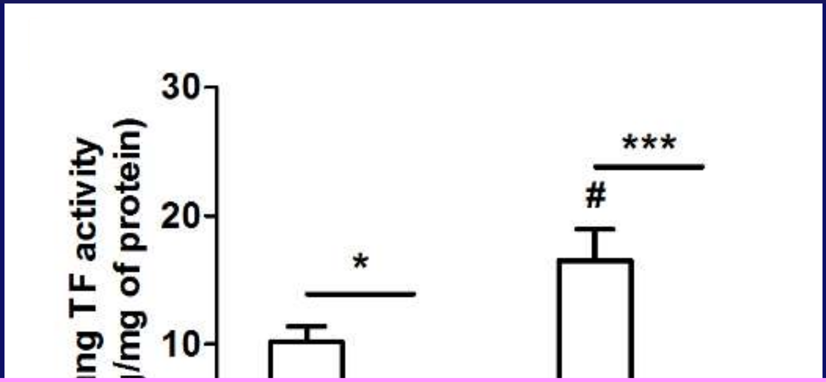
- Influenza A is a ssRNA virus.
- Influenza A/Puerto Rico/8/1934 H1N1.
- Mouse adapted and highly pathologic.
- Infected intranasally.

Influenza A Infection Induces TF Expression in $TF^{fl/fl}$ Mice but not in $TF^{fl/fl}$,SPC-Cre Mice

TF mRNA



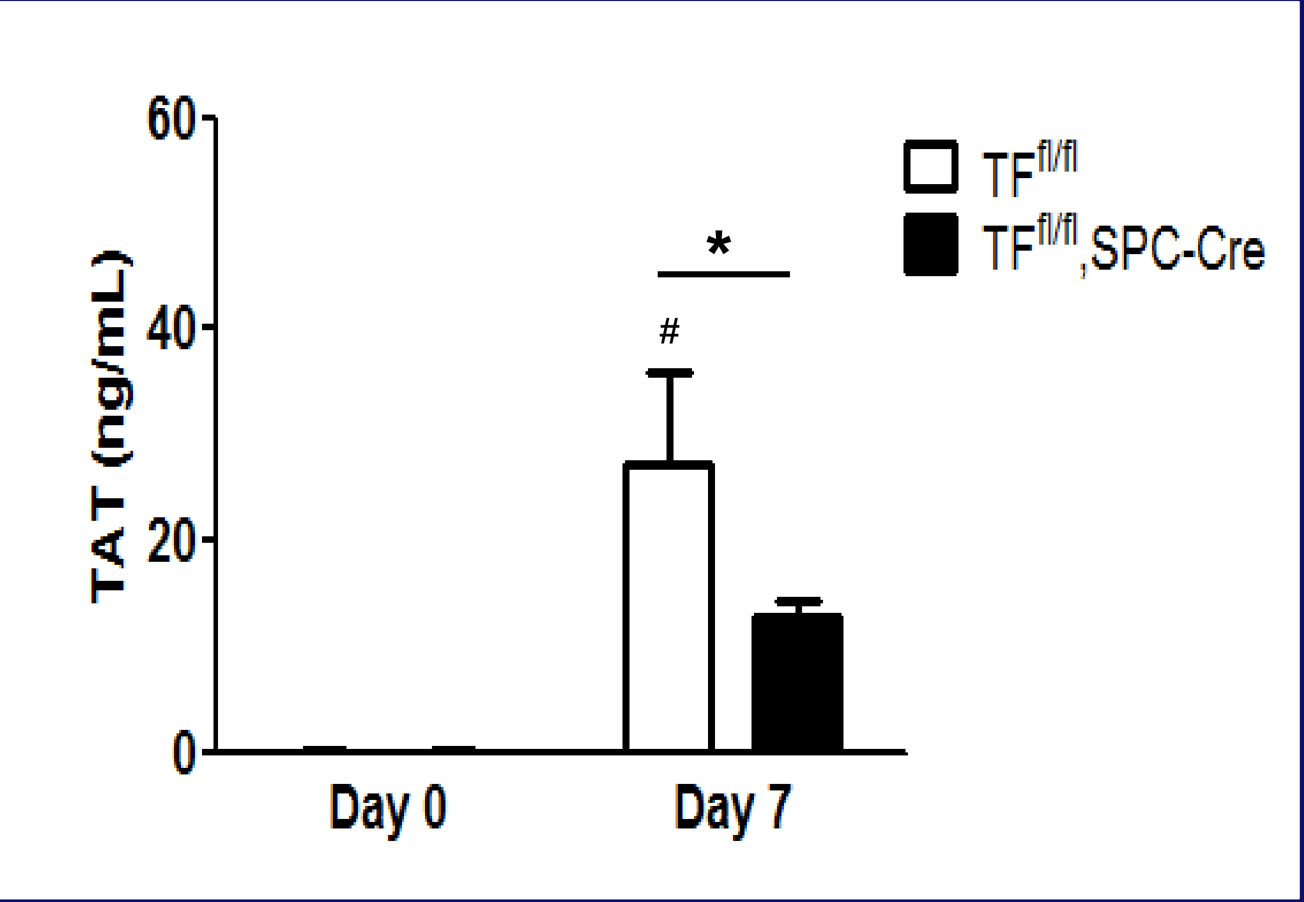
TF activity



Influenza A infection induces TF expression in lung epithelial cells- protective response

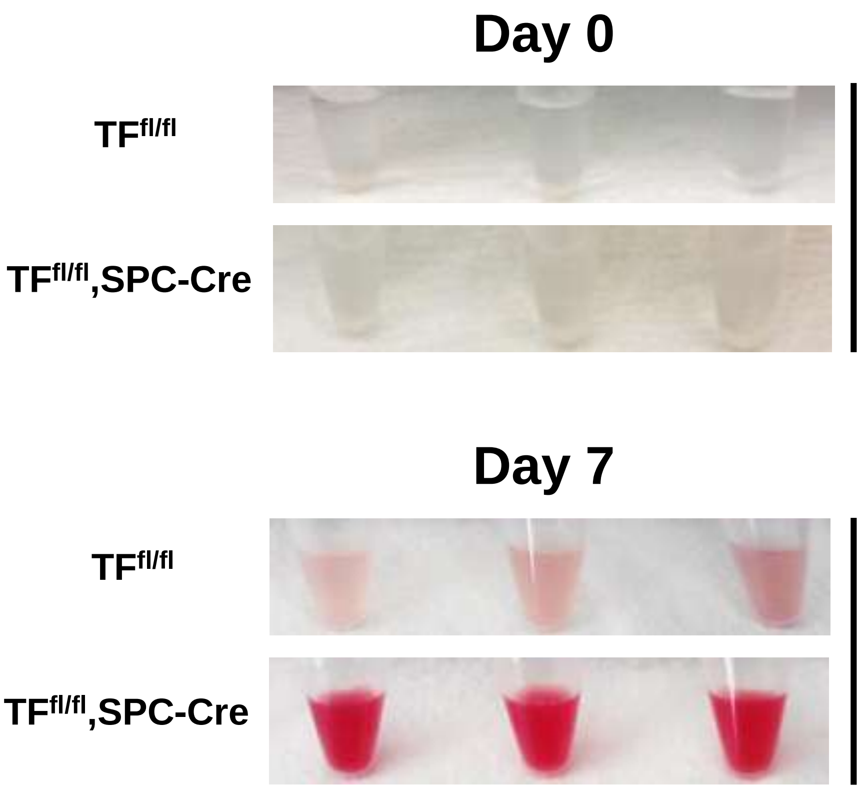
Antoniak S et al JTH 2016

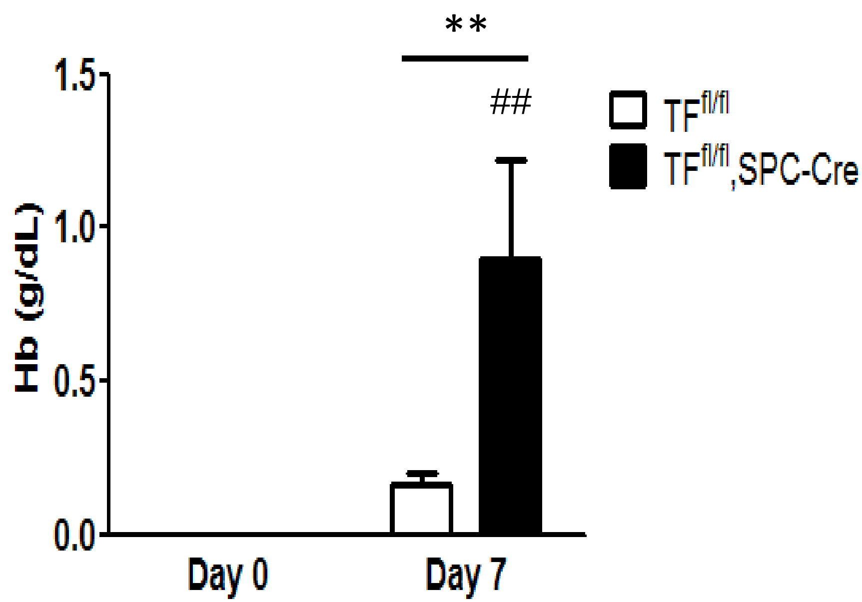
Reduced Activation of Coagulation in the Lungs of TF$^{fl/fl}$,SPC-Cre Mice after Influenza A Infection



Antoniak S et al JTH 2016

TF$^{fl/fl}$,SPC-Cre Mice have Increased Lung Hemorrhage after Influenza A Infection





Antoniak S et al JTH 2016

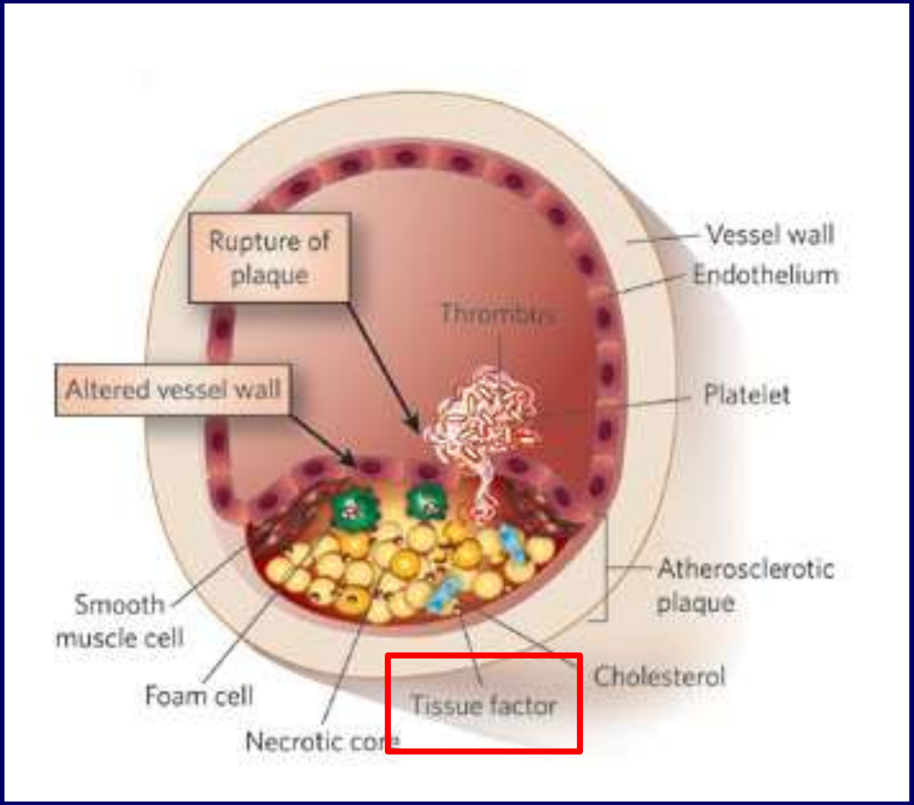
Conclusion

TF expression by lung epithelial cells is required to maintain hemostasis during influenza A infection

Thrombosis

- **Thrombosis** is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system. Wikipedia Arterial thrombois (MI and stroke) and venous thrombosis.

Arterial Thrombosis



- Plaque rupture
- Platelet-rich thrombus
- TF activates clotting
- Clot forms rapidly

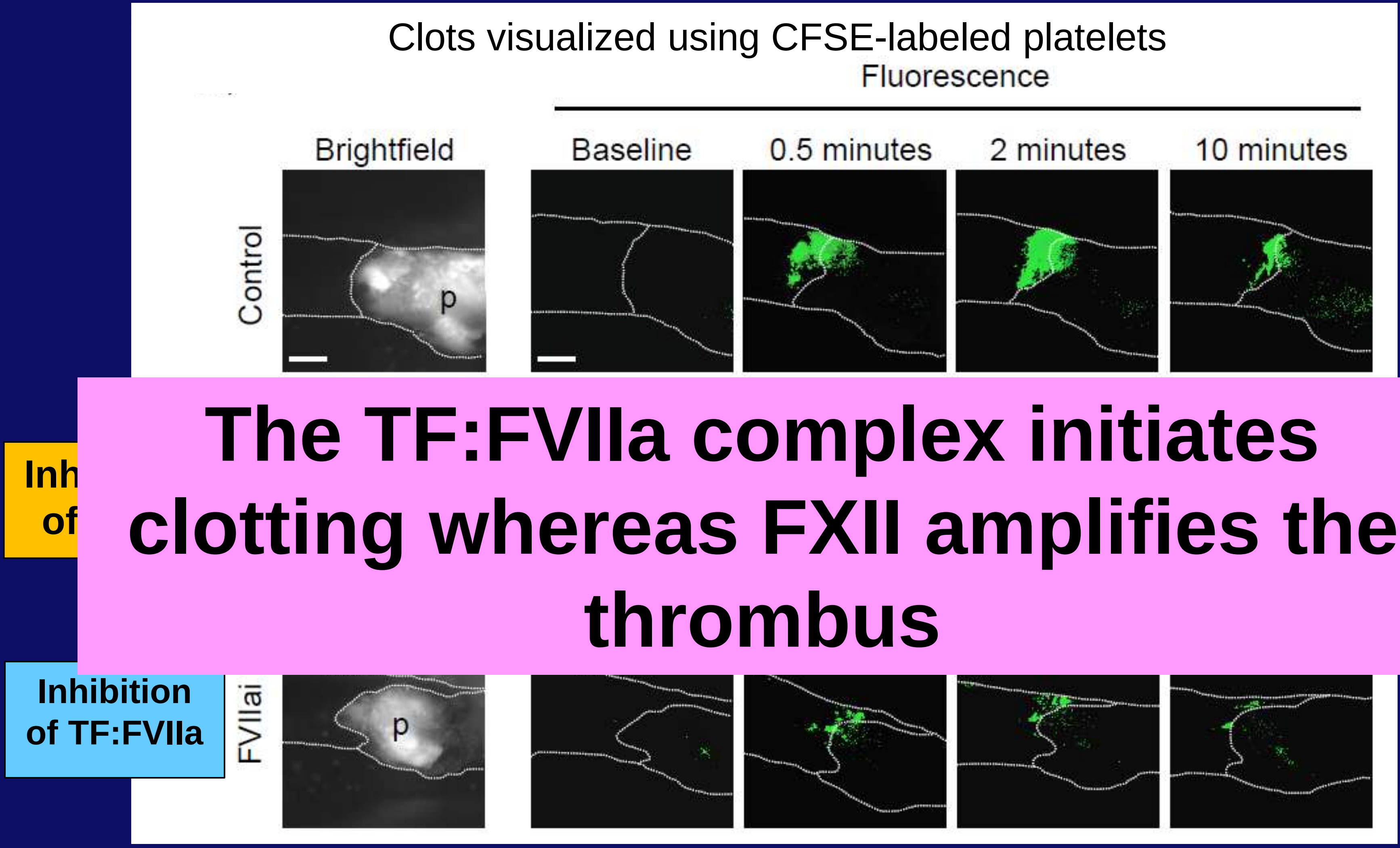
Mackman Nature 2008

Mouse Model of Atherothrombosis

- $ApoE^{-/-}$ mice fed a western diet to induce atherosclerosis
- Plaques in the carotid arteries ruptured using ultrasound

Kuijper M et al ATVB 2014
Van Montfoort M et al ATVB 2014
Editorial Mackman N ATVB 2014

Role of the Extrinsic (TF:FVIIa) and Intrinsic (FXIIa) Coagulation Pathways in Atherothrombosis



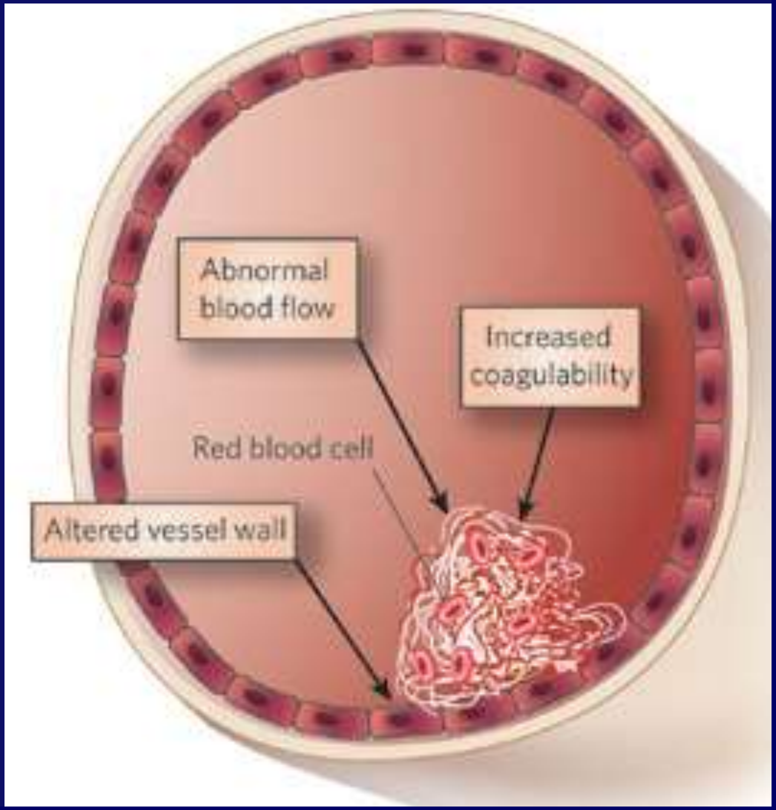
The TF:FVIIa complex initiates clotting whereas FXII amplifies the thrombus

Kuijper M et al ATVB 2014

Conclusion

TF in atherosclerotic plaques activates coagulation after plaque rupture

Venous Thrombosis



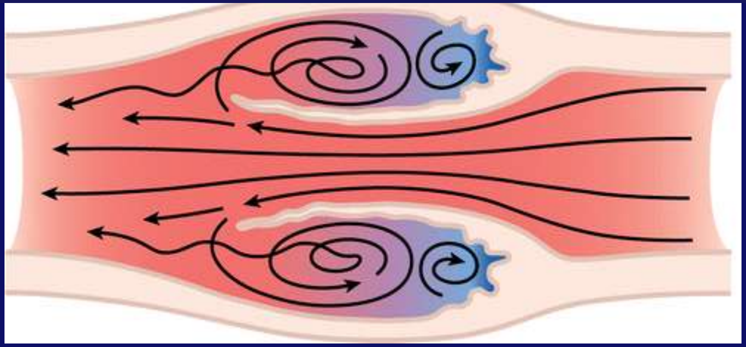
- Fibrin-rich
- Intact endothelium
- Form in valve pockets
- Form slowly (hours to days)

Venous thromboembolism (VTE) - DVT and PE

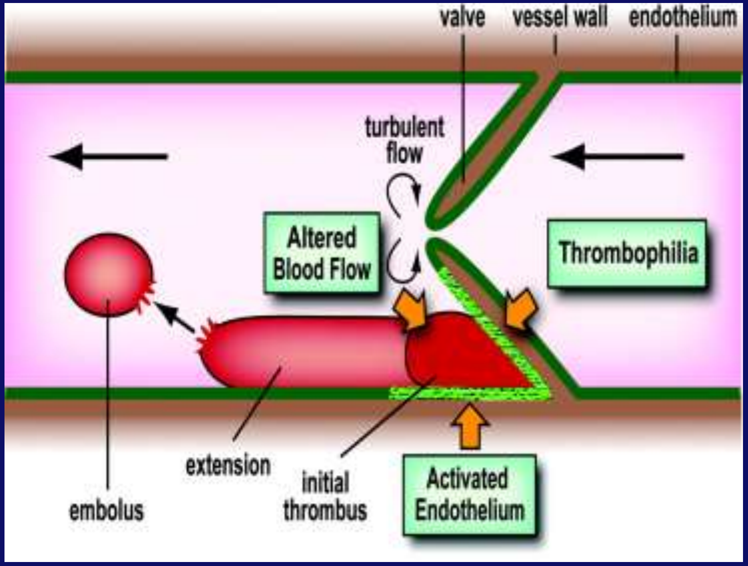
Mackman Nature 2008

Formation of a Deep Vein Thrombus

Hypoxia in valve pockets

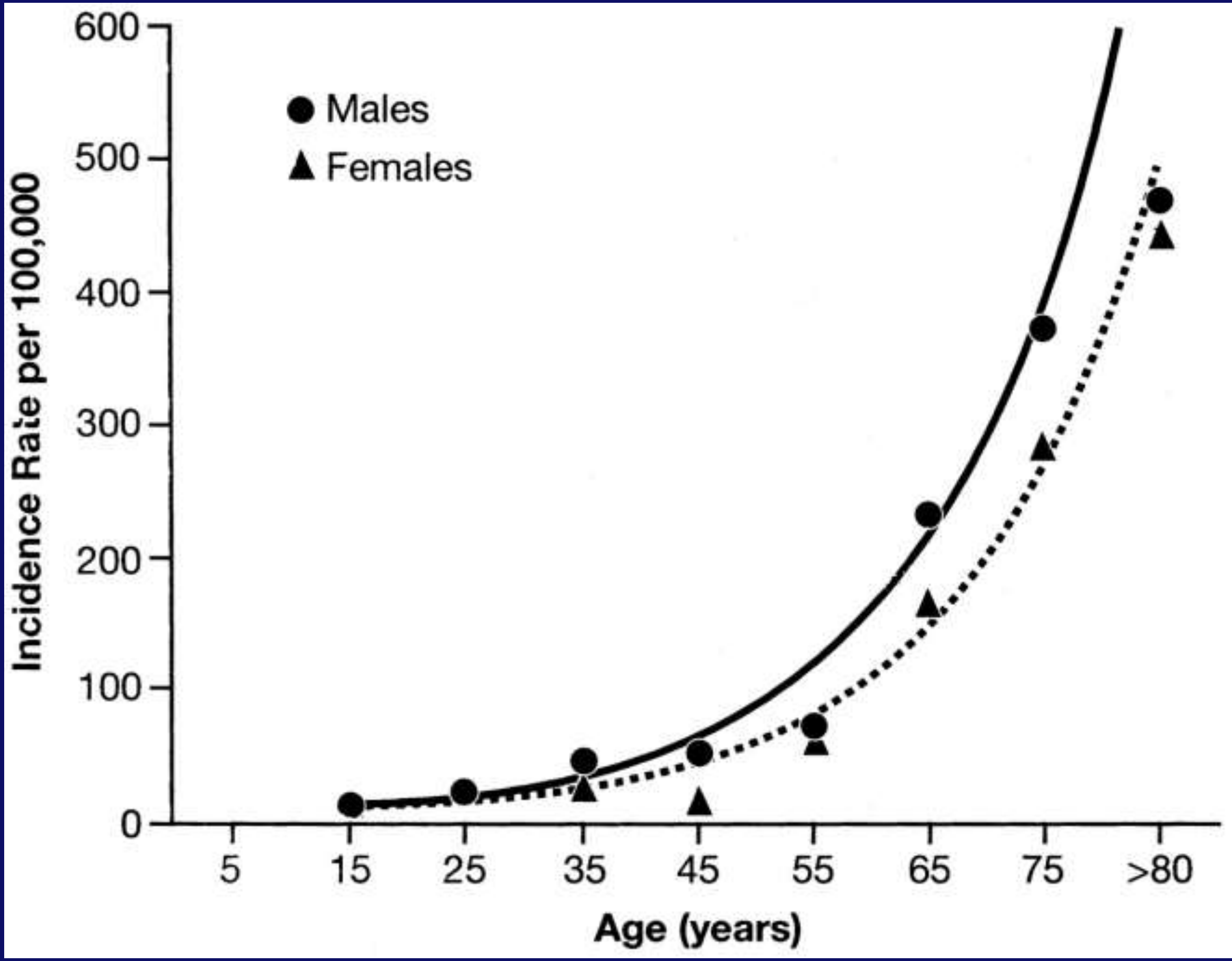


Bovill Ann Rev Physiol 2011



Moll and Mackman ATVB 2008

Annual Incidence of VTE by Age and Sex



Richard H. White Circulation. 2003;107:I-4-I-8

American Heart Association

Cancer and Venous Thrombosis

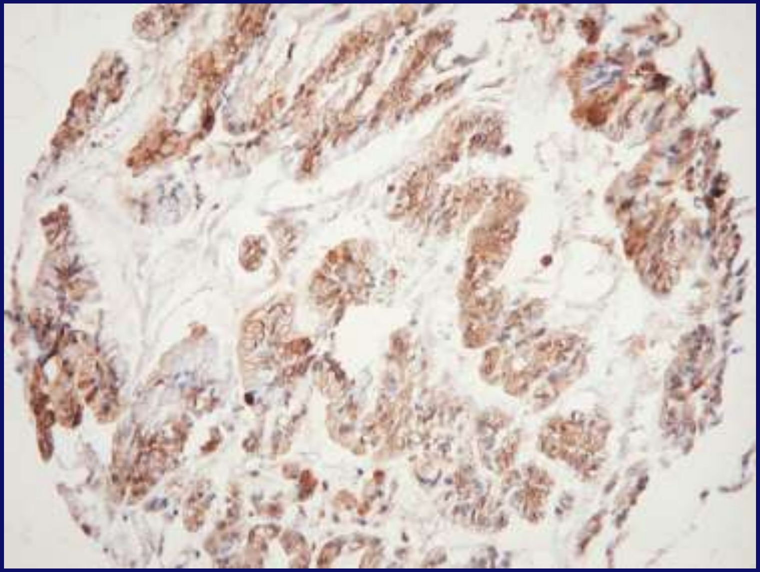
Cancer patients have an a 4-7 fold increased risk for venous thrombosis – cancer-associated thrombosis (CAT)

~20% of patients with idiopathic VTE have cancer

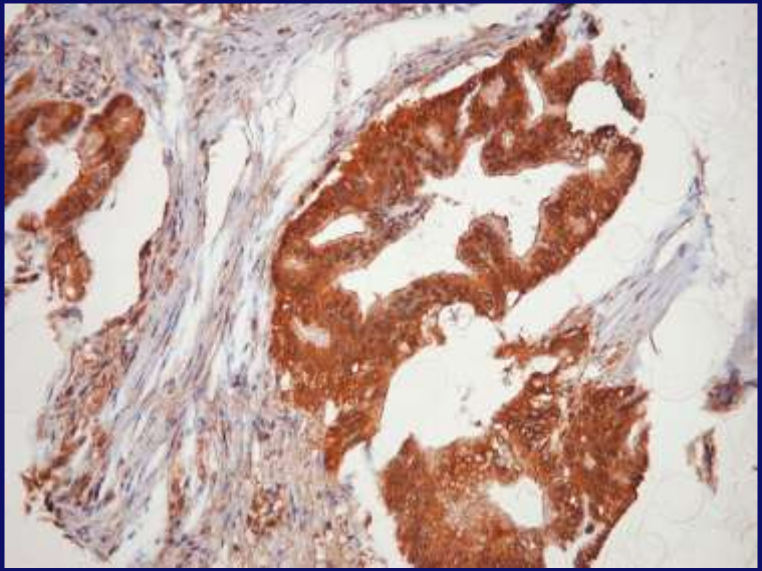
Incidence of Symptomatic VTE within One Year of Cancer Diagnosis

Cancer	Incidence	Risk
Pancreas	5.3- 26%	High
Brain	1.6- 26%	High
Lung	1.6- 13.6%	Medium
Colon	3.1- 10.3%	Medium
Breast	0.4- 8.1%	Low
Prostate	0.5- 1.4%	Low

TF Expression in Pancreatic Cancer



Grade 1



Grade 3

Khorana et al Clin Cancer Res 2007

Cancer Cells and Procoagulant Microvesicles

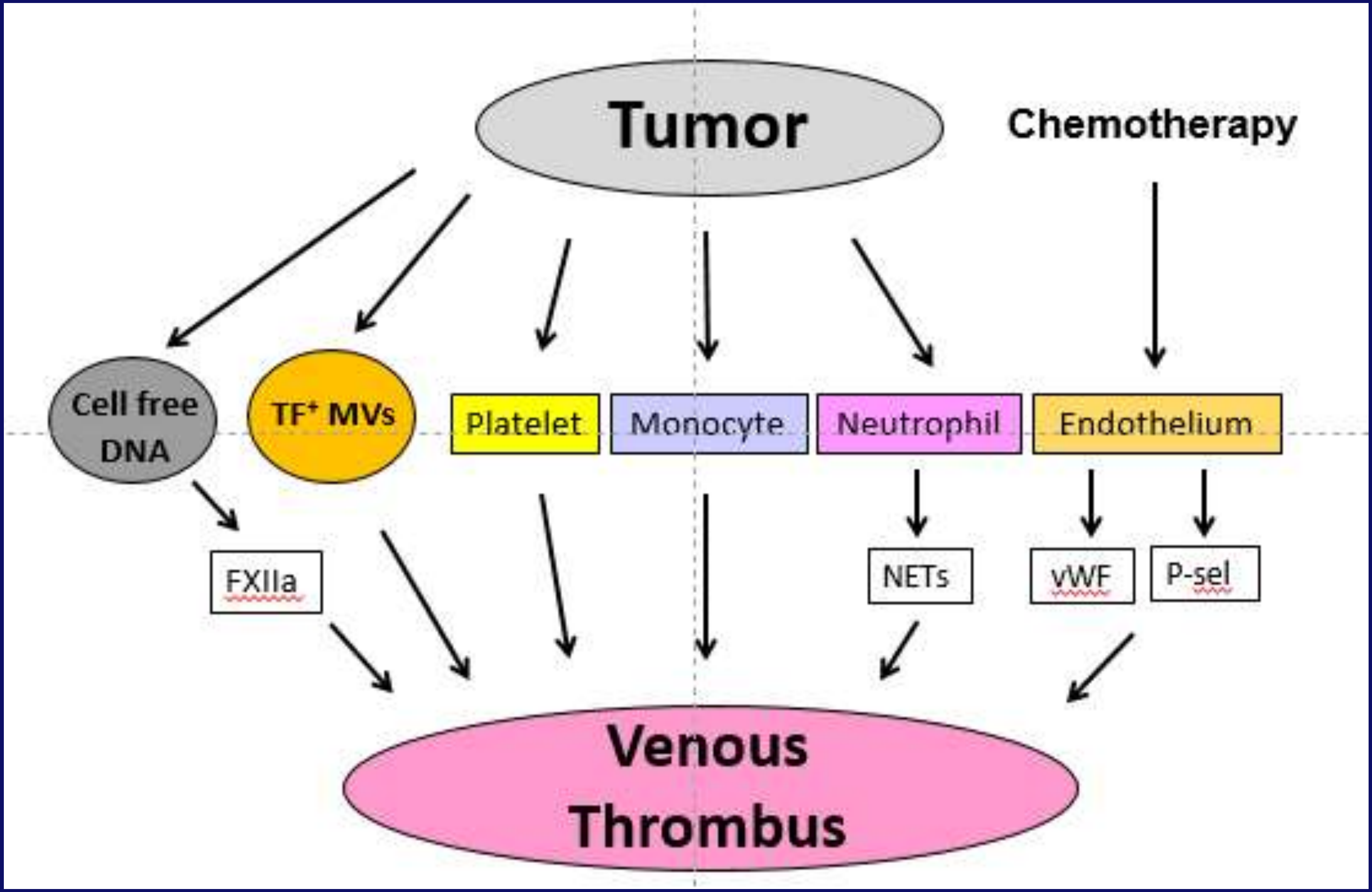
Tumor cells shed plasma membrane vesicles when cultured in vitro. Shed vesicles carrying procoagulant activity may account for activation of the clotting system associated with malignancy

Dvorak HF et al Science 1981

The procoagulant activity of tumor-derived MVs is due to TF

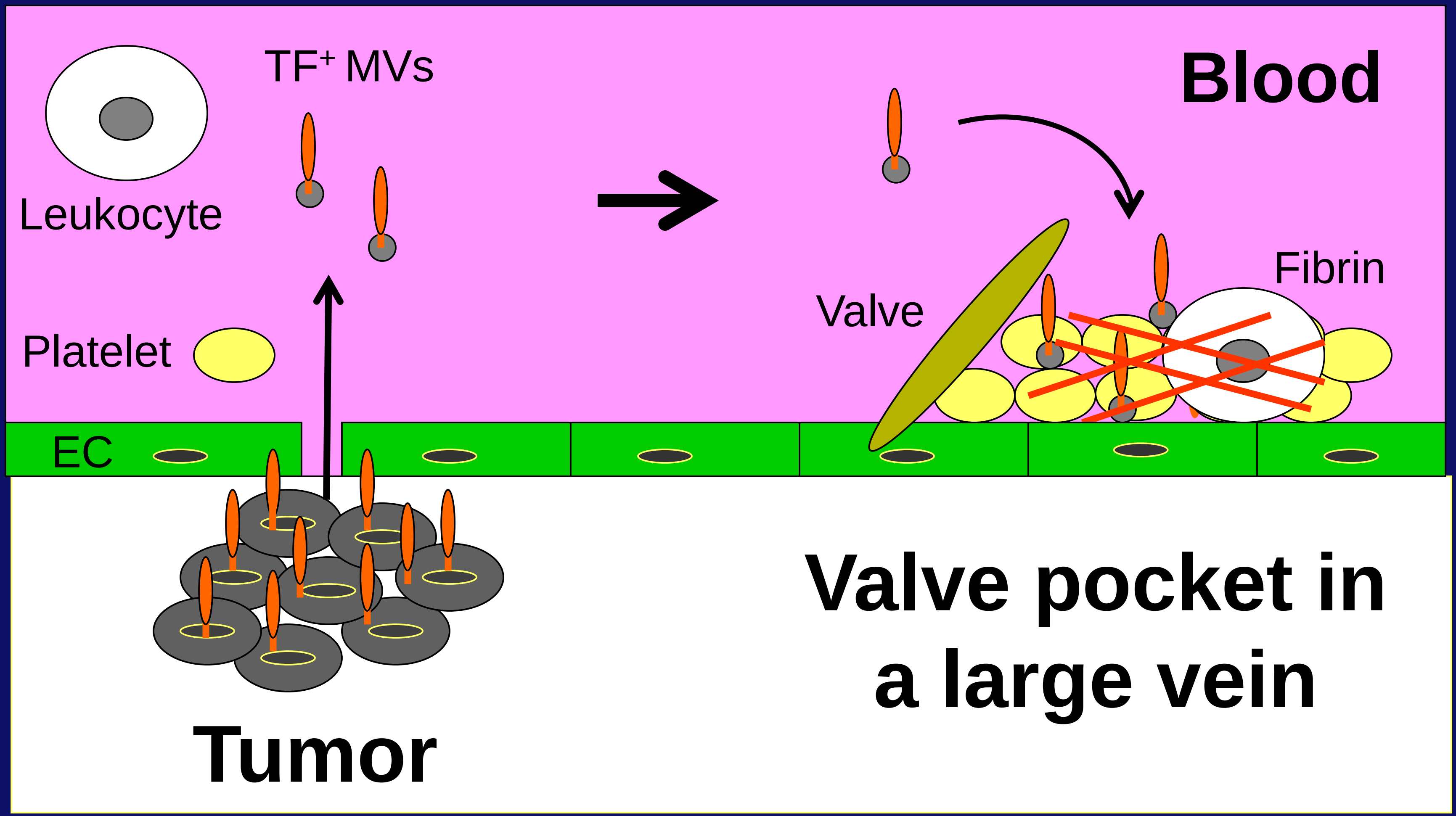
Dvorak et al Cancer Res 1983; Bastida et al Blood 1984;Yu and Rak JTH 2004

Mechanisms of Cancer-associated Thrombosis



Hisada Y et al JTH 2015

Hypothesis: Tumor-derived TF⁺ MVs Trigger Venous Thrombosis in Cancer Patients



Mouse Studies

Hypothesis

Tumor-derived, TF^+ MVs activate coagulation and increase clot size in a venous thrombosis model

Summary of Results with TF and Mouse Tumor Models

Human CRC and pancreatic tumors in mice release TF+ MVs into the blood.
Yu et al Blood 2005; Davila et al JTH 2008; Wang J-G et al Blood 2012.

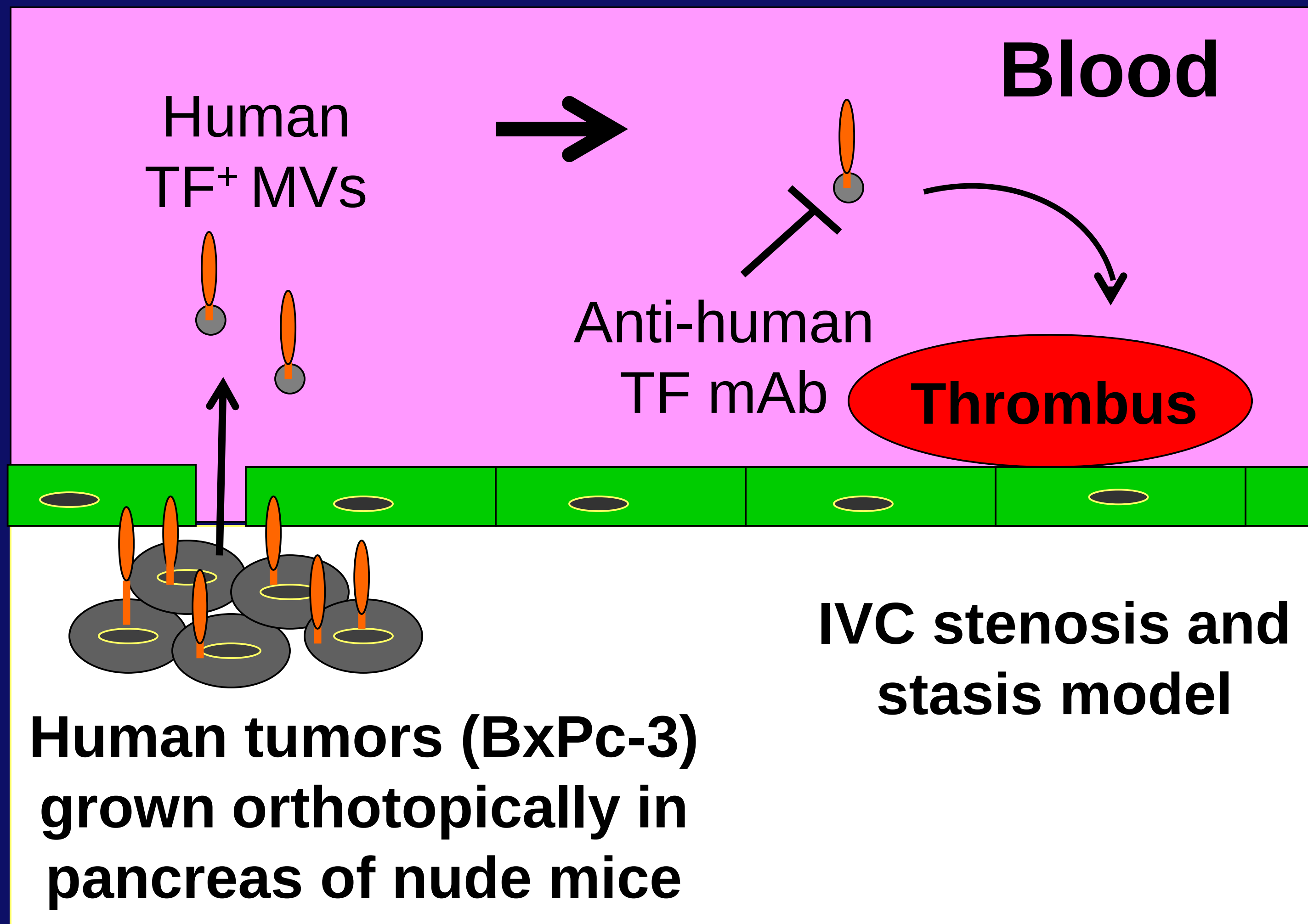
Mice with SQ murine pancreatic tumors have increased thrombosis. Human tumors in mice release MVs containin into the blood. **Thomas C et al JEM 2009, Thomas C et al JTH 2015.**

Do tumors enhance clot size in mice?

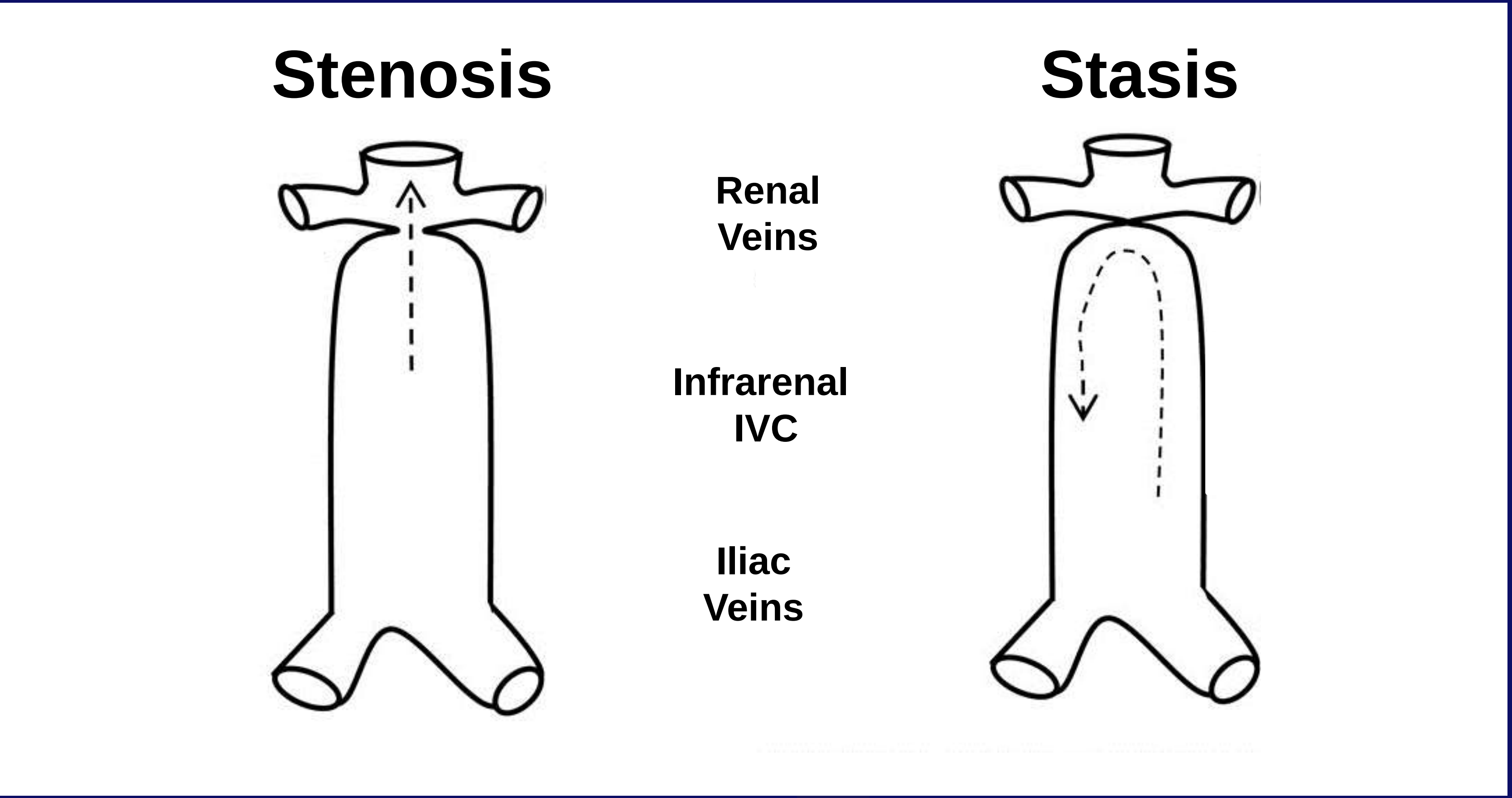
Model to Study the Role of Tumor-derived, TF^+ MVs in Venous Thrombosis in Mice

Julia Geddings

Yohei Hisada



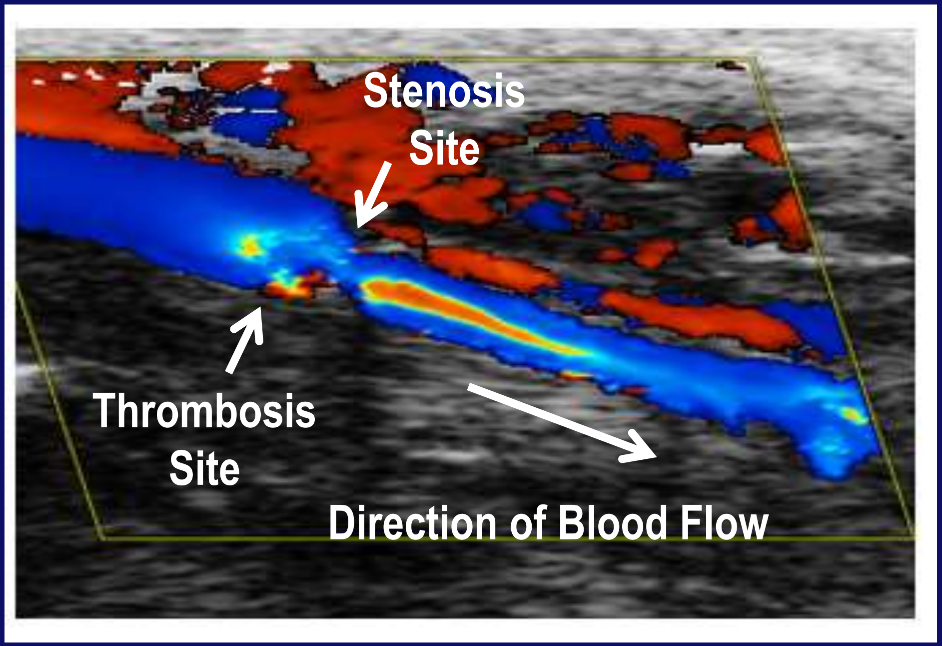
Mouse Venous Thrombosis Models: Infrarenal Vena Cava Stenosis and Stasis



Diaz J. et al. ATVB 2012

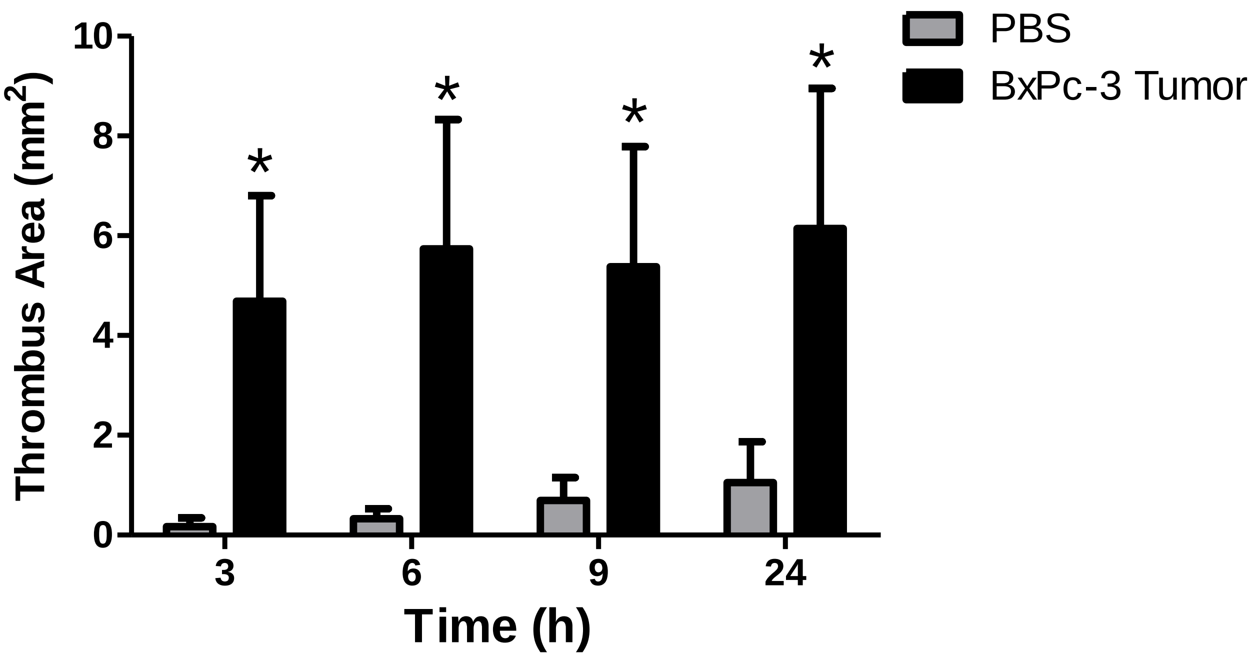
Kinetic Analysis of Thrombosis Formation

Color Doppler



Geddings J et al. JTH 2014

Human Pancreatic Tumors increase the Incidence of Thrombosis in the IVC Stenosis Model

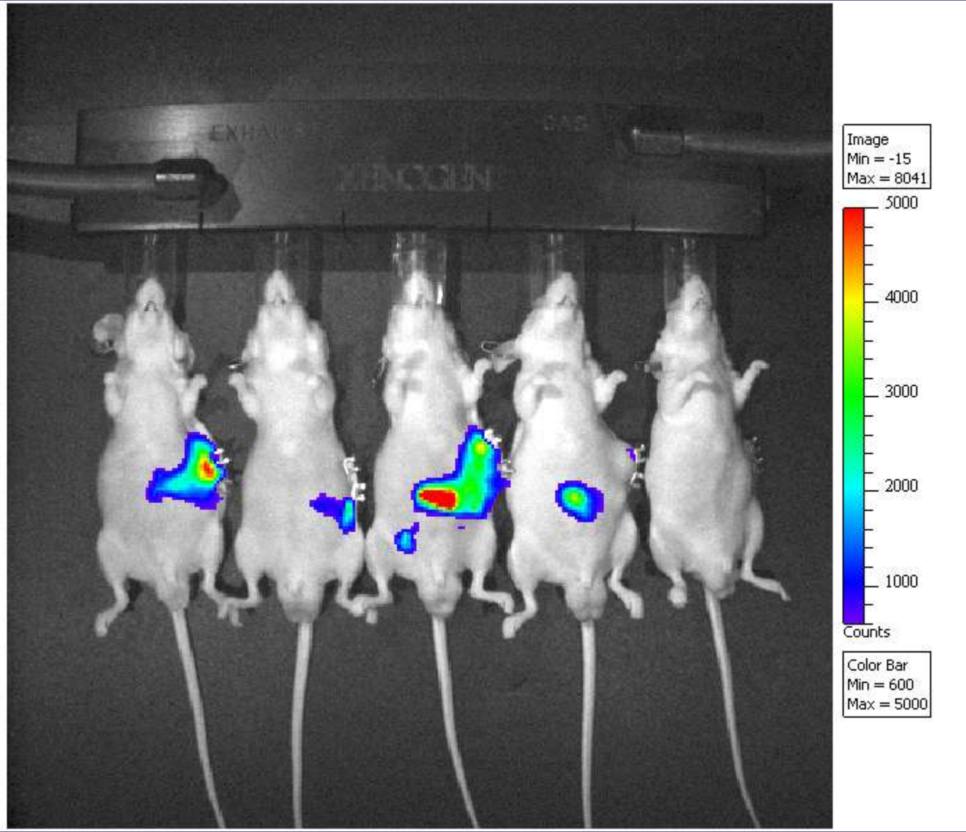


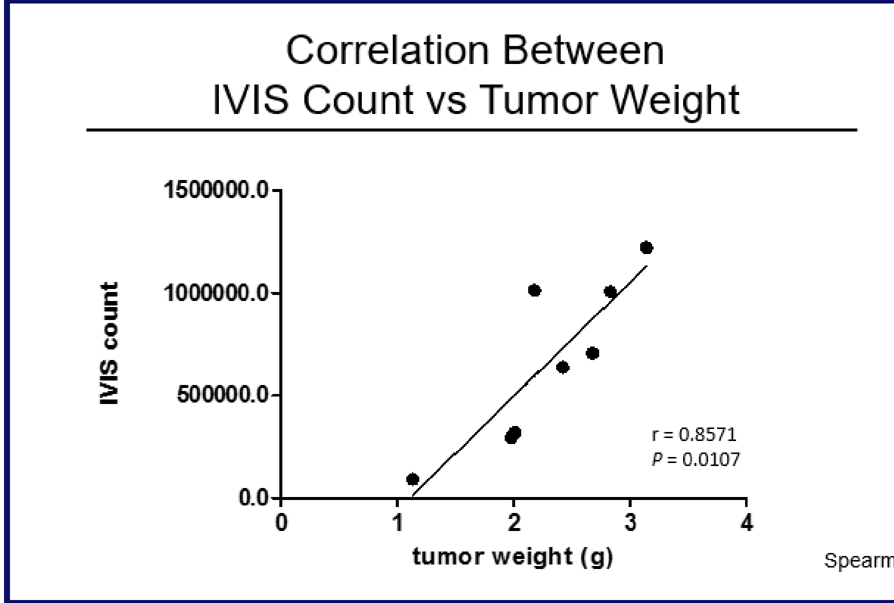
Thrombus Incidence

Time (h)	3	6	9	24
PBS	17%	33%	33%	33%
BxPc-3 Tumor	50%	50%	50%	50%

Geddings J et al. JTH 2015

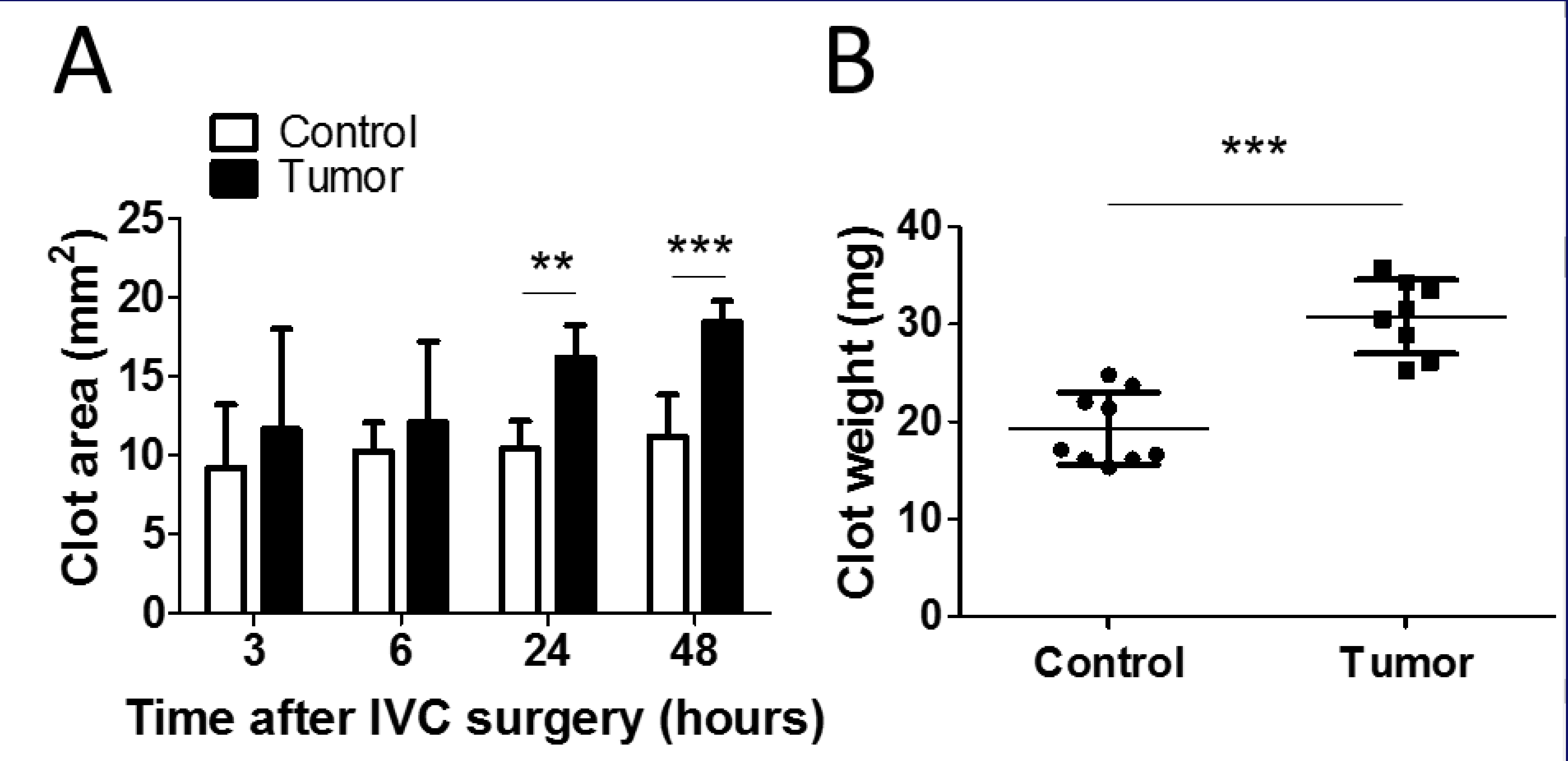
Imaging of BxPc-3-luciferase Tumors in the Pancreas of Mice





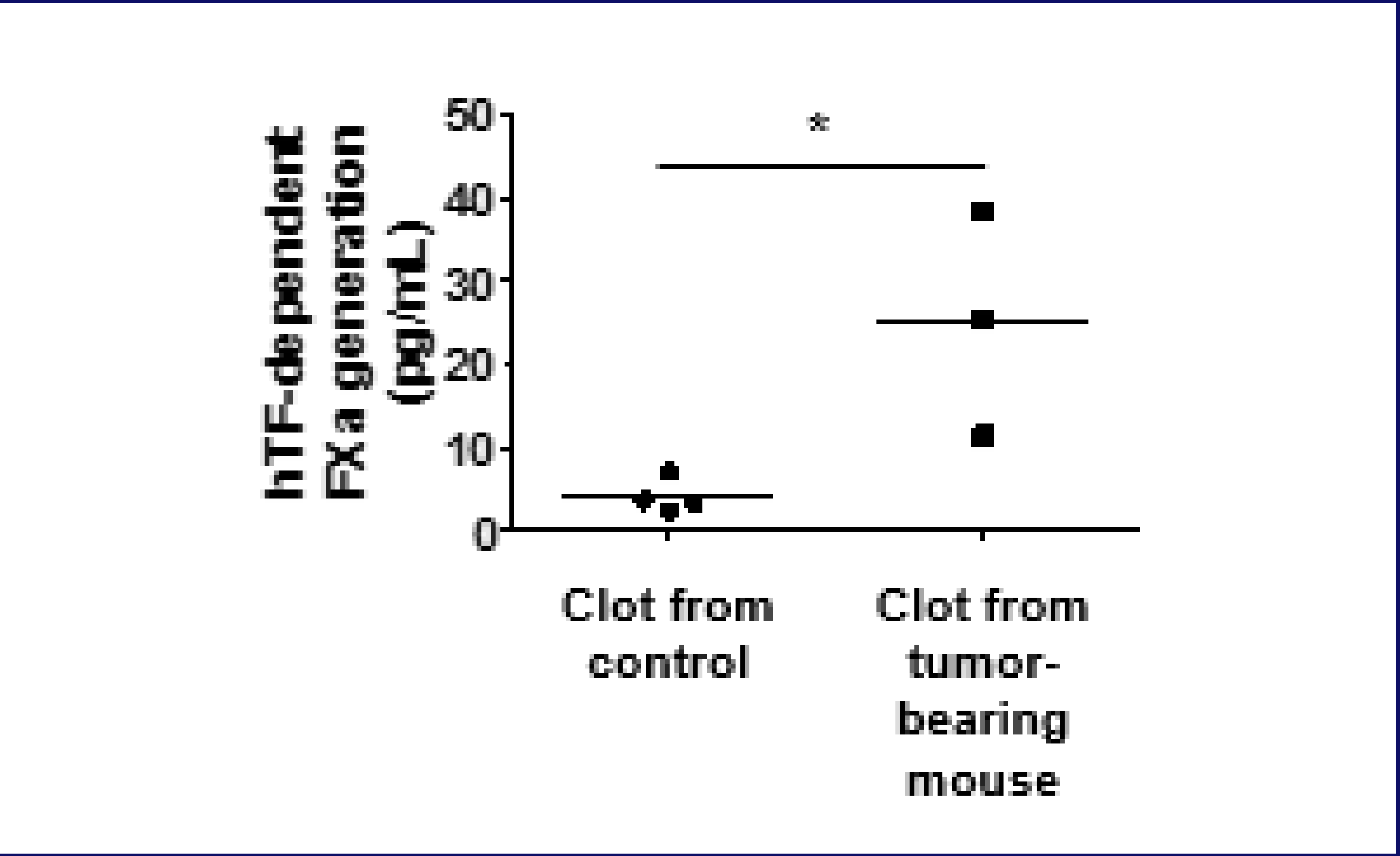
Hisada Y et al JTH 2017

Human Pancreatic Tumors Increase Clot Size in Nude Mice



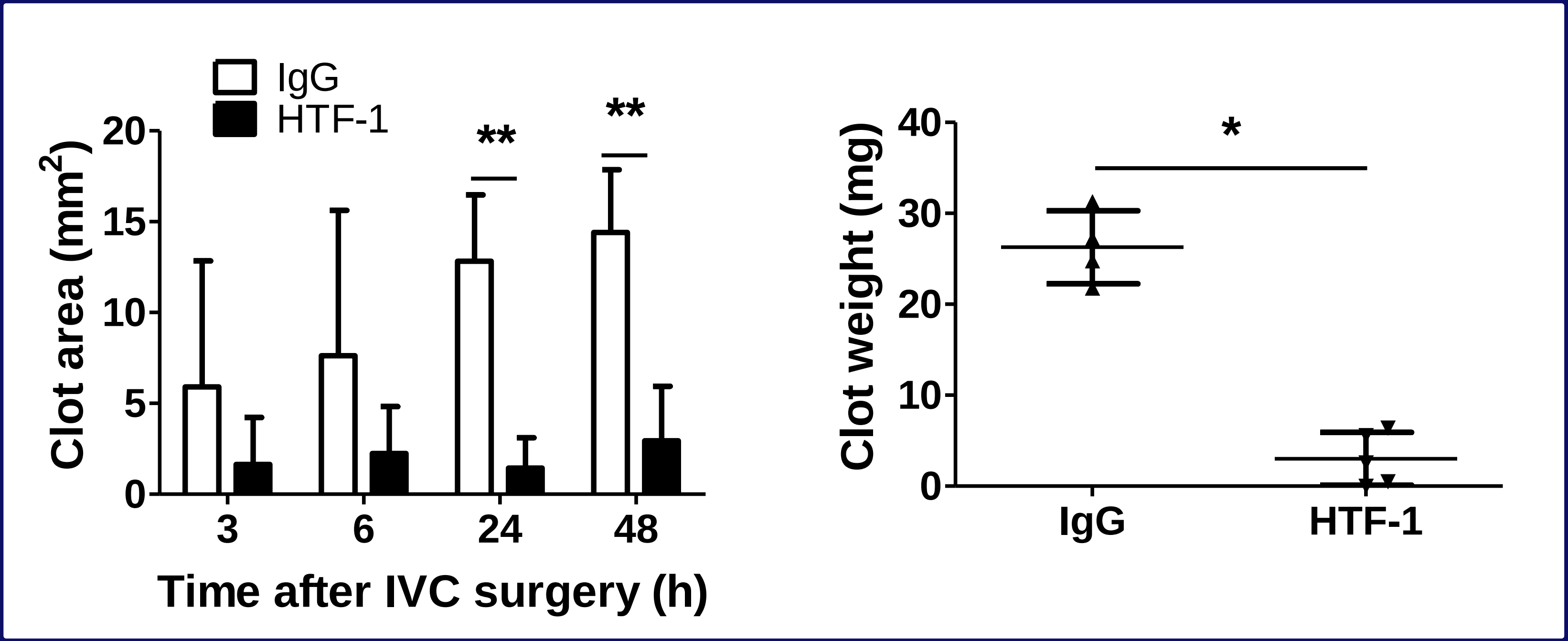
Hisada Y et al JTH 2017

Clots from Tumor-bearing Mice Contain Human TF



Hisada Y et al JTH 2017

Inhibition of Human TF Reduces Clot Size in Tumor-bearing Mice



HTF-1 does not affect thrombosis in control mice

Hisada Y et al JTH 2017

Conclusion

Tumor-derived TF^+ MVs enhance venous thrombosis in mice with pancreatic tumors

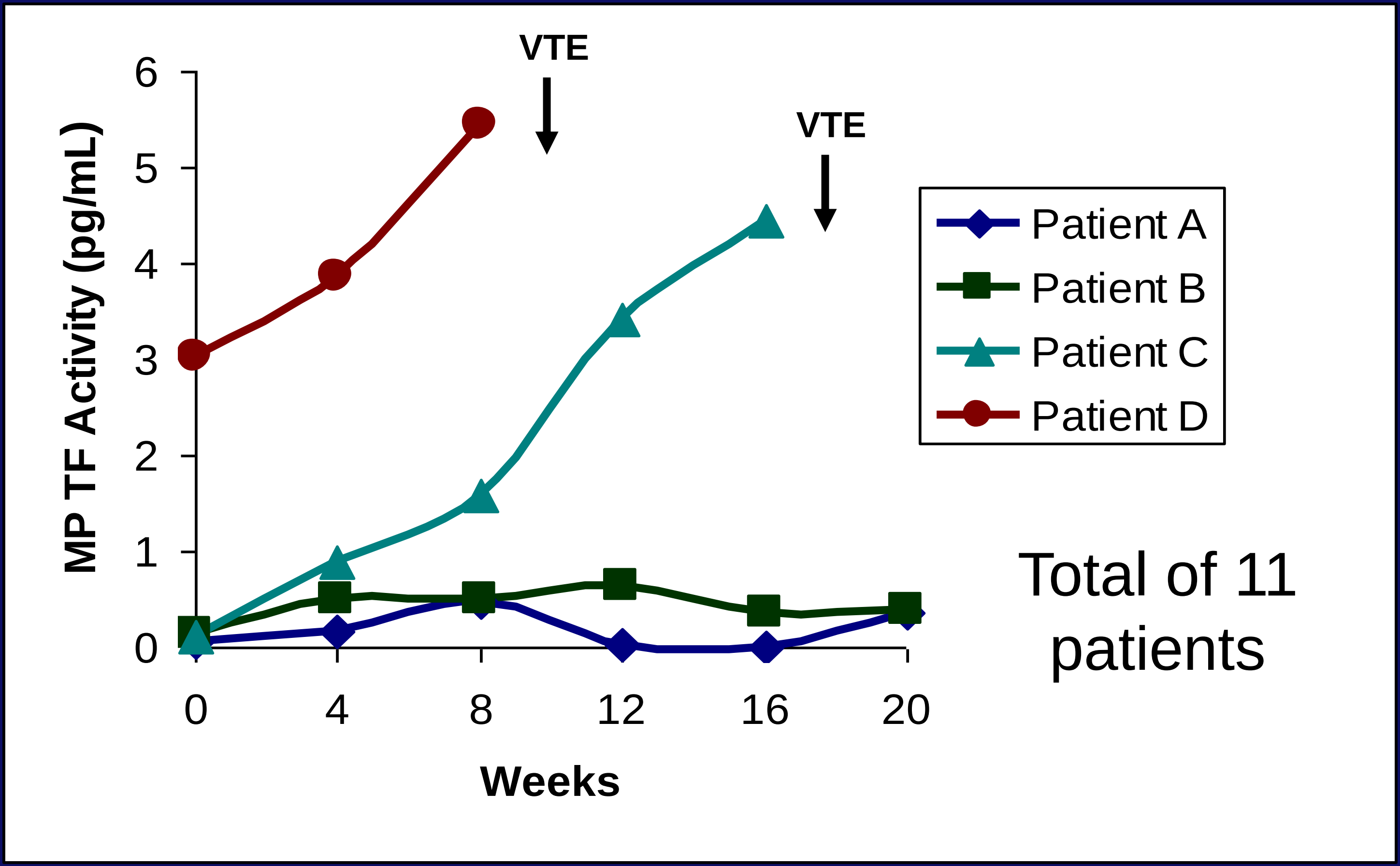
Clinical Studies

Hypothesis

MV TF activity may be a useful biomarker to identify cancer patients at risk for venous thrombosis

Perform prospective studies

Levels of MV TF Activity Increase in Pancreatic Cancer Patients before VTE



Khorana A et al JTH 2008

Association of MV TF Activity and VTE in Pancreatic Cancer Patients

Study	Patients	Time of Follow-up	Association between MV TF and VTE?
Khorana, 2008	2/9 patients developed VTE	5 months	Yes
Van Doormaal, 2012	3/13 patients developed VTE	6 months	Yes
Thaler, 2012	12/60 patients developed VTE	2 years	Weak
Bharthuar, 2013	*52/117 patients developed VTE	6 months	Yes
Van Es, submitted	9/89 patients developed VTE	6 months	Weak
Khorana, submitted	7/39 patients developed VTE	3 months	Yes
Ilich, In preparation	4/22 patients developed VTE	6 months	Yes

*Pancreaticobiliary cancer patients

Levels of MV TF Activity in 4 Cancers with a High Rate of VTE: Vienna CATS

	VTE	MV TF activity	Association with VTE
Pancreatic	12/60 (20%)	0.1 (0.04-0.19)	Yes
Stomach	6/43 (14%)	0.07 (0.0-0.17)	No
Colorectal	12/126 (10%)	0.05 (0.01-0.15)	No
Brain	19/119 (16%)	0.04 (0.0-0.08)	No

Thaler J et al JTH 2012

Conclusion

MV TF activity may be a useful biomarker to identify pancreatic cancer patients at risk for VTE

General Conclusions

- TF provides a hemostatic envelope around blood vessels and at organ and body surfaces.
- TF expression provides additional hemostatic protection to vital organs, such as the heart, lung and brain.
- TF is expressed in atherosclerotic plaques and contributes to arterial thrombosis after plaque rupture.
- In pancreatic cancer, levels of tumor-derived circulating TF+ MVs are associated with VTE.

Mackman Lab 2017

NATIONAL INSTITUTES OF HEALTH

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MYOCARDITIS FOUNDATION

TREC
K.G. JEBSEN THROMBOSIS RESEARCH AND EXPERTISE CENTER

TraCS

Re-balancing the Clotting Cascade in Mice

Can we rescue the lethality of embryos lacking different anticoagulants with low levels of TF?

Cross =

$TFPI^{+/-}$,$mTF^{+/-}$,hTF^{+} x $TFPI^{+/-}$,$mTF^{+/-}$,hTF^{+}

Mice

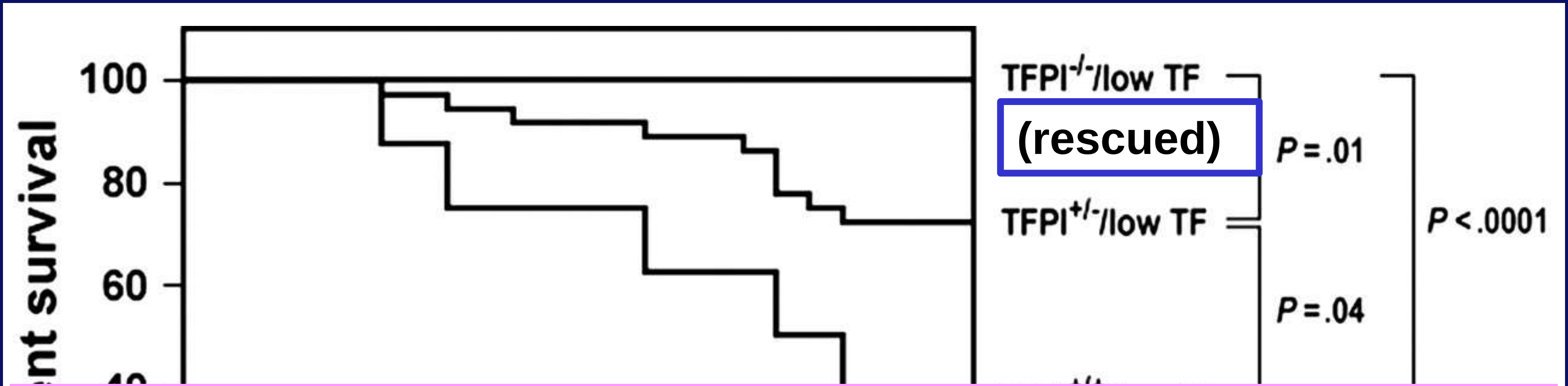
Low TF, $TFPI^{+/+}$

Low TF, $TFPI^{+/-}$

1 slide

Low TF, $TFPI^{-/-}$ (rescued mice)

Balancing TF and TFPI in Mice

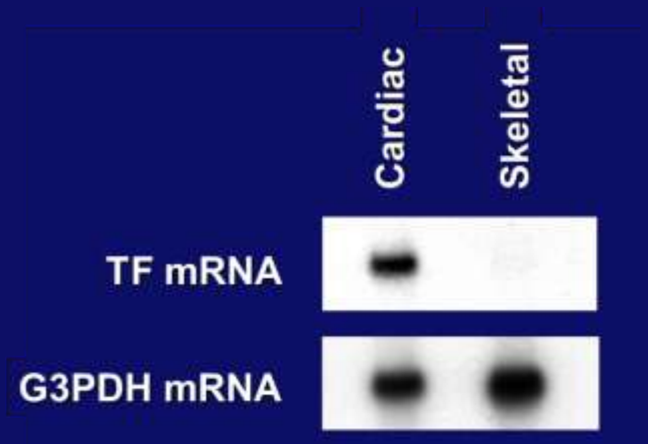


1/ TFPI$^{-/-}$ embryos are rescued by reducing the level of TF.
2/ Fatal bleeding in low TF mice is rescued by reducing the level of TFPI.

Pedersen B et al Blood 2005

TF Expression in Mouse and Human Cardiac and Skeletal Muscle

Mouse

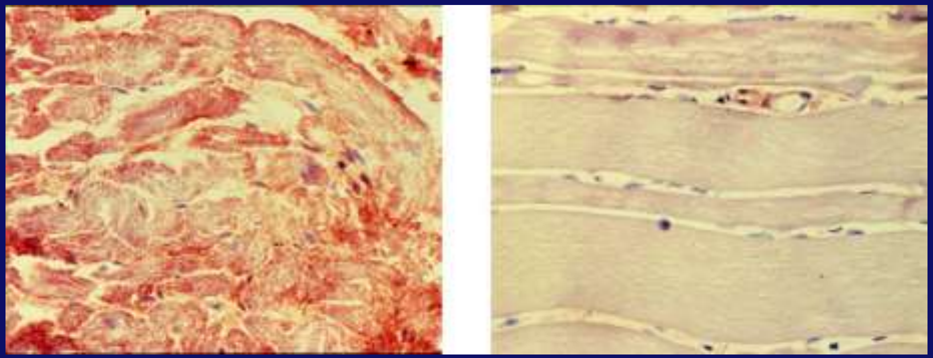


Mackman et al AJP 1993

Human

Cardiac muscle

Skeletal muscle



Drake, Morrissey & Edgington (1989)
A J P 134:1087-1097

move

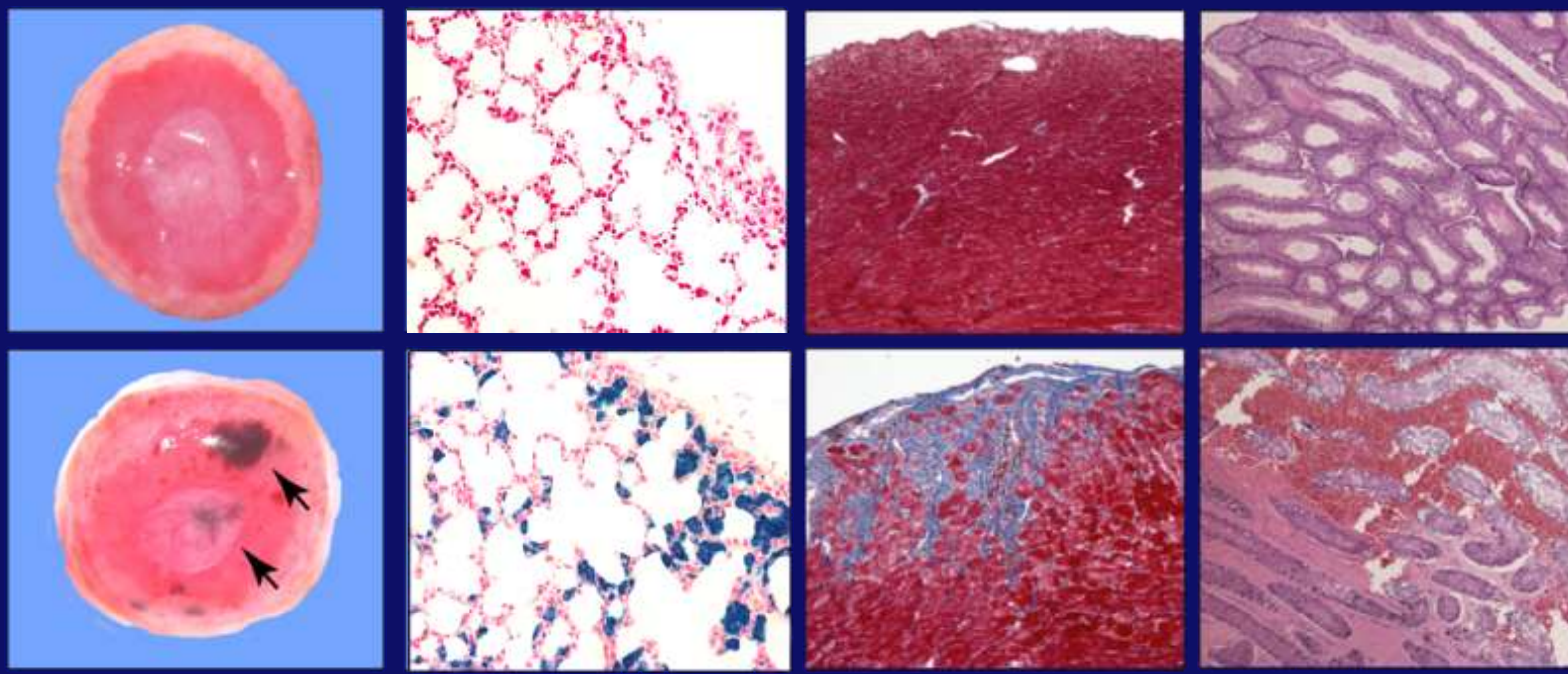
from Jim Morrissey, University of Illinois

Hemostatic Defects in Low TF Mice

Placenta | Lung | Heart | Testis

WT

Low TF



Erlich et al.

Pedersen et al. Blood 2005

Pawlinski et al. PNAS 2002

Mackman JTH 2007

move

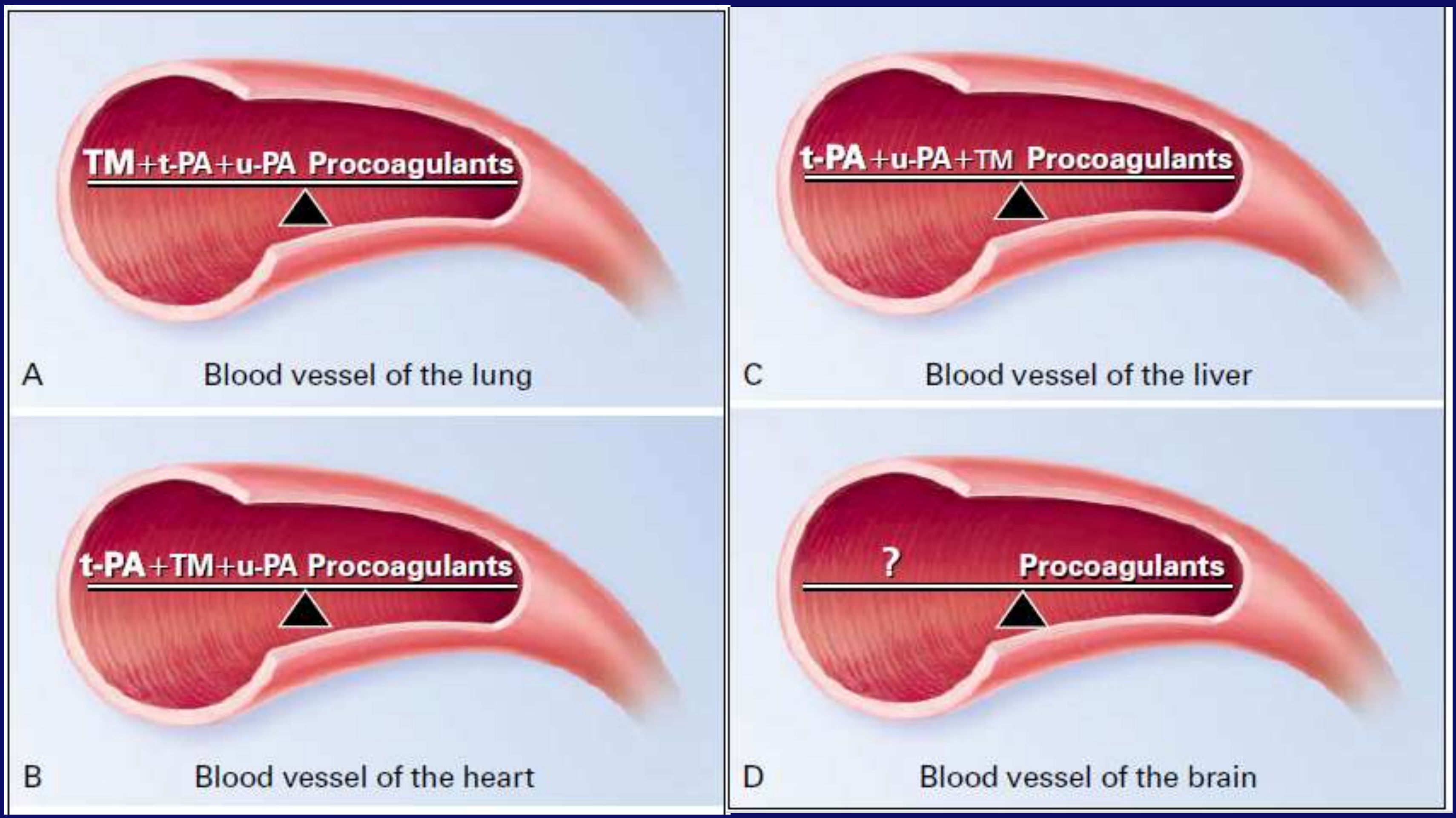
Role of Uterine TF in Hemostasis During Pregnancy

Breeding strategy	Level of TF expression		[redacted]	Fatal post-partum hemorrhage
	Mother	Embryo		

Low levels of TF in [redacted]her leads to fatal post-partu[redacted]rrhage, whereas low leve[redacted] in the mother and embry[redacted]s to fatal mid-gestational [redacted]hage.

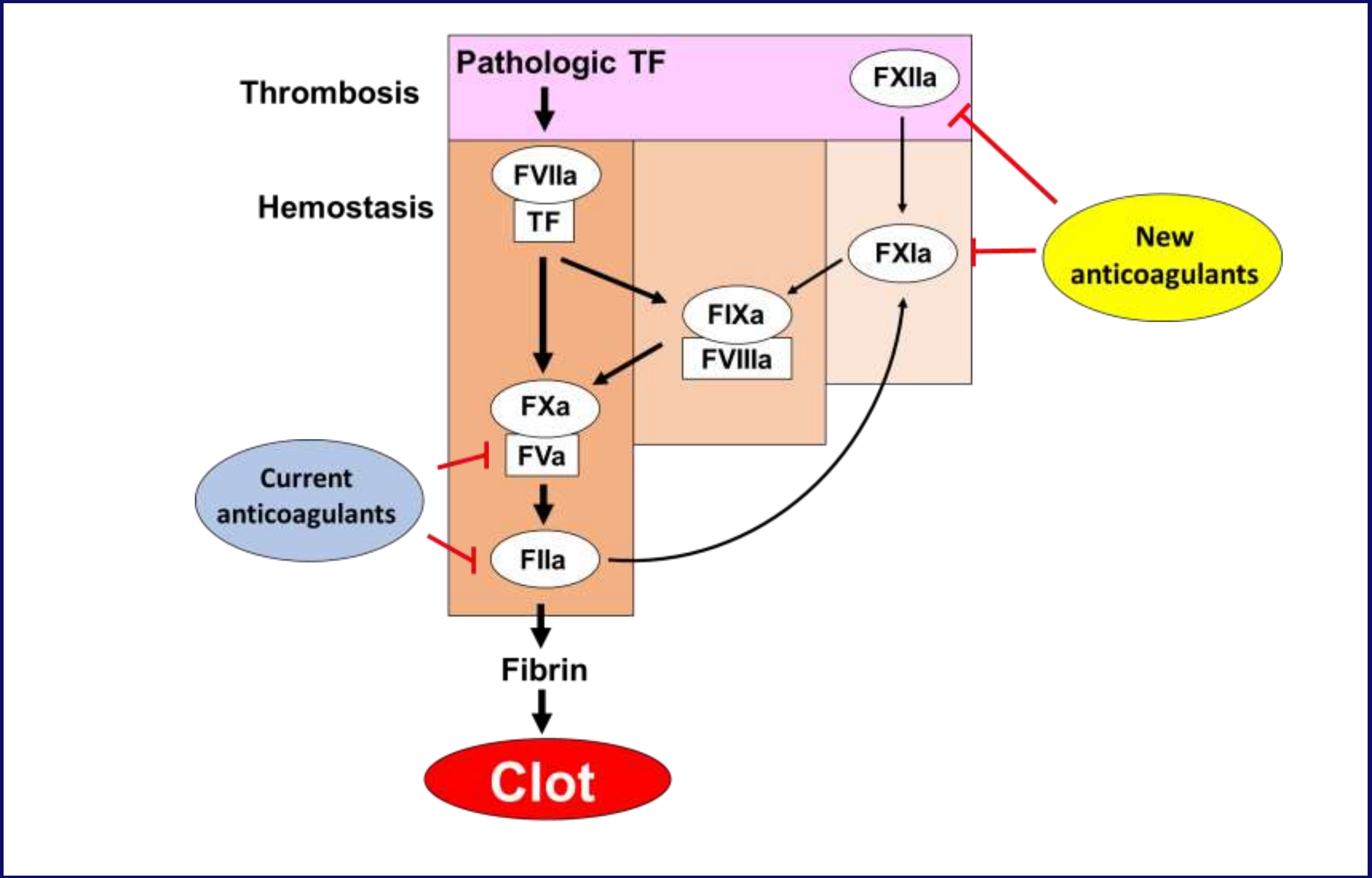
F move IX$^{-/-}$ have normal pregnancies

Vascular Bed-specific Hemostasis



Aird and Rosenberg NEJM 1999

Development of New Anticoagulant Drugs



The NEW ENGLAND JOURNAL of MEDICINE

Factor XI Antisense Oligonucleotide for Prevention of Venous Thrombosis

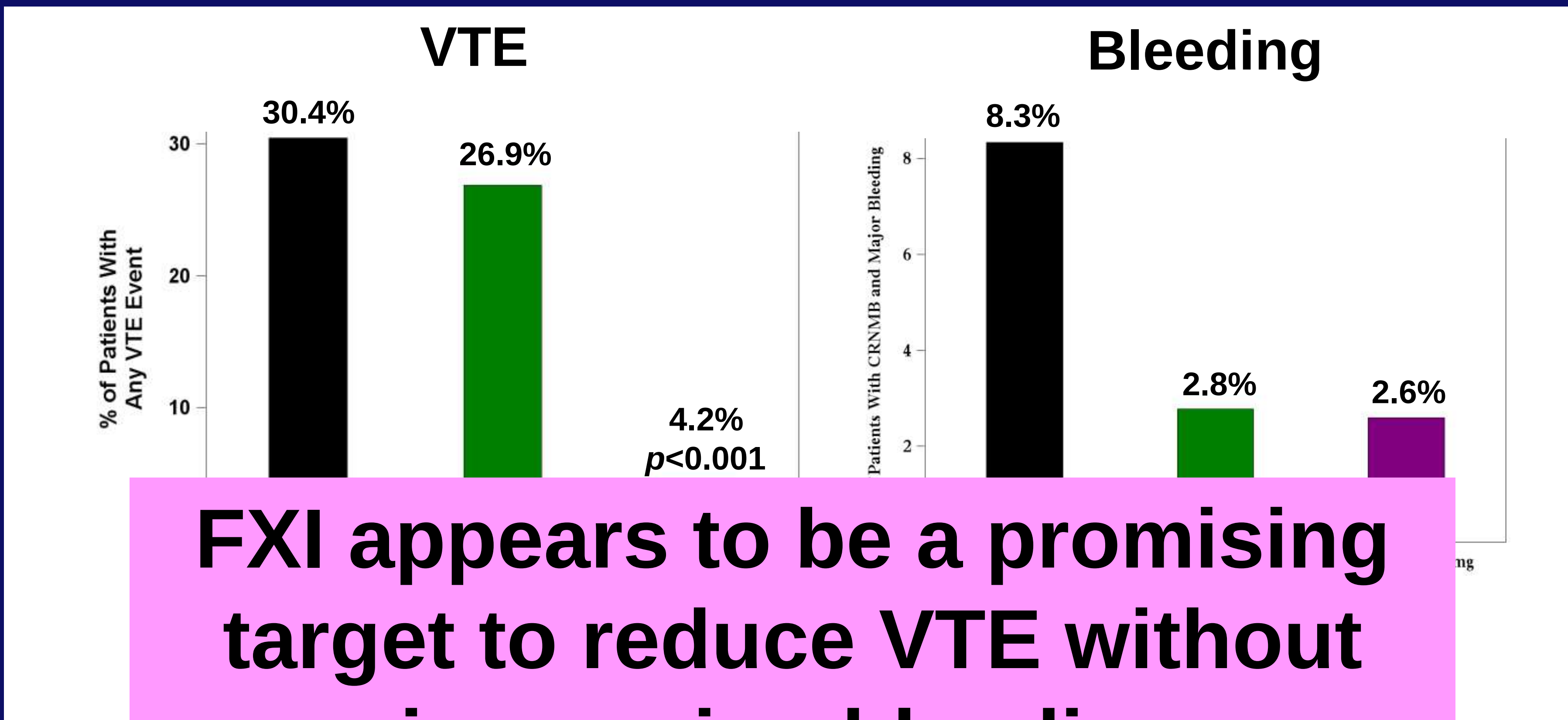
Harry R. Büller, M.D., Claudette Bethune ,Ph.D., Sanjay Bhanot, M.D., Ph.D, David Gailani, M.D., Brett P. Monia, Ph.D.,Gary E. Raskob, Ph.D., Annelise Segers, M.D., Peter Verhamme, M.D., and Jeffrey I. Weitz, M.D.

2015;372:232-240

- **Effect of decreased levels of FXI on VTE in pts undergoing total knee replacement**
- **Two doses of a FXI ASO were used: 200 mg ~41% FXI; 300 mg ~22% FXI.**
- **Enoxaparin was used as a control.**

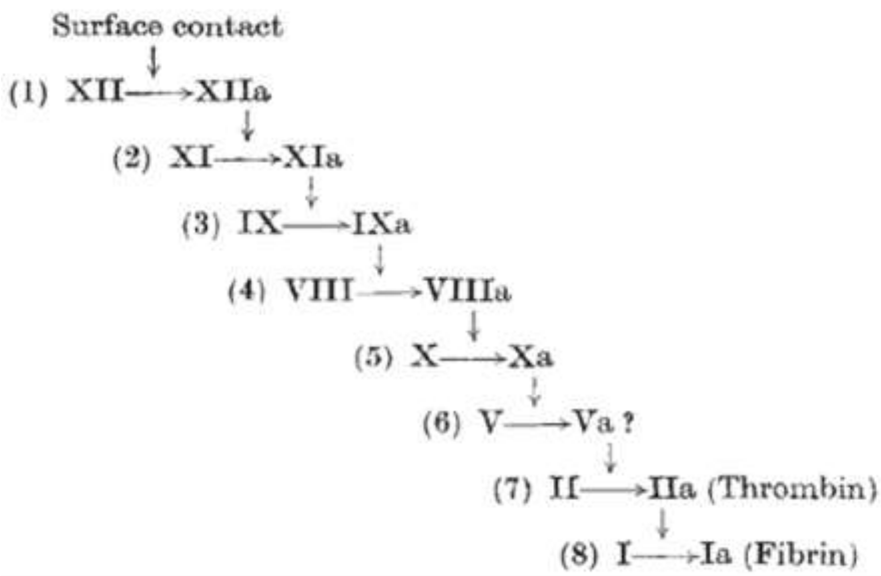
Slides from Dr. J. Weitz

Effect of Reducing FXI Levels on VTE and Bleeding after Total Knee Replacement

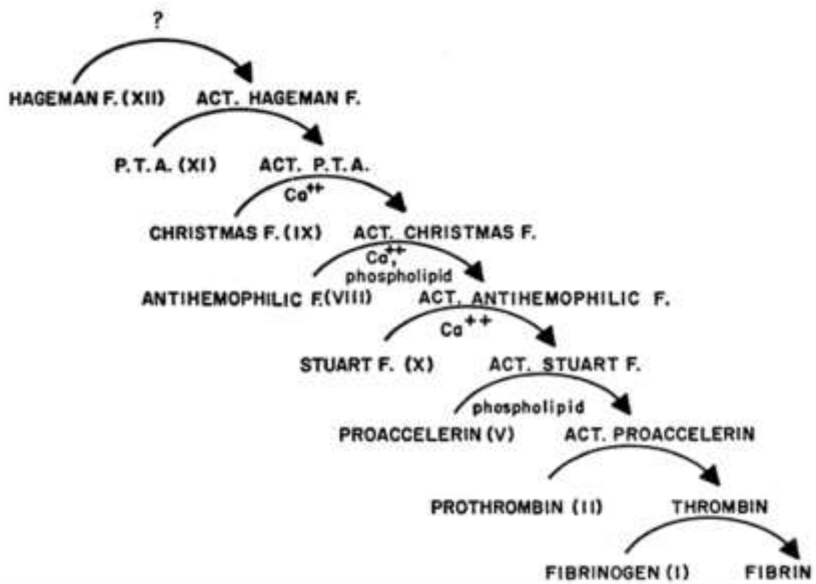


FXI appears to be a promising target to reduce VTE without increasing bleeding

Buller H et al N Engl J Med 2015



Macfarlane Nature 1964

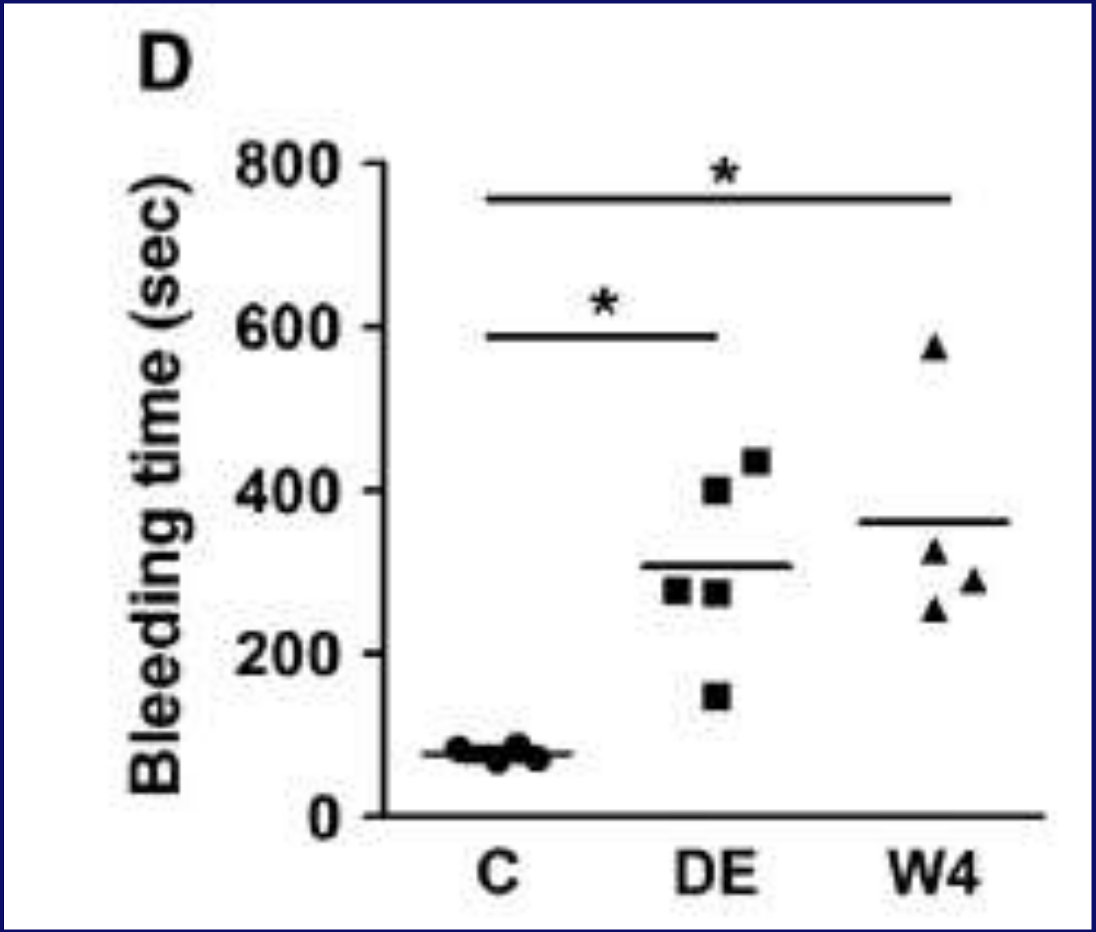


Davie Science 1964

Determine the effect of anticoagulants on alveolar hemorrhage in mice after influenza A infection

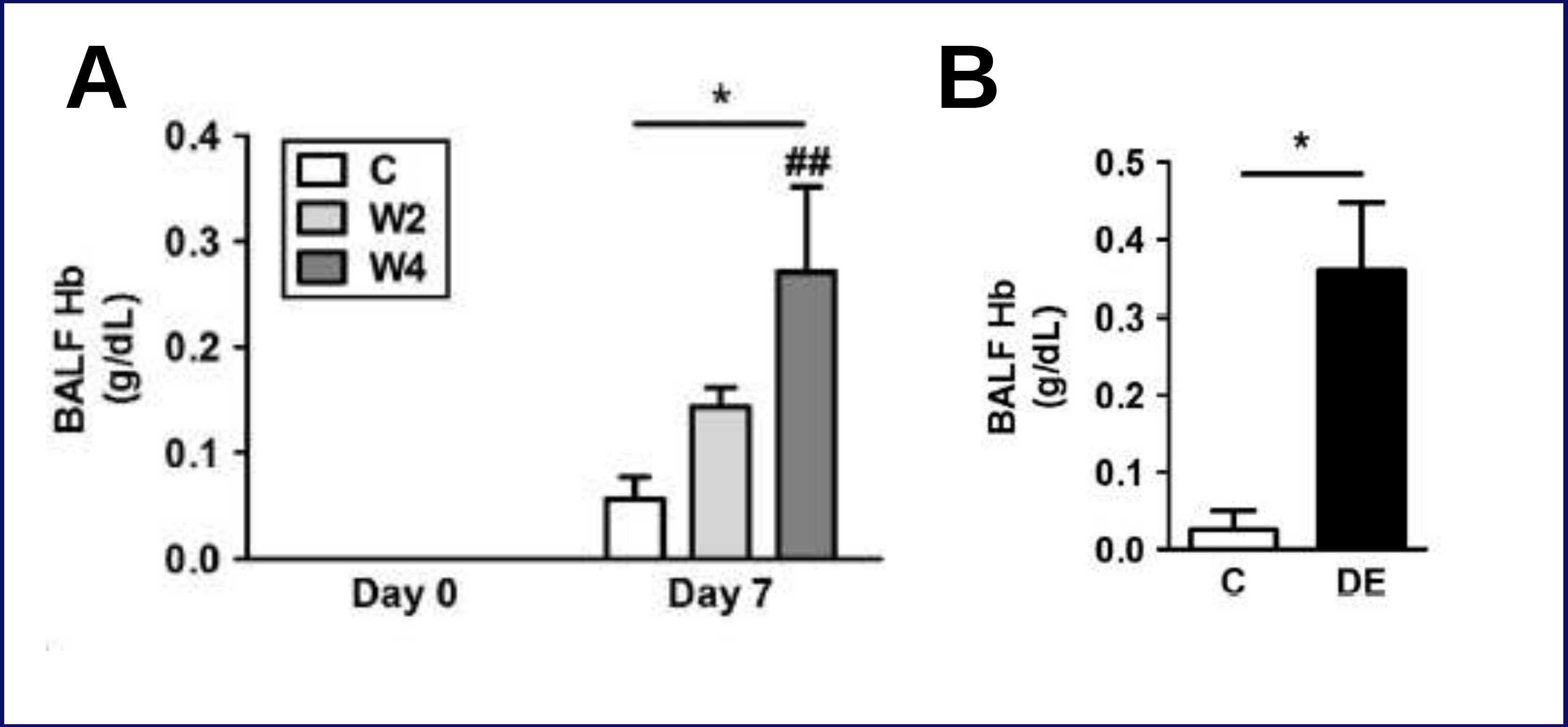
Kohei Tatsumi

Anticoagulating Mice with Dabigatran or Warfarin



Tatsumi K et al Physiol Reports 2016

Warfarin and Dabigatran Increase Lung Hemorrhage After H1N1 Infection



Tatsumi K et al Physiol Reports 2016

Conclusion

Anticoagulants may increase alveolar hemorrhage and morbidity in patients infected with influenza A

Use of anticoagulants in the US

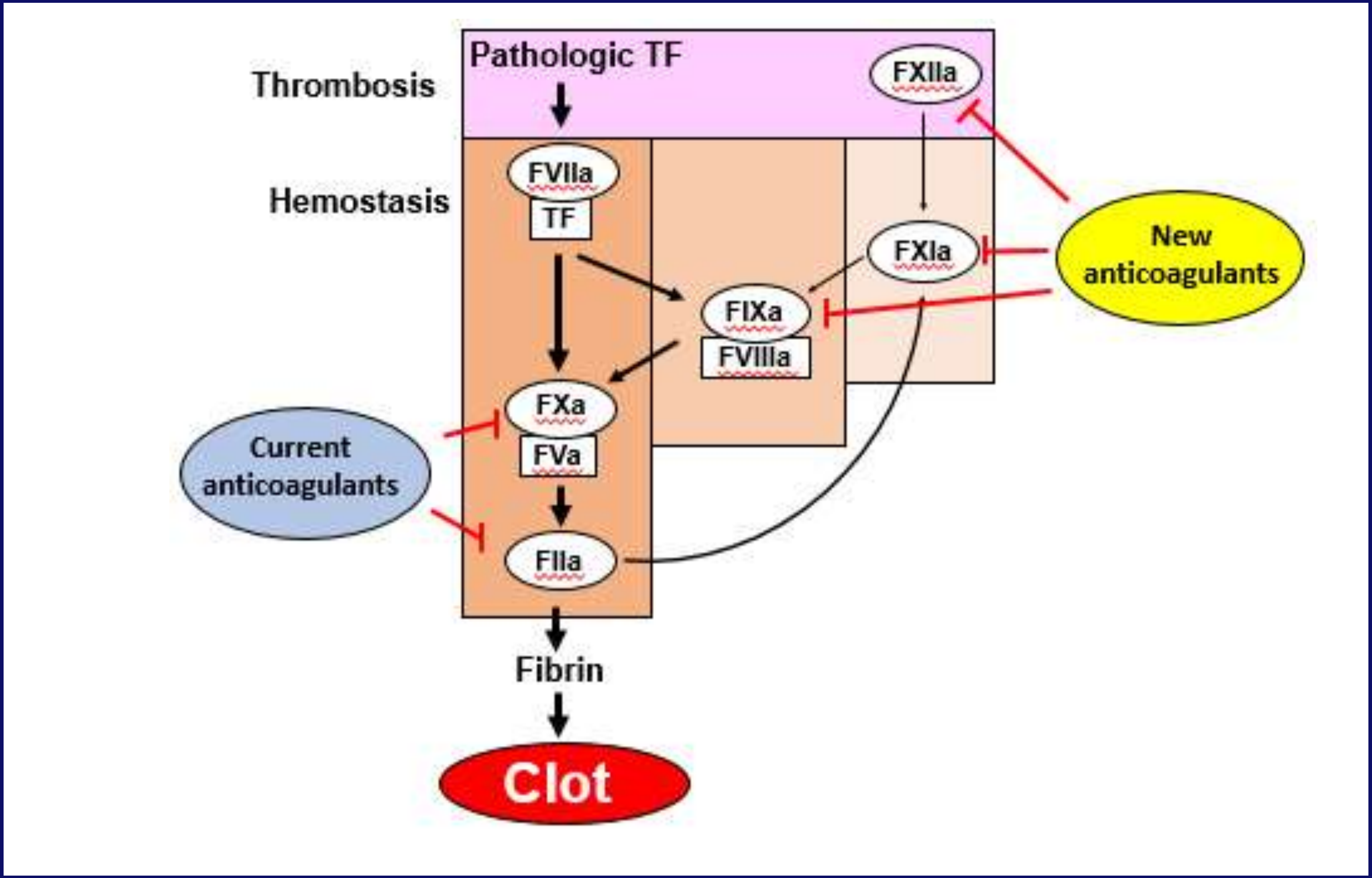
<1% under 65

5% 65-74

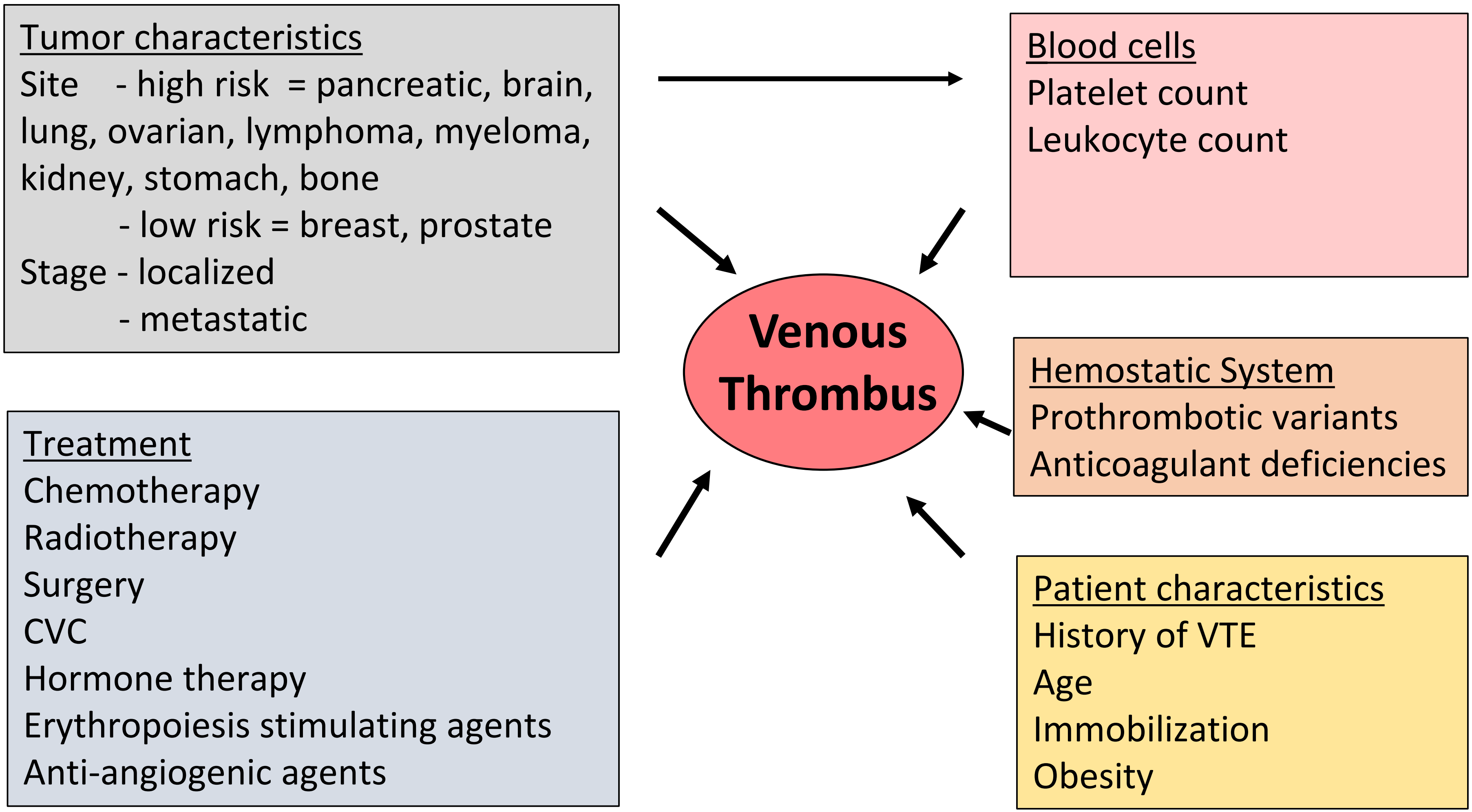
10% >75

Medical Expenditure Panel Summary Oct 2009

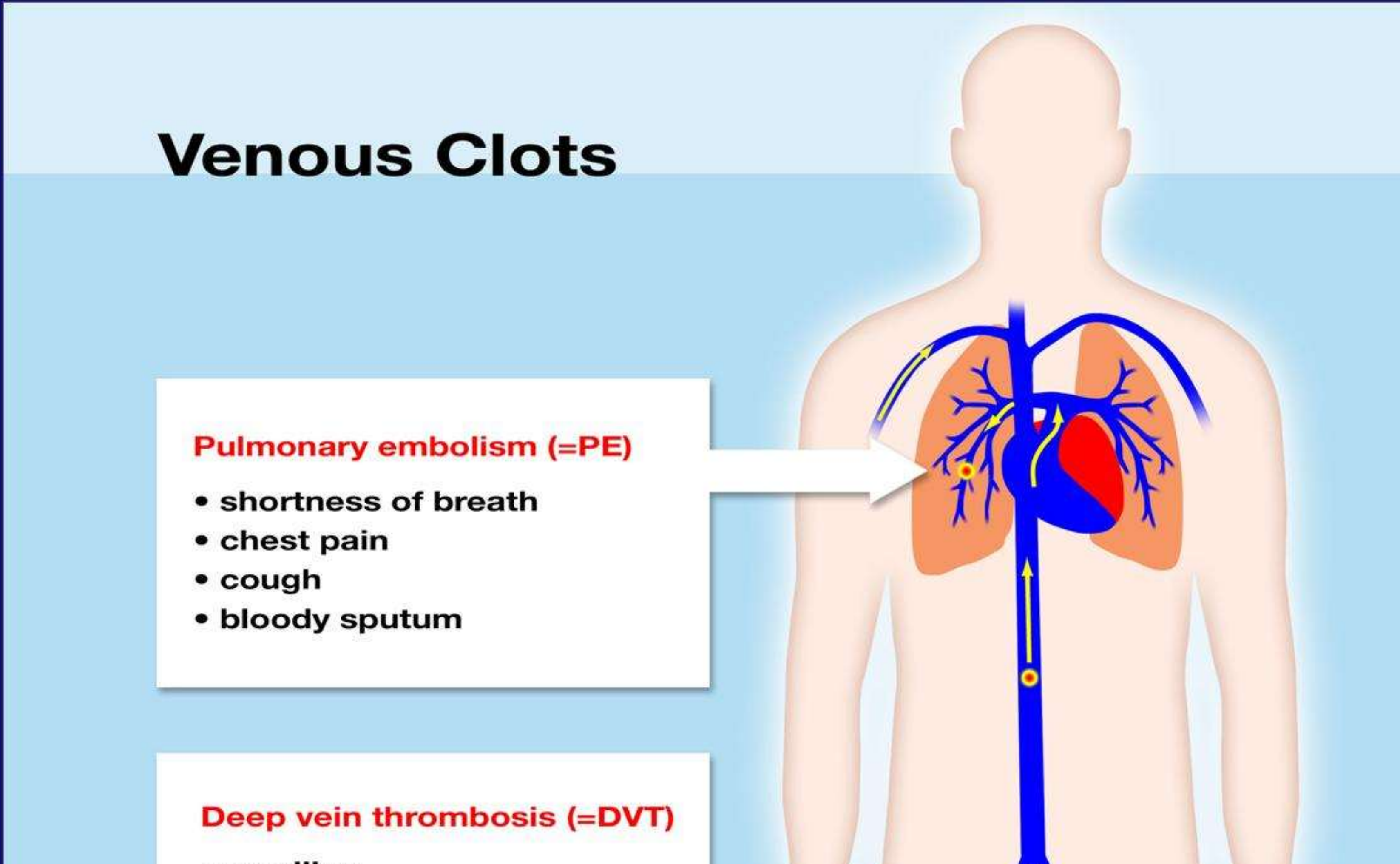
Development of New Anticoagulant Drugs



Risk Factor for Cancer-associated Thrombosis



Hisada Y, Geddings J and Mackman N JTH 2015



Venous thromboembolism (VTE) = DVT + PE

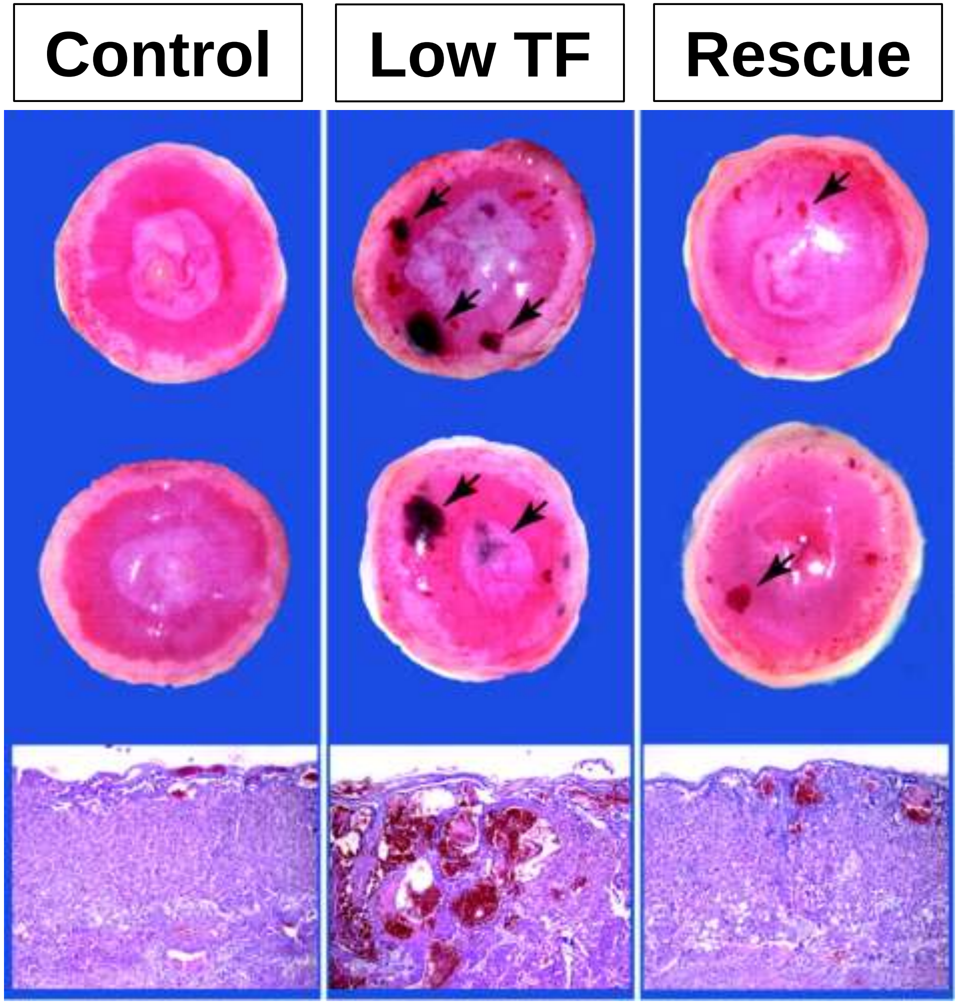
Slide from Dr. Stephan Moll clotconnect.org

Arterial Thrombosis

Thrombosis in a human coronary artery

provided by Dr. Taubman

Reduced Blood Pools in "Rescued" Placentas



Pedersen B et al Blood 2005

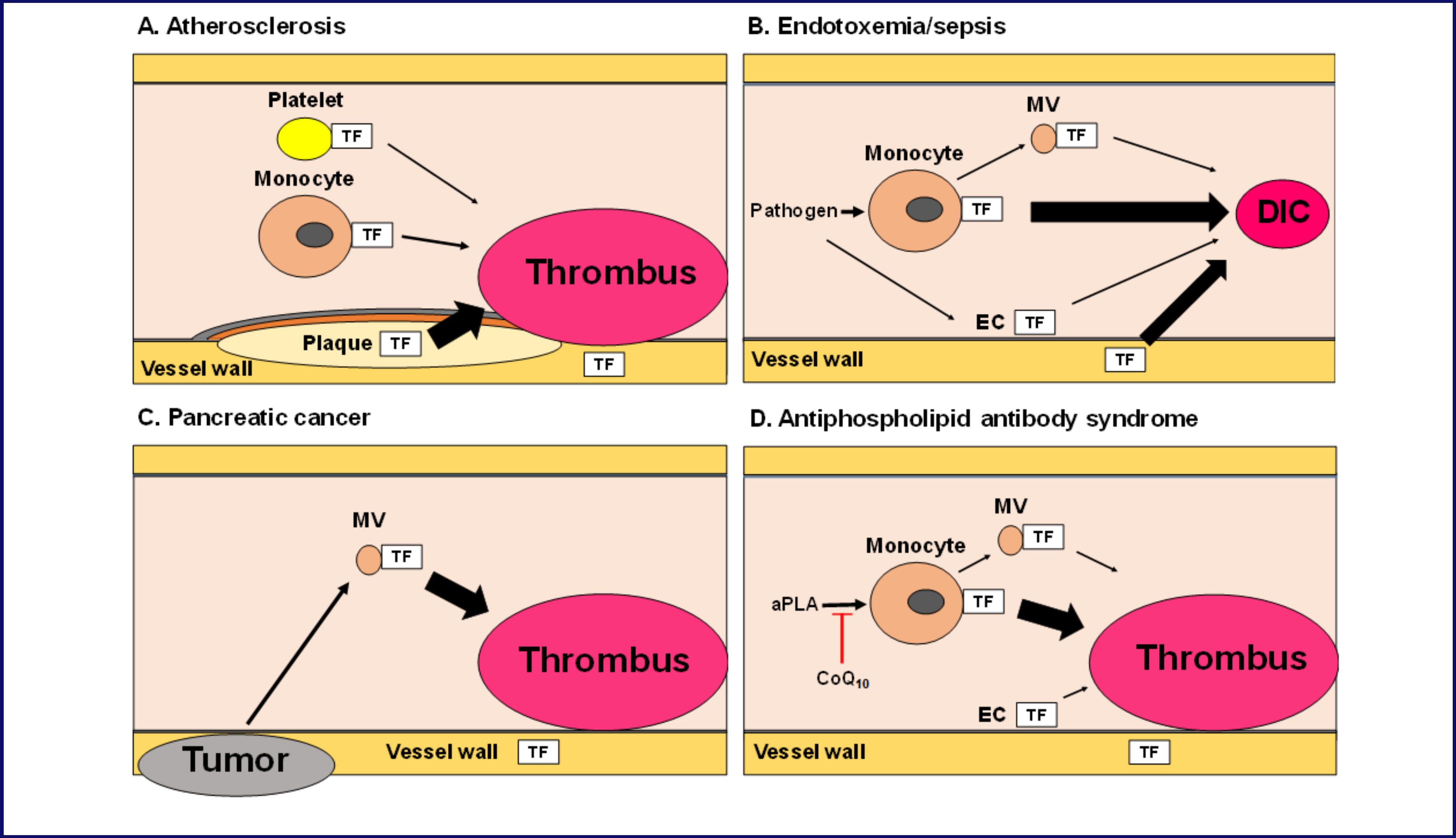
History of Tissue Factor-Protein

- **Purification of human TF** (Broze G et al JBC 1985).
- **Crystal structure** (Harlos K et al Nature 1994).

History of Tissue Factor-Gene

- **Human TF cDNA** (Spicer E et al PNAS 1987; Morrissey J et al Cell 1987; Scarpati E et al Biochem 1987; Fisher K et al Thromb Res 1987).
- **Human TF gene** (Mackman N et al Biochem 1989).
- **Promoter characterization** (Mackman N et al JEM 1991).
- **KO of the mouse TF gene** (Bugge T et al PNAS 1996; Carmeliet P et al Nature1996; Toomey J et al Blood, 1996)

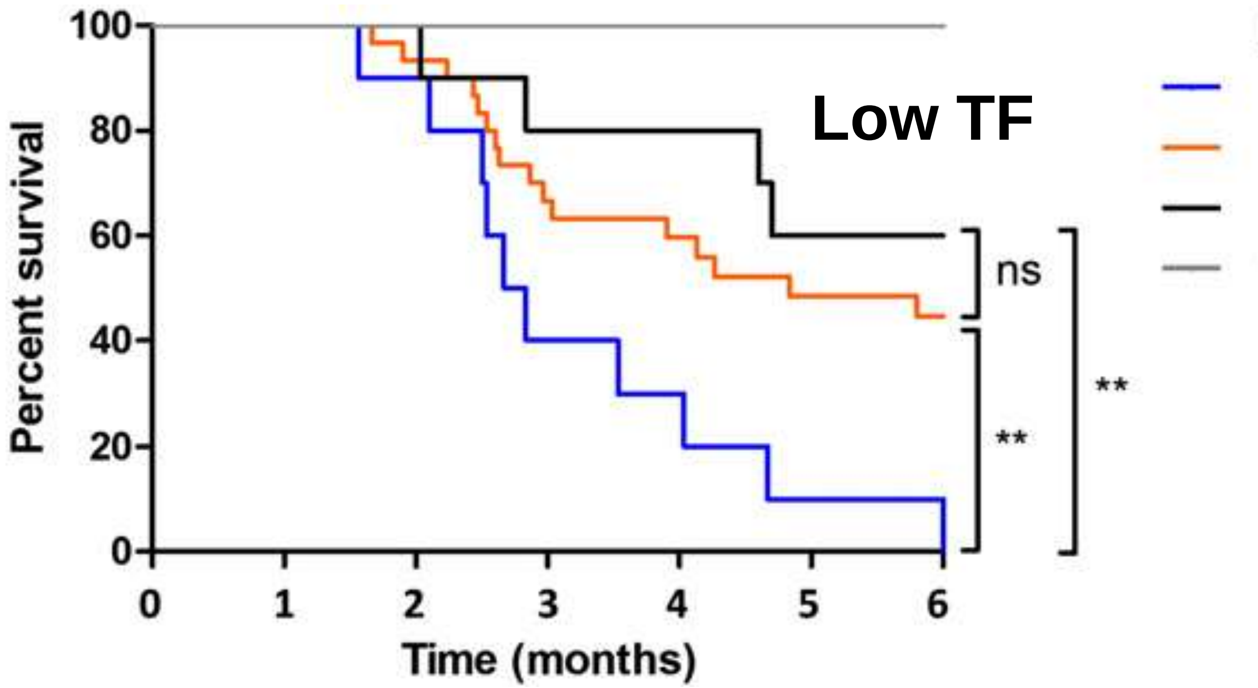
Roles of TF in Thrombosis and Disseminated Intravascular Coagulation



Grover and Mackman ATVB in press

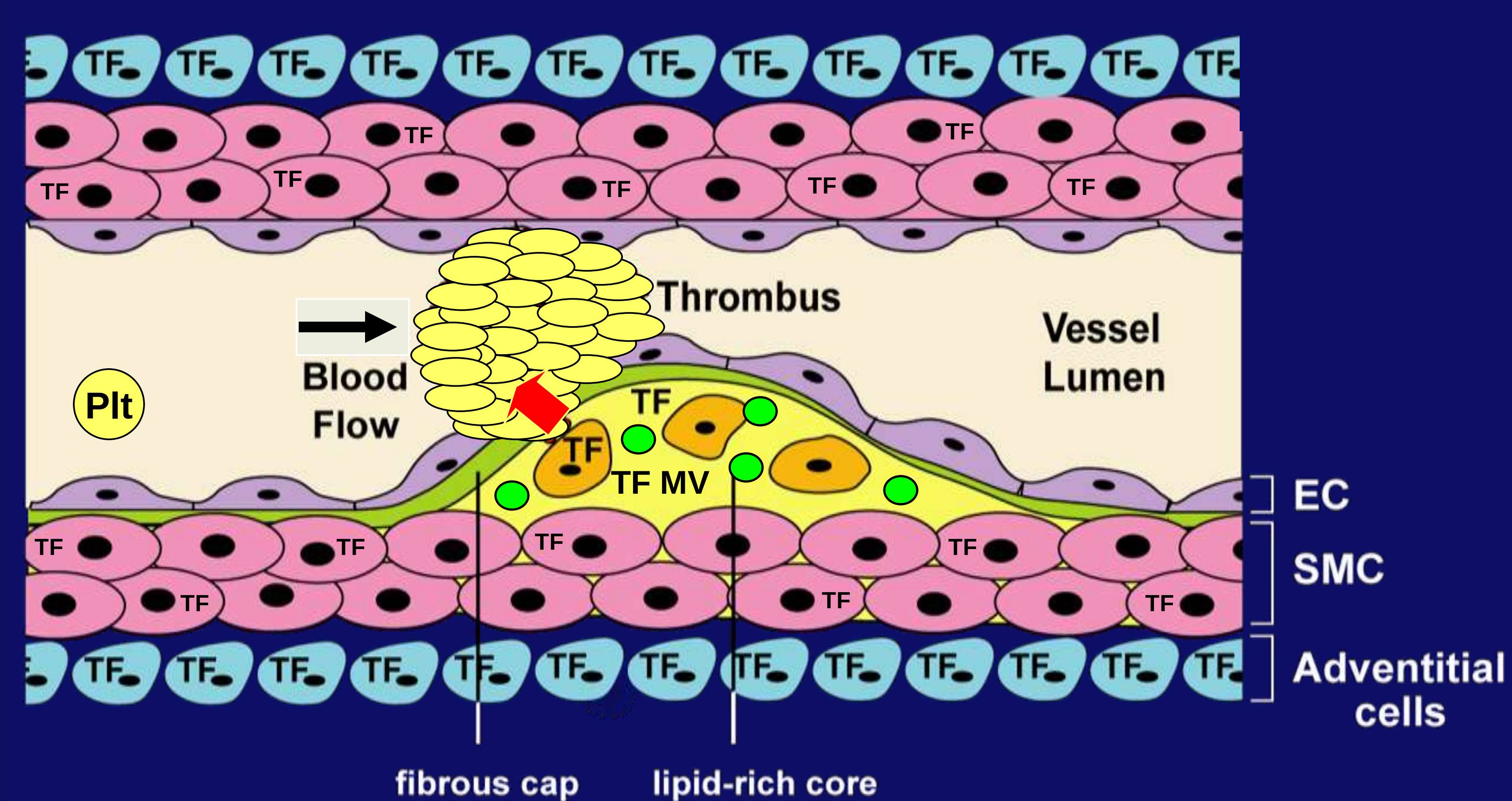
Effect of Combining Low Levels of TF with PAR4 Deficiency

Expected numbers of low TF, PAR4 deficient mice at wean



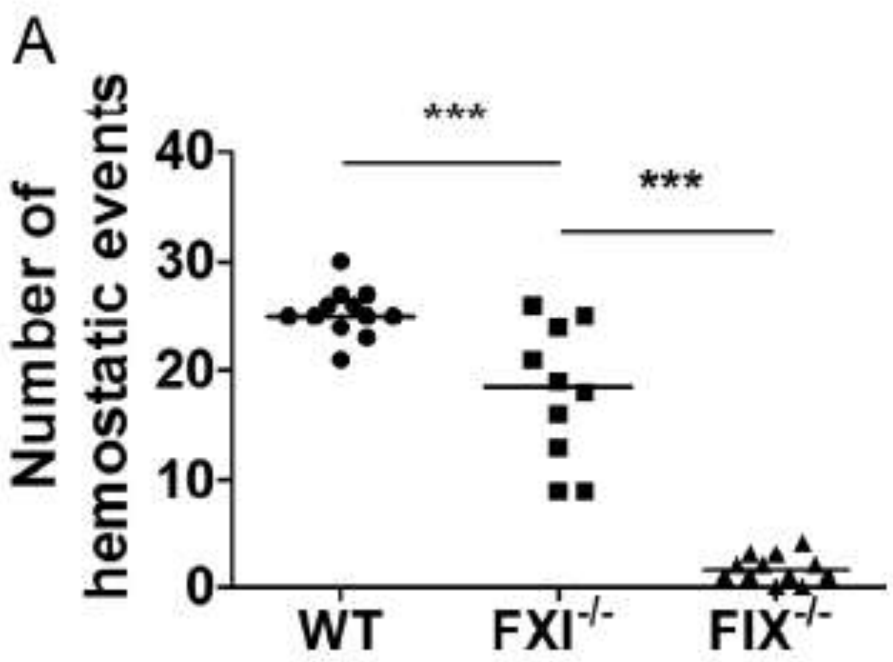
Bode M and Mackman N Thromb Res 2016

Role of TF in Thrombosis After Rupture of an Atherosclerotic Plaque



Mackman ATVB 2004

FXI$^{-/-}$ Mice Exhibit Increased Bleeding in the Saphenous Vein Model



Ay C et al JTH 2017

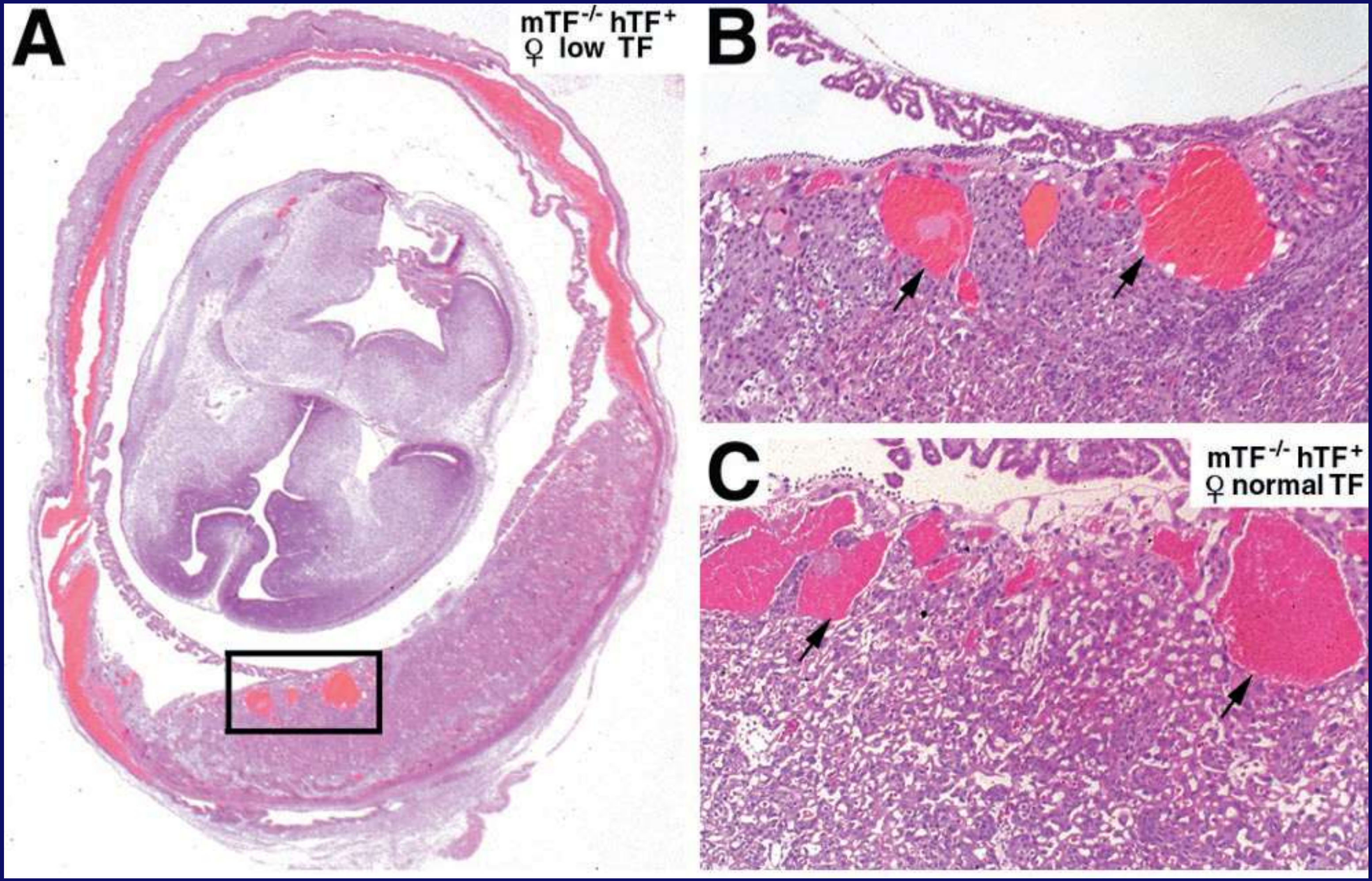
Effect of Combining Low Levels of TF with FXI Deficiency on Blood Pools in the Placenta

$mTF^{+/-}$, $FXI^{+/+}$ $mTF^{-/-}$, $FXI^{+/-}$ $mTF^{-/-}$, $FXI^{-/-}$

Grover unpublished

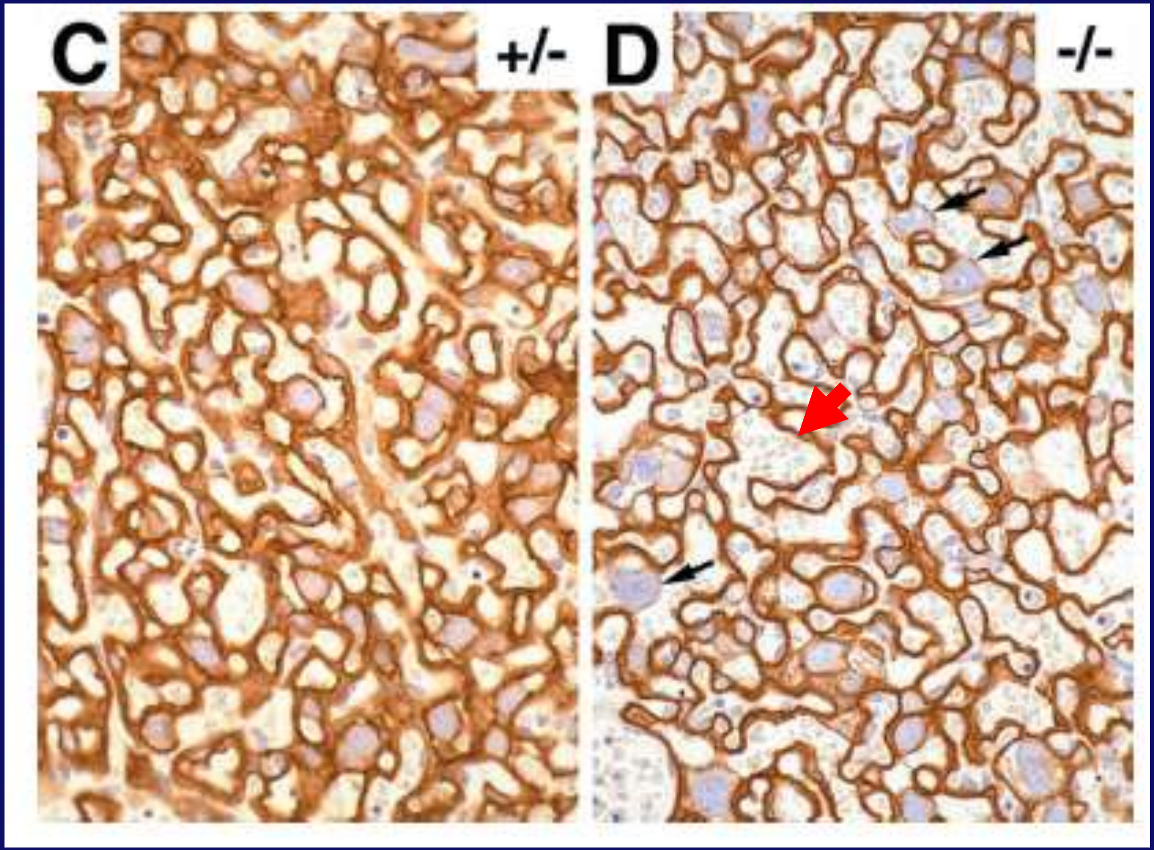
Are the blood pools in low TF placentas due to a hemostatic defect?

Low TF Placentas Contain Blood Pools Regardless of the Level of TF in the Mother



Erlich et al PNAS 1999

Cytokeratin Staining in Placenta



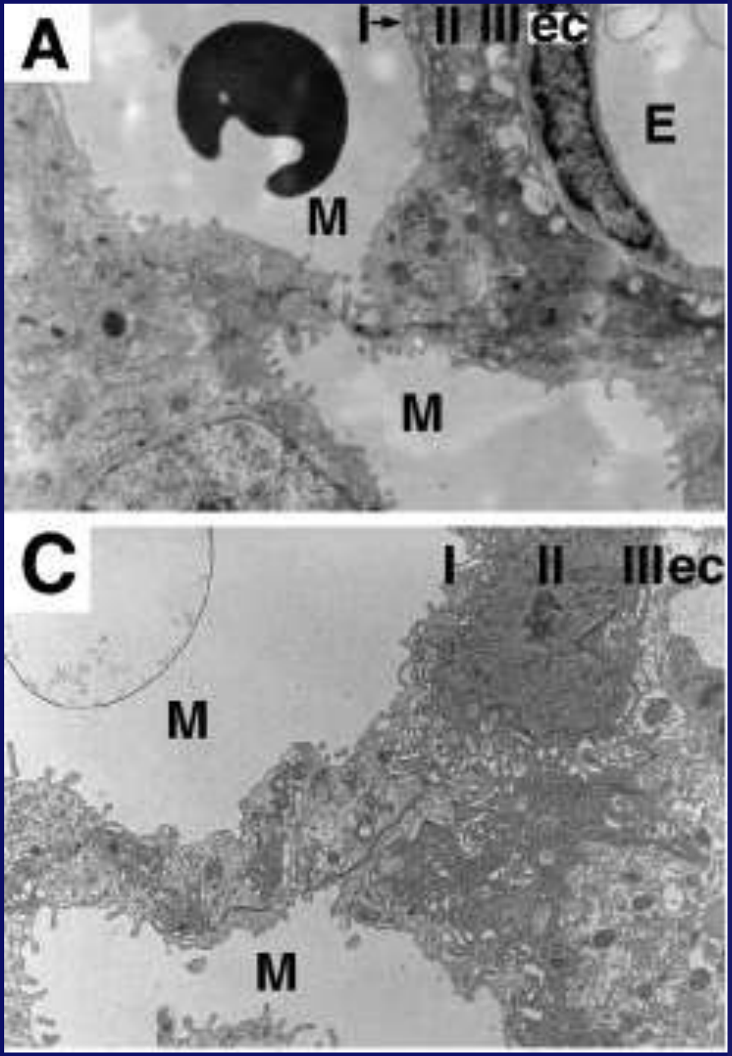
Reduced cytokeratin staining in layer I trophoblasts-multiple attachments to adjacent trabeculae

Larger maternal lacunae in low TF placentas

Erlich et al PNAS 1999

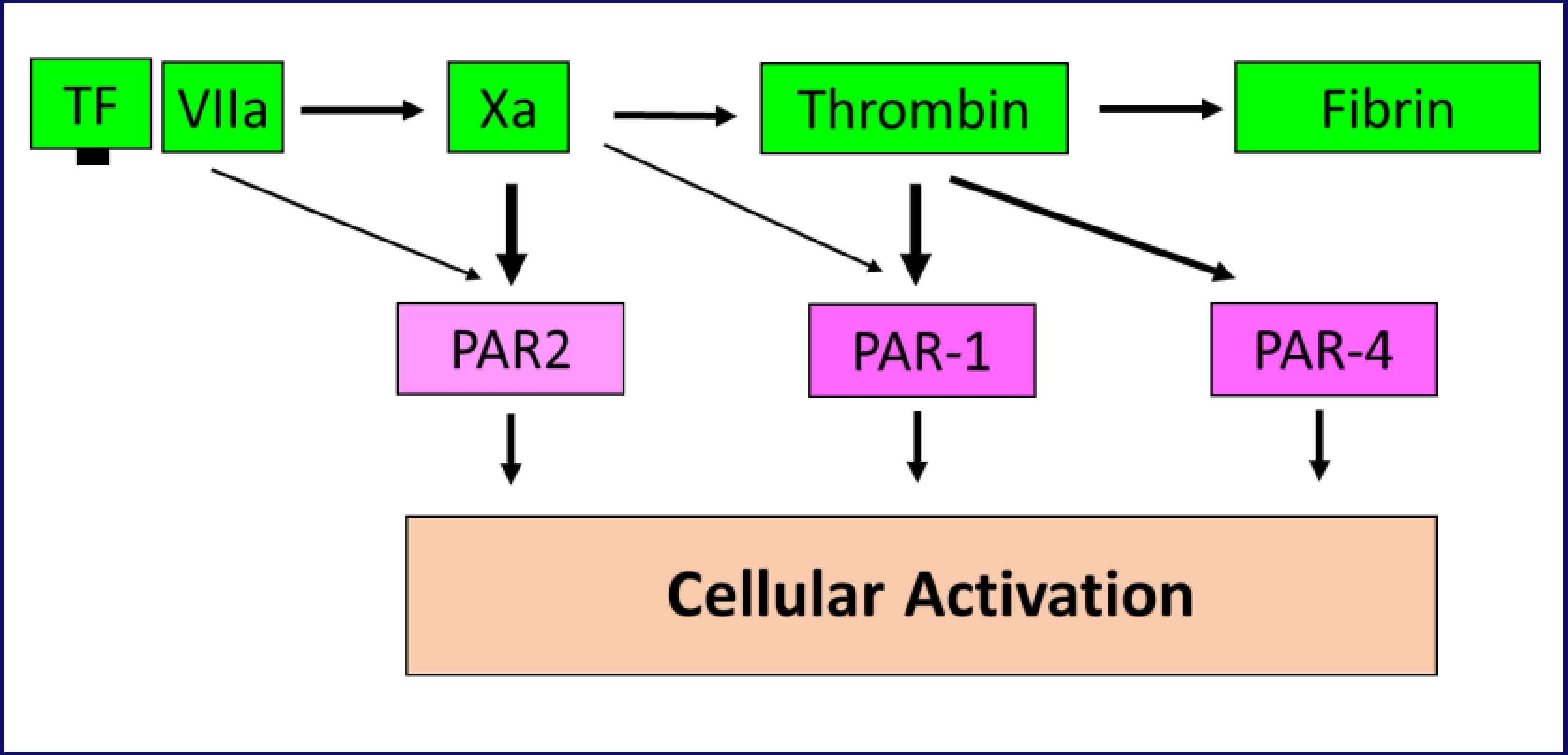
Ultrastructural of the Placenta

Reduced areas of contact between layer I trophoblasts that subdivide maternal lacunae

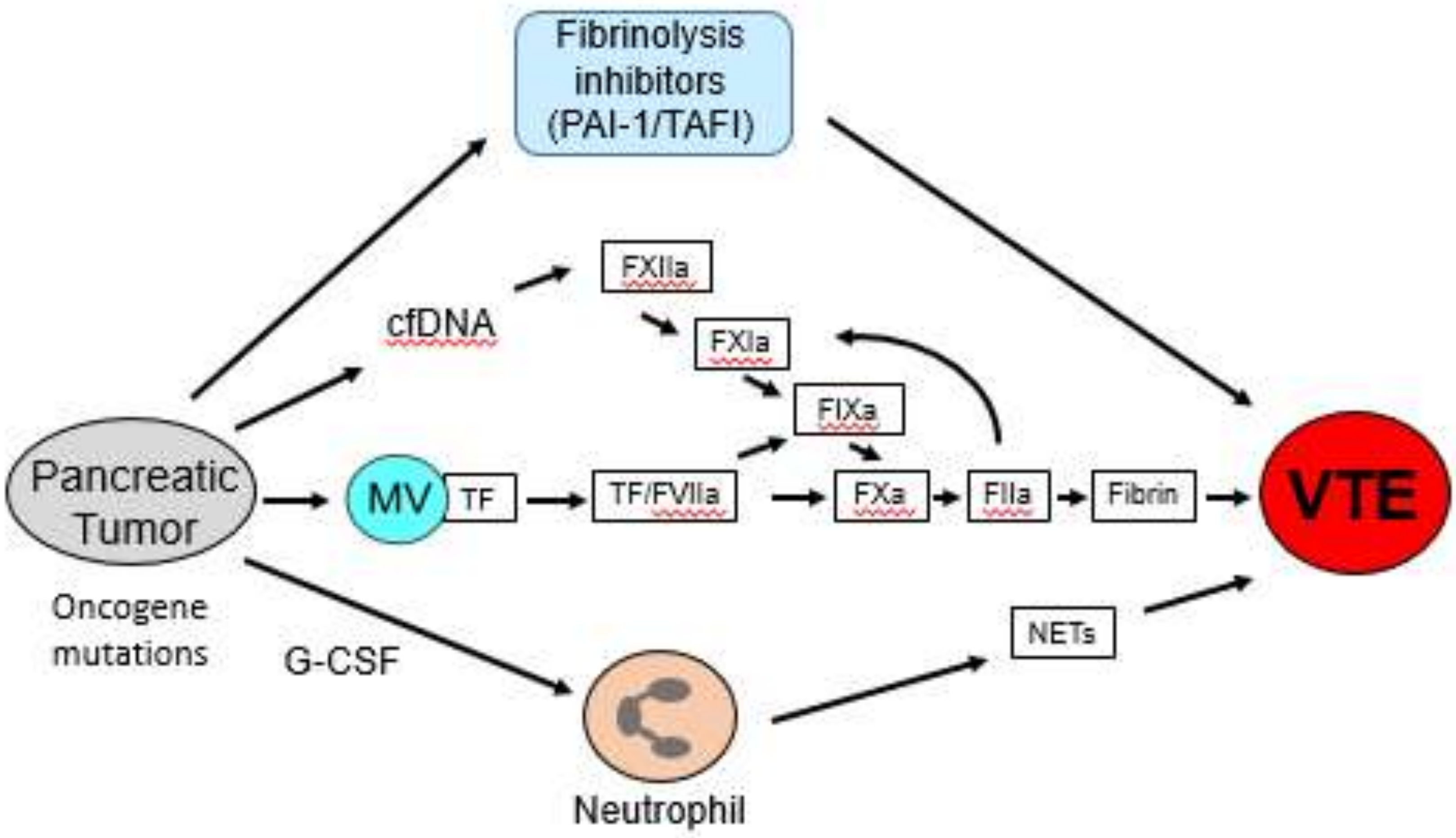


Erlich et al PNAS 1999

Coagulation Proteases Activate PARs



Pathways that Increase VTE in Pancreatic Cancer Patients



Effect of Crossing Low TF Mice with Mice Deficient in Genes of the Intrinsic Pathway

Low Tissue Factor and...	Survival
FXII Deficiency	Yes
FXI Deficiency	No
FIX Deficiency	No

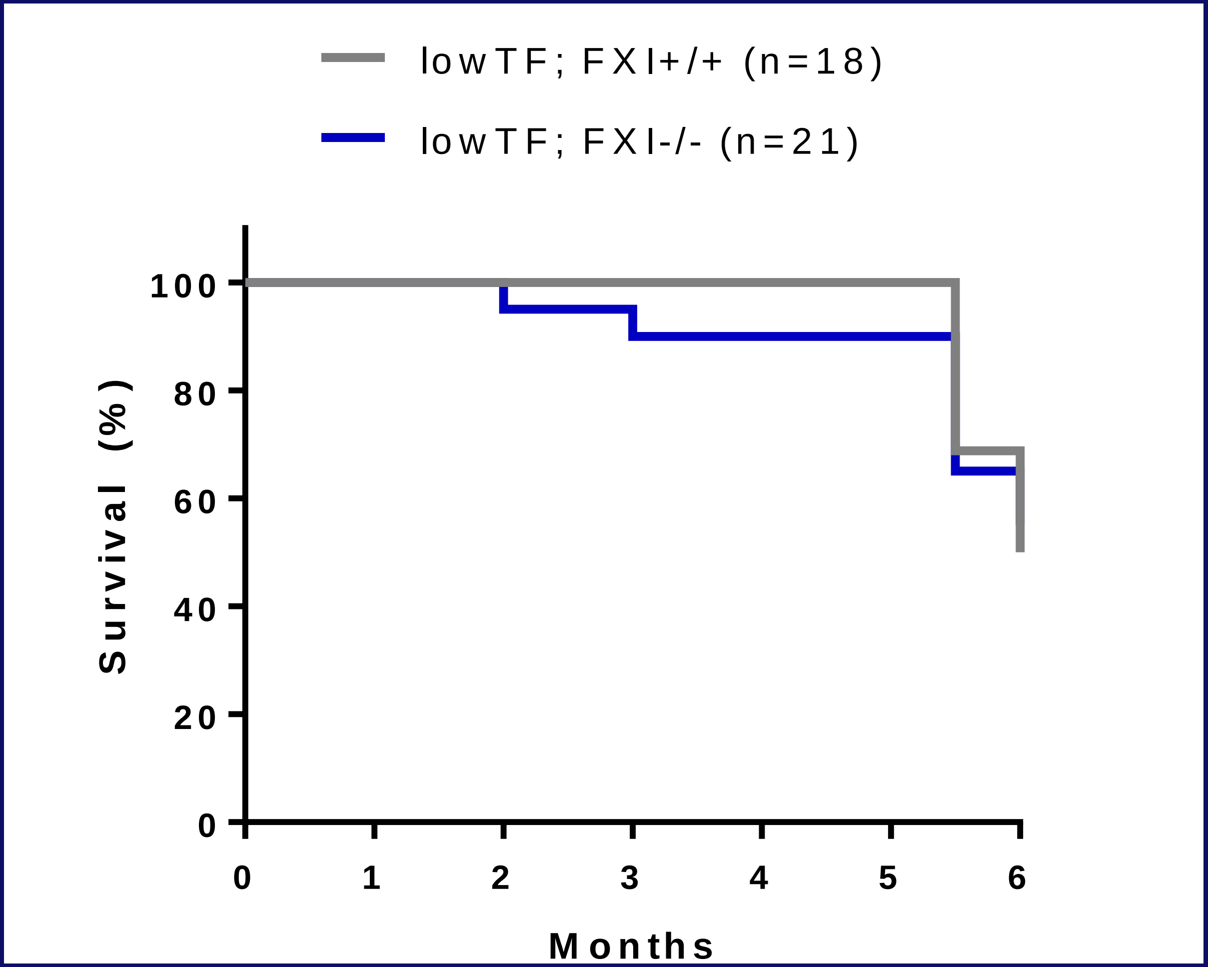
Spronk H et al ISTH 2009

Low Tissue Factor and...	Survival	
FXII Deficiency	Yes	
FXI Deficiency	Yes*	Loop 2
FIX Deficiency	No	Loop 1

Mackman N 2017

*increased placental blood pools and maternal death.

Survival of Low TF Mice without FXI$^{-/-}$ Mice



Jamsa A et al unpublished data

Ongoing Studies

- Determine the cause of death of mTF$^{+/-}$,hTF^{+}, FXI$^{+/-}$ mothers carrying Low TF, FXI$^{-/-}$ embryos. Placental hemorrhage?
- Determine when Low TF, FIX$^{-/-}$ embryos die.

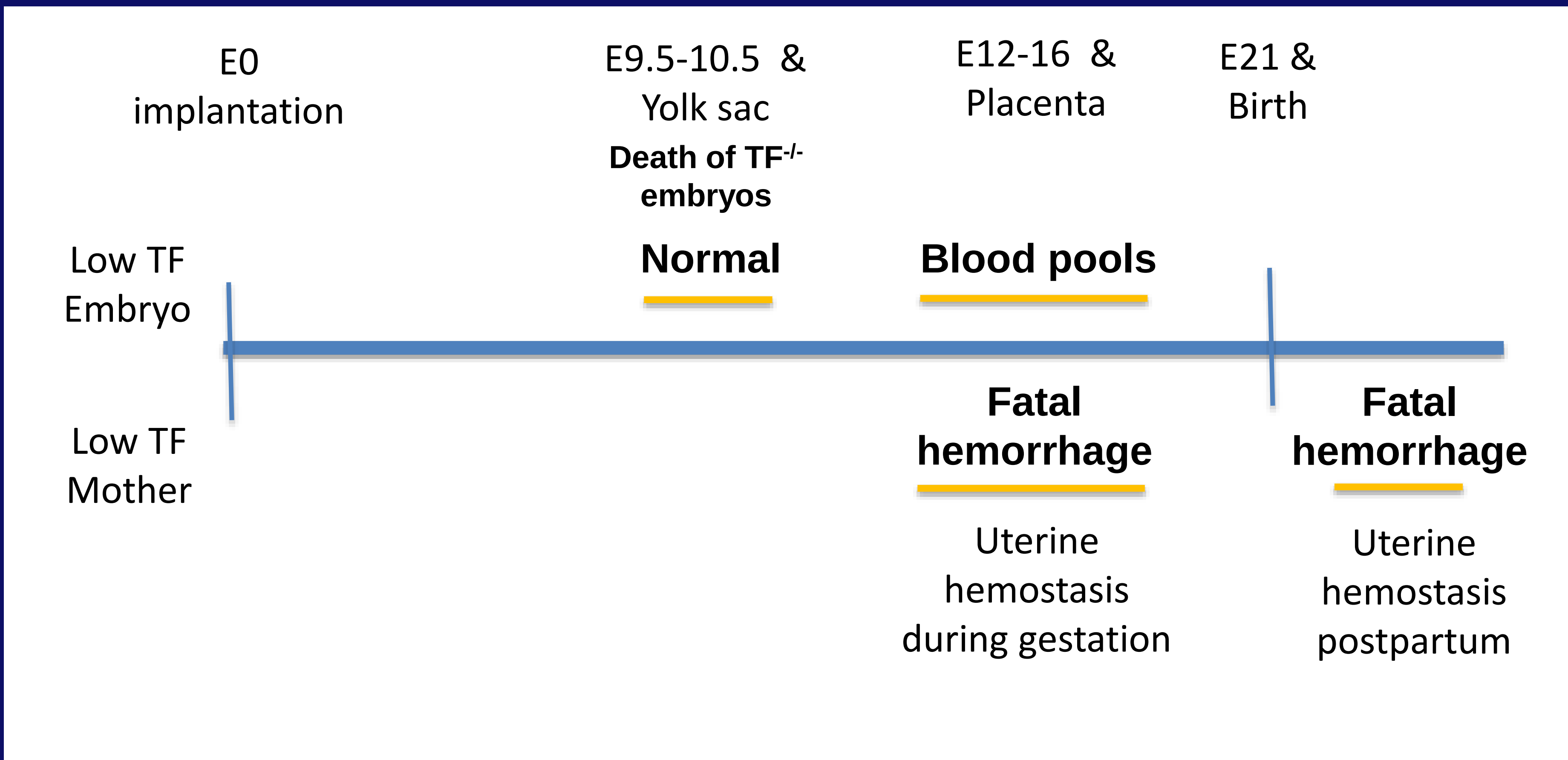
Effect of Crossing Two Prohemorrhagic Mice

Can we exacerbate the bleeding in Low TF mice by adding another prohemorrhagic genetic defect?

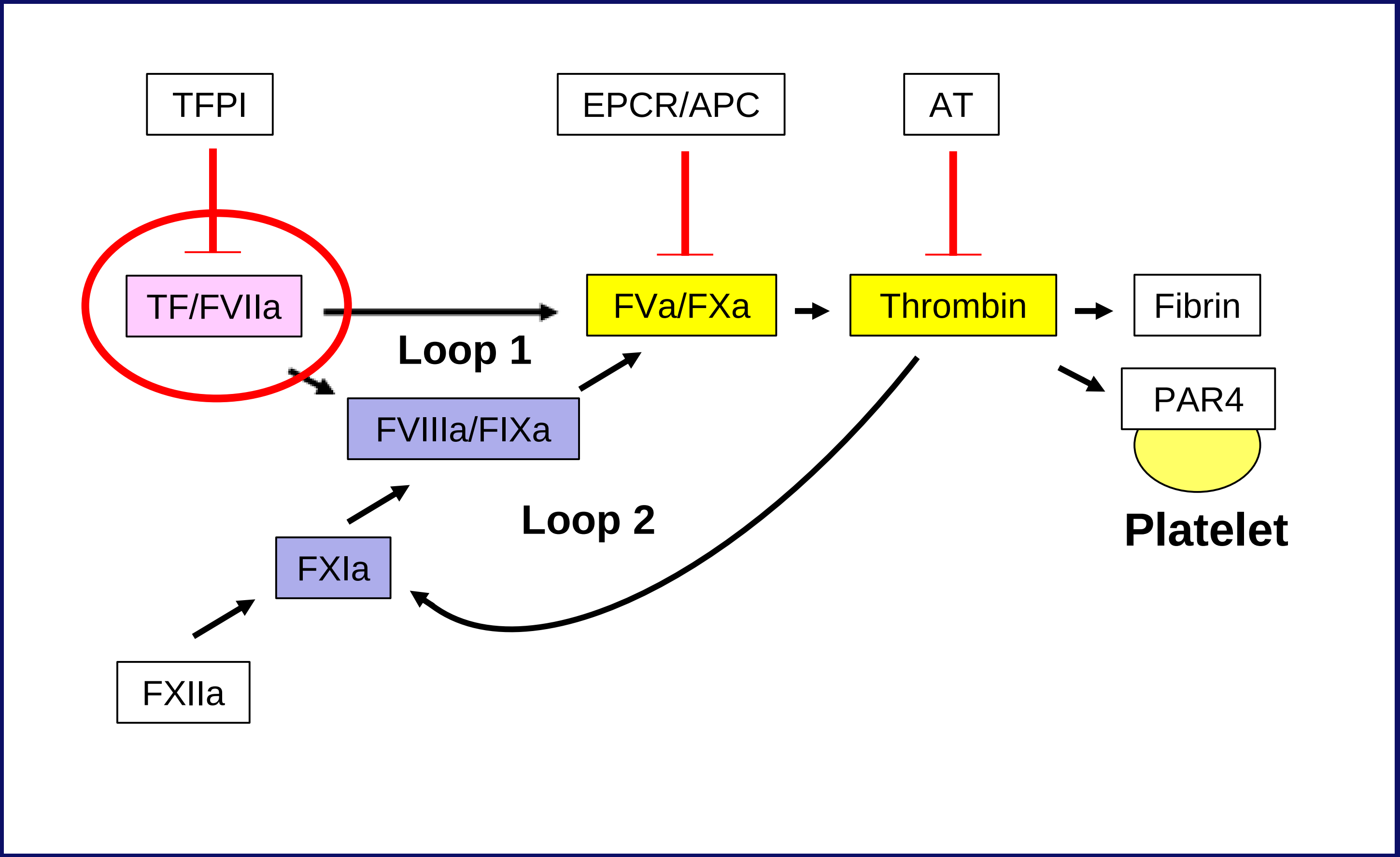
Combine Low TF with PAR-4 deficiency (no thrombin activation of platelets).

Bode & Mackman Thromb Res 2016

Summary of the Hemostatic Defects in Low TF Placentas and Mothers

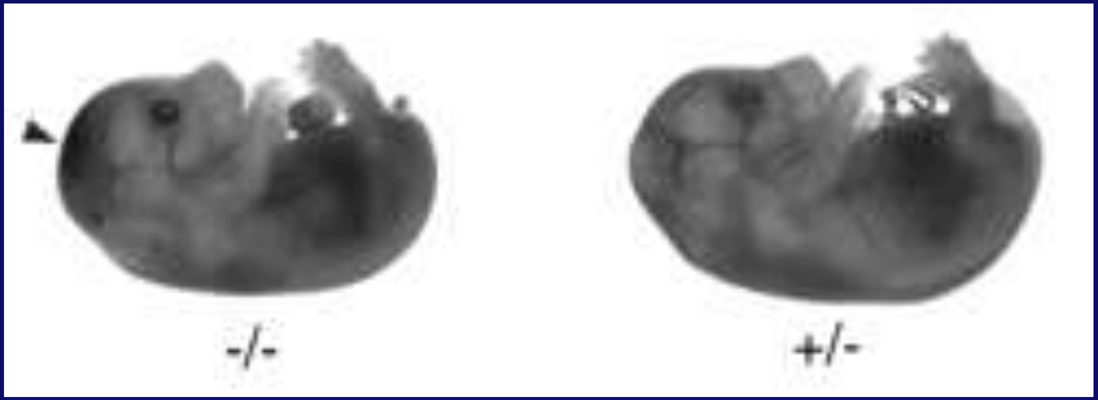


Simplified Version of the Clotting Cascade



Effect of Inactivation of the TFPI Gene in Mice

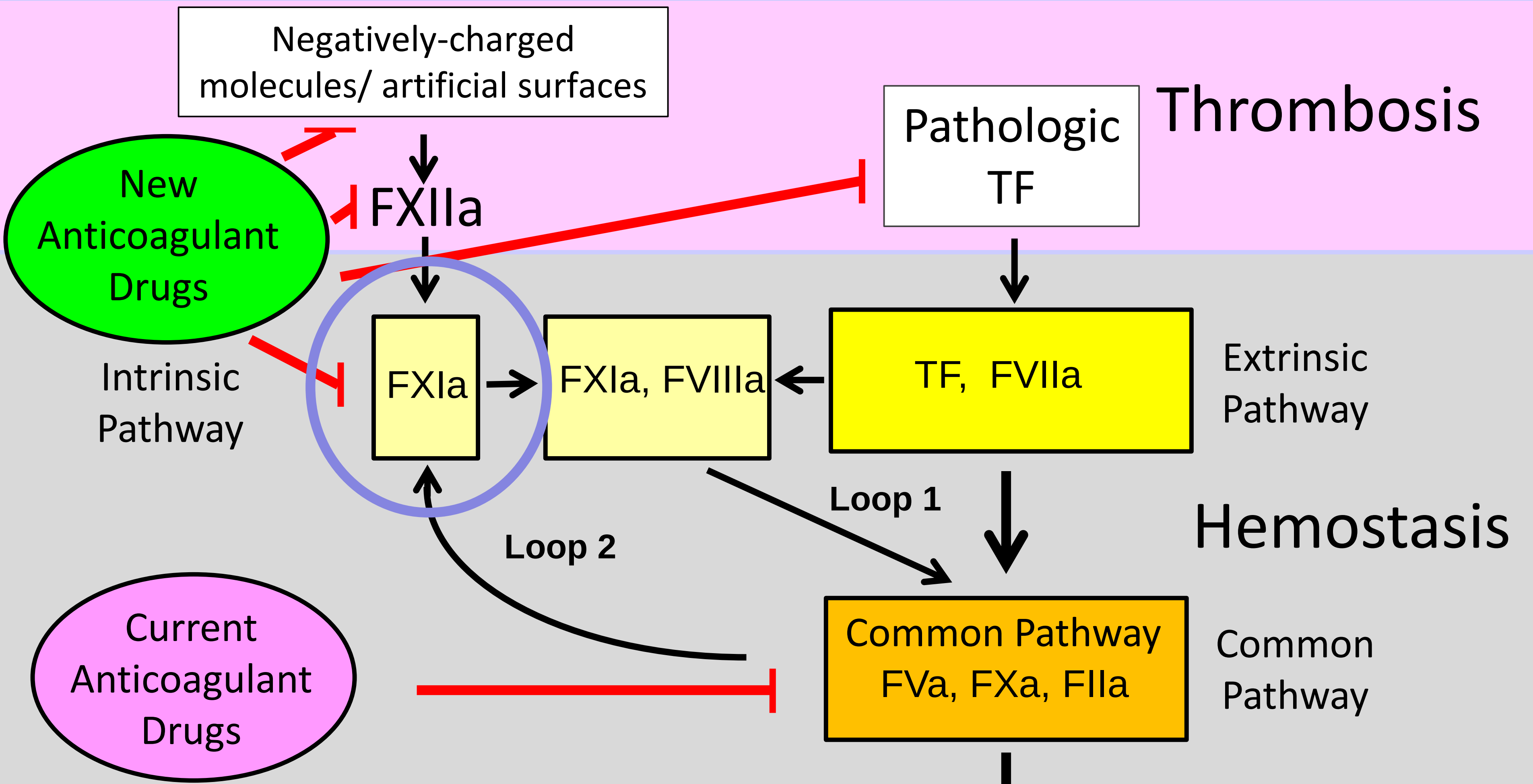
TFPI null embryos die during embryonic development



TFPI is essential for survival

Huang Z et al Blood 1997

Development of New Anticoagulant Drugs



Extrinsic = initiation
Intrinsic = amplification

Modified from Geddings and Mackman Thromb Haemost 2014

The NEW ENGLAND JOURNAL of MEDICINE

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Factor XI Antisense Oligonucleotide for Prevention of Venous Thrombosis

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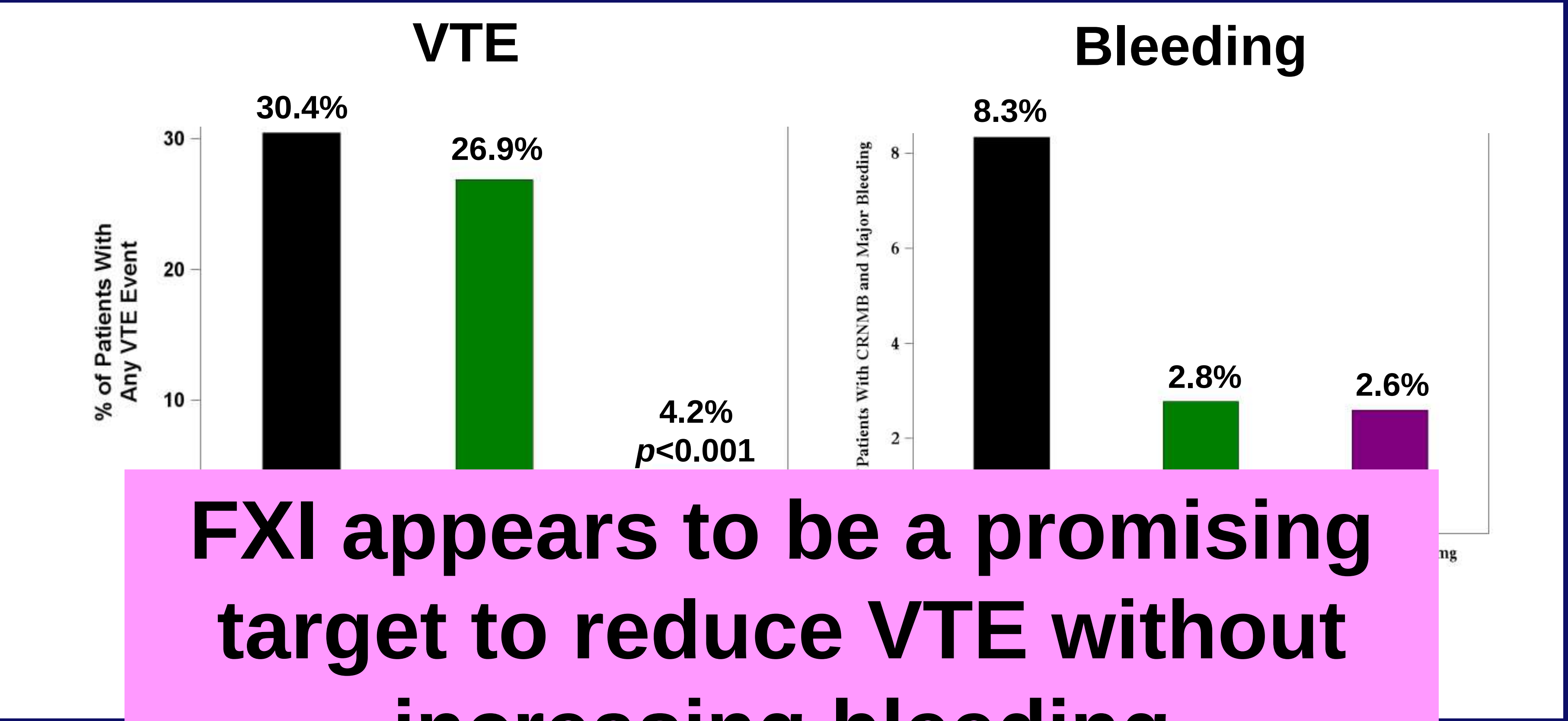
Slides from Dr. J. Weitz

Effect of Reducing FXI Levels on VTE and Bleeding after Total Knee Replacement



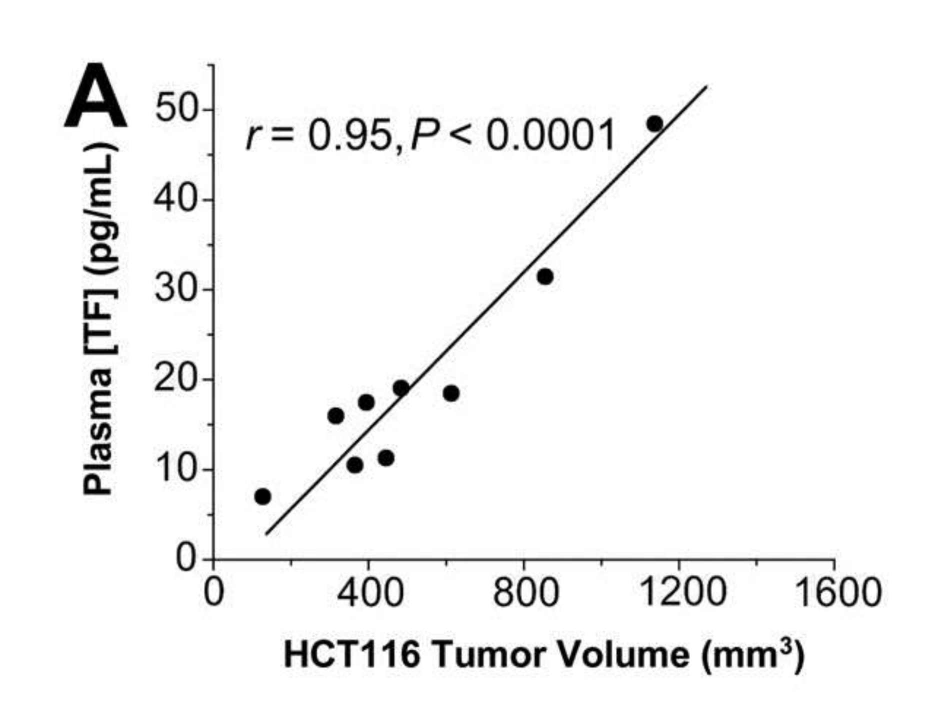
FXI appears to be a promising target to reduce VTE without increasing bleeding

Buller H et al N Engl J Med 2015

Do human tumors grown in mice release TF^{+} MV into the blood?

Move

Tumors Release Human TF into the Circulation in SCID Mice containing Human Tumors



Move

uman colorectal cell line

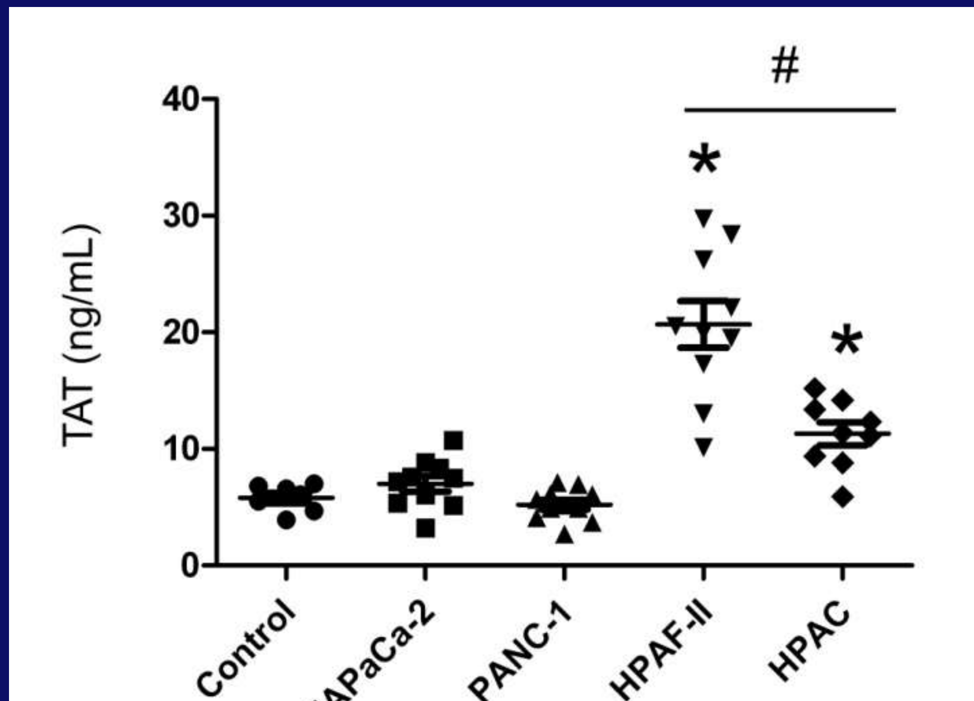
Yu et al Blood 2005

Do TF^{+} human pancreatic tumors grown in mice activate coagulation?

Move

Activation of Coagulation in Nude Mice containing Human Pancreatic Tumors

Jianguo Wang



An a… TF Ab abolishes the activation … on in tumor-bearing mice

Move

Wang JG et al Blood 2012

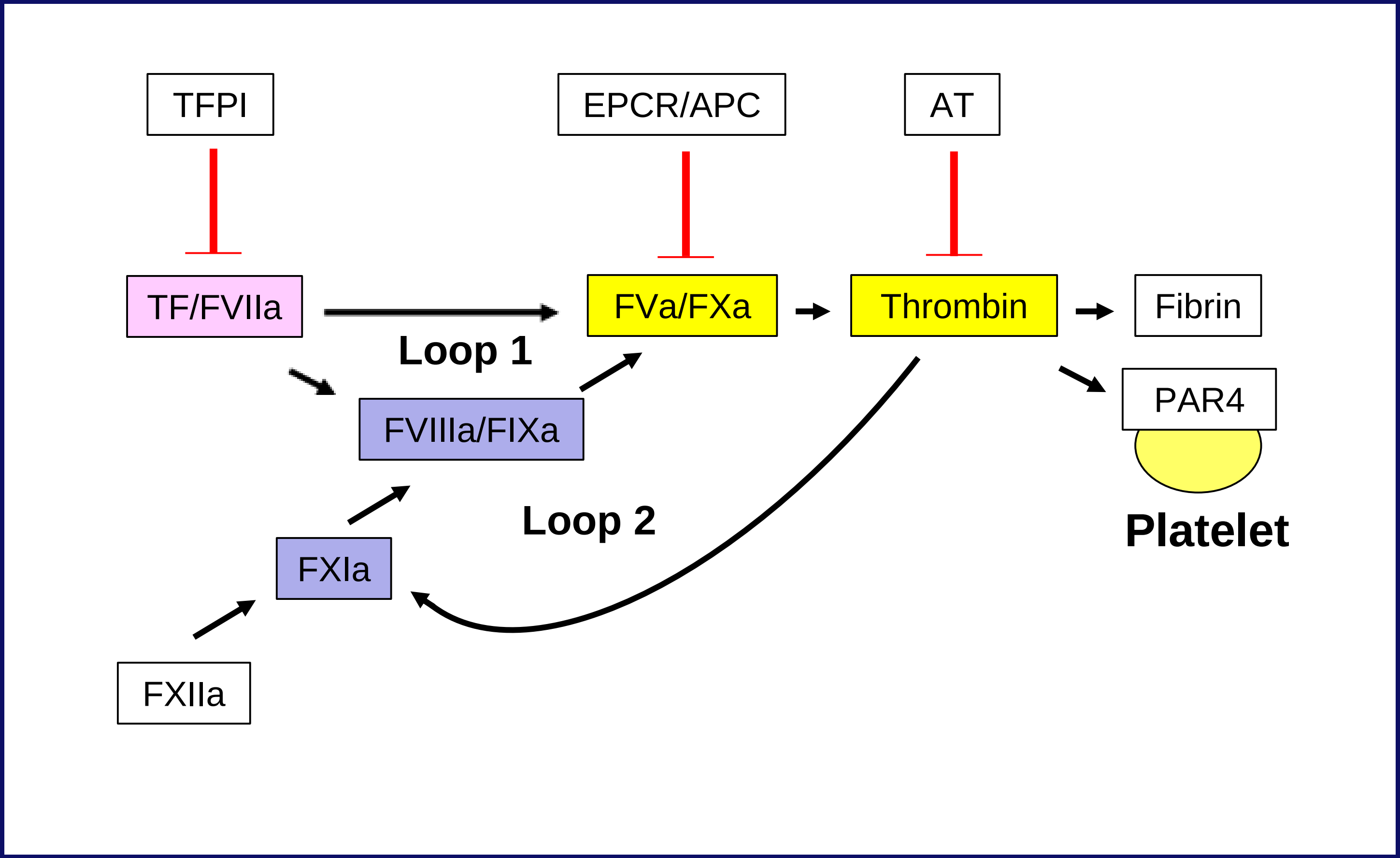
Effect of Inactivation of the TF Gene in Mice

TF null embryos die during embryonic development

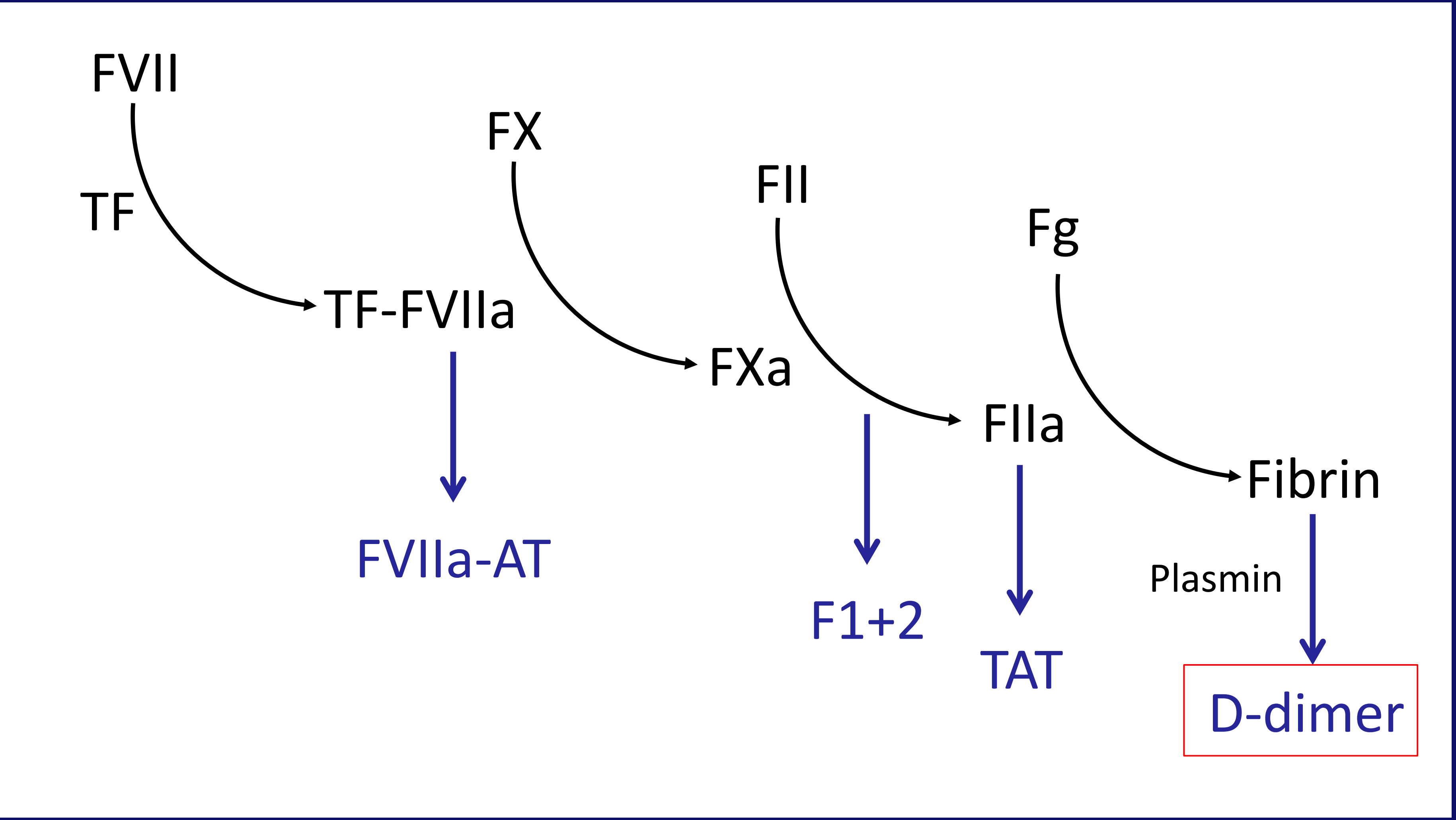
- **Carmeliet, Mackman et al *Nature* 1996**
- **Bugge et al *PNAS* 1996**
- **Toomey et al *Blood* 1996**

TF are essential for survival

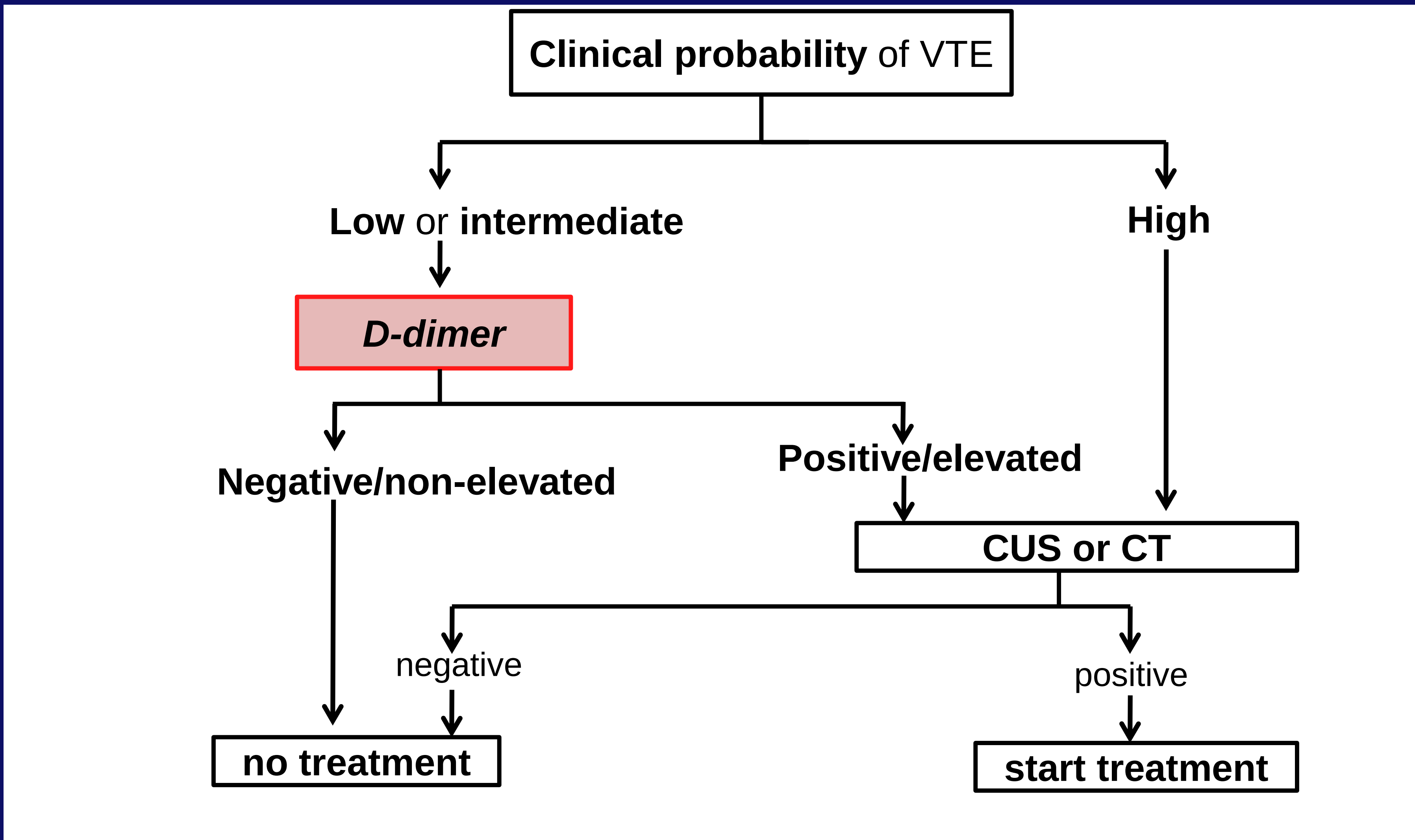
Simplified Version of the Clotting Cascade



Biomarkers Associated with Activation of the Clotting Cascade

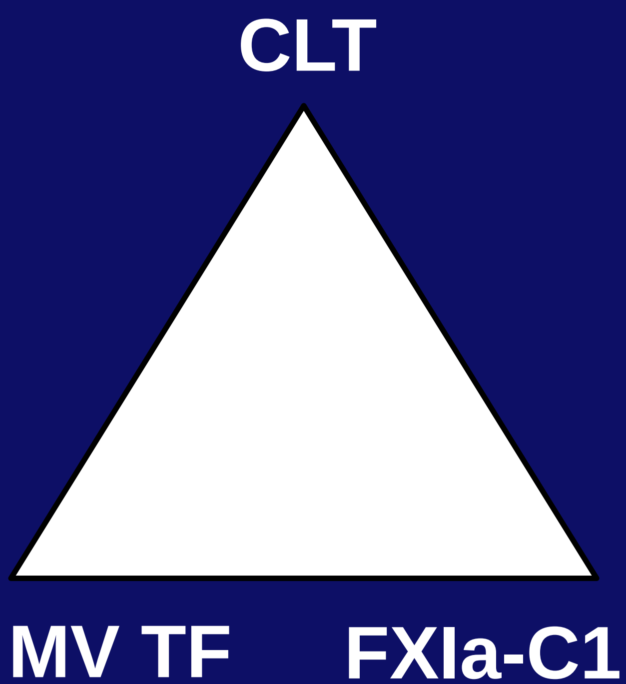


Measurement of D-dimer in the Diagnostic Work-up of Suspected Venous Thromboembolism (VTE)

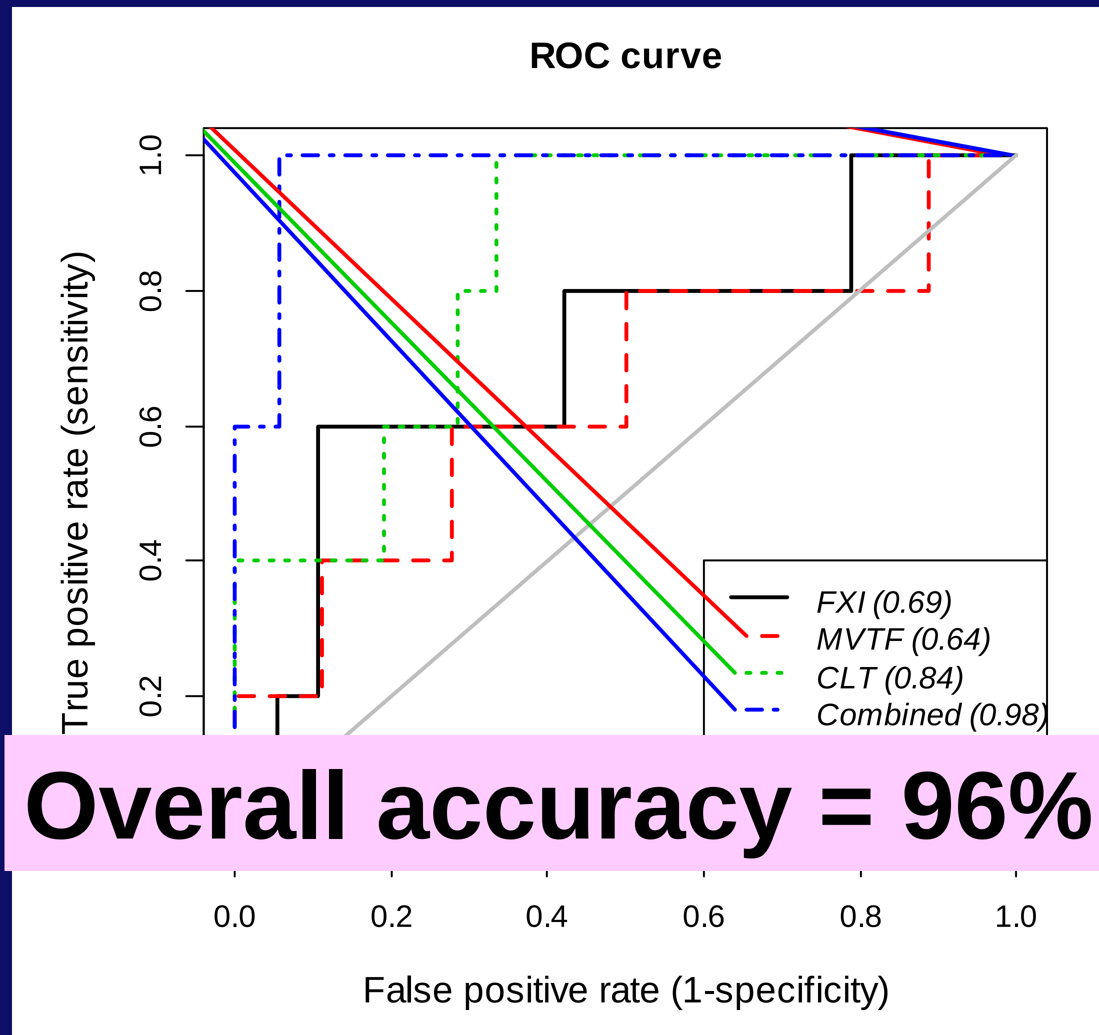


Slide provided by Dr C Ay

Biomarkers for Predicting VTE in Pancreatic Cancer Patients

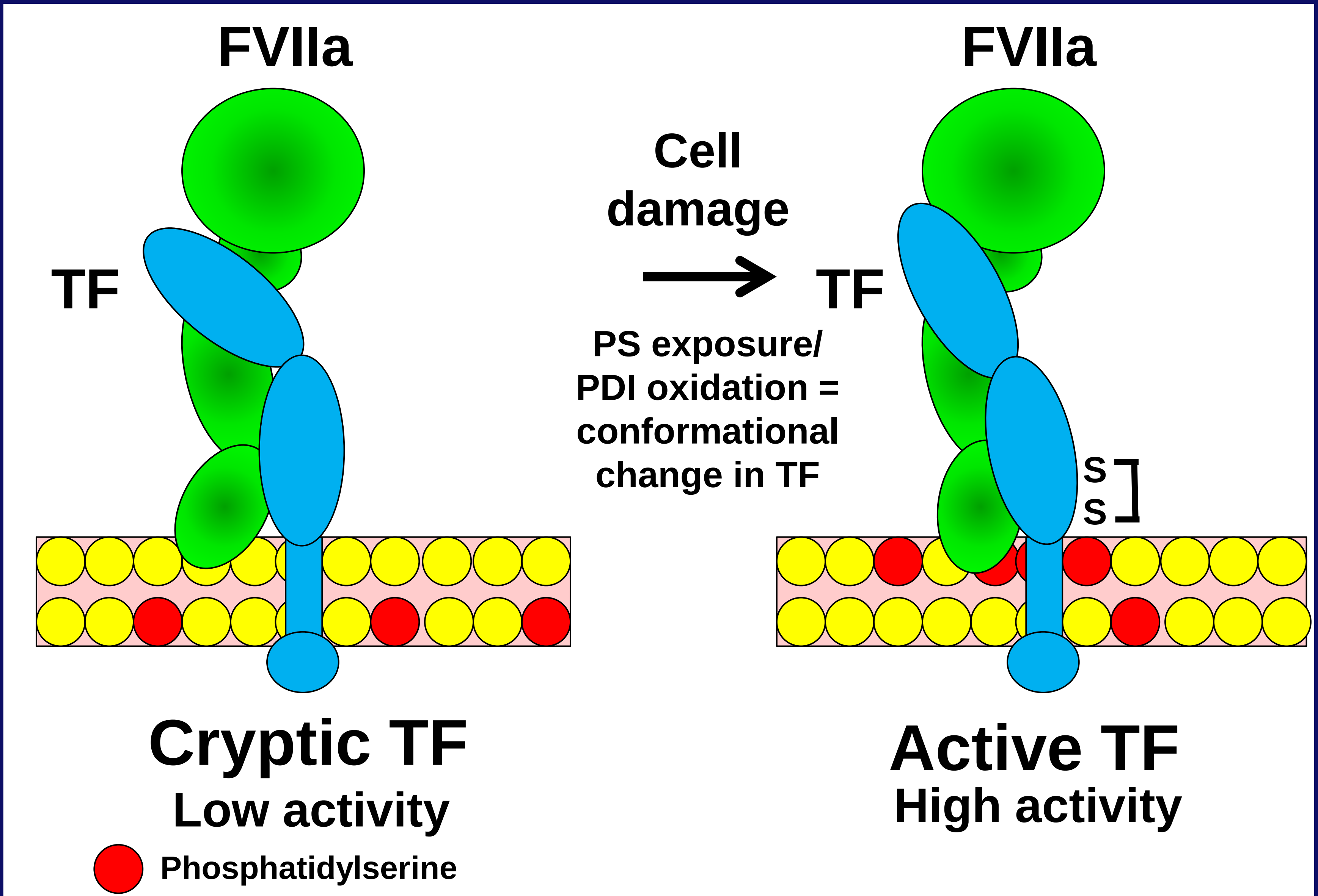


Data from 26 pancreatic cancer pts (5 developed VTE)

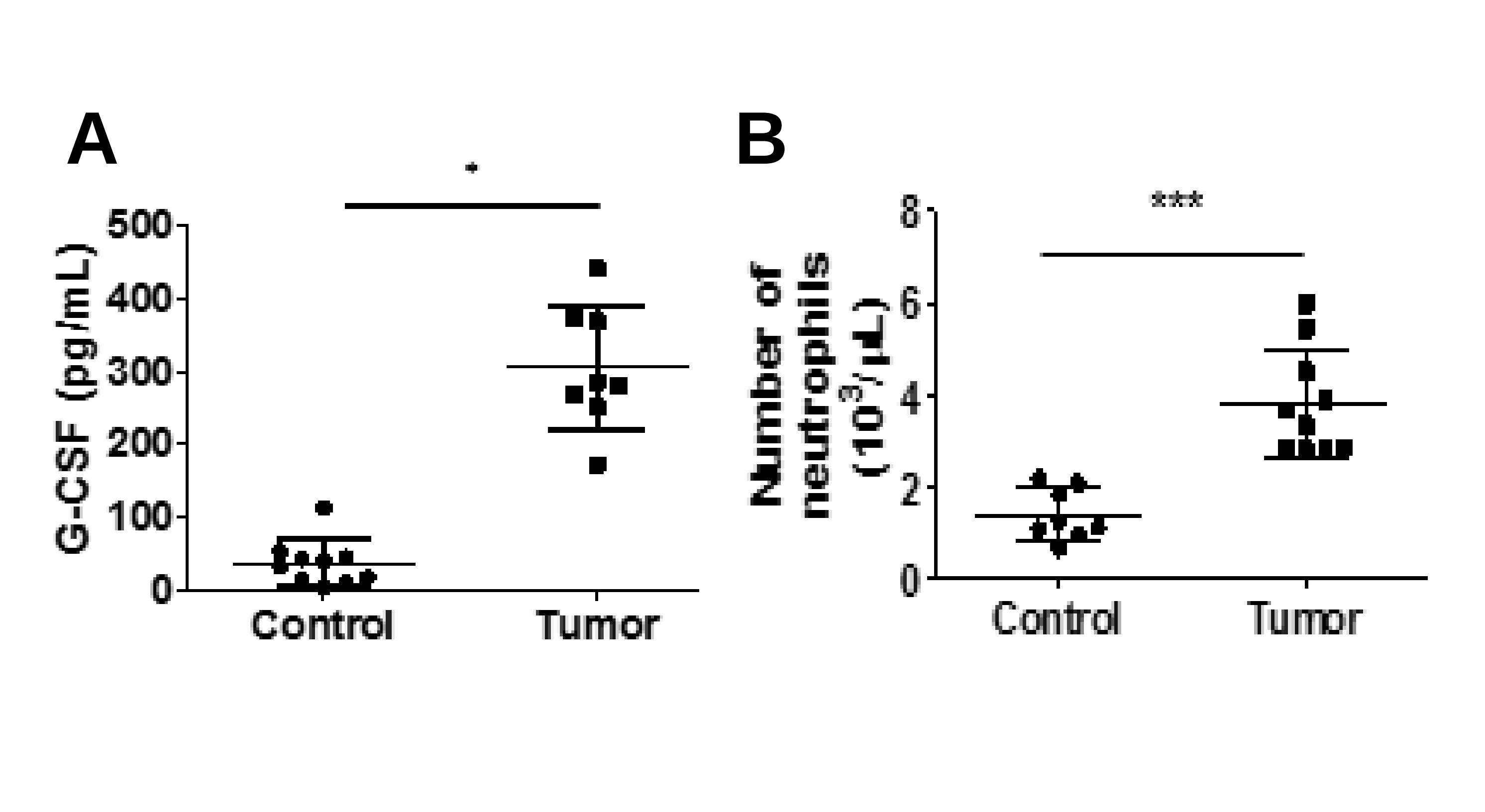


Overall accuracy = 96%

Regulation of TF Activity by PS

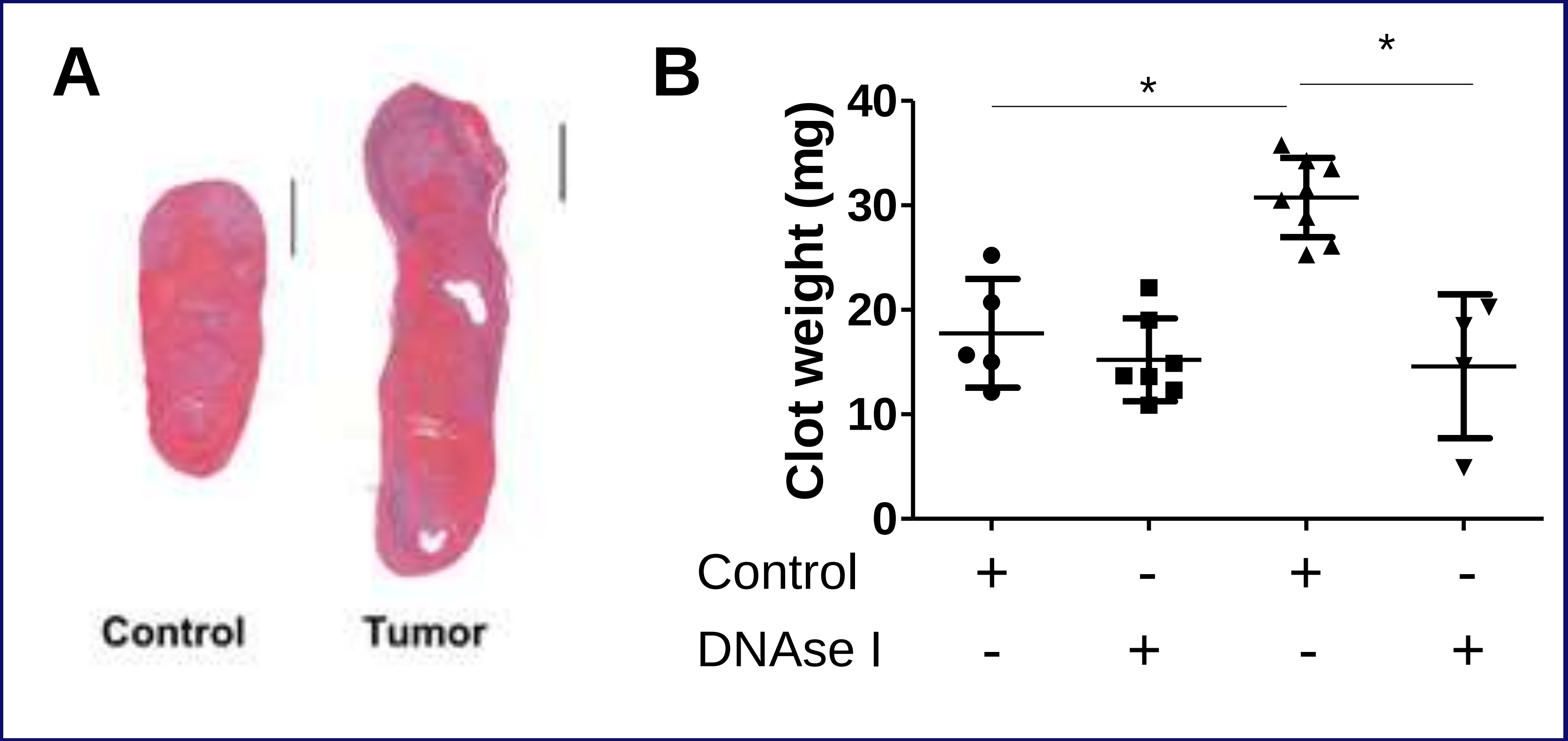


Tumor-bearing Mice have Increased Levels of G-CSF and Neutrophils



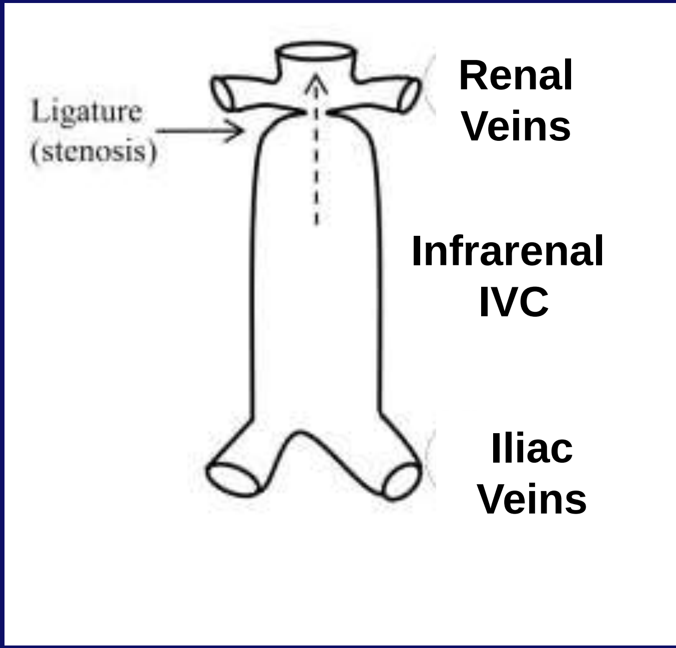
Hisada Y et al JTH 2017; unpublished data

Effect of DNAse 1 Treatment on Clot Size In Tumor-bearing Mice

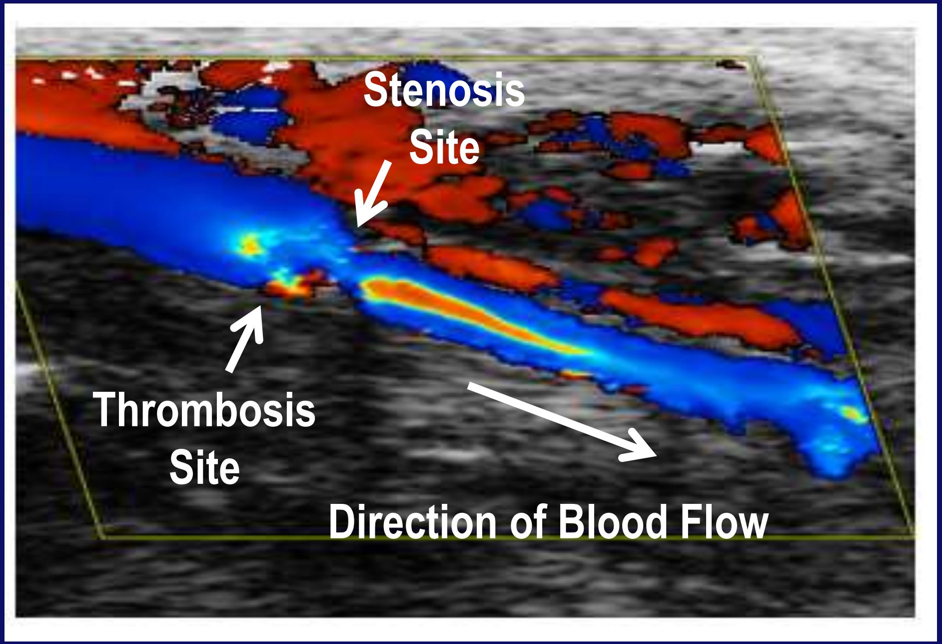


Hisada Y et al JTH 2017; unpublished data

Mouse Model of Venous Thrombosis: Inferior Vena Cava Stenosis or Stasis



Color Doppler

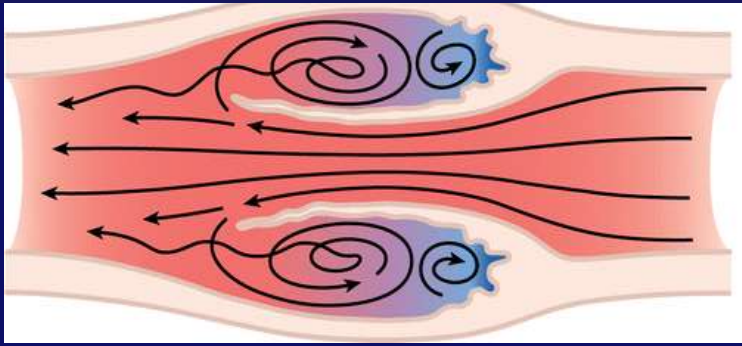


Can perform kinetic studies on thrombus formation

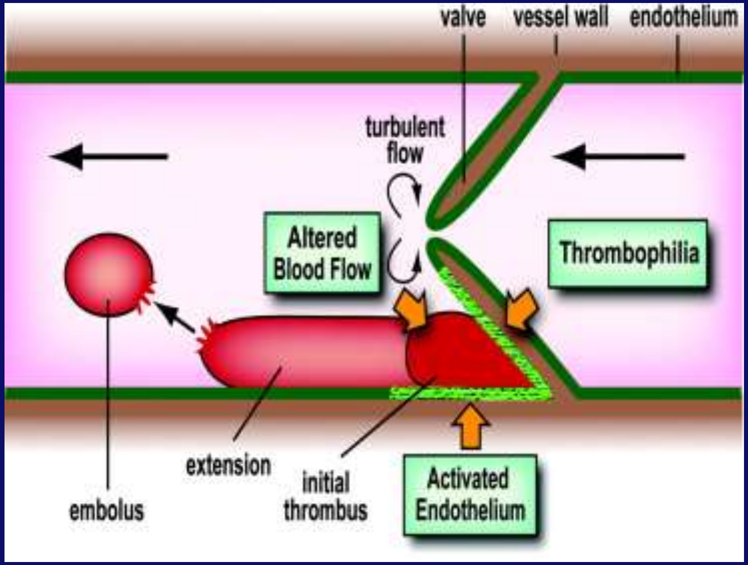
Brill A et al. Blood 2011; von Brühl M et al. J Exp Med 2012; Geddings J et al. JTH 2014

Formation of a Deep Vein Thrombus

Hypoxia in valve pockets

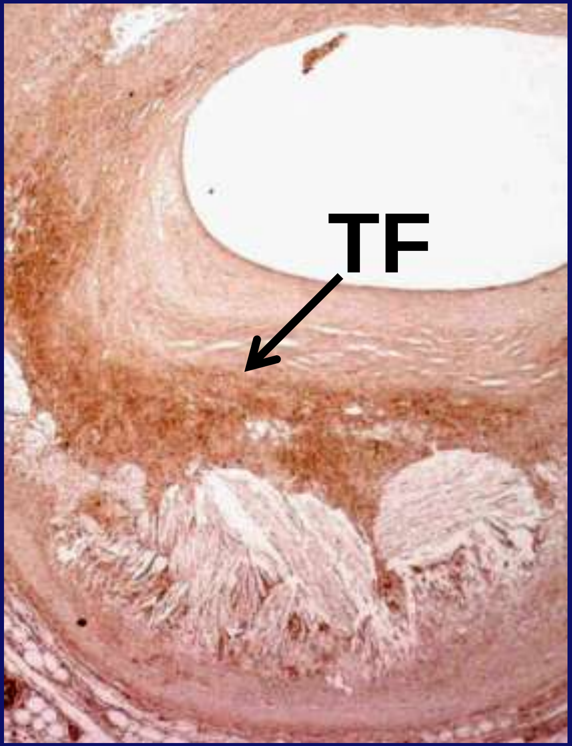


Bovill Ann Rev Physiol 2011



Moll and Mackman ATVB 2008

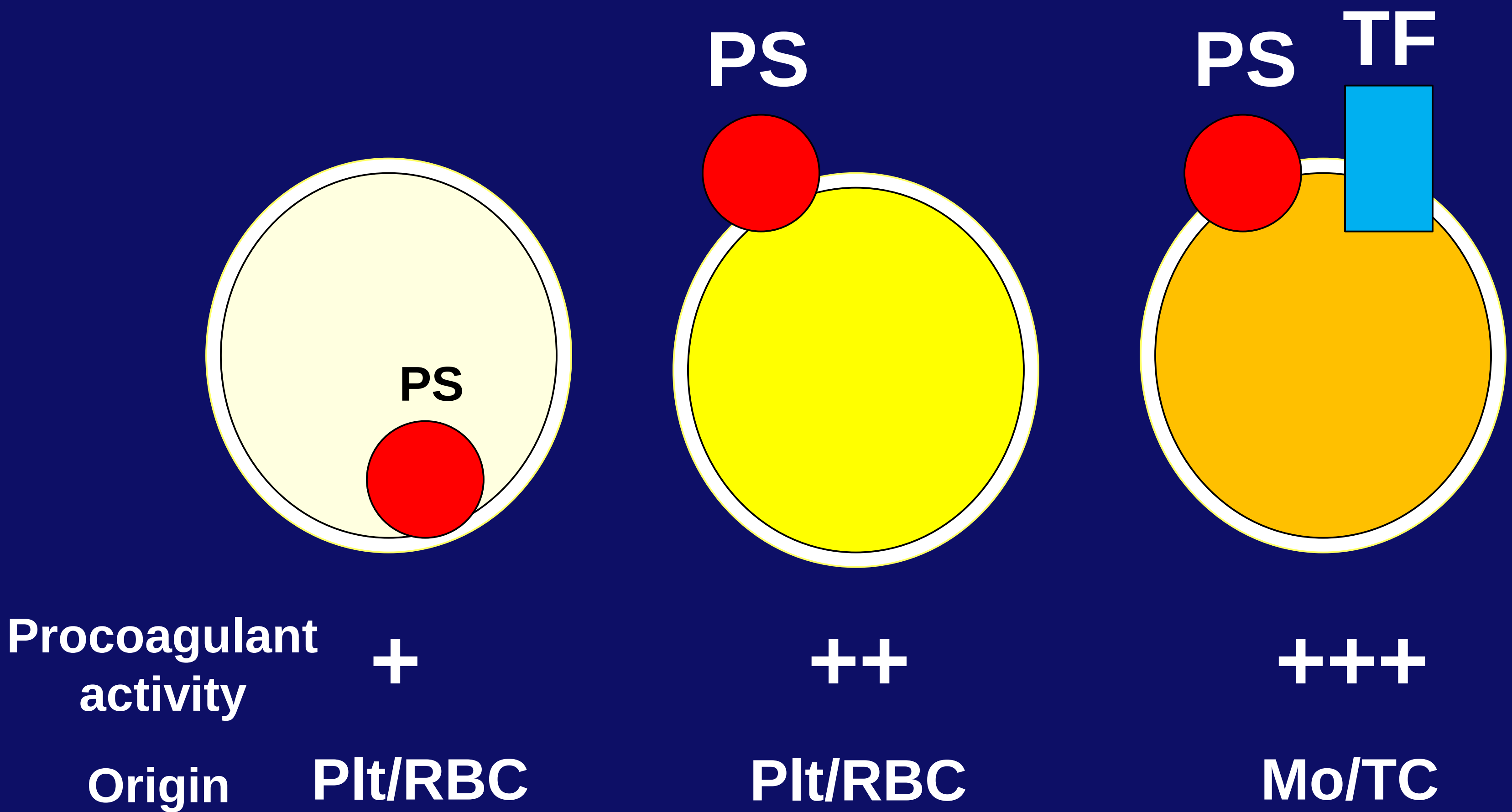
High Levels of TF are Present in Atherosclerotic Plaques



"Thrombotic" TF

Slide provided by Dr. Y. Asada

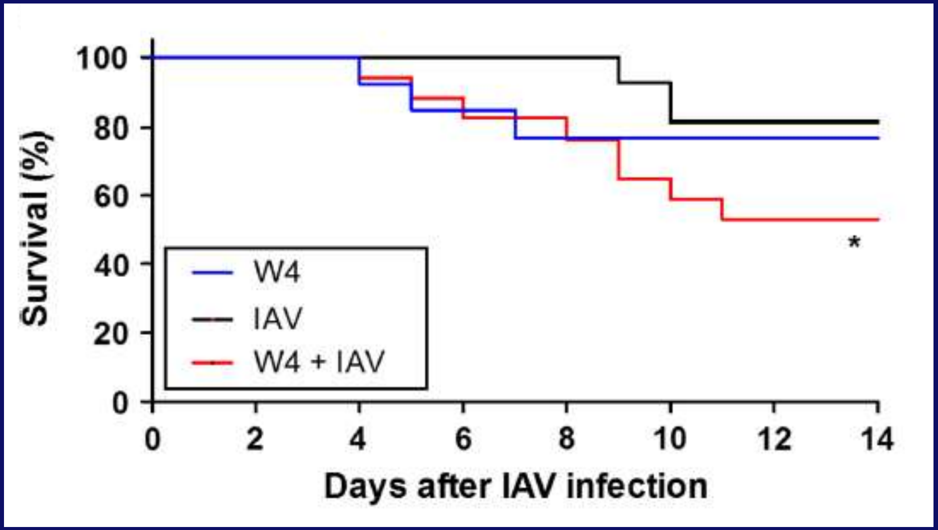
Types of Procoagulant EVs



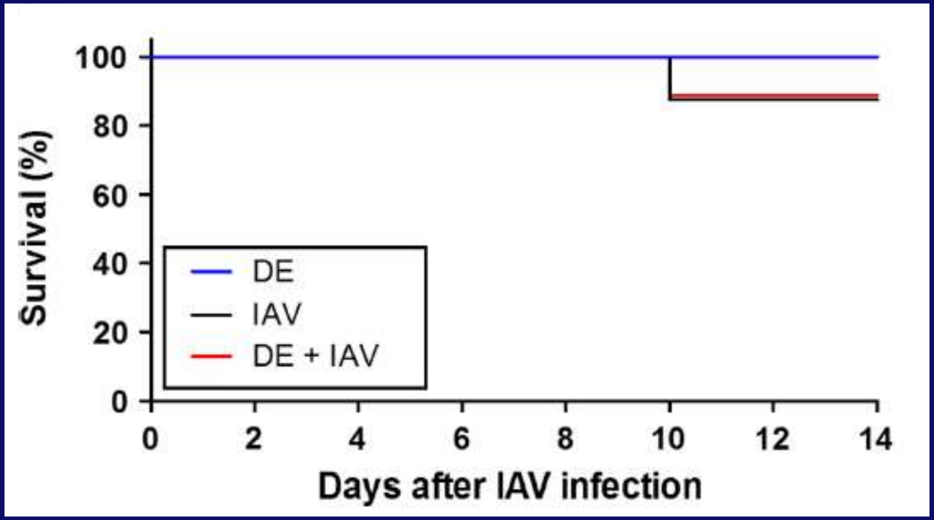
Plt, platelet; RBC, red blood cell, Mo, monocyte; TC, tumor cell

Effect of Warfarin and Dabigatran on the Survival of Mice Infected with H1N1

Warfarin

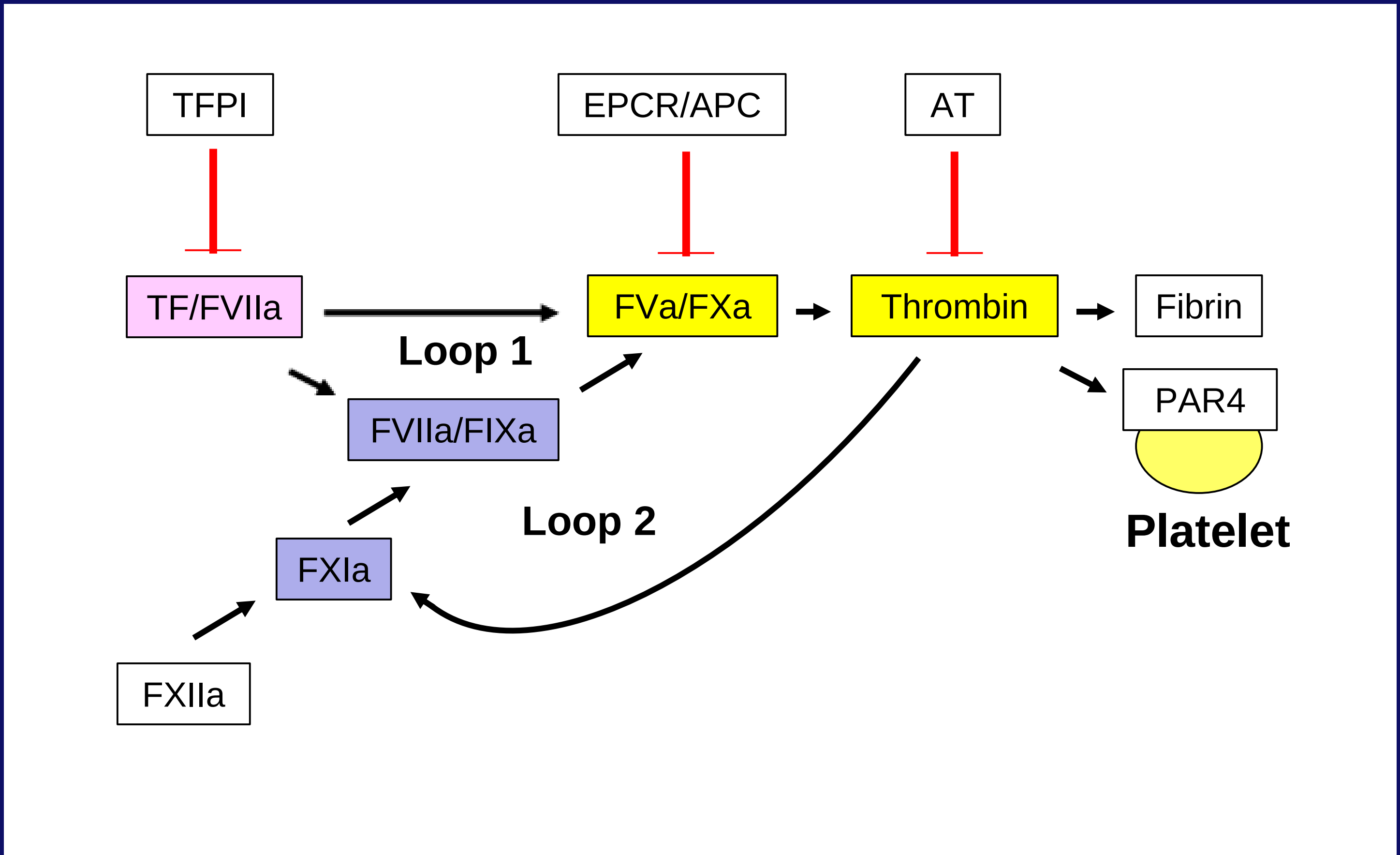


Dabigatran



Tatsumi K et al Physiol Reports 2016

Simplified Version of the Clotting Cascade



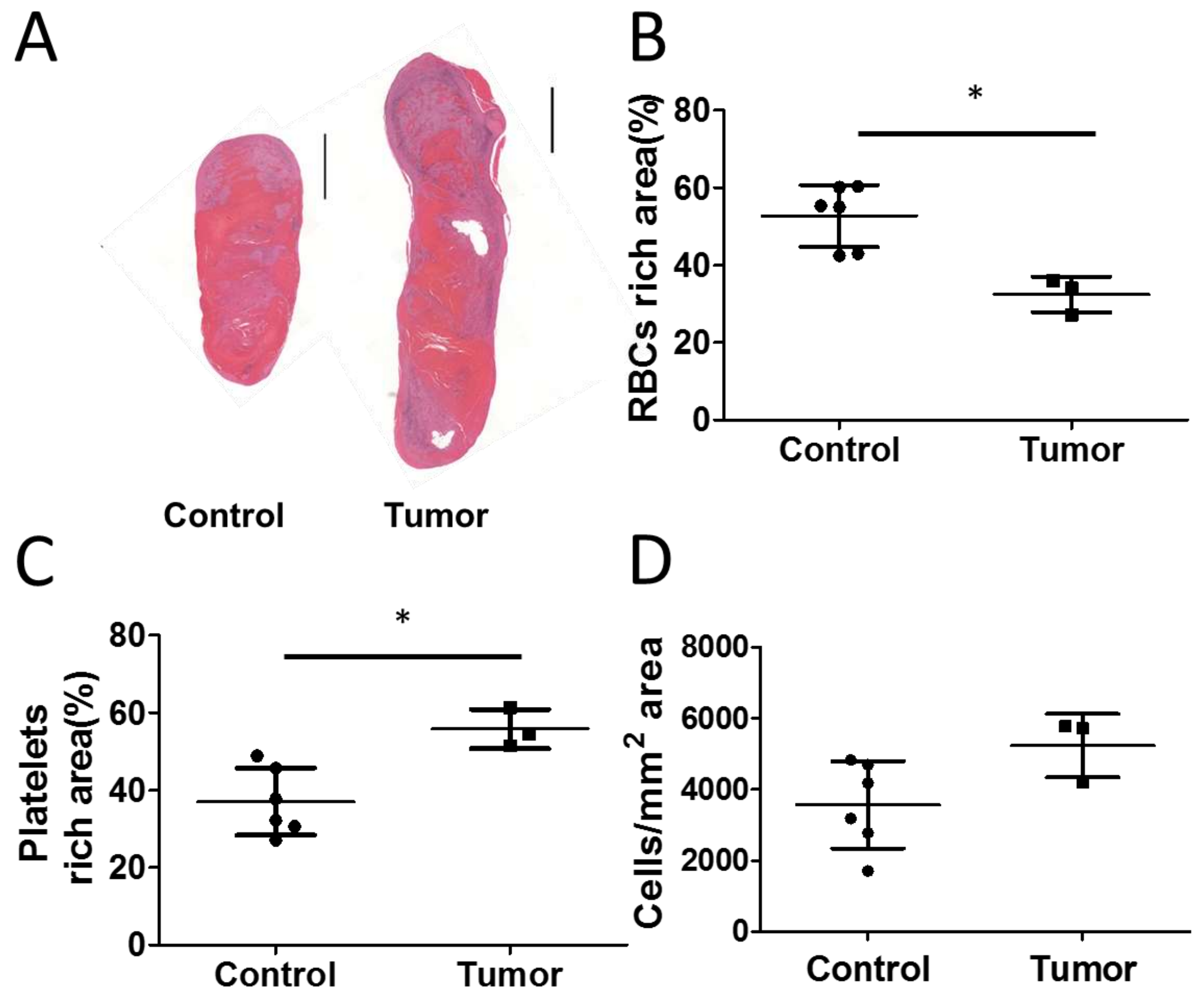
Clinical Characteristics and Biomarkers that are Predictive of VTE in Pancreatic Cancer

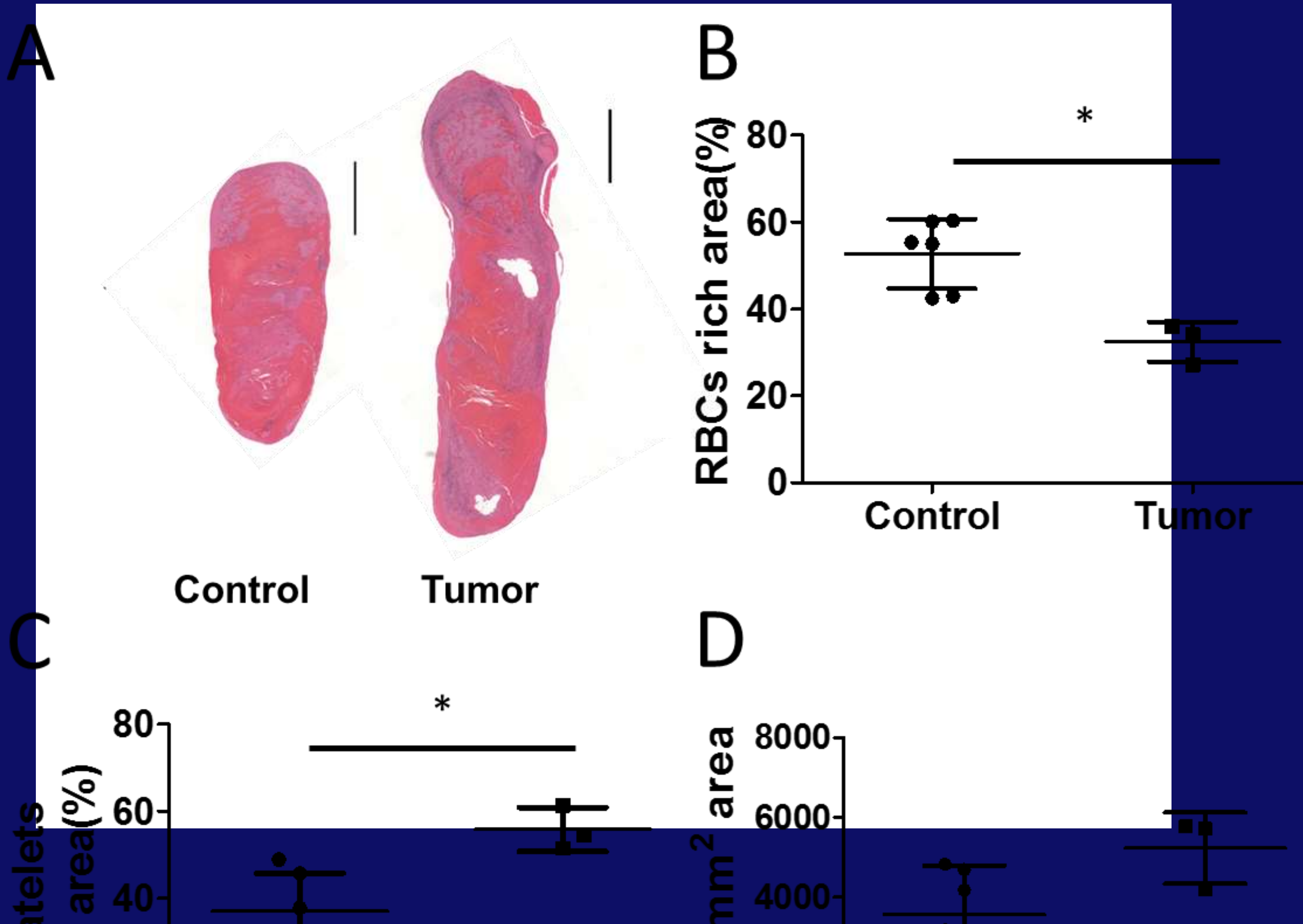
Patient	Death	VTE	Elevated WBC	Elevated Hbg	D-dimer (ng/mL)	MV-TF activity (pg/mL)	PAI-1 (ng/mL)	Clot lysis time (min)
6	n	n	n	n	0.9	0.1	9.25	2.18
7	n	n	n	n	-	0.08	-	-
8	n	n	n	n	-	0.32	-	-
9	n	n	n	n	1.93	0.16	-	1.97
12	n	n	n	n	0.74	0.06	1.58	3.52
14	n	n	n	n	0.74	0.16	0.32	2.07
15	n	n	n	n	1.12	0.01	15.46	3.17
20	y	y	y	y	36.42	1.23	31.78	23.97
22	n	n	n	n	2.24	0.39	3.97	2.18
27	y	n	y	y	12.34	0.94	19.33	5.92
35	n	n	n	n	0.85	-	0.06	2.15
38	n	n	n	n	0.76	0.03	2.24	2.47
40	n	n	n	n	0.96	0.02	2.07	1.62

Kasthuri R et al in prep

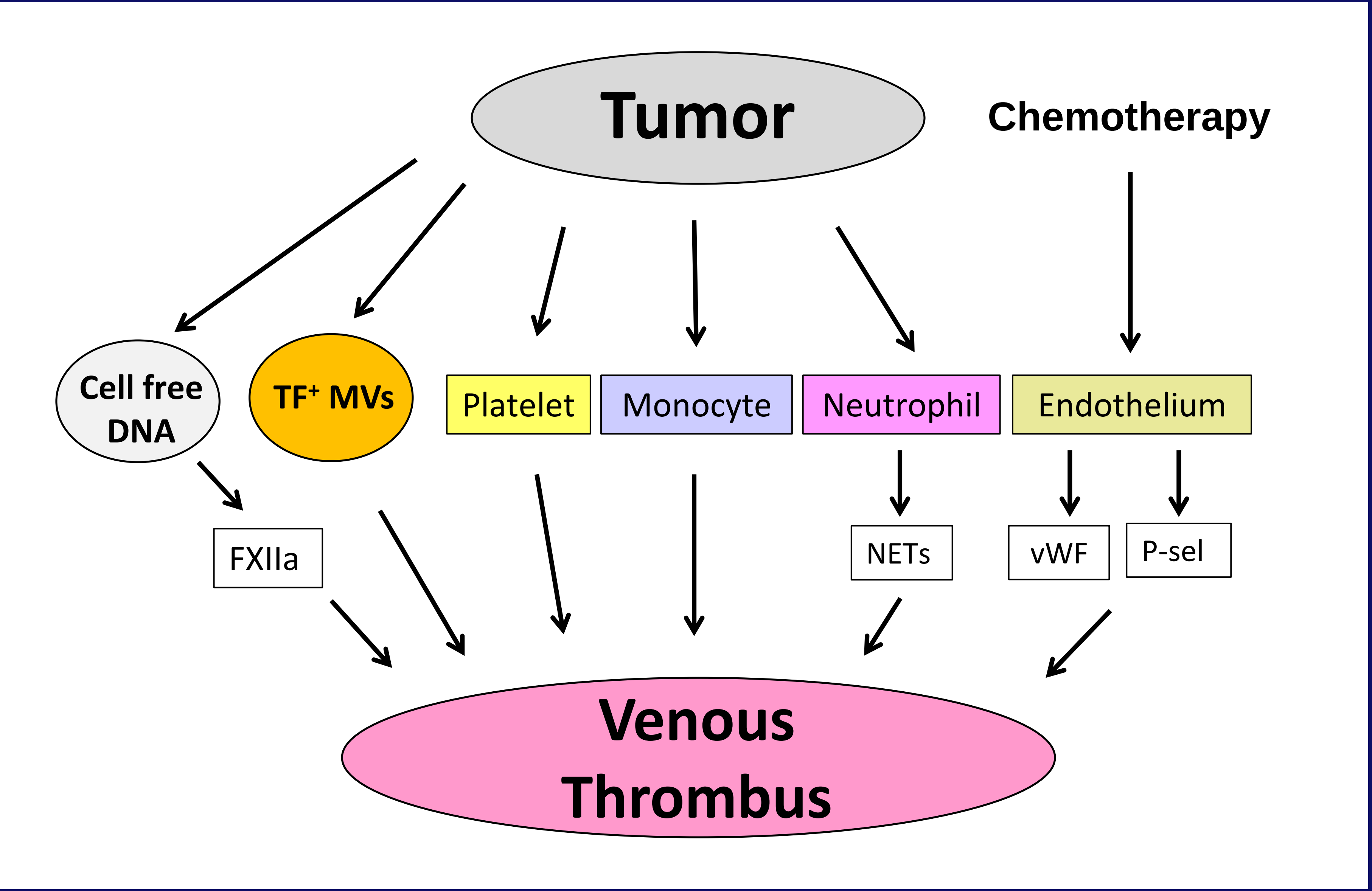
General Conclusions

Clots from Tumor-bearing Mice have more Platelets/Inflammatory Cells



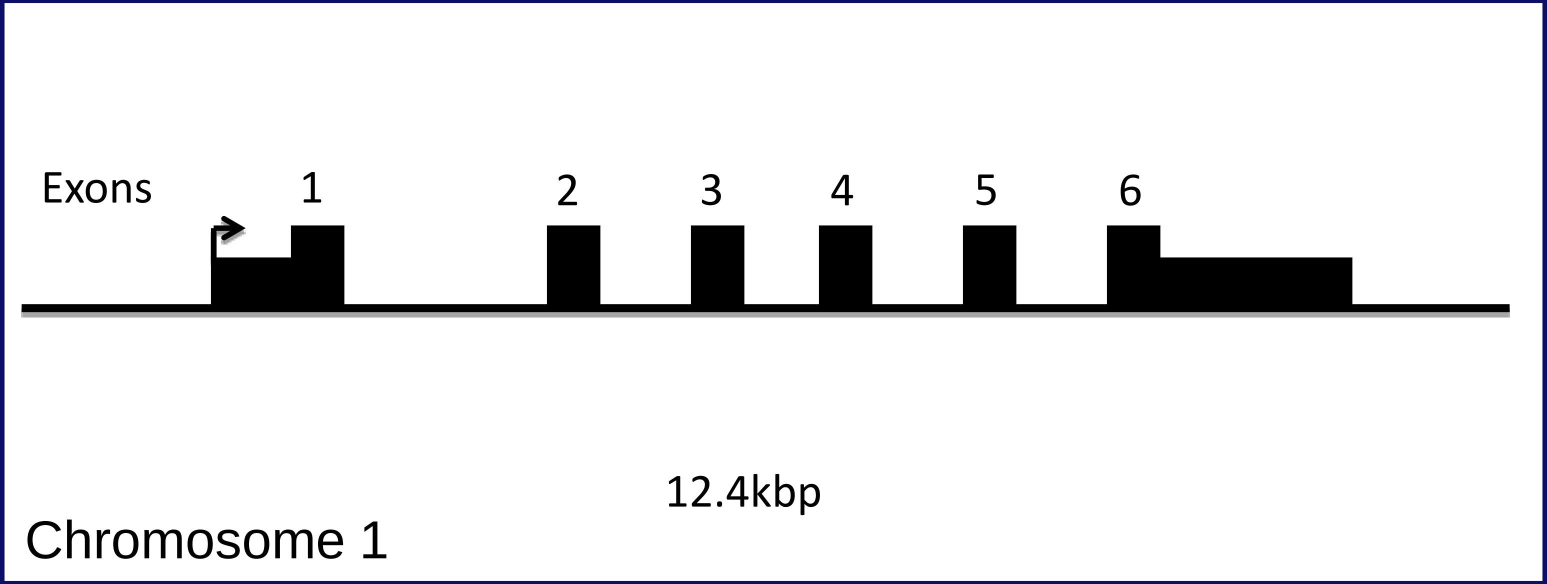

A
Control
Tumor
B
RBCs rich area(%)
80
60
40
20
0
*
Control
Tumor
C
80
60
40
*
area(%)
D
area
8000
6000
4000

Possible Mechanisms of VTE in Cancer Patients



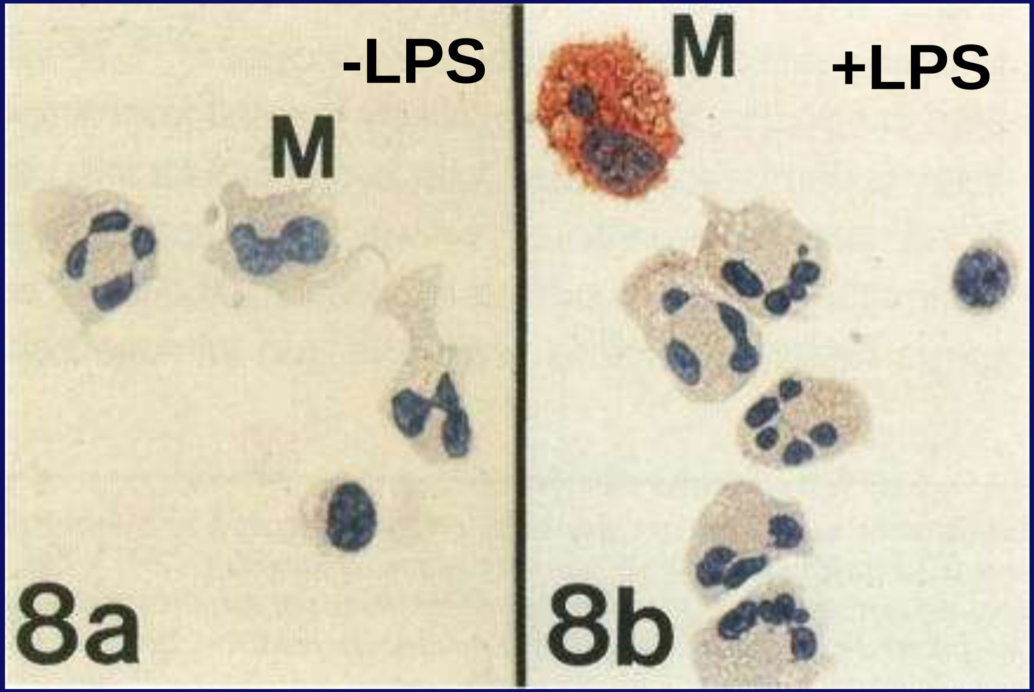
Hisada Y, Geddings J and Mackman N JTH 2015

The Human Tissue Factor Gene



Mackman et al Biochemistry 1989

LPS Induction of TF Expression in Monocytes

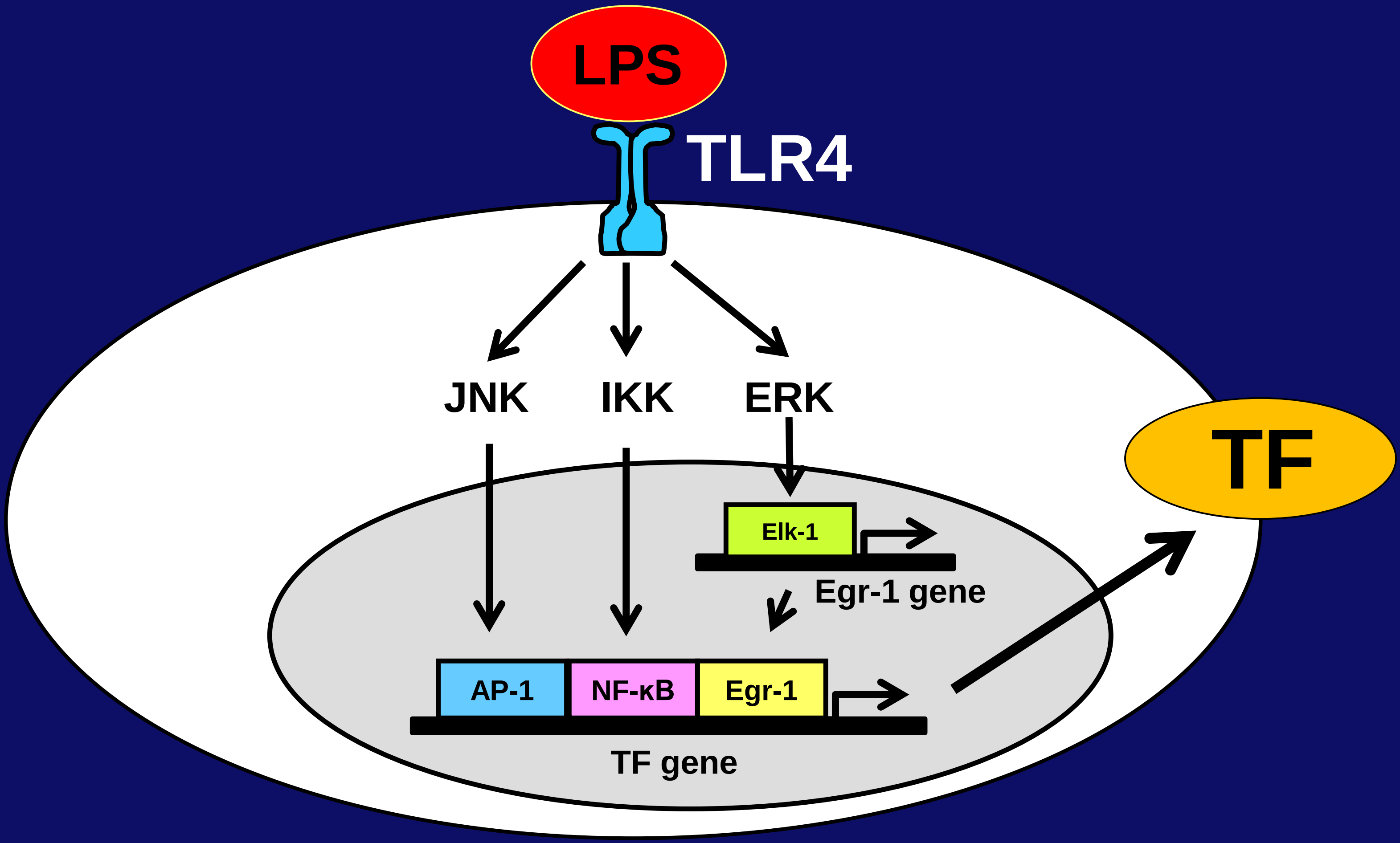


6 hrs

Host Defense

Drake et al AJP 1989

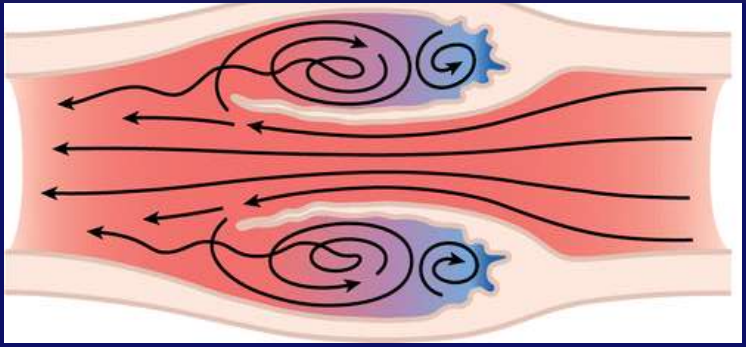
LPS Induction of the TF Gene in Monocytic Cells



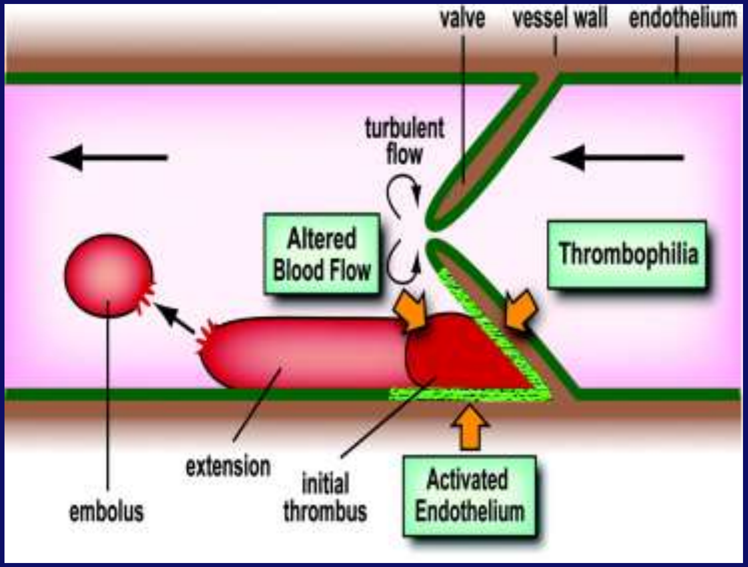
Mackman et al, JEM 1991; Mackman JBC 1994; Parry and Mackman JBC 1994; Oeth et al, MCB 1994; Guha et al, Blood 2002

Formation of a Deep Vein Thrombus

Hypoxia in valve pockets

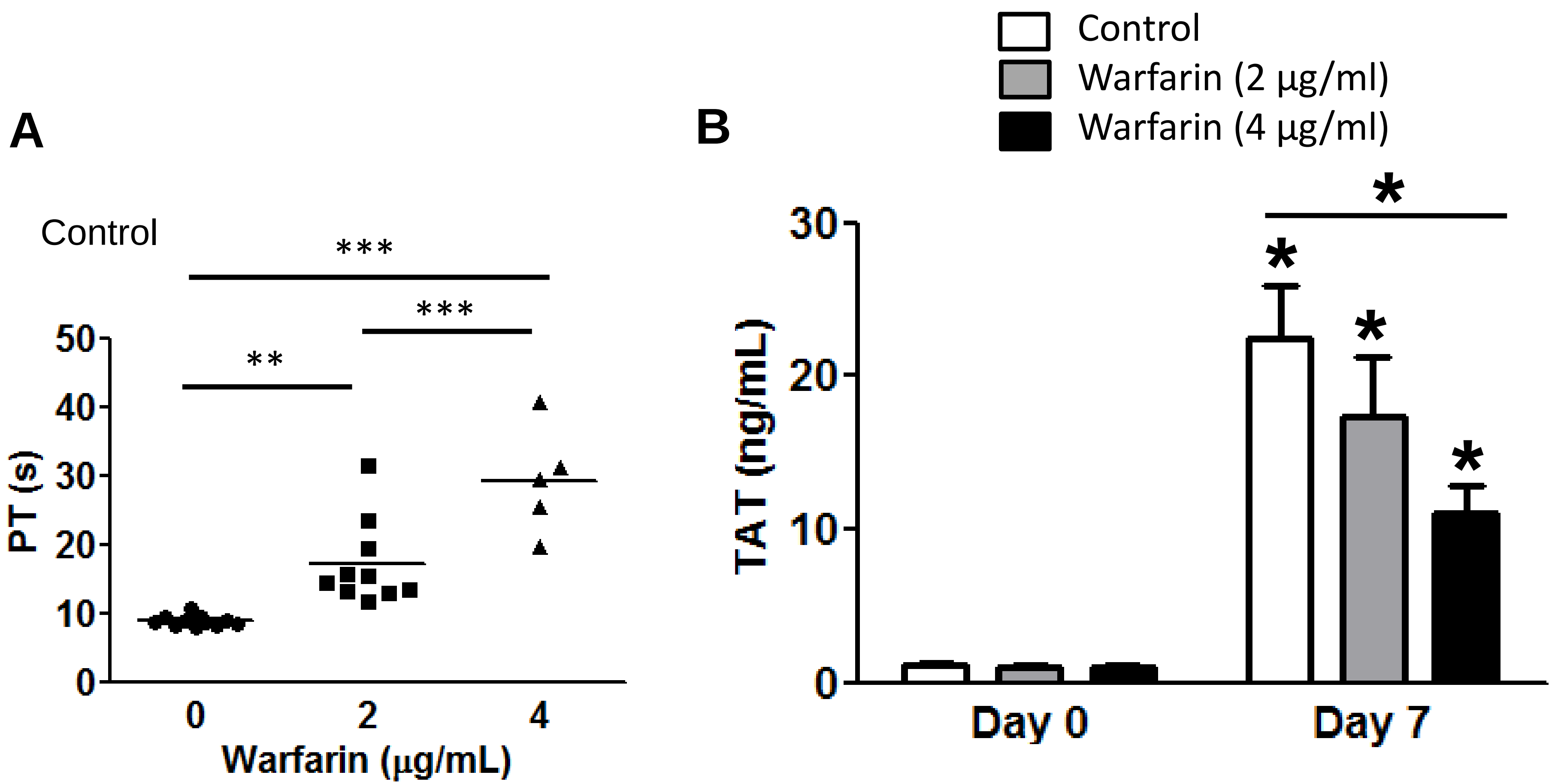


Bovill Ann Rev Physiol 2011



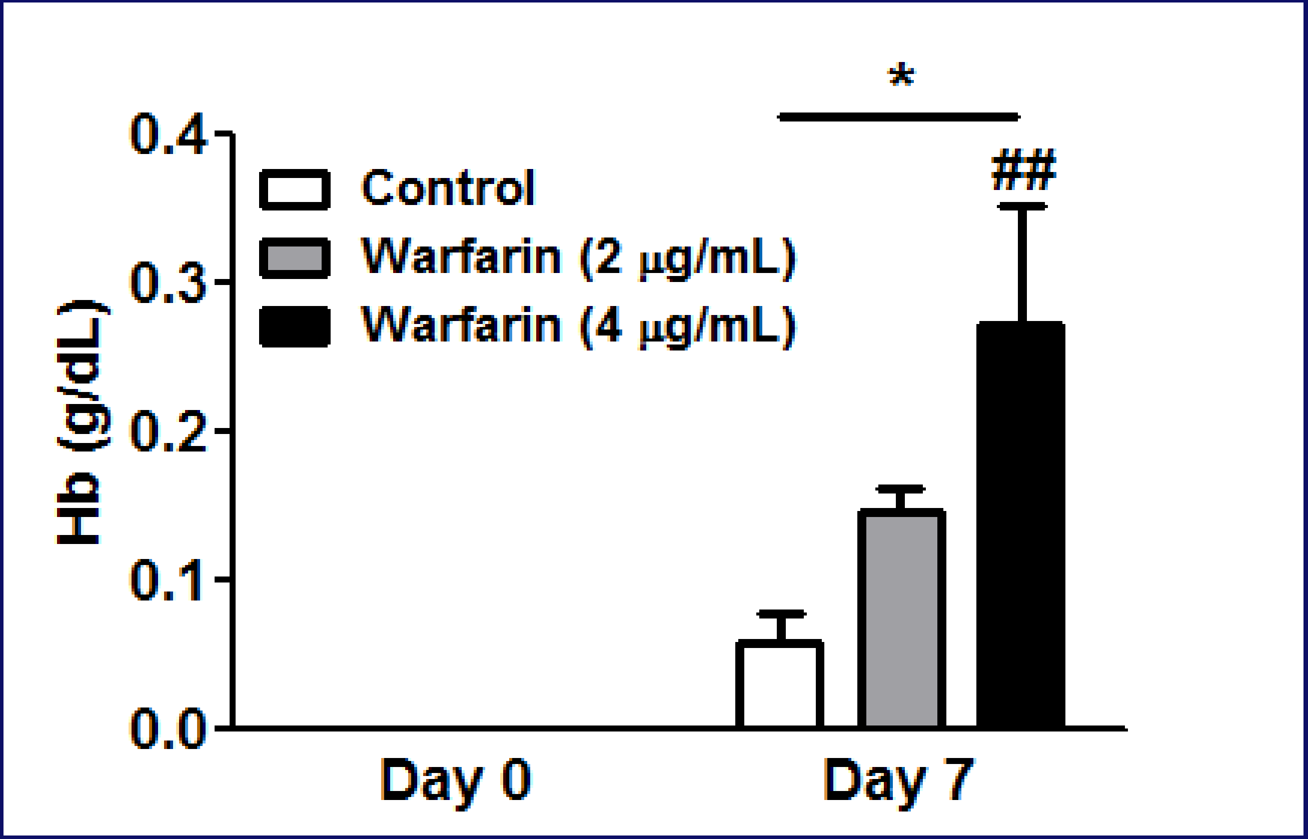
Moll and Mackman ATVB 2008

Anticoagulating Mice with Warfarin



Tatsumi K et al Physiol Reports 2016

Warfarin Increases Lung Hemorrhage After IAV Infection



Tatsumi K et al Physiol Reports 2016

TF Expression in Pancreatic Cancer and VTE

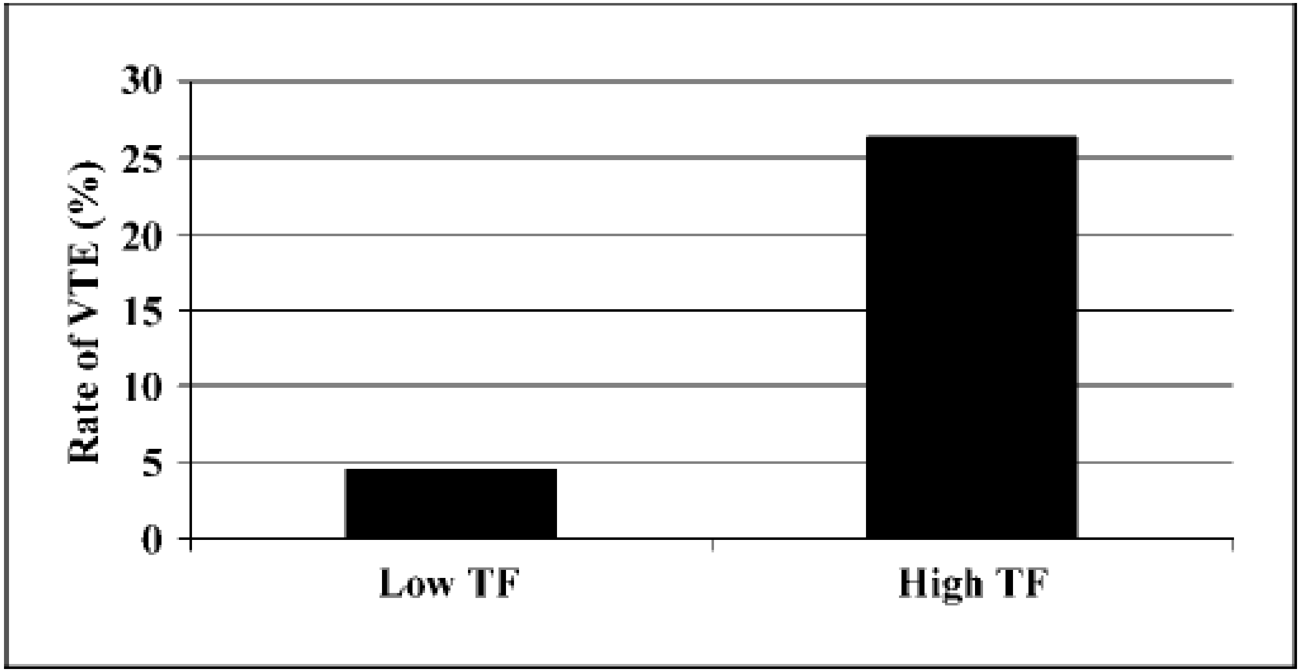
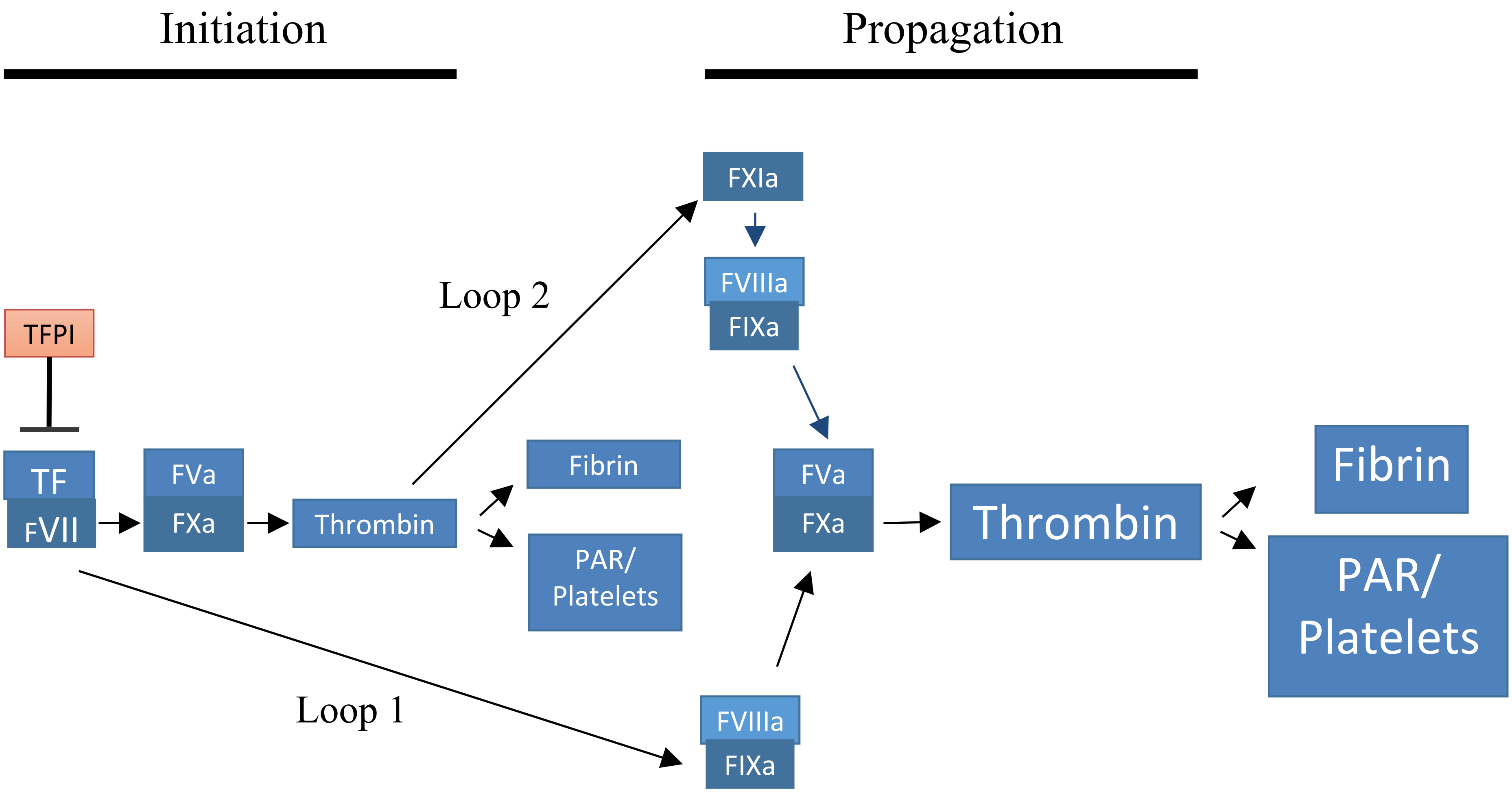


Fig. 2. TF expression and symptomatic venous thromboembolism (*VTE*) in pancreatic cancer. Pancreatic cancer patients with low TF expression had a venous thromboembolism rate of 4.5%, and this was elevated 4-fold to 26.3% in patients with high TF expression ($P = 0.04$).

Khorana A et al Clin Cancer Res 2007

Amplification Loops in the Clotting Cascade



Effect of Crossing Two Prohemorrhagic Mice

Can we exacerbate the bleeding in Low TF placentas and mice by adding another prohemorrhagic genetic defect?

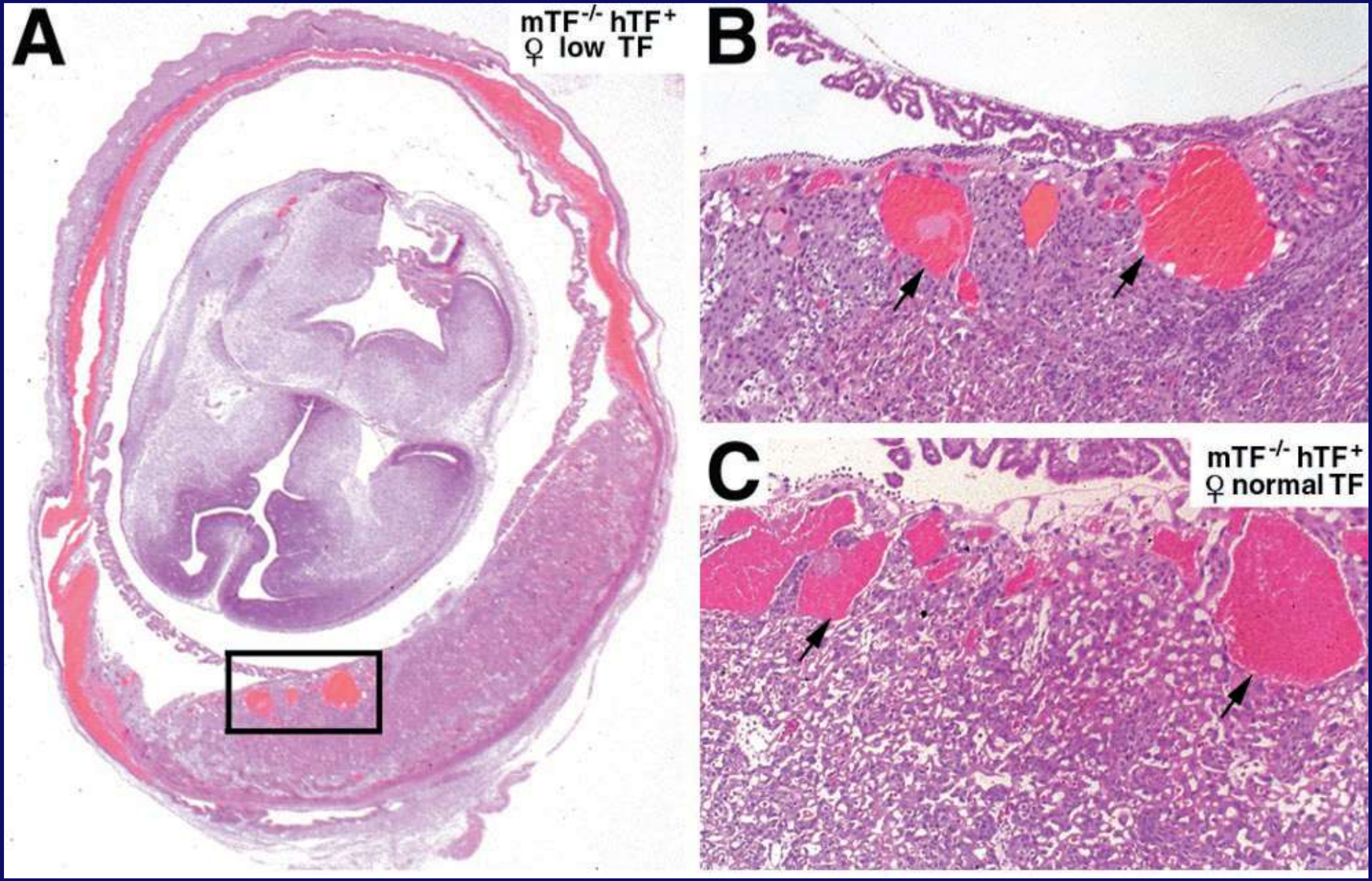
Low TF x PAR4$^{-/-}$ - increased fatal lung hemorrhage Bode & Mackman Thromb Res 2016

Low TF x FIX$^{-/-}$ - embryonic death.

Low TF x FXI$^{-/-}$ - expected number of mice at wean but increased placental blood pools and maternal death.

Low TF x FXII$^{-/-}$ - expected number of mice at wean.

Low TF Placentas Contain Blood Pools in Low TF and Control Mothers



Erlich et al PNAS 1999

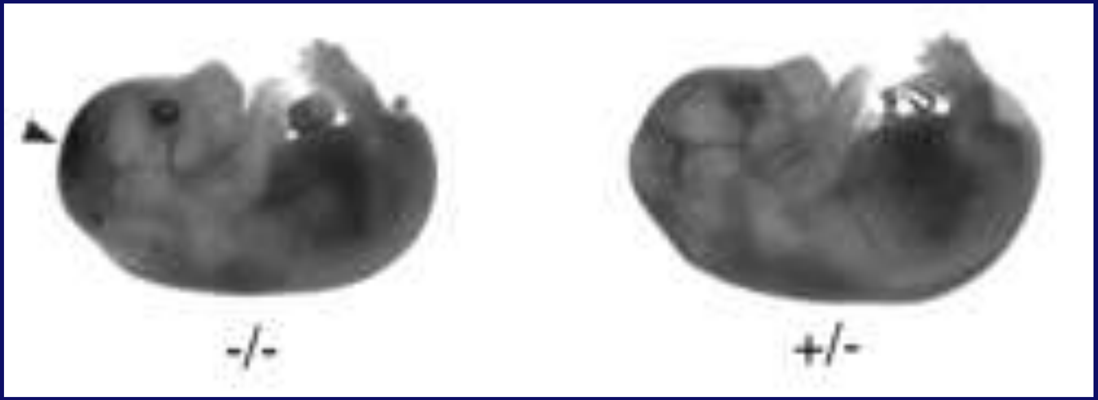
Roles of TF in the Uterus and Placenta During Gestation and Post-Partum

Breeding strategy	Level of TF expression		Fatal mid-gest hemorrhage	Fatal post-partum hemorrhage
	Mother	Embryo		
♂ -/-, h^+ X ♀ -/-, h^+	1%	1%	17/41 (42%)	4/22 (18%)
♂ +/+ X ♀ -/-, h^+	1%	50%	0/43 (0%)	6/43 (14%)

FVIII$^{-/-}$ and FIX$^{-/-}$ have normal pregnancies

Effect of Inactivation of the TFPI Gene in Mice

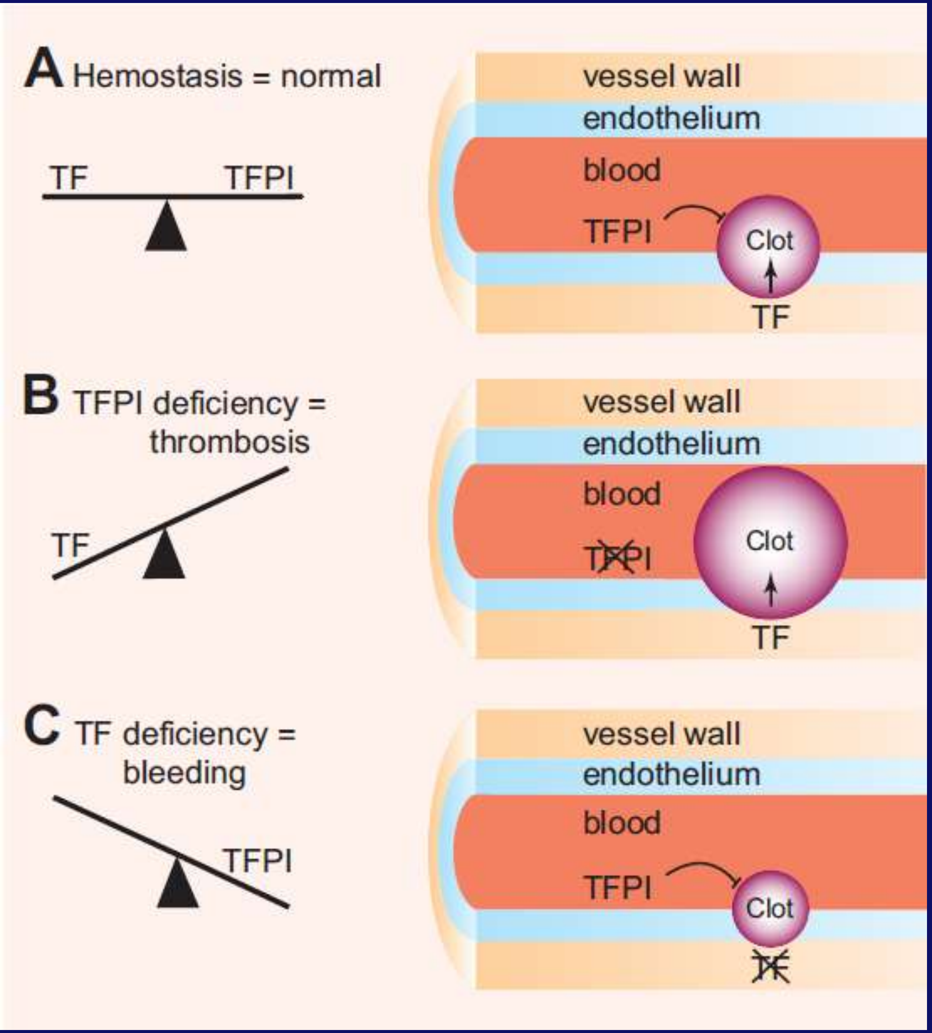
TFPI null embryos die during embryonic development



TFPI is essential for survival

Huang Z et al Blood 1997

Balancing the TF Pathway

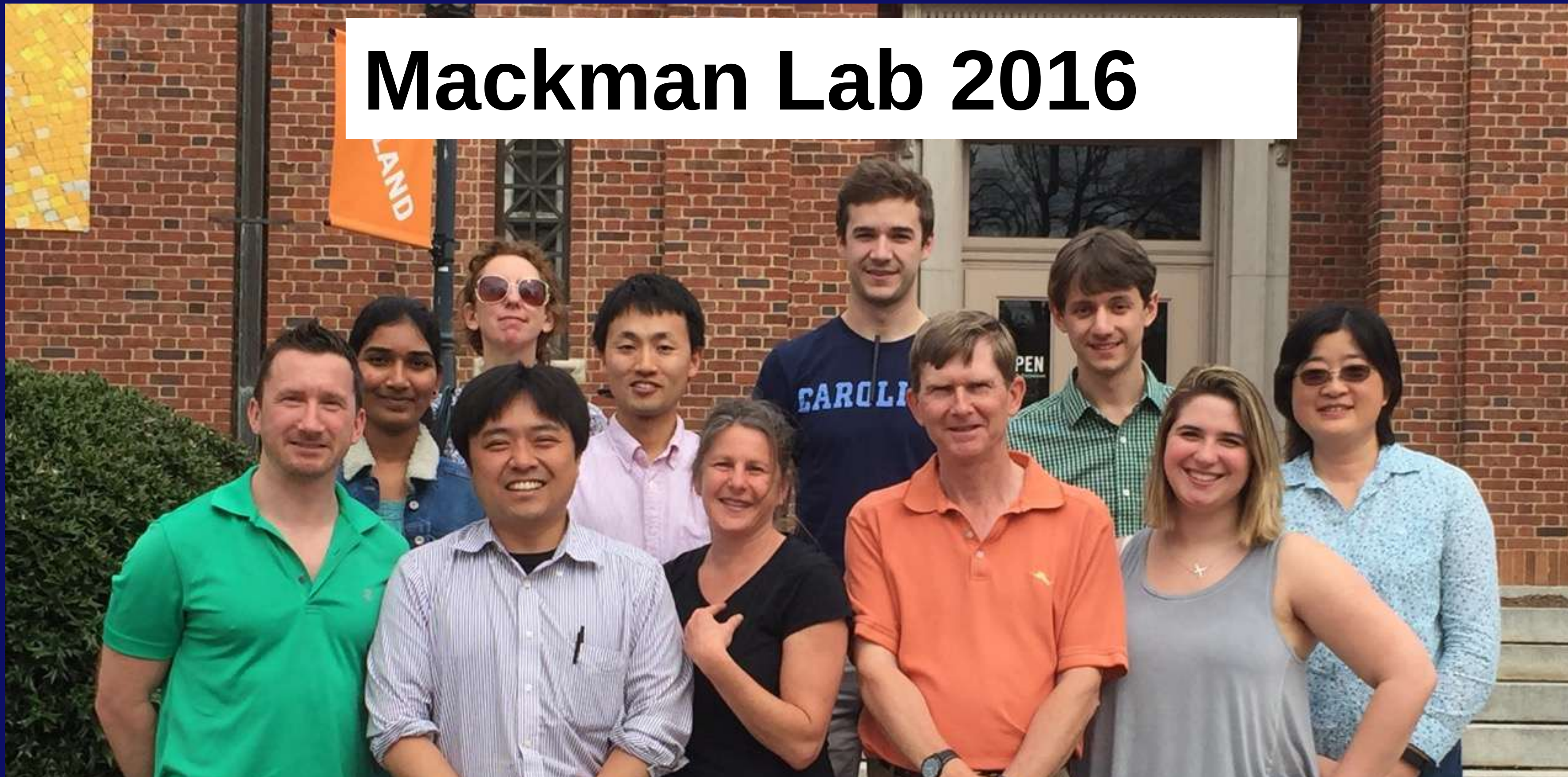


Normal

Thrombosis

Bleeding

Mackman Lab 2016



NATIONAL INSTITUTES OF HEALTH

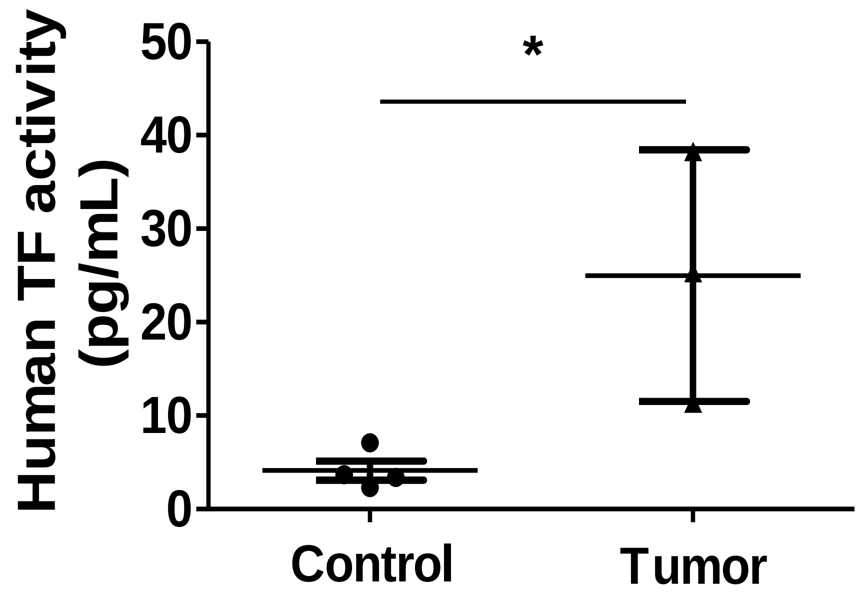
American Heart Association.
Learn and Live.

MYOCARDITIS FOUNDATION

TREC
K.G. JEBSEN THROMBOSIS RESEARCH AND EXPERTISE CENTER

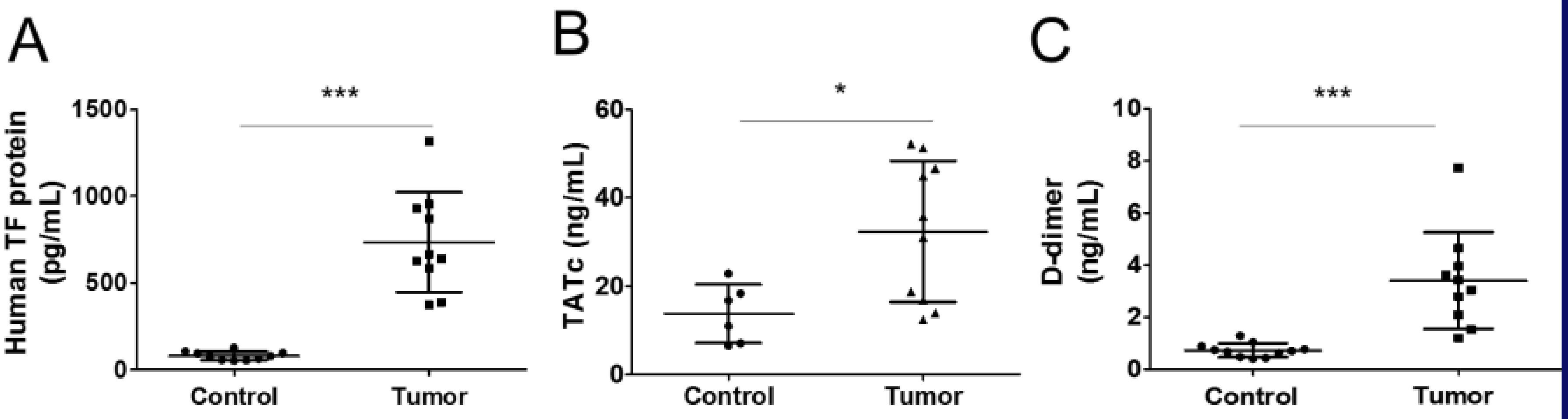
TraCS

Tumor-derived Human TF is Present in Venous Clots in Mice



Hisada Y et al JTH 2017

Levels of Human TF and D-dimer in Tumor-bearing Mice



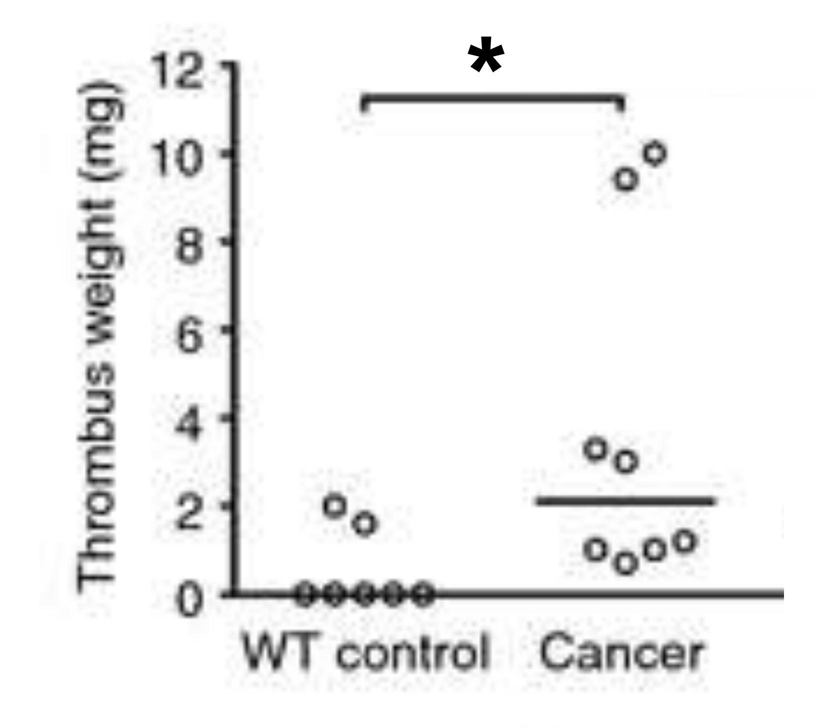
Hisada Y et al JTH 2017

Effect of Crossing Low TF Mice with FXI$^{-/-}$ Mice

FXI	mTF	hTF	N	Observed (%)	Expected (%)
+/+	+/+	-	5	7.813	1.5625
+/-	+/+	-	1	1.563	3.125
-/-	+/+	-	2	3.125	1.5625
+/+	+/+	+	6	9.375	4.6875
+/-	+/+	+	1	1.563	9.375
-/-	+/+	+	0	0.000	4.6875
+/+	+/-	-	1	1.563	3.125
+/-	+/-	-	6	9.375	6.25
-/-	+/-	-	1	1.563	3.125
+/+	+/-	+	8	12.500	9.375
+/-	+/-	+	14	21.875	18.75
-/-	+/-	+	6	9.375	9.375
+/+	-/-	-	0	0.000	1.5625
+/-	-/-	-	0	0.000	3.125
-/-	-/-	-	0	0.000	1.5625
+/+	-/-	+	5	7.813	4.6875
+/-	-/-	+	4	6.250	9.375
-/-	-/-	+	4	6.250	4.6875
Total			64	100	100

Thrombosis is Increased by the Presence of a Mouse Pancreatic Tumor

Clot measured at 3 h

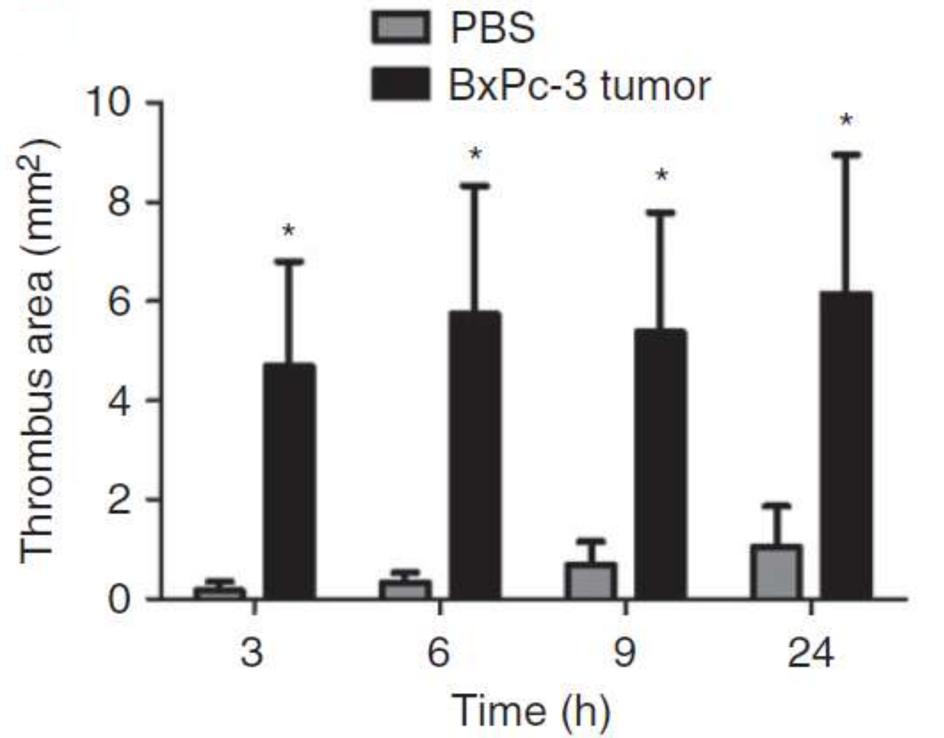


Panc02 is a mouse pancreatic cancer cell line

Thomas G et al JTH 2015

Thrombosis is Increased by the Presence of Human Pancreatic Tumors Grown in Nude Mice

Julia Geddings



Incidence (%)	3 h		6 h		9 h		24 h	
	17	50	33	50	33	50	33	50

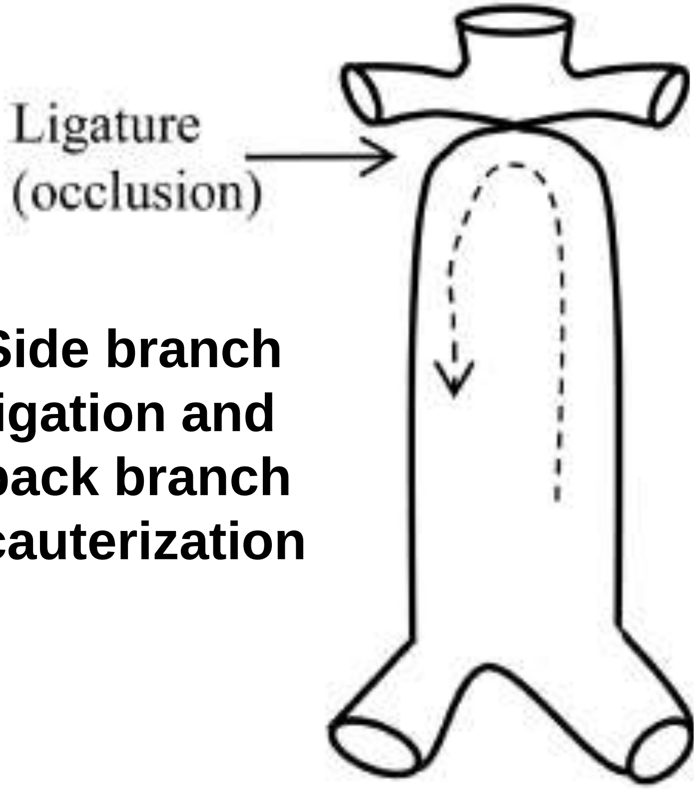
BxPc-3 is a human pancreatic tumor

Geddings J et al JTH 2016

Limitations of the Current Studies

- The incidence and clot size in the IVC stenosis model is quite variable.
- These studies do not specifically analyze the role of tumor-derived, TF^{+} MVs in thrombosis.

IVC Stasis Model



Side branch ligation and back branch cauterization

The incidence is ~100% and the clot size is ~20 mg at 48 h

Diaz J et al ATVB 2012

TF Expression in Pancreatic Cancer and VTE

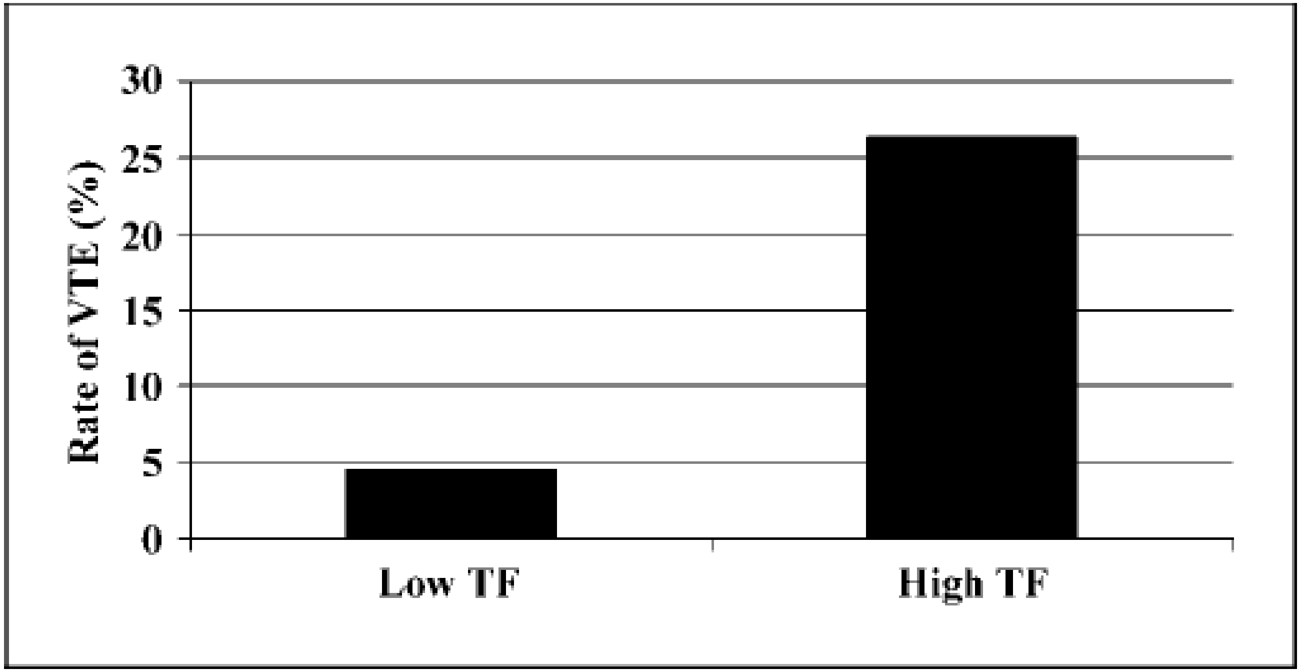
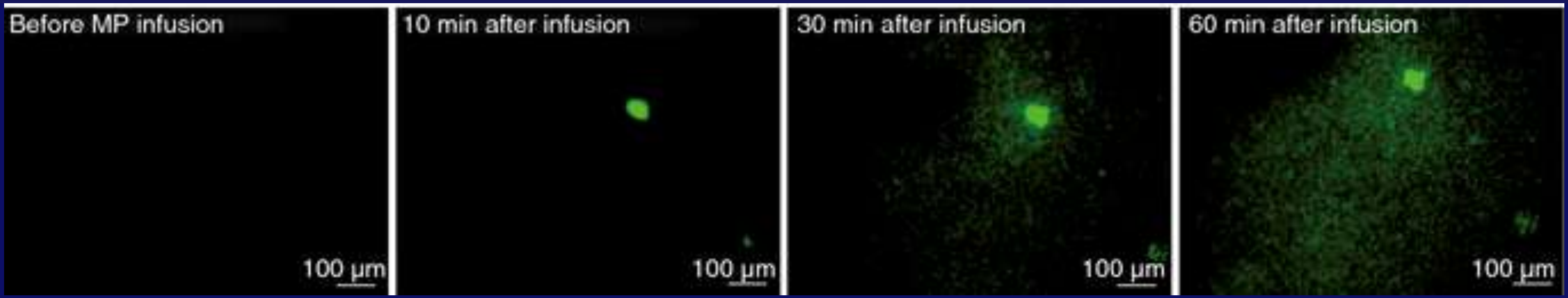


Fig. 2. TF expression and symptomatic venous thromboembolism (*VTE*) in pancreatic cancer. Pancreatic cancer patients with low TF expression had a venous thromboembolism rate of 4.5%, and this was elevated 4-fold to 26.3% in patients with high TF expression ($P = 0.04$).

Khorana A et al Clin Cancer Res 2007

Effect of injection of exogenous tumor-derived, TF^+ MVs on venous thrombosis in mice

Injected Tumor MVs Localize to the Site of Thrombosis in an IVC Stenosis Model

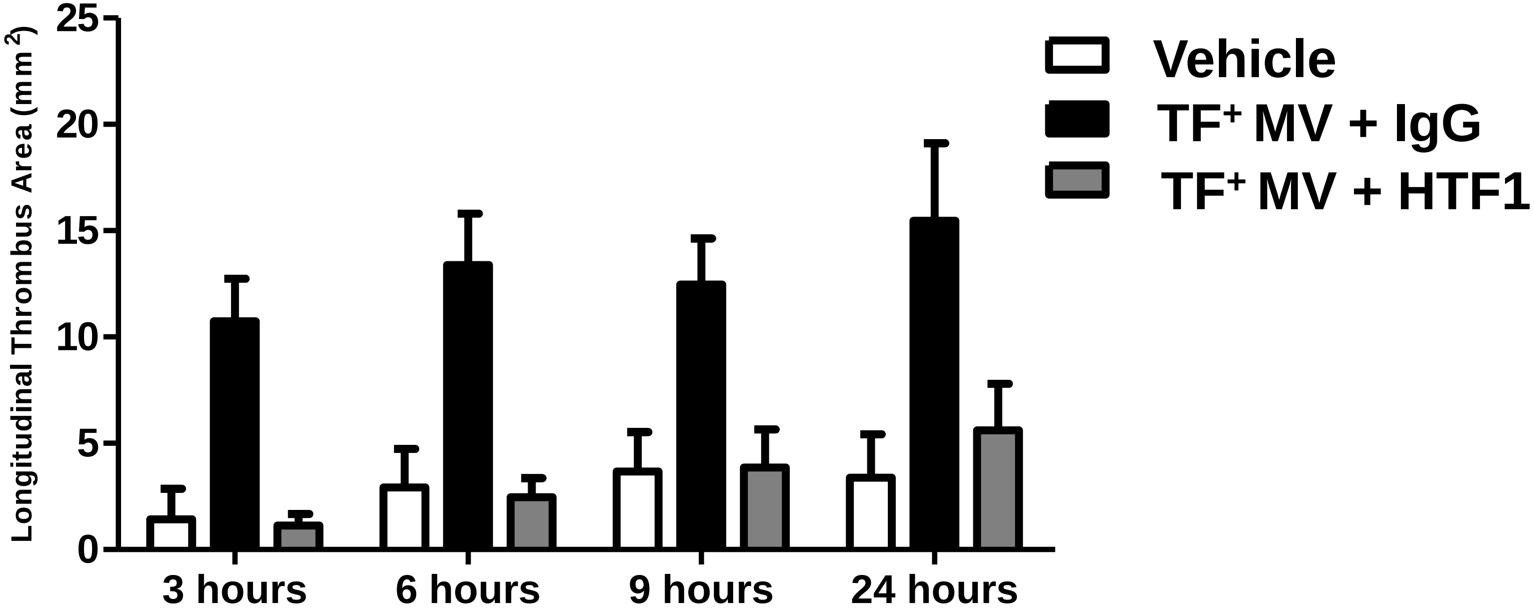


Panc02 = mouse pancreatic cancer cell line

Thomas G et al JTH 2015

Exogenous TF$^+$ Tumor MVs Enhance Thrombosis in the IVC Stenosis Model

Julia Geddings



MVs isolated from a human pancreatic tumor cell line BxPc-3

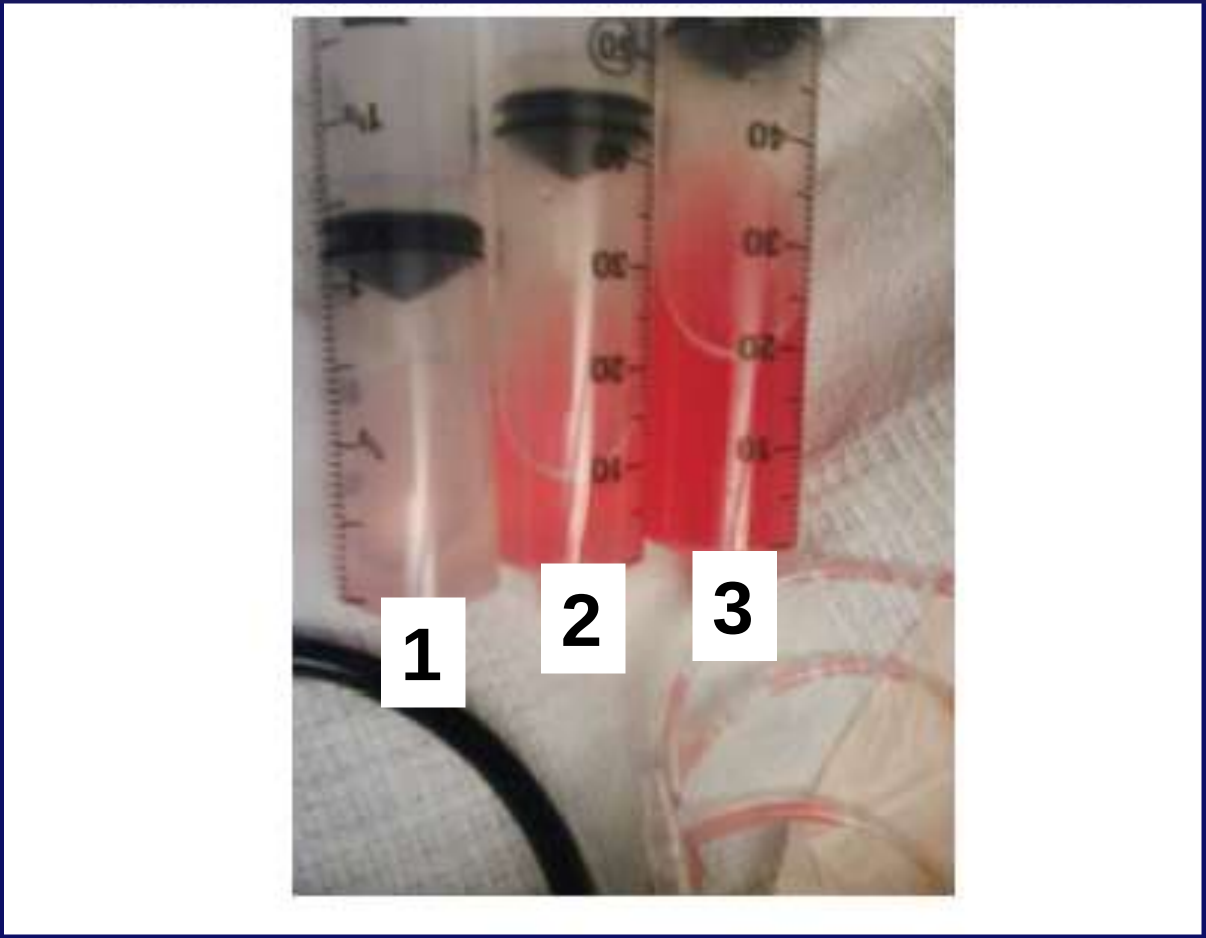
TF on tumor MVs is required for the enhancement of thrombosis

Robert G MacFarlane
(1907-1987)

Rosemary Biggs
(1912-2001)

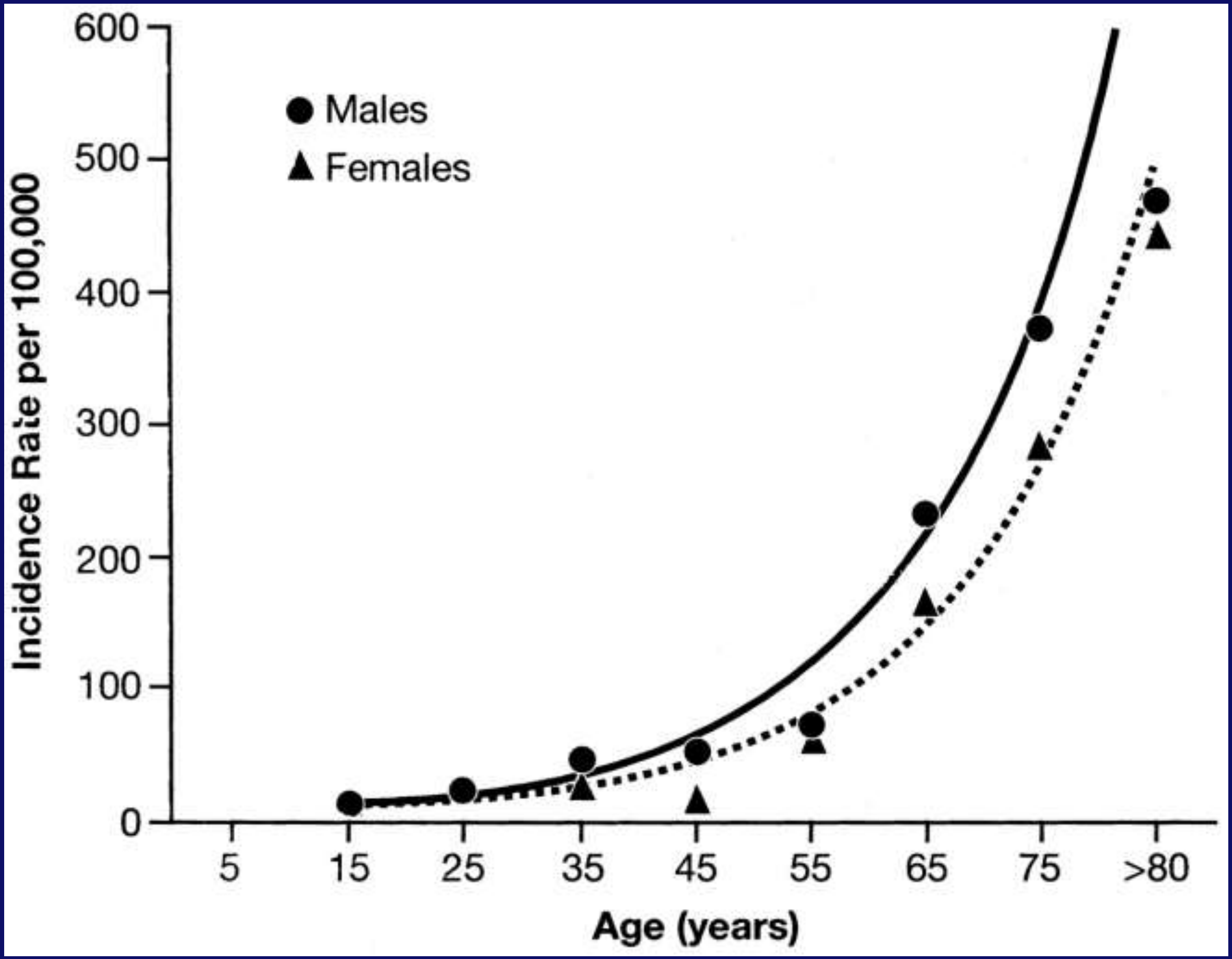
Alveolar Hemorrhage in Patients with Severe Influenza Infection

Bronchiolar lavage fluid from a patient infected with influenza A



Gilbert et al Respit Care 2010

Annual Incidence of VTE by Age and Sex



Richard H. White Circulation. 2003;107:I-4-I-8

Risk Factors for Venous Thrombosis

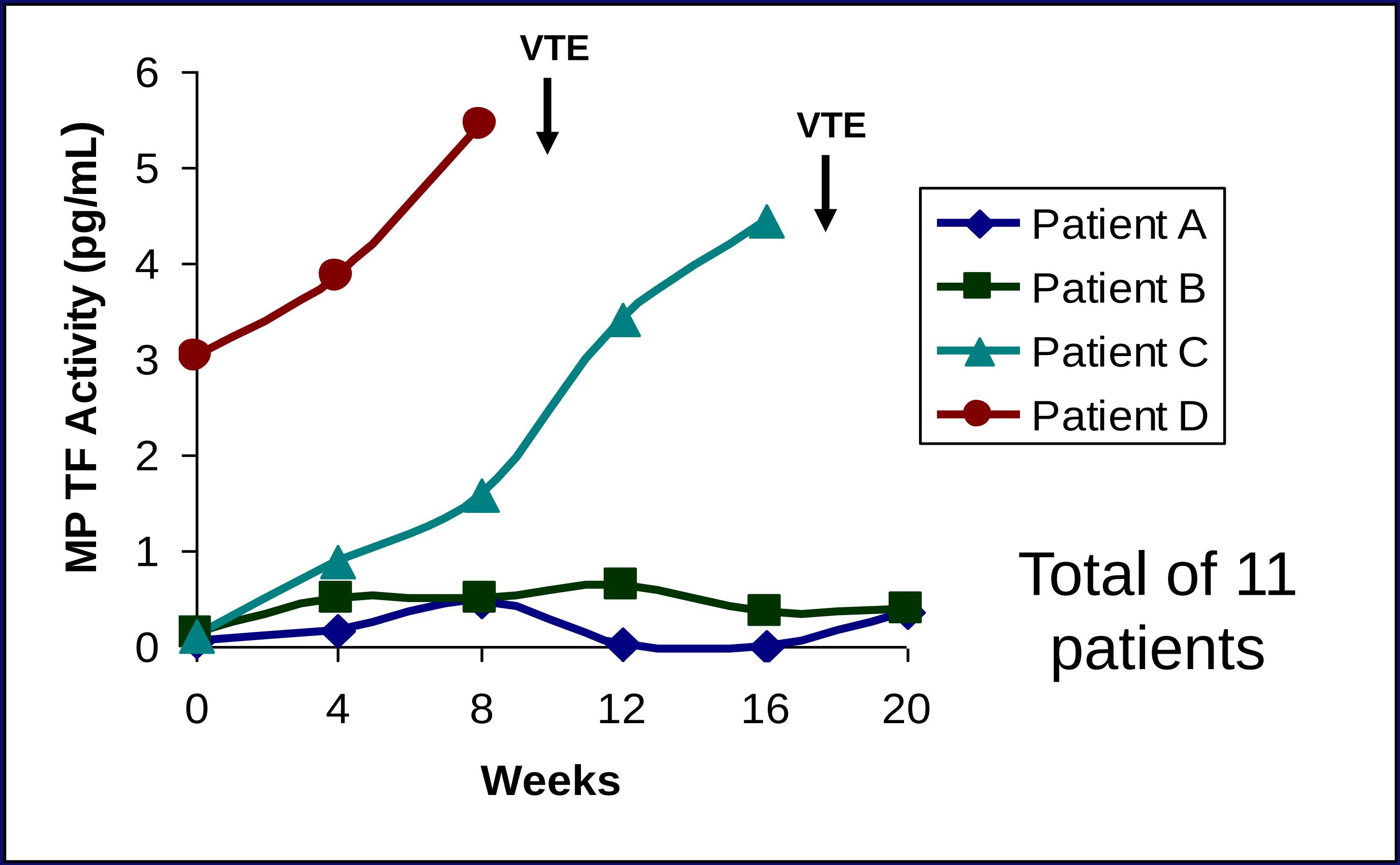
ACCP Practice Guidelines

Padua Prediction Risk Score – high risk defined as a score ≥ 4

Risk Score	Points
Active cancer	3
Previous VTE	3
Reduced Mobility (bed rest ≥ 3 days)	3
Known thrombophilic condition	3
Recent trauma and/or surgery	2
Elderly age (≥ 70 years)	1
Heart and/or respiratory failure	1
Acute MI or ischemic stroke	1
Obesity (BMI ≥ 30)	1
Ongoing hormone treatment	1

Barbar S et al JTH 2010- Identification of hospitalized medical patients at risk for VTE

Levels of MV TF Activity Increased in Pancreatic Cancer Patients before VTE



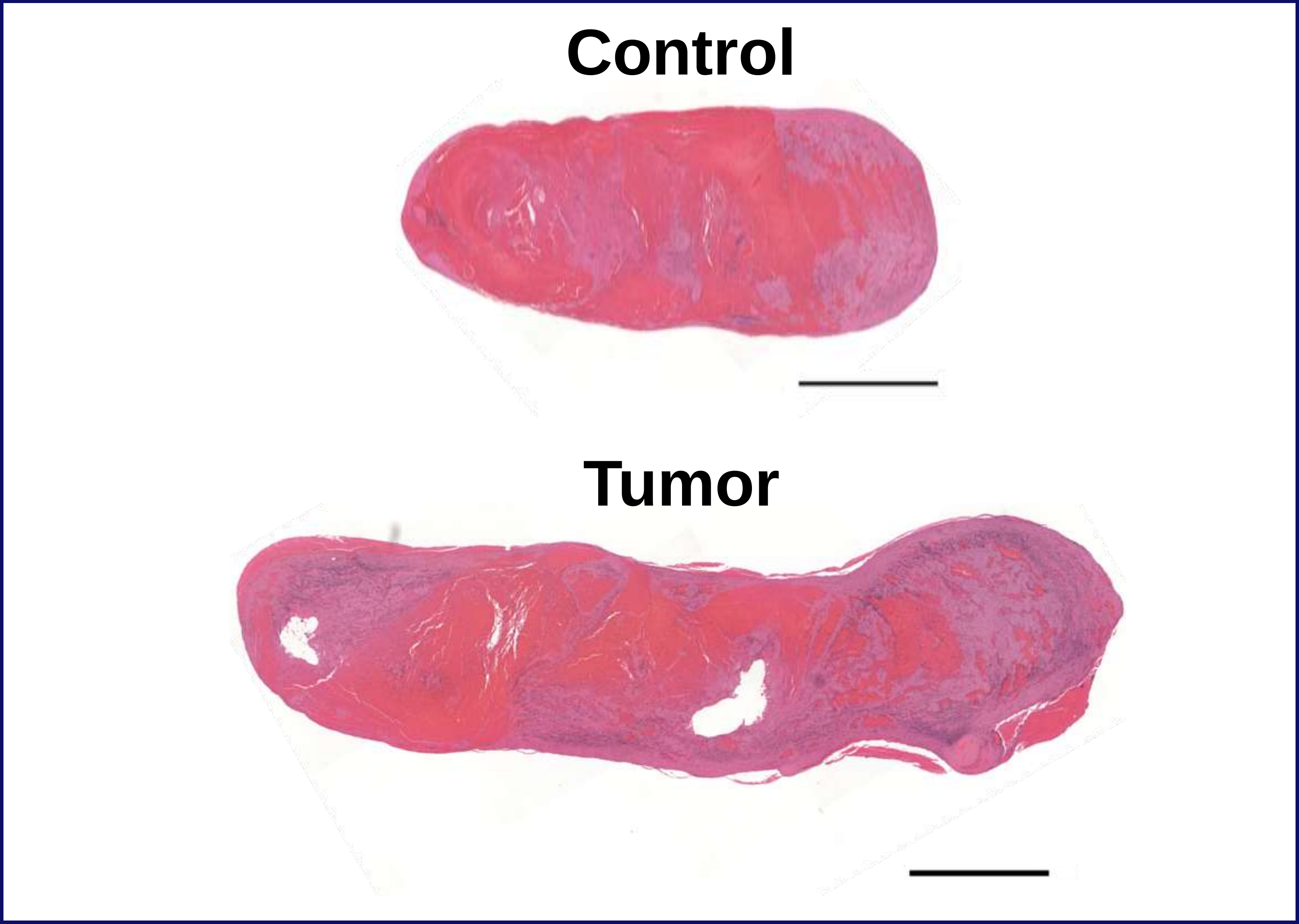
Khorana A et al JTH 2008

TF^+ MPs and VTE in Cancer Patients: Prospective Studies

Study	Method	Patients	Time of Follow-up	Association between MP TF and VTE?
Khorana, 2008	MP TF activity TF ELISA	2 pancreatic cancer patients who develop VTE 8 pancreatic cancer patients without VTE	Every 4 weeks for 20 weeks	Yes
Zwicker, 2009	impedance-based flow cytometry	4 cancer patients who develop VTE 56 cancer patients without VTE	1 year	Yes
Bharthuar, 2010	MP TF activity	52 Pancreaticobiliary cancer patients with VTE 65 Pancreaticobilliary cancer patients without VTE	6 months	Yes
Van Doormaal, 2012 [64]	MP TF activity MP-dependent fibrin generation flow cytometry TF ELISA	5 cancer patients who develop VTE (3 pancreatic) 38 cancer patients without VTE (10 pancreatic)	6 months	Yes by functional assay No by antigen assay
Thaler, 2012 [60]	MP TF activity	49 cancer patients who develop VTE (12 pancreatic) 299 cancer patients without VTE (48 pancreatic)	2 years	No

Geddings J and Mackman N Blood 2014

Clots from Tumor-bearing Mice are Larger but have a Similar Composition

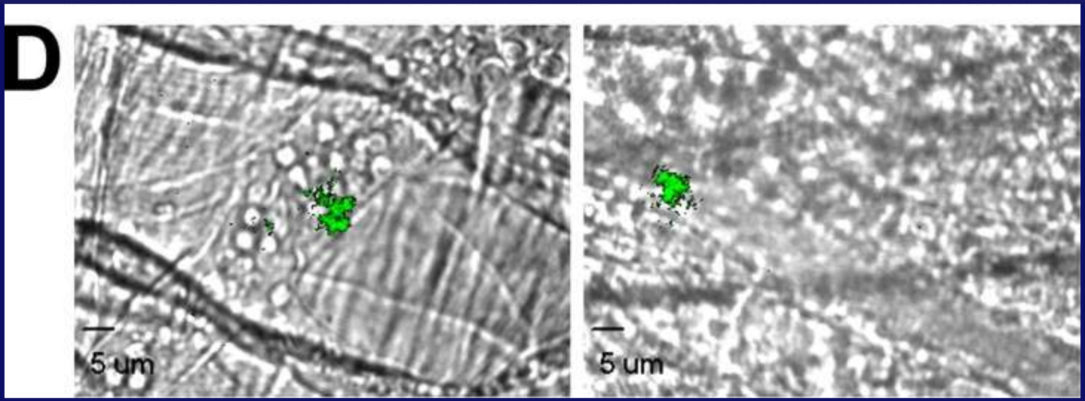


Models to Study the Role of Tumor-derived MVs in Thrombosis in Mice

- Injection of exogenous tumor MVs injected into healthy mice
- Endogenous MVs in tumor-bearing mice
- Different mouse models of thrombosis- small (mesenteric) versus large (inferior vena cava) vessels

Tumor-derived MPs Accumulate at the Site of Thrombus Formation In Vivo

Mouse Panc02 pancreatic cells labeled with pEGFP



Venule **Arteriole**

JEM

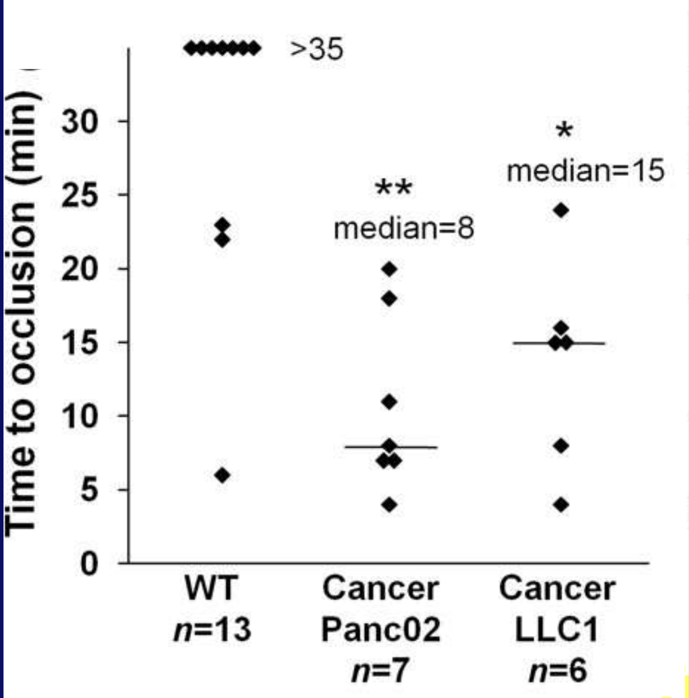
Thomas et al JEM 2009

Tumor-bearing Mice have Shorter Occlusion Times Larger in a Mesenteric Vessel Model

Panc02 = mouse pancreatic carcinoma

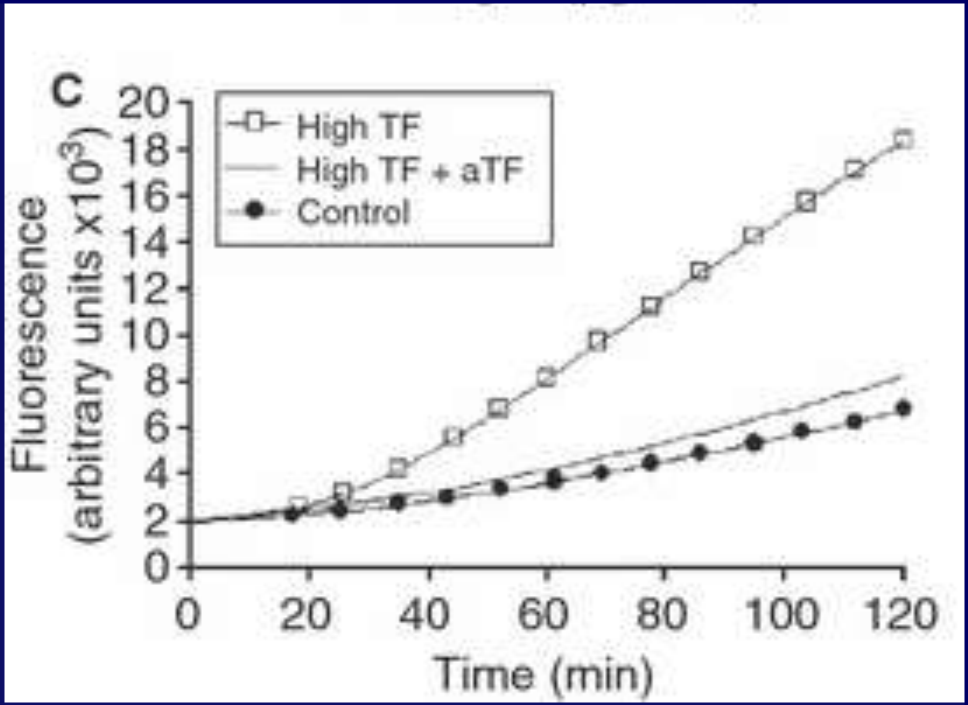
LLC = mouse lewis lung carcinoma

s.c.



Thomas et al JEM 2009

Tumor-derived Human TF is Functional in an In Vitro Assay



Human pancreatic L3.6pl cells grown orthotopically in nude mice

Davila et al JTH 2008

Conclusions

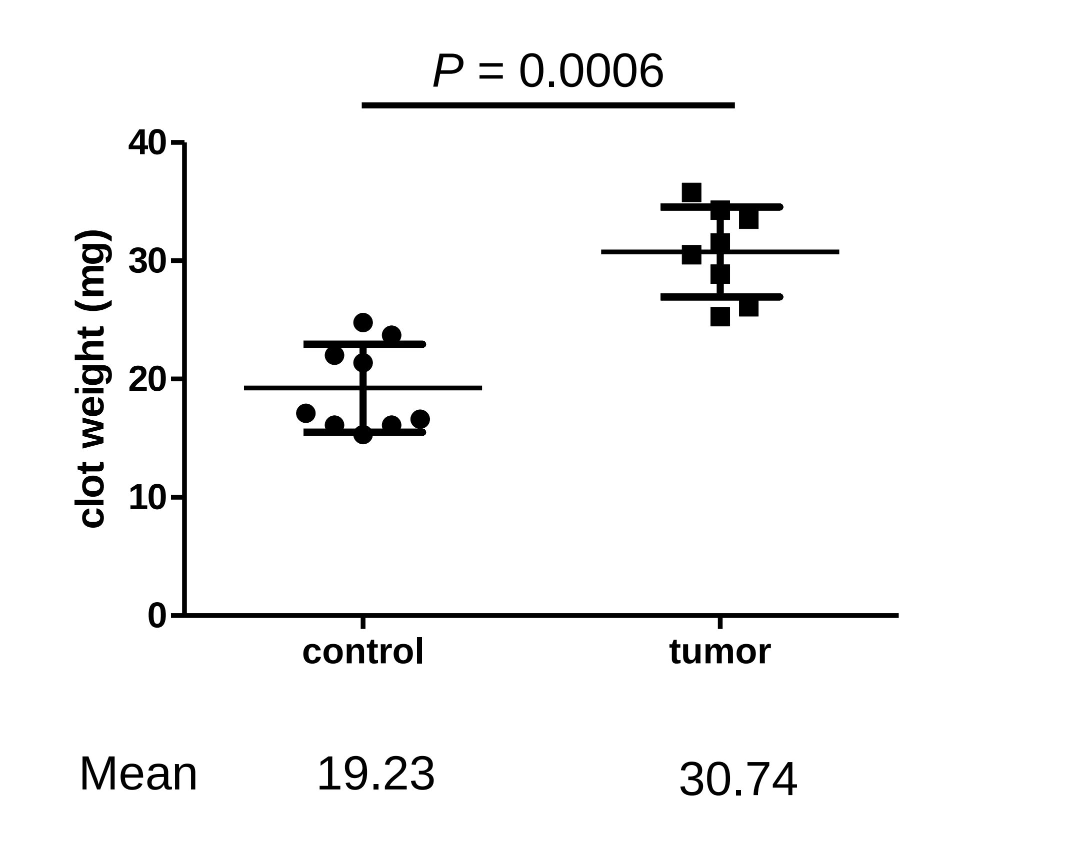
- Human pancreatic tumors grown orthotopically released TF^{+} MVs into the blood
- Tumor-bearing mice have bigger clots than controls
- Human TF is present in clots from tumor-bearing mice
- Inhibition of human TF reduces thrombosis

Conclusions

- Human pancreatic tumors grown orthotopically released TF^+ MVs into the blood
- Human TF is present in clots from tumor-bearing mice
- Inhibition of human TF reduces thrombosis suggesting that tumor-derived, human TF enhances thrombosis

Myocardial Infarction

Human Pancreatic Tumors Increase Thrombosis in Nude Mice



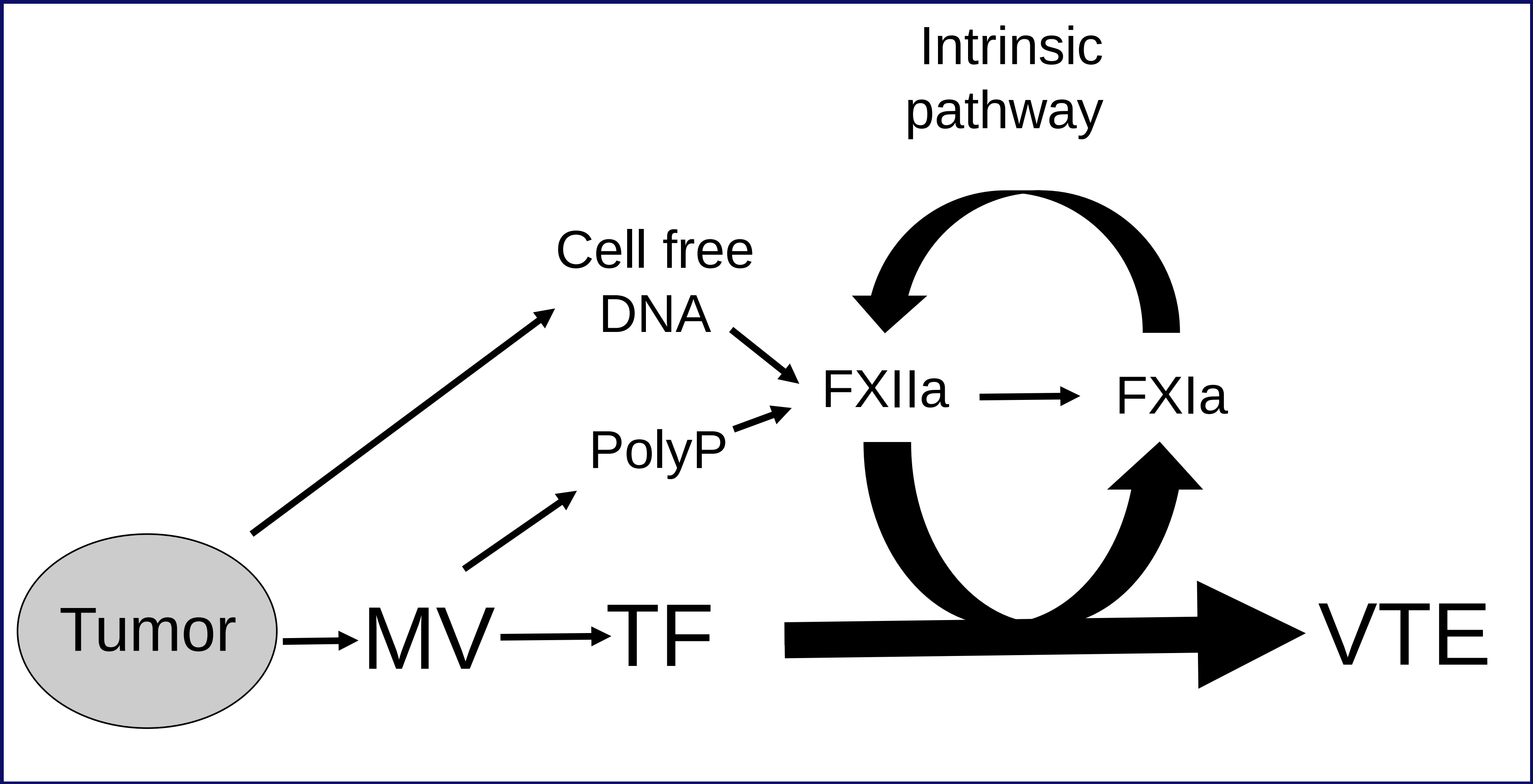
The ant-human TF antibody HTF-1 reduces thrombosis

IVC ligation model- 48 h

(Brian Cooley- Advanced rodent surgery Core)

Hisada Y et al unpublished data

Role of the Intrinsic Coagulation Pathway in Cancer Associated Thrombosis



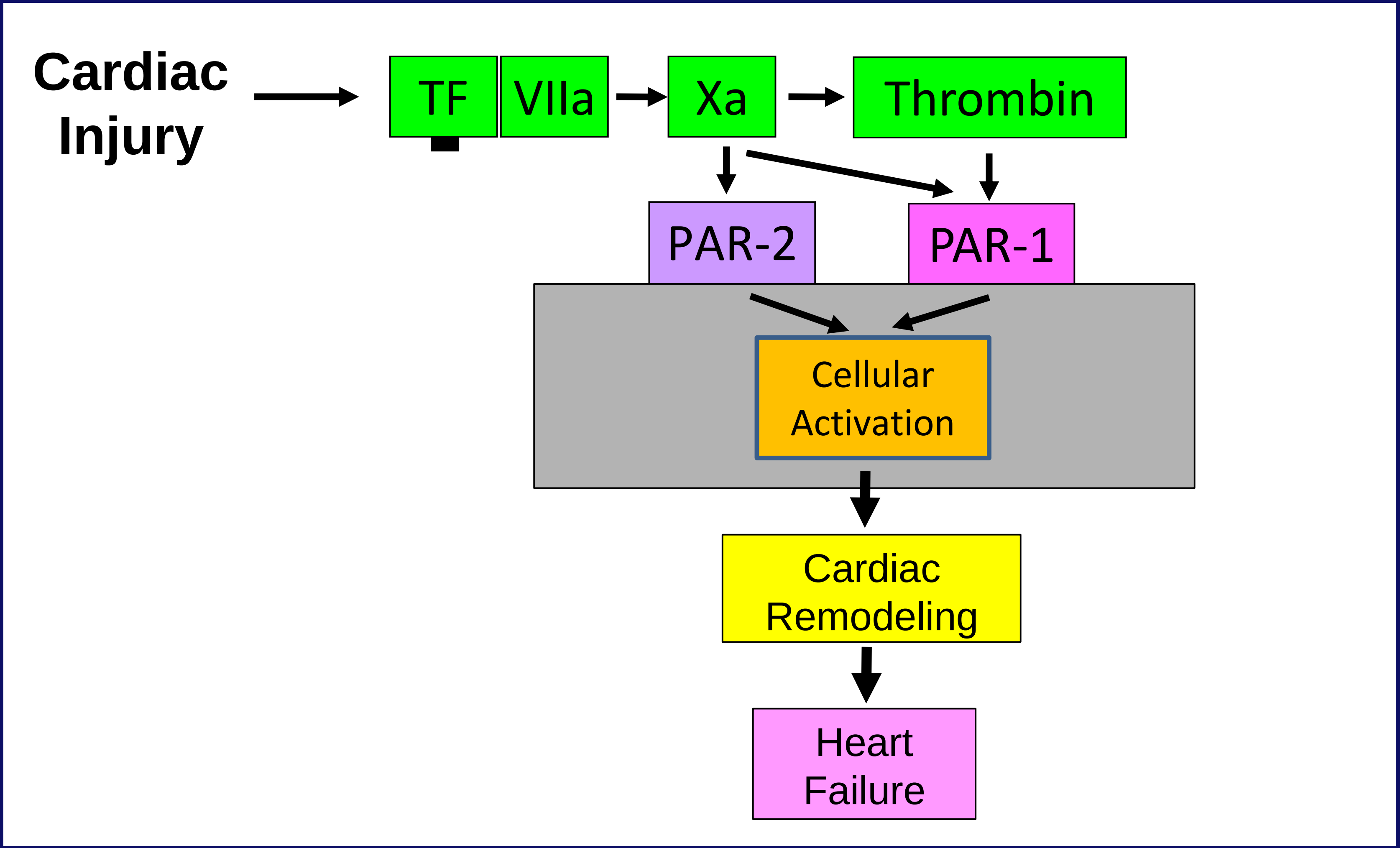
Conclusion

Anticoagulants may increase alveolar hemorrhage and morbidity in patients infected with influenza A

Mouse Models of Cardiac Injury

- Ischemia-reperfusion (I/R) injury
- LAD ligation
- Doxorubicin (anthracycline)
- Transaortic constriction – pressure overload
- Viral myocarditis (CVB3)

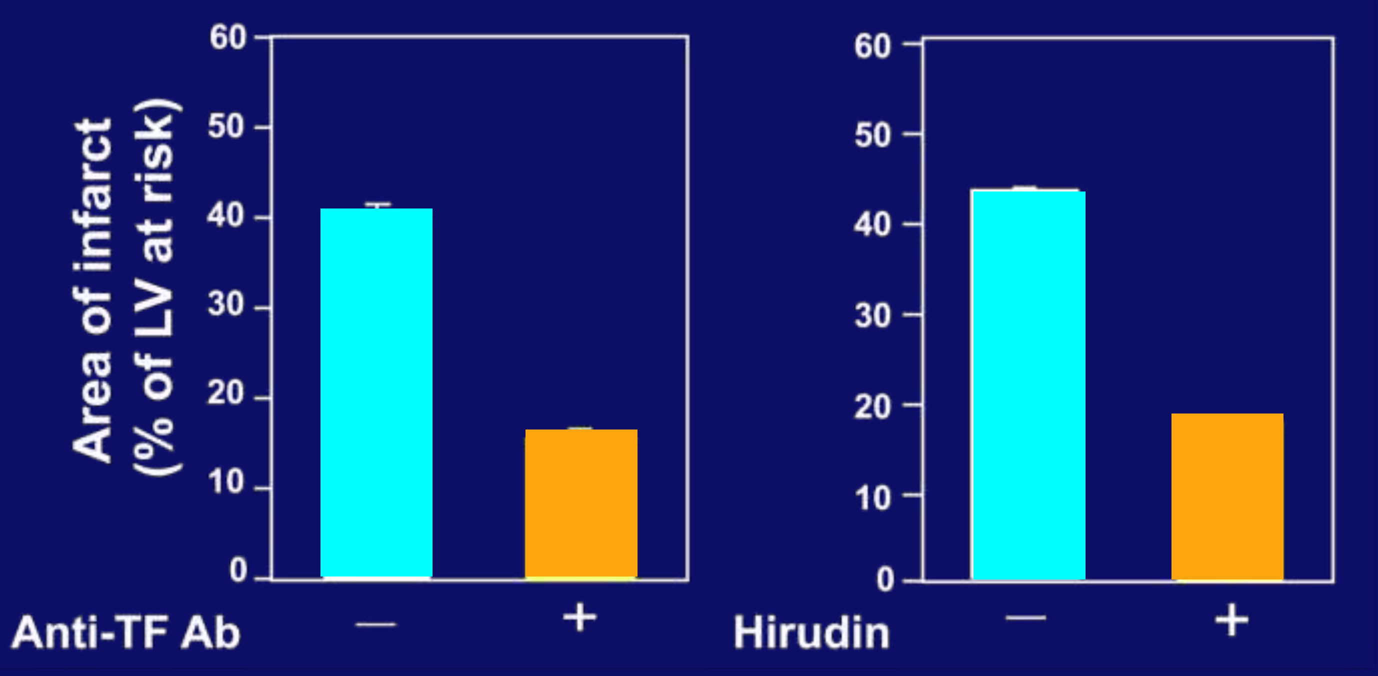
Role of the Clotting Cascade and PARs in Cardiac Remodeling and Heart Failure



Hypothesis

Inhibition of TF and coagulation proteases or a deficiency in PAR-1 or PAR-2 will reduce infarct size and cardiac remodeling after cardiac injury in animal models

Effect of Inhibition of TF or Thrombin on Infarct Size in a Rabbit Cardiac I/R Model



Inhibition of TF or thrombin also reduced inflammation

Erlich et al., *Am J Path* 2000

Clinical Studies with Rivaroxaban

ATLAS ACS-2-TIMI-51 trial- the factor Xa inhibitor rivaroxaban reduced death due to cardiovascular causes (MI and stroke) in patients with a recent ACS event **Mega J et al NEJM 2012.**

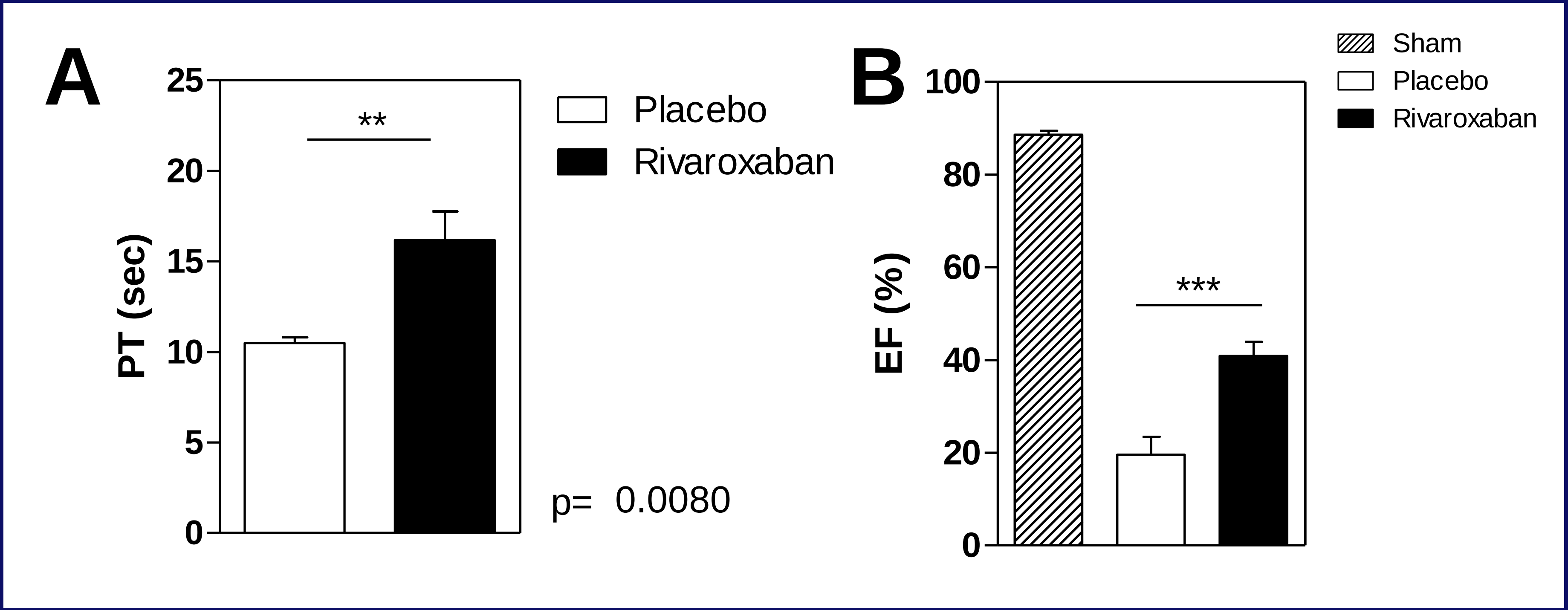
In a subgroup analysis of HF patients, treatment with rivaroxaban 2.5 mg twice daily plus antiplatelet therapy demonstrated decreased rates of cardiovascular events and death compared with antiplatelet therapy alone.

COMMANDER-HF is a phase III clinical trial to evaluate the effect of the new oral anticoagulant rivaroxaban in patients with chronic heart failure.

Hypothesis

Inhibition of FXa with rivaroxaban will reduce cardiac remodeling and heart failure in mice

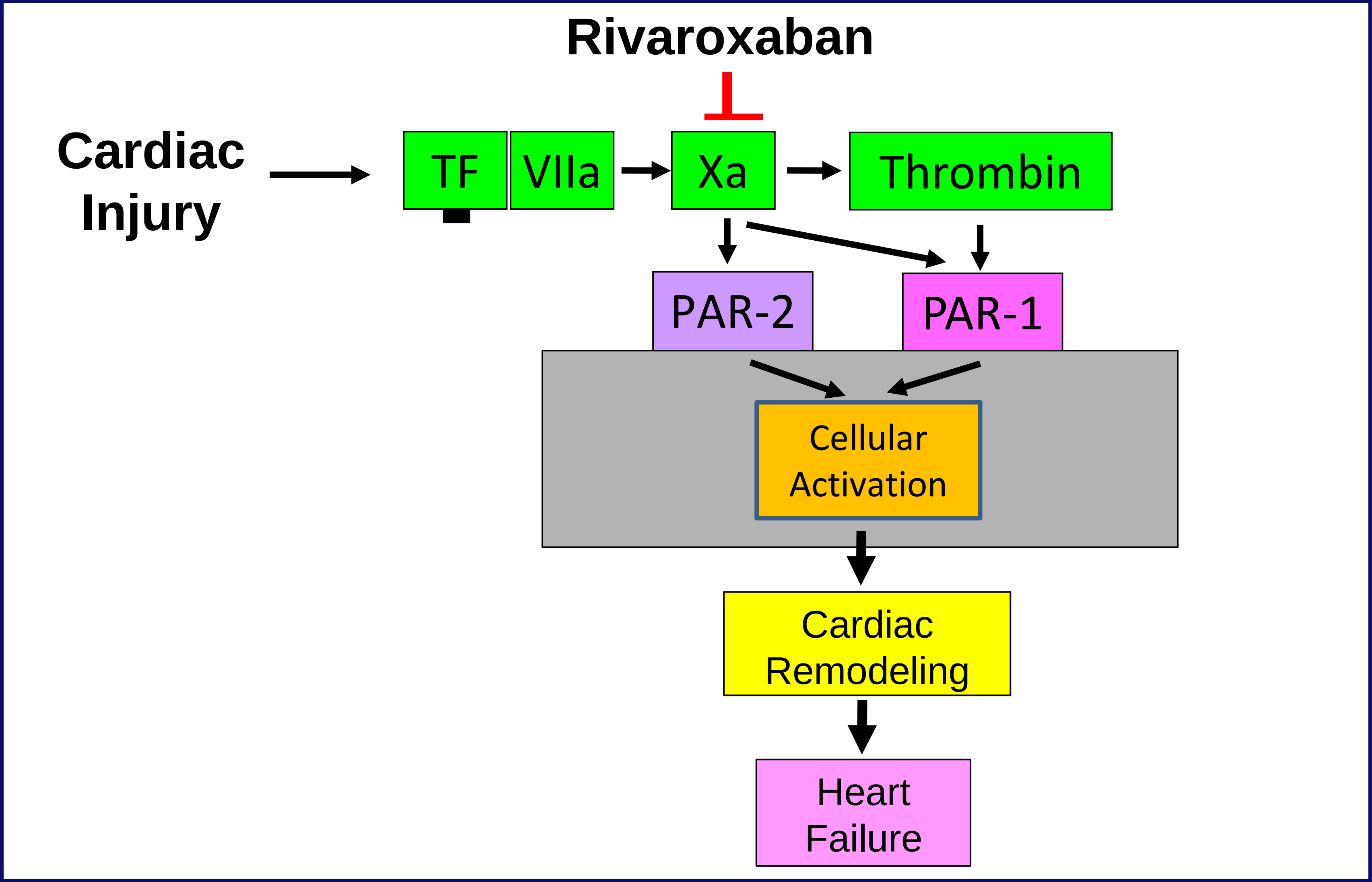
Effect of Rivaroxaban on Cardiac Remodeling and Heart Failure in a Mouse Model of LAD Ligation



28 days after LAD ligation

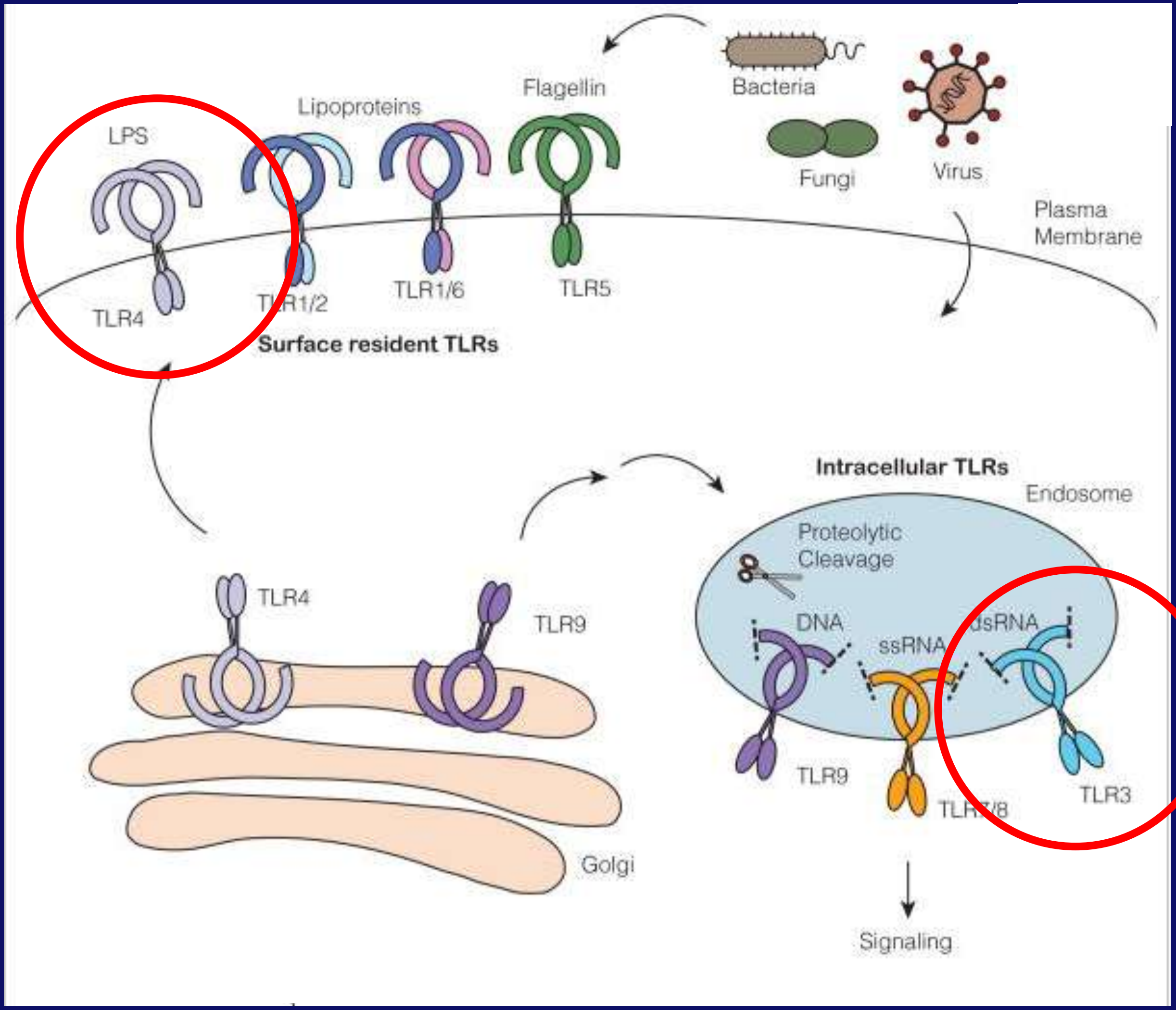
Bode M unpublished results

Effect of the FXa Inhibitor Rivaroxaban on Cardiac Remodeling and Heart Failure



TLRs and PARs in host defense

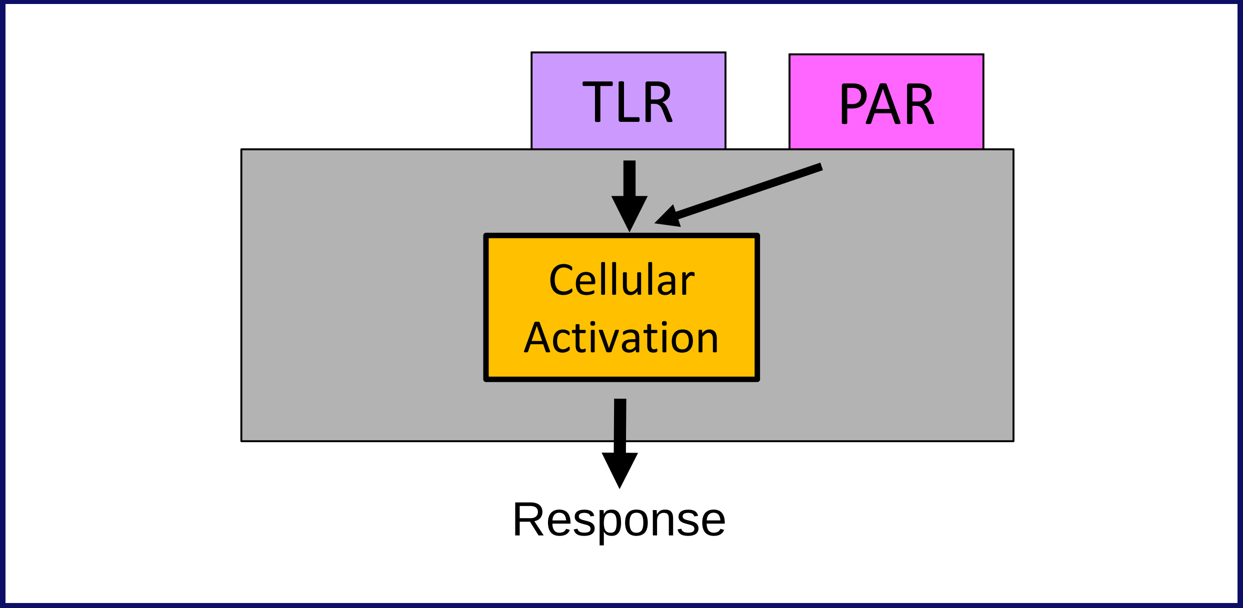
Detection of Microbial Pathogens by the TLR Receptor Family



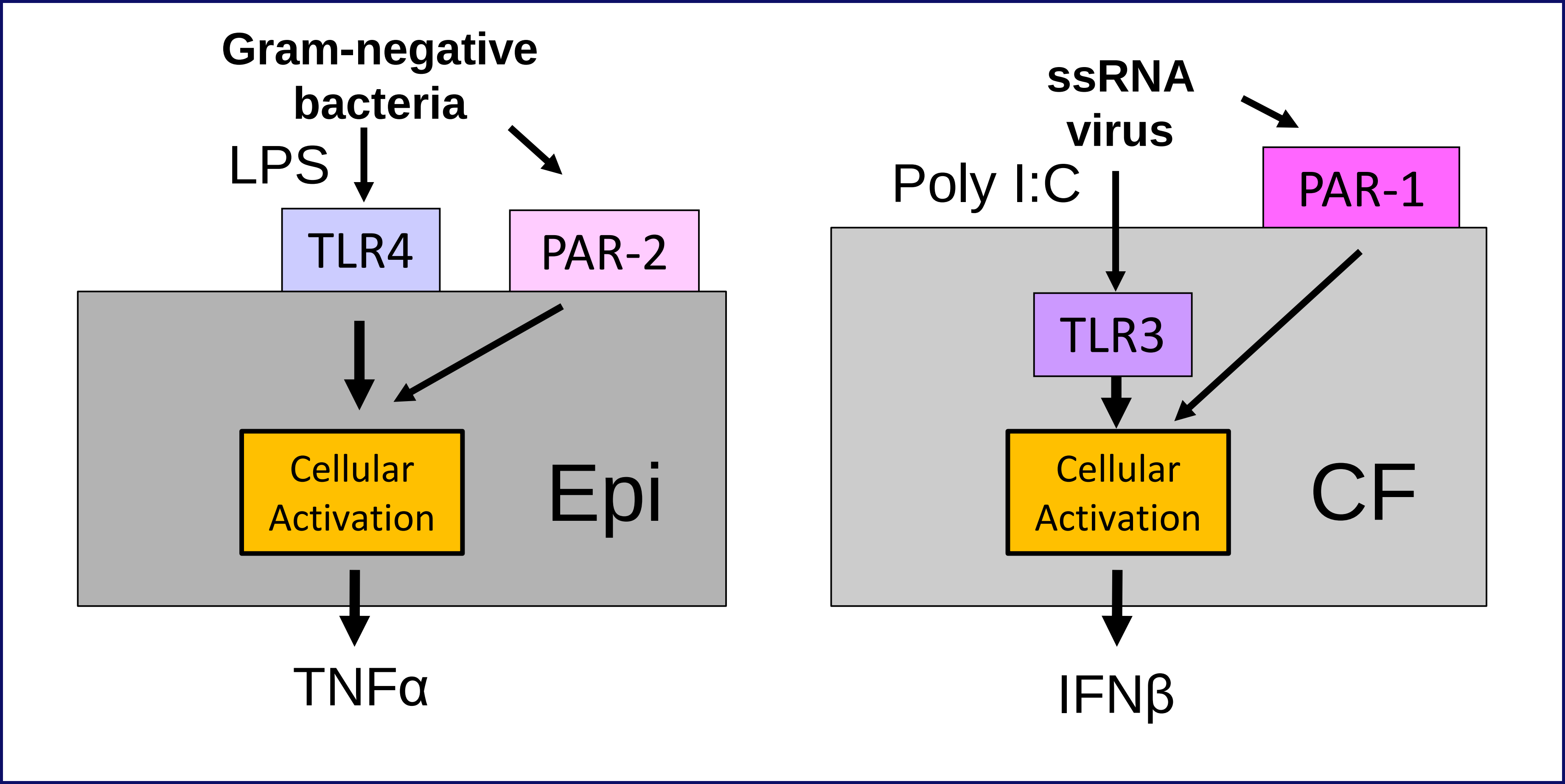
Lee and Barton Trend in Cell Biol 2014

TLRs and PARs as Co-receptors in the Detection of Pathogens

- **It has been proposed that the TLR family and the PAR family of receptors act as a dual-sensor system to detect pathogens.** **(Moretti S et al Mucosal Immunol 2008).**



TLRs and PARs in the Detection of Bacterial and Viral Pathogens



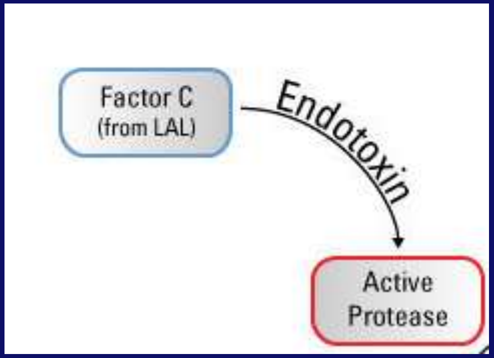
Rallabhandi P et al JBC 2008

Antoniak S et al JCI 2013

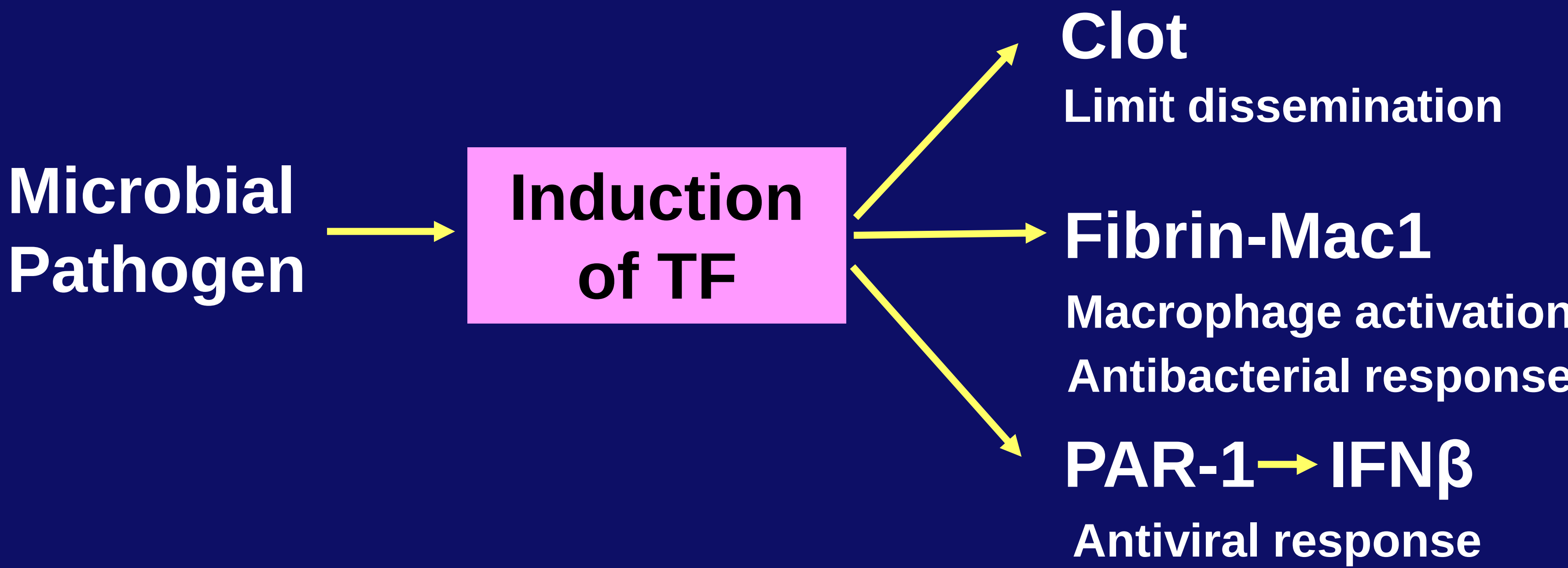
Why is the clotting system activated during microbial infection?

Hemolymph Coagulation

Amebocytes in the horseshoe crab (*Limulus polyphemus*) detect bacterial infection and trigger clotting of the hemolymph. Basis of the Limulus Amebocyte Assay (LAL) for detecting bacterial LPS.



The Clotting Cascade and Host Defense

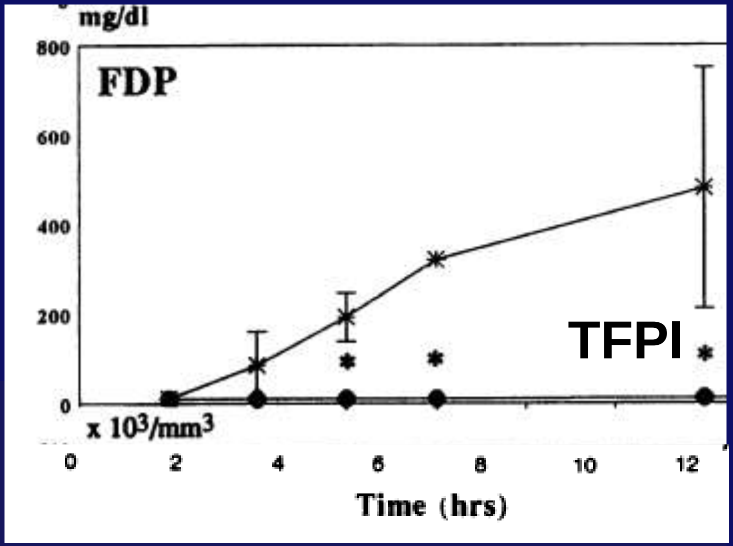


Parallels between Gram-Negative Bacterial Sepsis and Ebola Hemorrhagic Fever

- Localized coagulation is part of the host response to a microbial infection and is protective.
- However, systemic activation of coagulation during sepsis leads to disseminated intravascular coagulation (DIC) that consumes clotting factors (consumptive coagulopathy) and can lead to secondary bleeding.

Petechial rash in a monkey infection with Ebola virus (Giesbert et al J Infect Dis 2007)

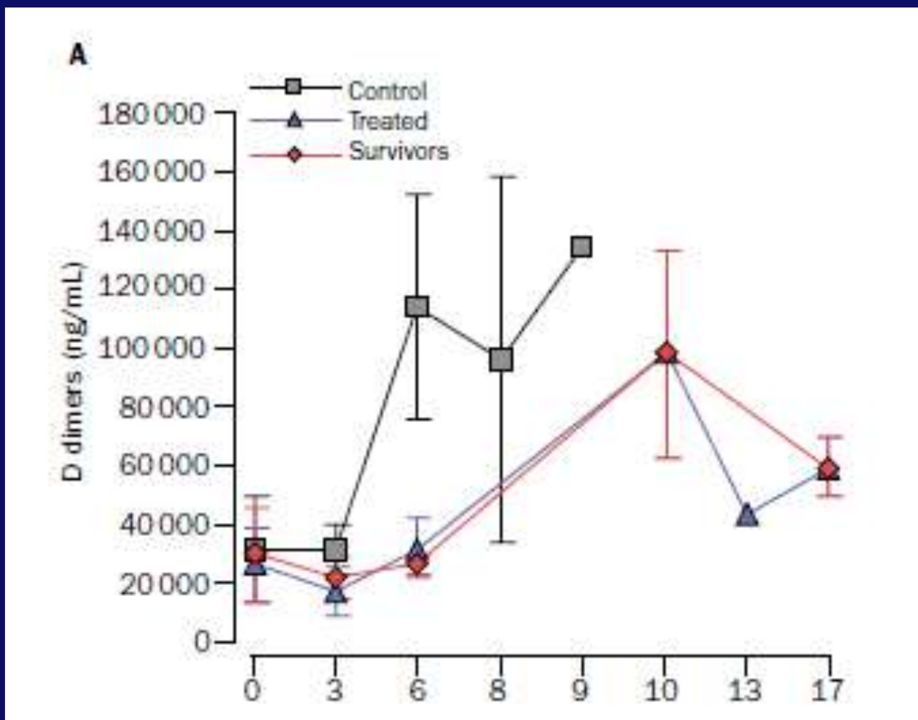
Role of Tissue Factor in a Lethal Baboon Model of *E.coli* Sepsis

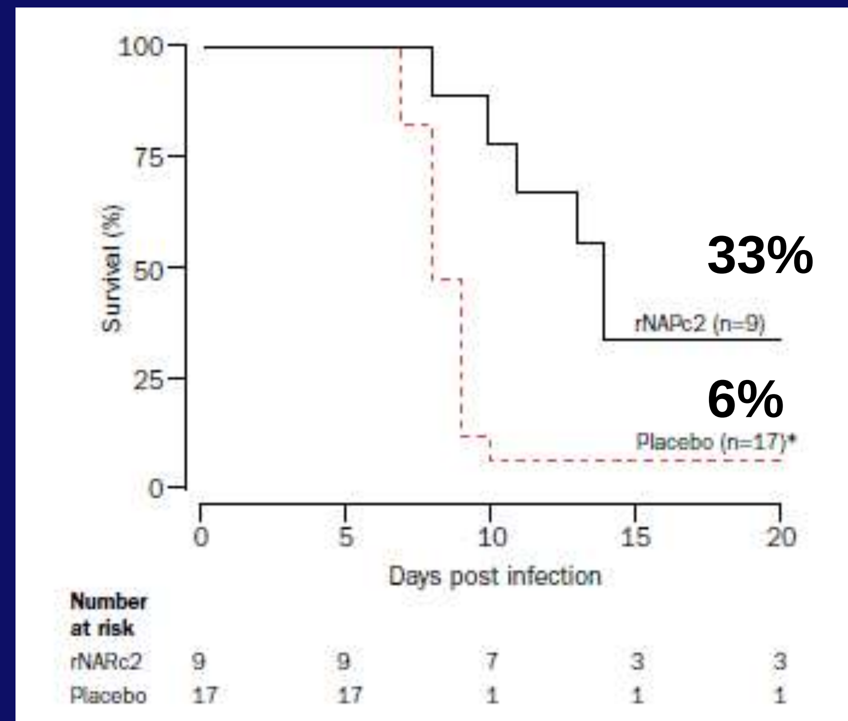


- Controls (n=5) mean survival was 39.9 h.
- Animals (n=5) treated with TFPI inhibitor all survived.

Creasey A et al JCI 1993

Role of Tissue Factor in a Monkey Model of Ebola Virus Infection

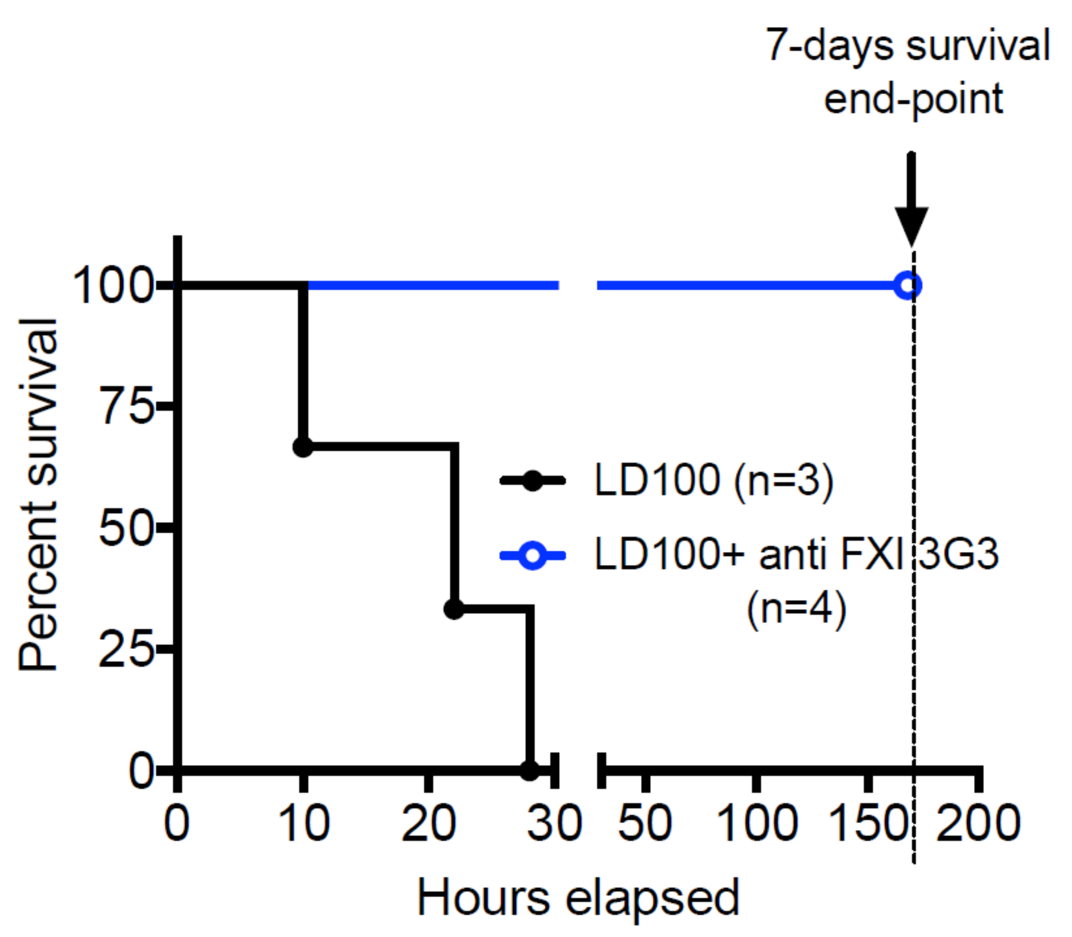




Monkeys treated with NAPc2

Geisbert T et al Lancet 2003

Effect of Inhibition of the Formation of FXIa on Survival in a Baboon Model of *S.aureus* Sepsis



McCarty O et al unpublished data

Cellular Sources of Tissues Factor that Activate Coagulation in Bacterial and Viral Infections

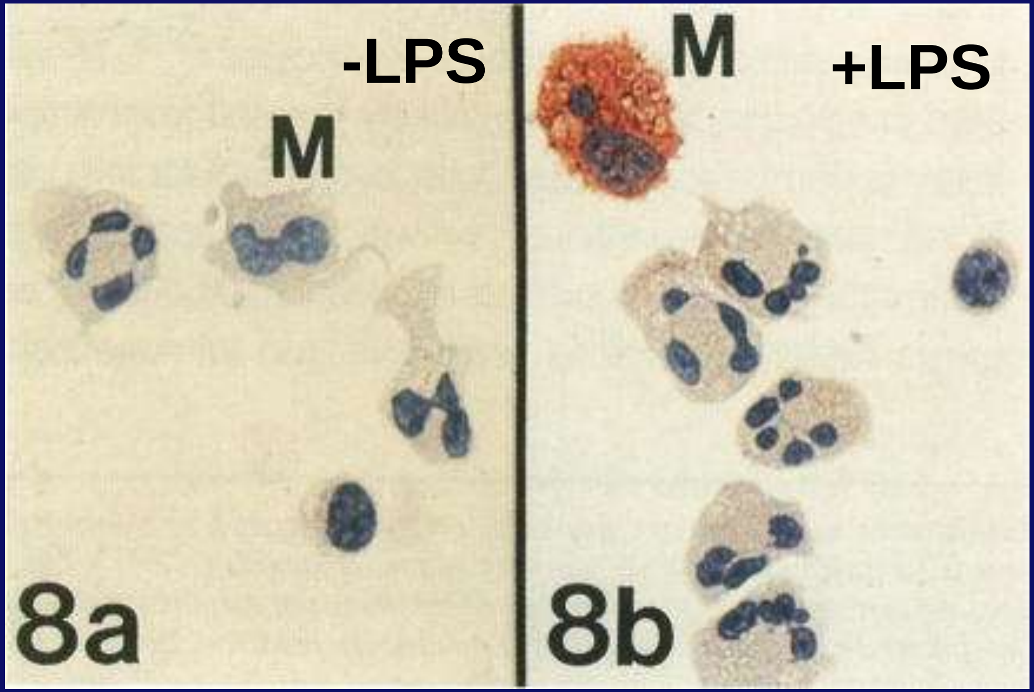
TF induced on monocyte

TF induced on endothelial cells

LPS - used to model Gram-negative sepsis (TLR4)

Poly I:C - used to model infection with a ssRNA virus (TLR3)

LPS Induction of TF Expression in Monocytes

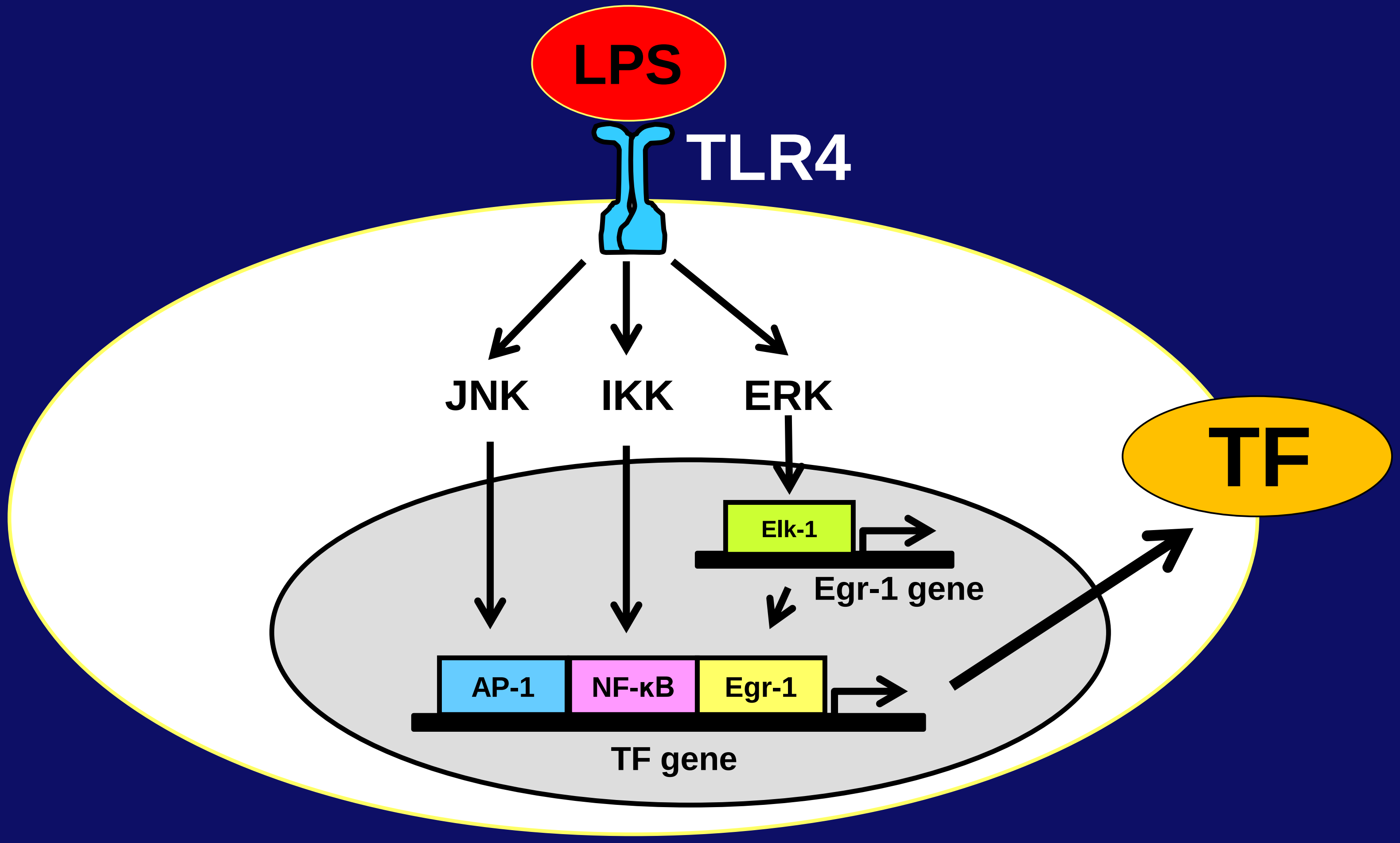


6 hrs

Host Defense

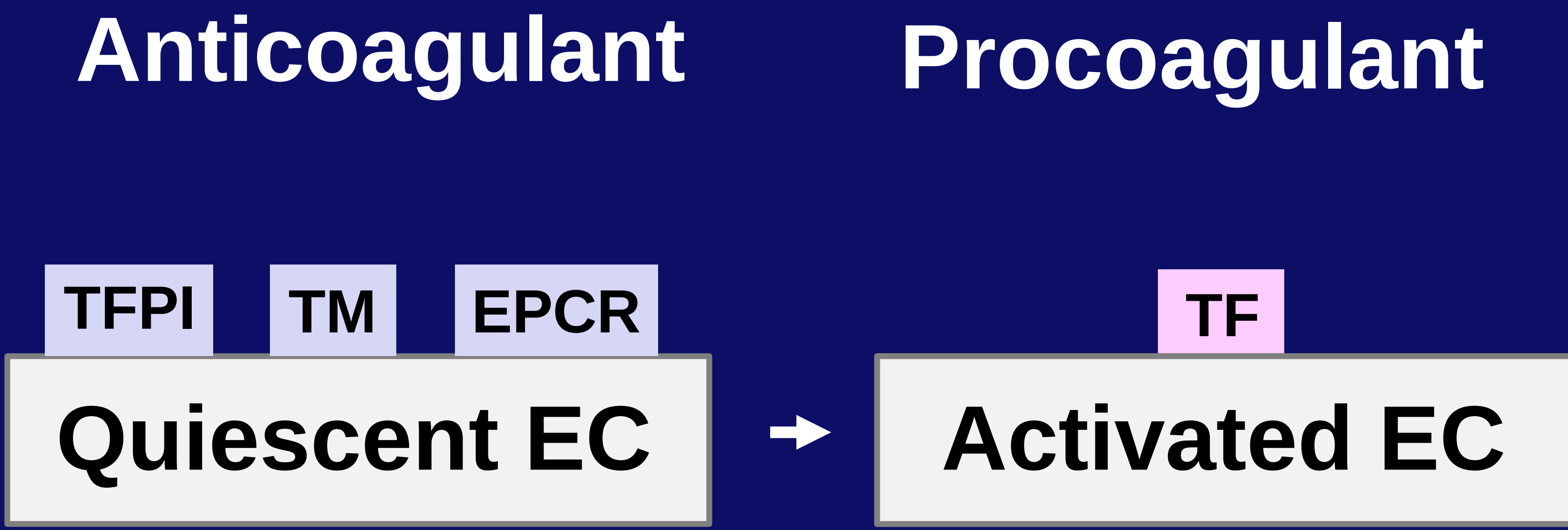
Drake et al AJP 1989

LPS Induction of the TF Gene in Monocytic Cells



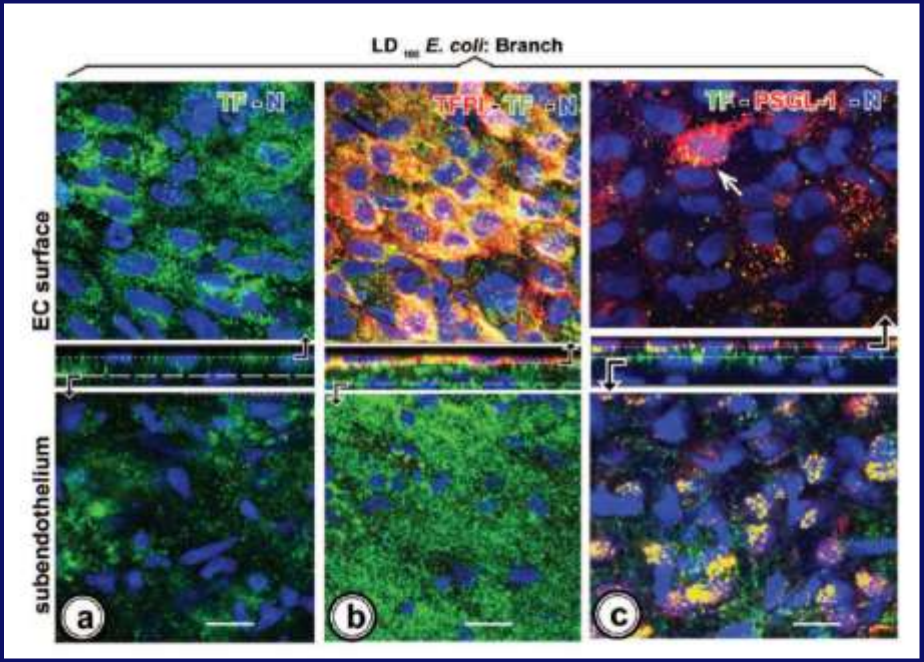
Mackman et al, JEM 1991; Mackman JBC 1994; Parry and Mackman JBC 1994; Oeth et al, MCB 1994; Guha et al, Blood 2002

Expression of Anticoagulant and Procoagulant Proteins by ECs



LPS, inflammatory mediators and various viruses induce TF expression in cultured Ecs. However, there is limited evidence that TF is expression by the endothelium in vivo.

TF Expression in the Endothelium of Arterial Branching Areas in Baboons with *E.coli* Sepsis



TF expression in the endothelium is difficult to detect in septic baboons, and may be due to binding of monocyte-derived TF^{+} MVs

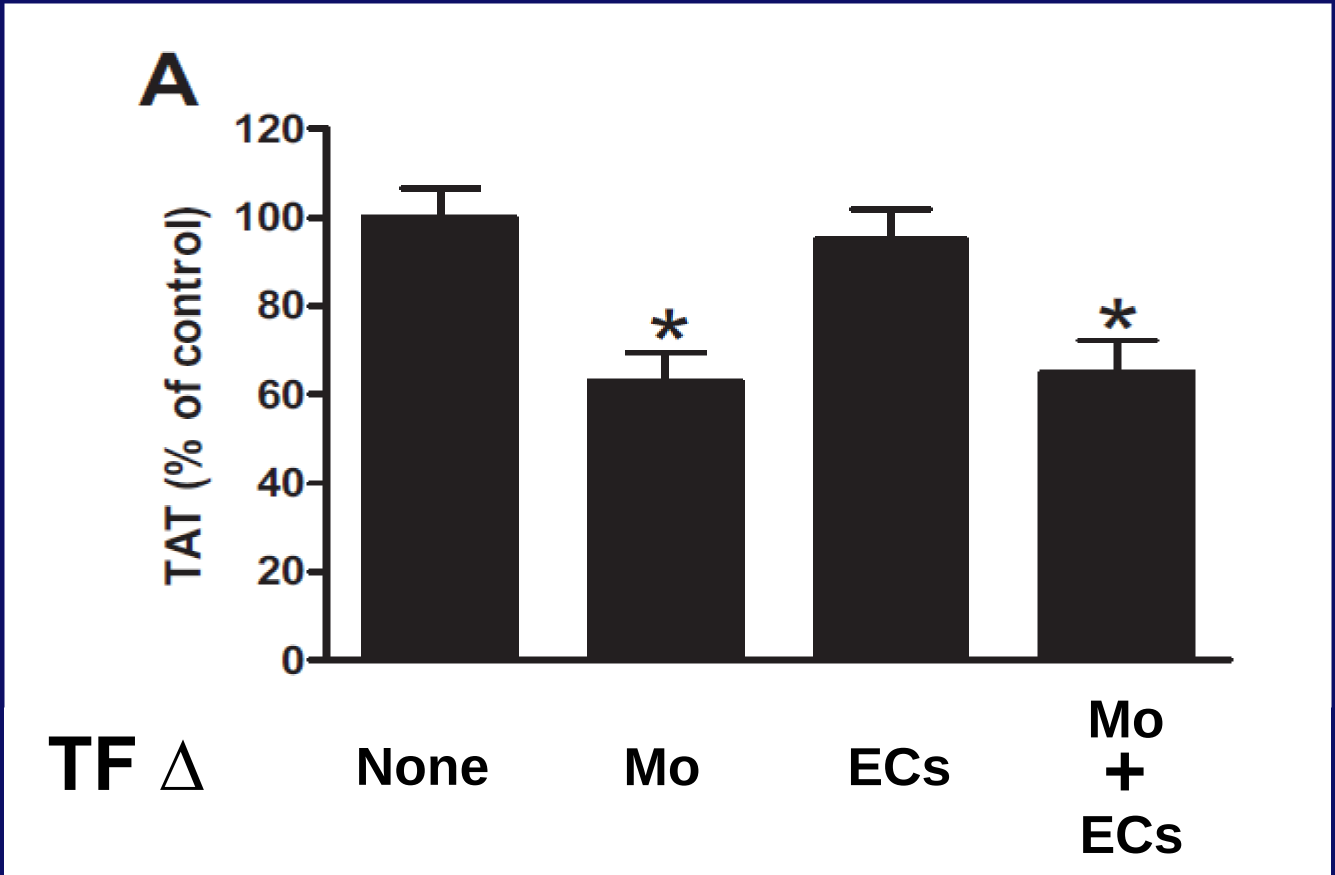
Lupu C et al Am J Path 2005

Role of Monocyte and Endothelial Cell TF in the Activation of Coagulation in a Mouse Model of Endotoxemia

- **$TF^{fl/fl}$,LysMcre mice (deletion of TF in myeloid cell), decrease in TAT.**
- **$TF^{fl/fl}$,Tie2Cre mice (deletion of TF in ECs and hematopoietic cells), decrease in TAT.**
- **Transplant $TF^{fl/fl}$,Tie2Cre mice with WT BM to look at contribution of EC TF.**

Pawlinski R et al Blood 2010

Role of TF Expression by Monocytes and EC in the Activation of Coagulation in a Mouse Endotoxemia Model



Model – LPS (5 mg/kg) IP for 8 h

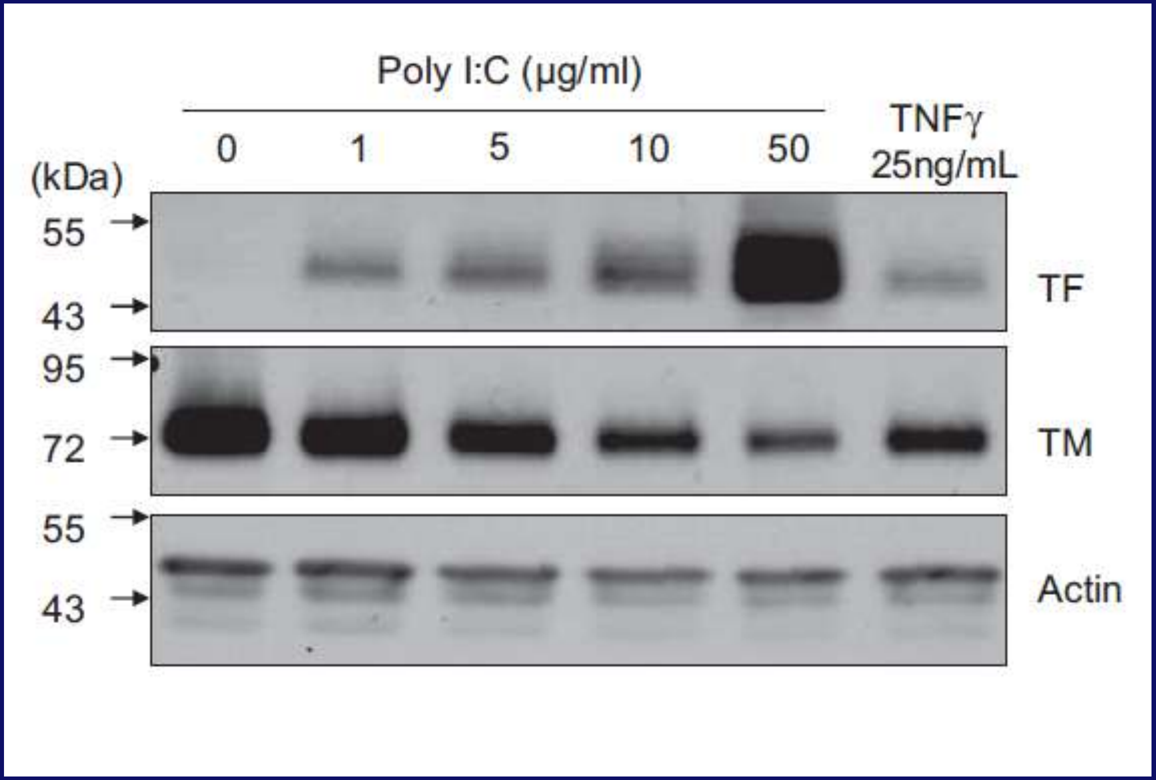
Pawlinski R et al Blood 2010

Conclusion

Monocytes are a major source of inducible TF expression that activates the coagulation cascade in endotoxemia

Poly I:C Induction of TF Expression in HUVECs

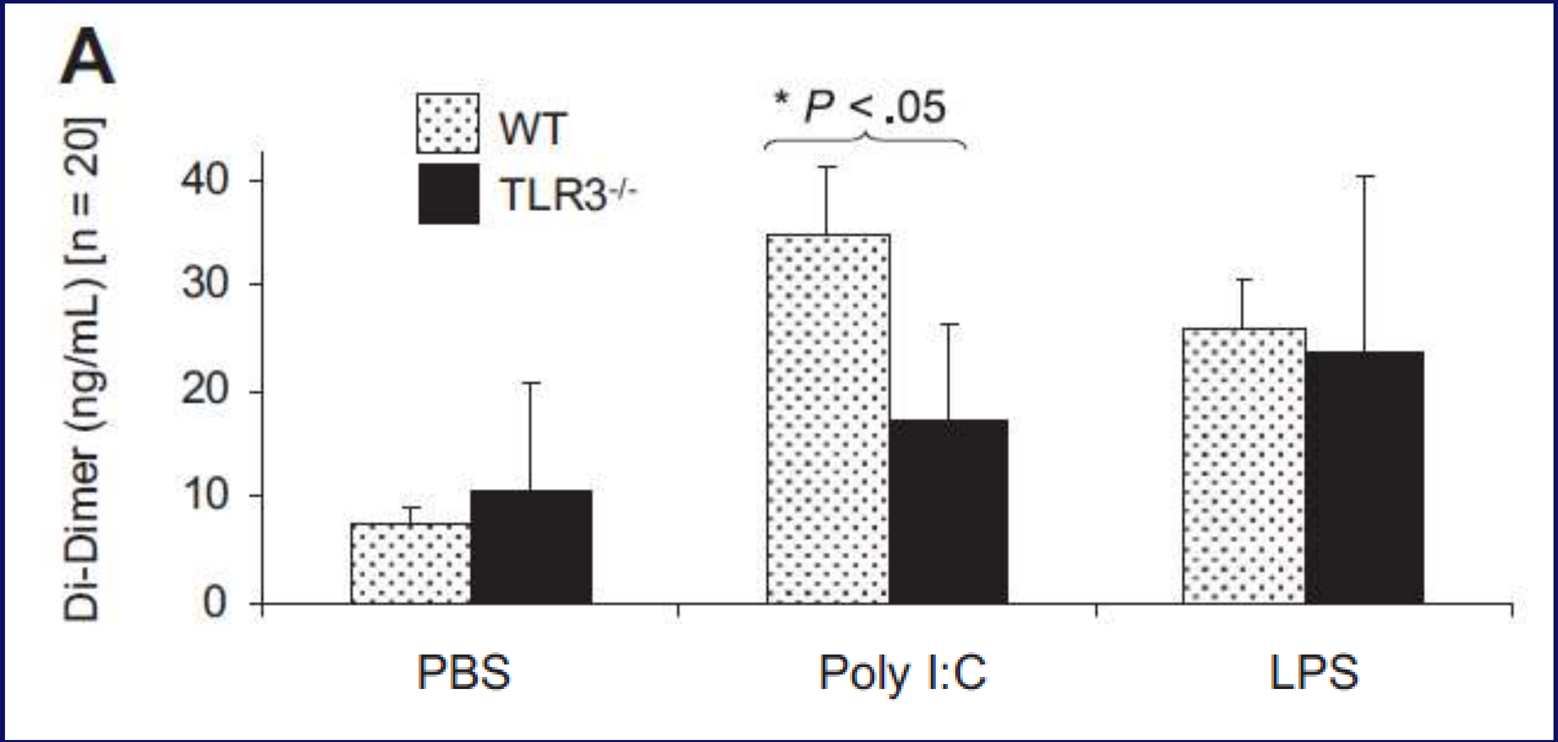
Saravanan Subramanian



Poly I:C does not induce TF expression in monocytes

Kanse S et al Blood 2009

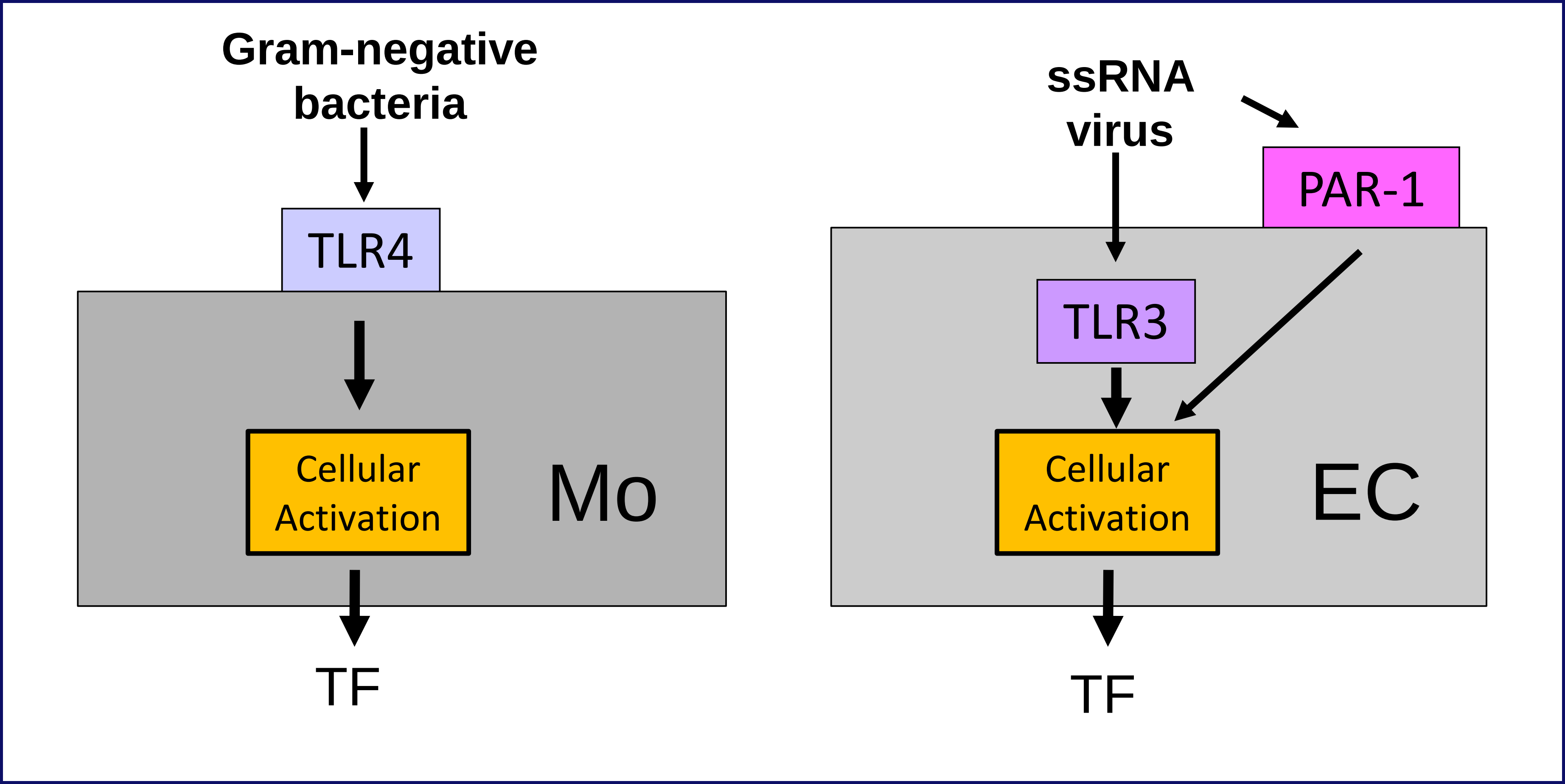
Role of TLR3 in Poly I:C Activation of Coagulation in Mice



We are currently determining the role of TF expression by monocytes and ECs in poly I:C activation of coagulation in mice

Kanse S et al Blood 2009

TF Expression by Monocytes and ECs during Bacterial and Viral Infections



Rallabhandi P et al JBC 2008

Antoniak S et al JCI 2013

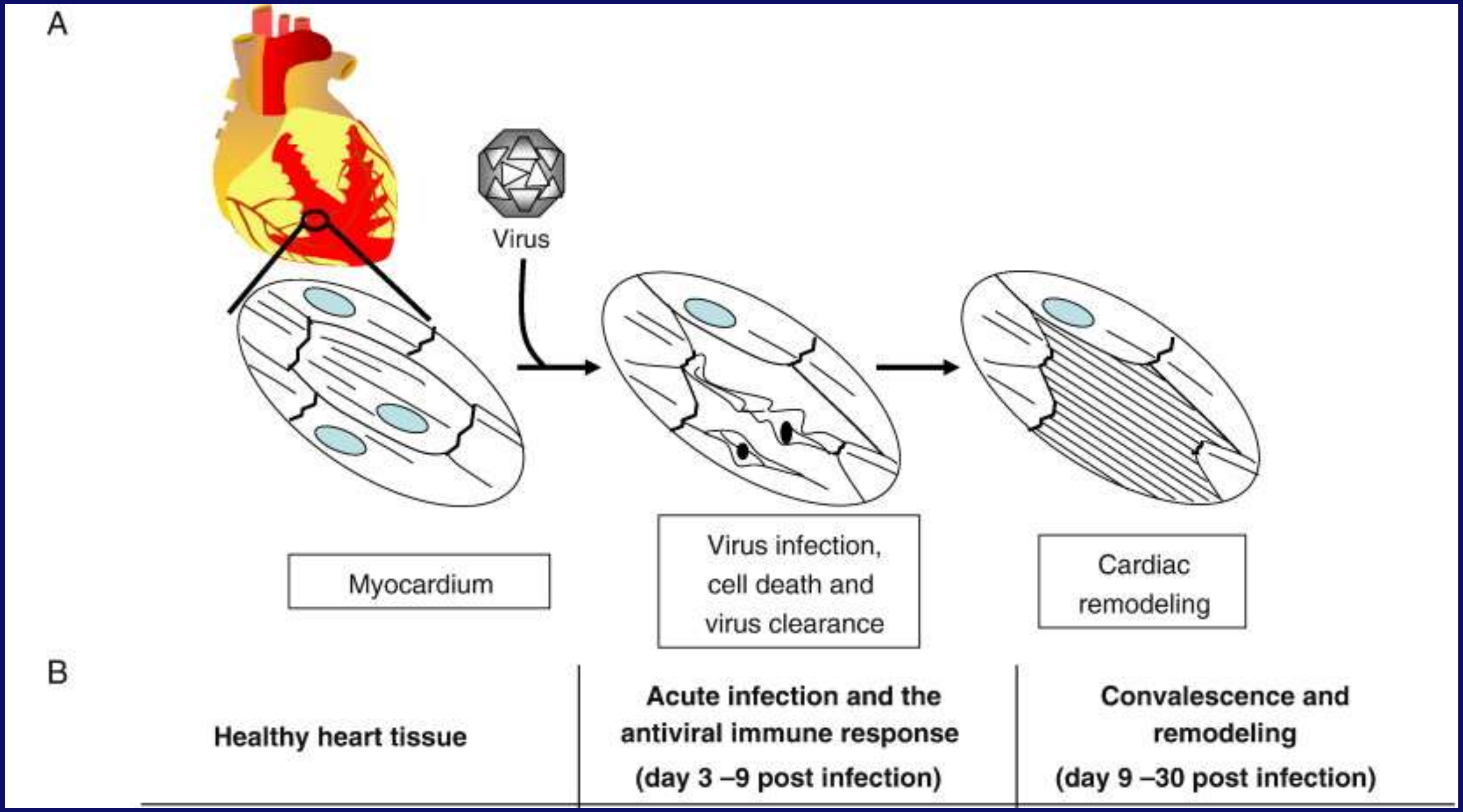
Role of the TF-thrombin-PAR-1 pathway in the antiviral response

Outline

- **Coxsackievirus B3 virus model**
- **Influenza A virus model**

Viral Myocarditis

Accounts for up to 20% of sudden cardiac death among young people (<40 years of age)



Mouse Model of CVB3-induced Myocarditis

- Coxsackievirus B3 (CVB3) = cardiotrophic ssRNA(+) virus

Silvio Antoniak

CVB3 ($1x10^5$ PfU i.p.)

0d **2-4d** **8d** **28d** **time points**

early acute chronic

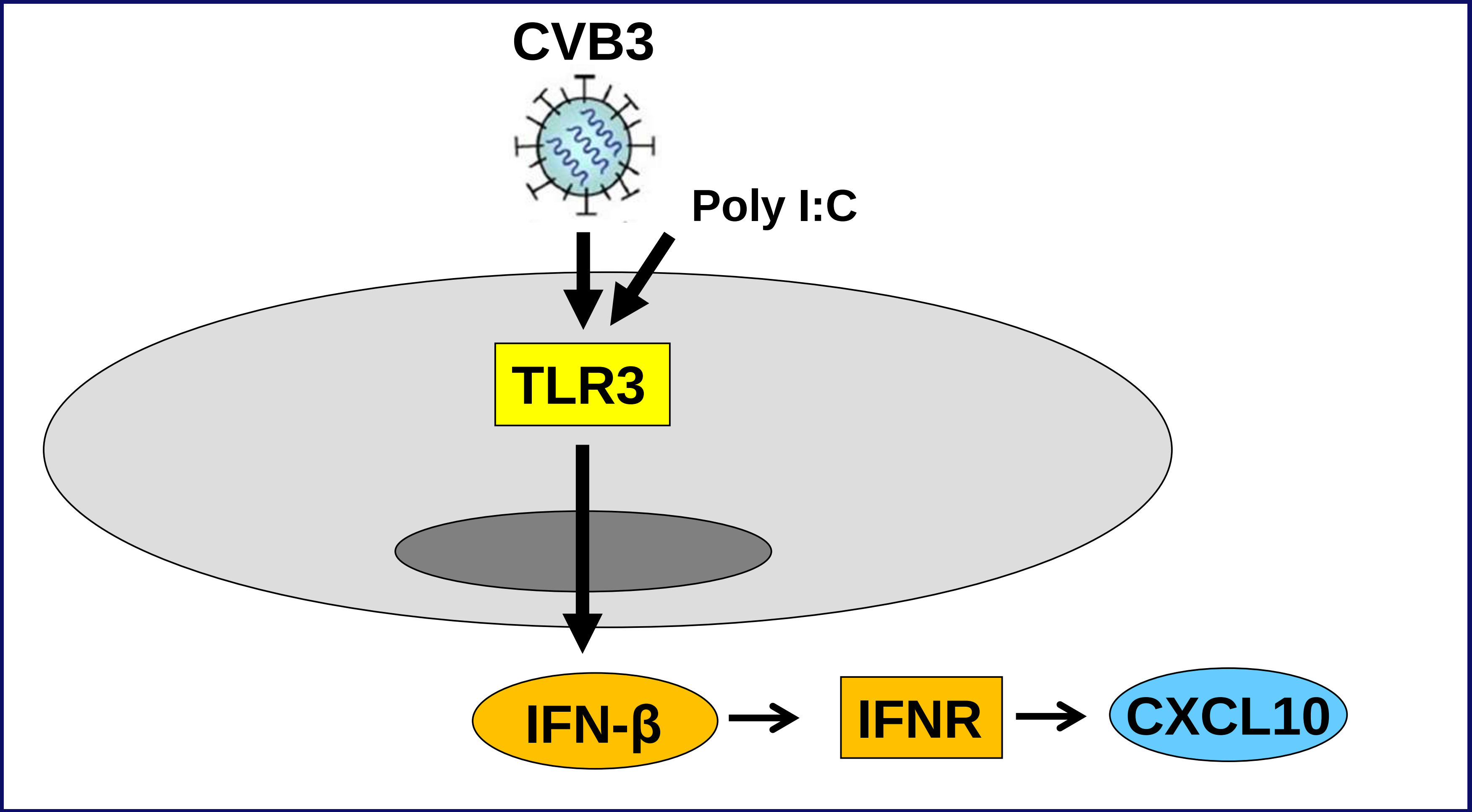
Immune response
IFNβ and CXCL10

Cardiac injury and
viral replication

Heart failure

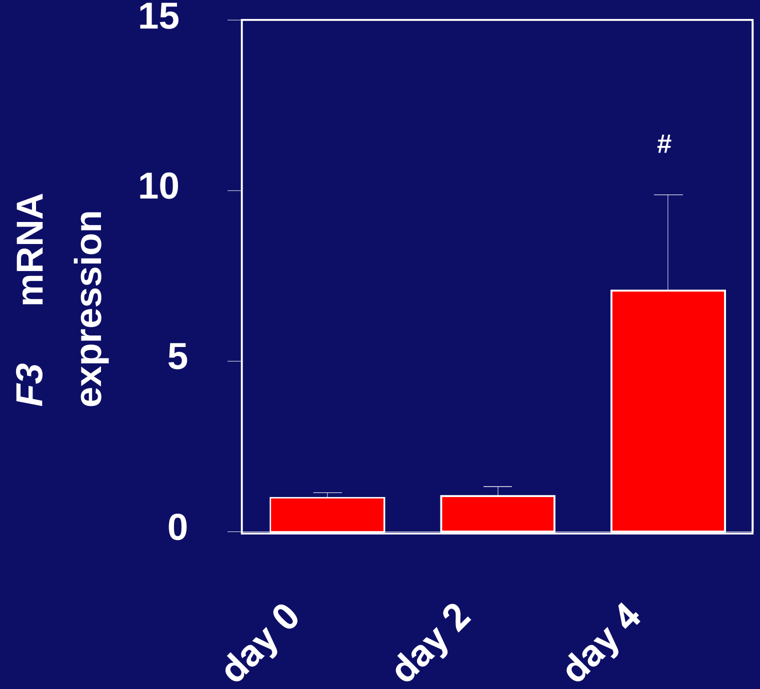
Silvio Antoniak

Innate Immune Response to CVB3 Infection

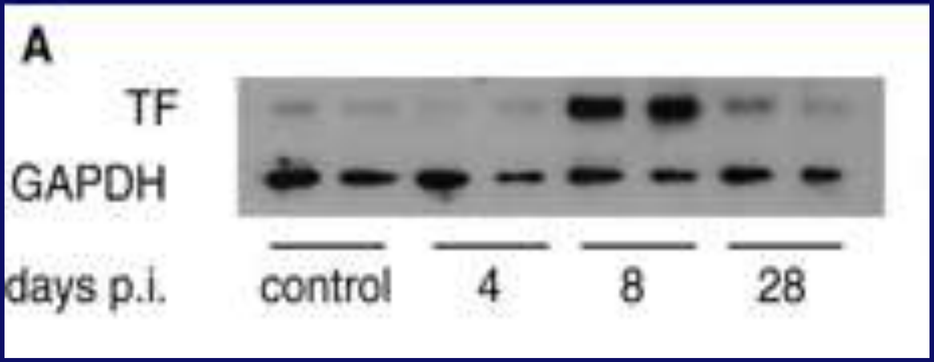


CVB3 Infection of Mice Increases TF Expression in the Heart

TF mRNA



TF Protein

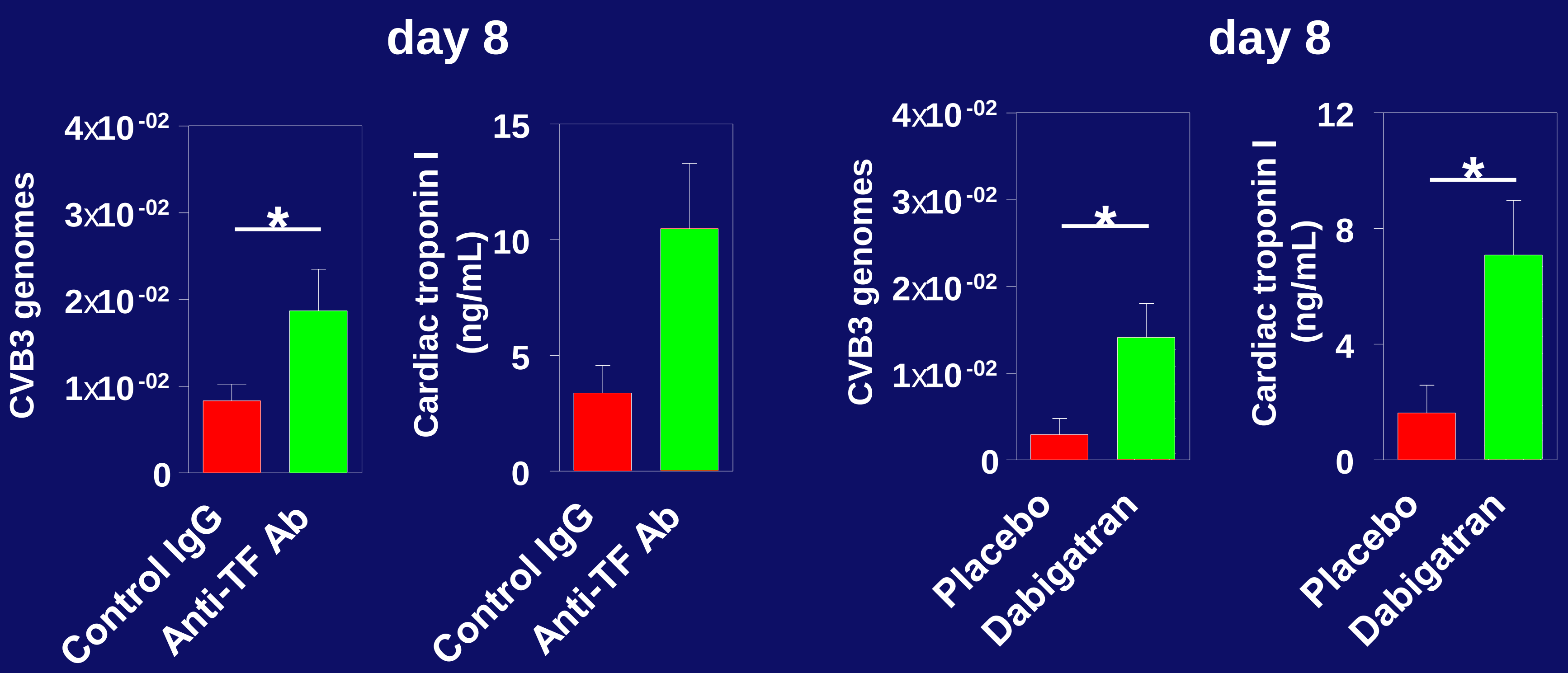


Antoniak S et al JMCC 2008; Antoniak S et al JCI 2013

Effect of Inhibition of TF or Thrombin on CVB3-induced Myocarditis

Anti-TF Ab

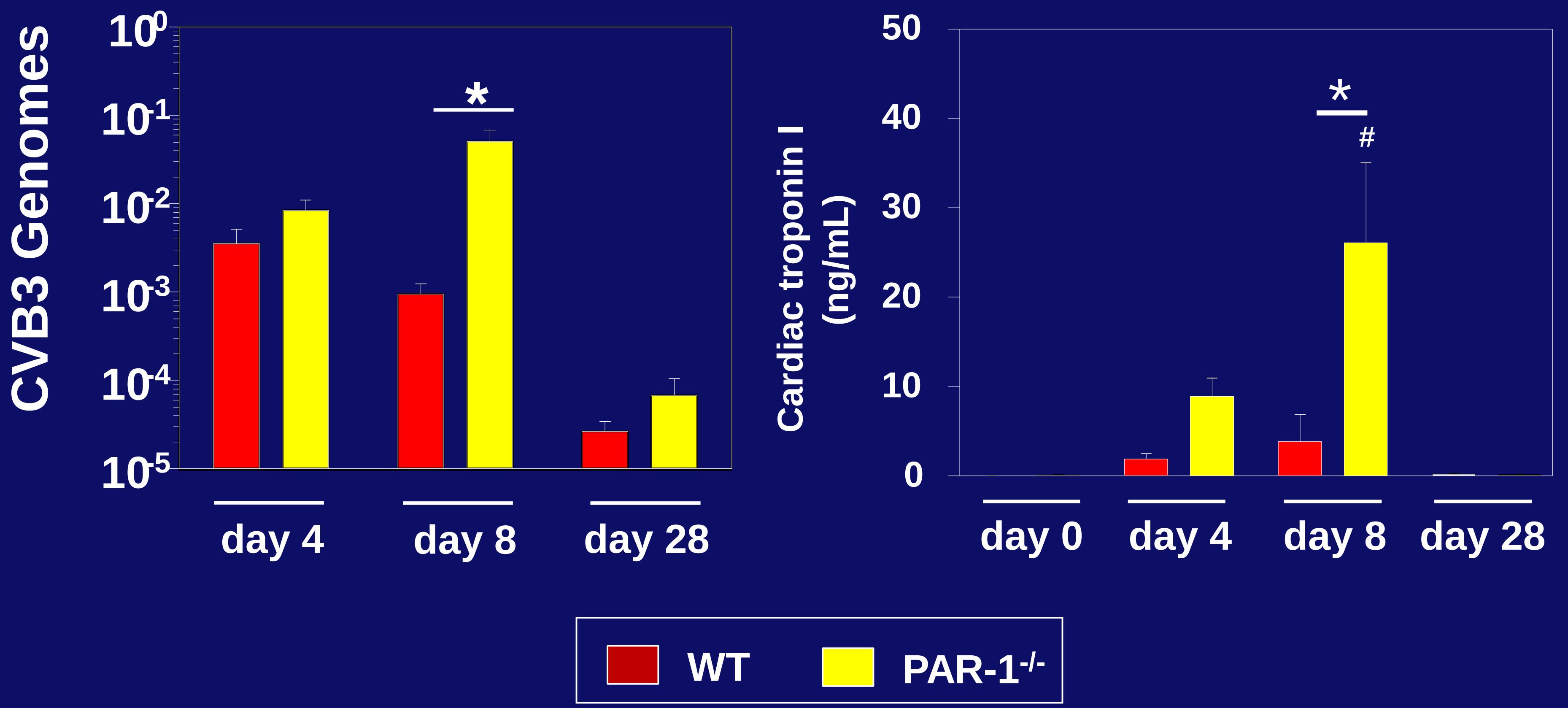
Dabigatran



*P<0.05
Student's *t*-test

Antoniak et al, JCI 2013

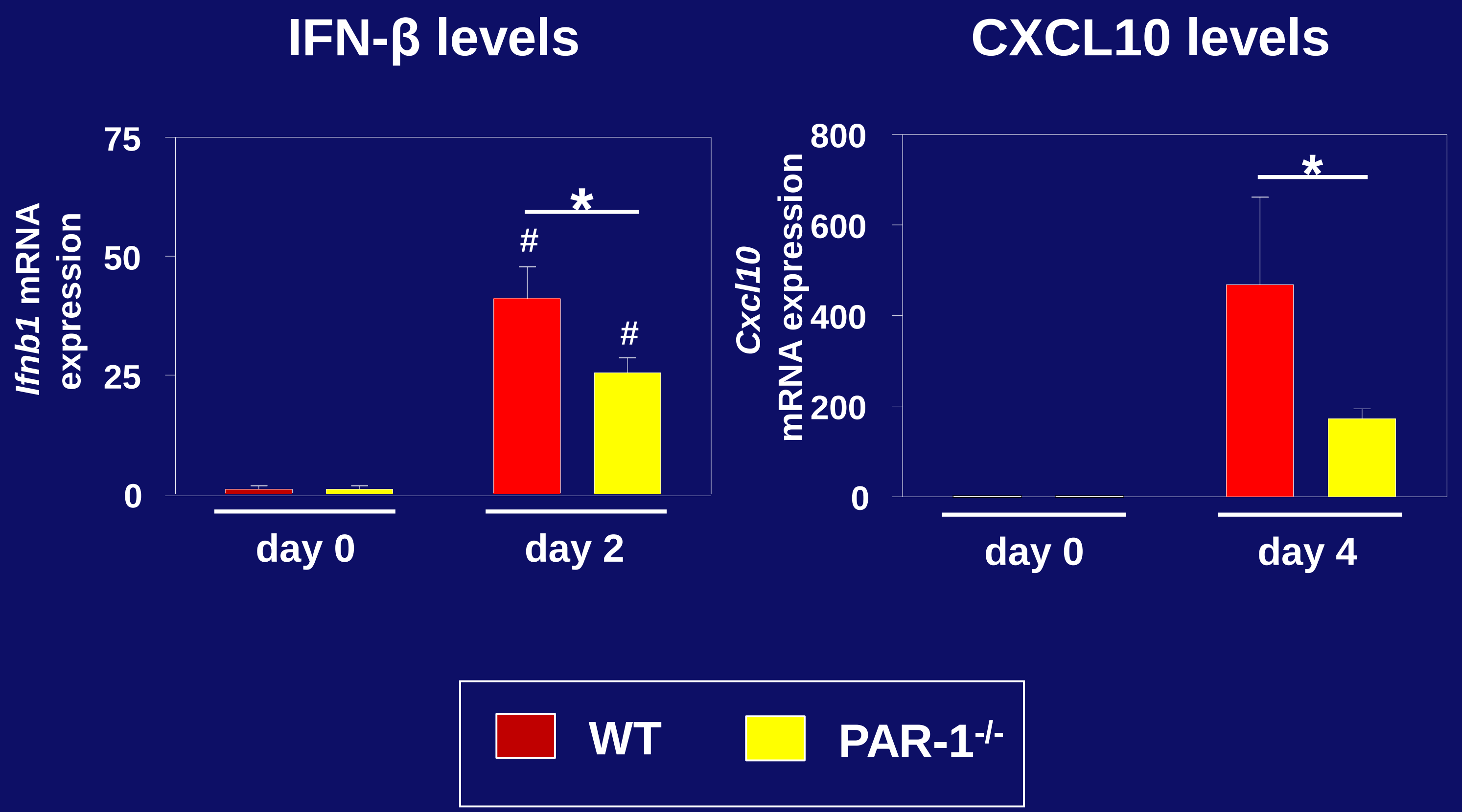
PAR-1 Deficiency is Associated with Increased Viral Load and Cardiac Injury after CVB3 infection



*P<0.05
Two-Way ANOVA

Antoniak et al, JCI 2013

PAR-1 Deficiency is Associated with Reduced IFN-β and CXCL10 expression After CVB3 Infection



*P<0.05; #P<0.05 vs. day 0
Two-Way ANOVA

Antoniak et al, JCI 2013

BMT studies indicated that PAR-1 on both hematopoietic and non-hematopoietic cells contributed to the protection from CVB3 infection.

Establishing a Cell Model to Study the Role of PAR-1 in CVB3 Infection

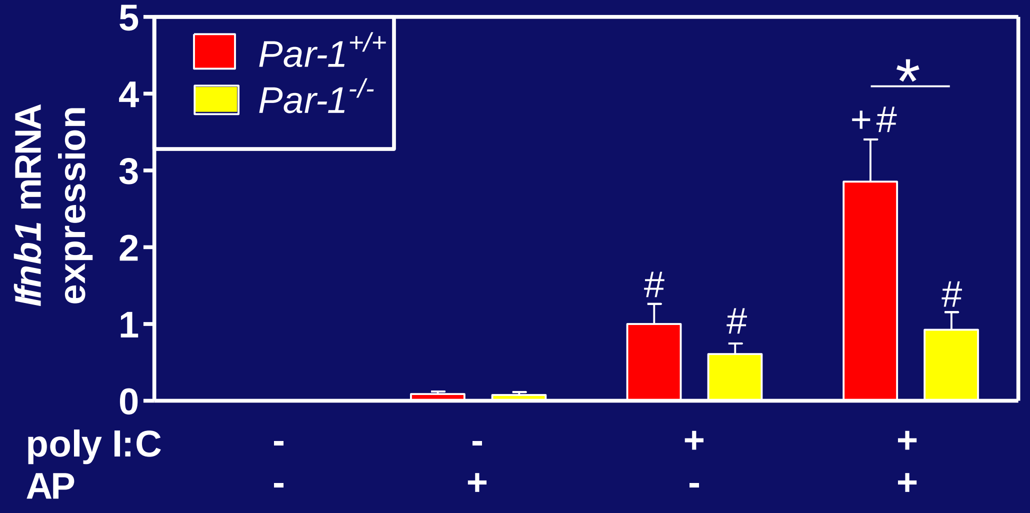
Poly I:C (dsRNA mimetic was used to stimulate TLR3.

PAR-1 agonist peptide (AP) was used to stimulate PAR-1.

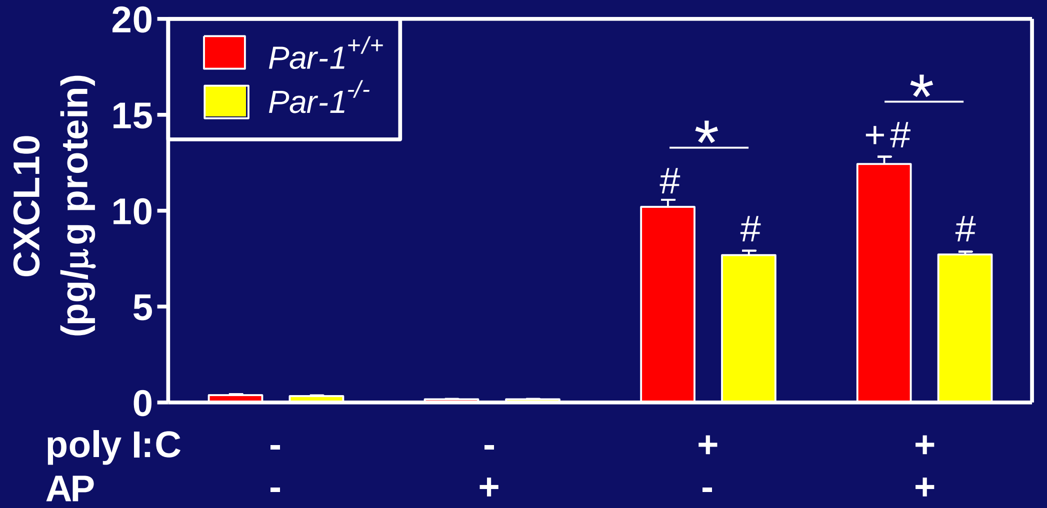
Use mouse cardiac fibroblasts.

PAR-1 Activation Enhances Poly I:C Induction of IFN-β and CXCL10 Expression in Cardiac Fibroblasts

IFNβ mRNA

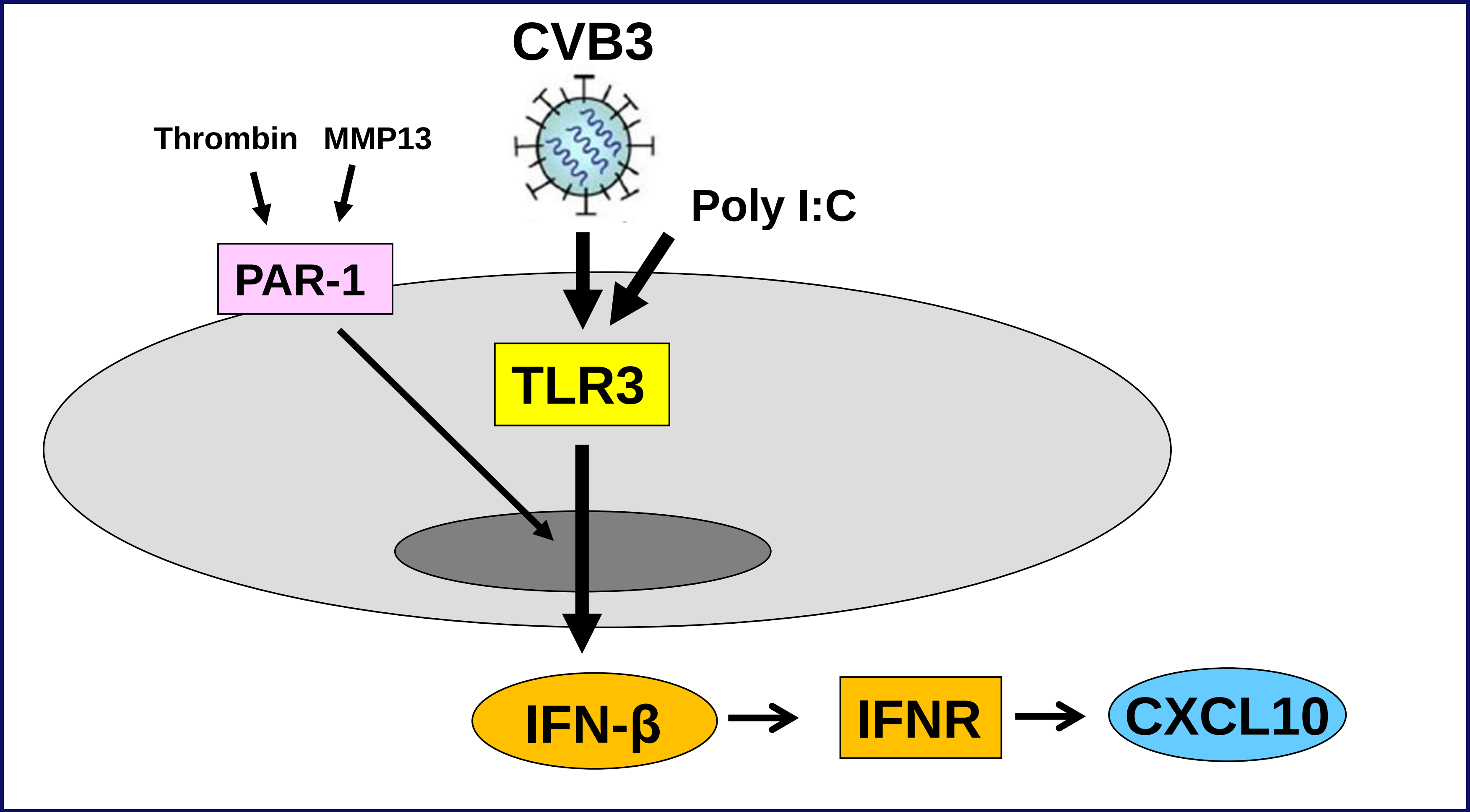


CXCL10 Protein

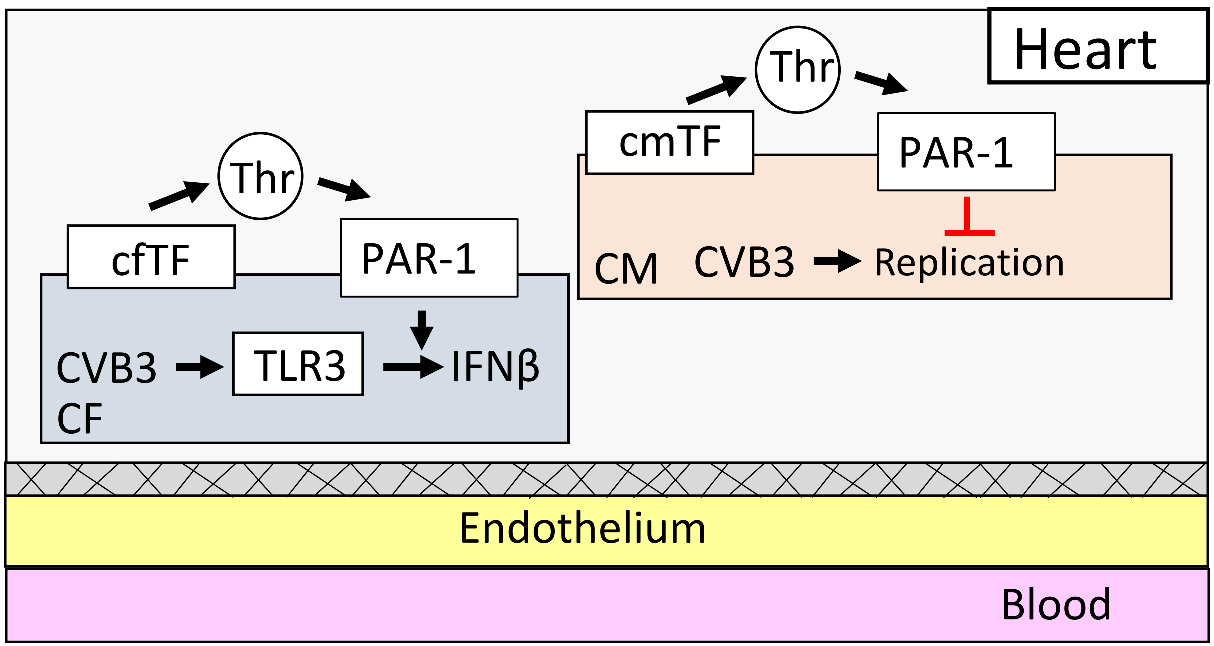


Antoniak et al, JCI 2013

Innate Immune Response to CVB3 Infection



Roles of the TF-Thrombin-PAR-1 Pathways in CVB3-induced Myocarditis



Influenza A Infection

- Influenza A (IAV) is a respiratory pathogen
- IAV causes acute infection of the upper respiratory tract.
- Severe IAV infection can cause infection of the lower respiratory tract, resulting in viral pneumonia.
- Damage to the barrier formed by epithelial and endothelial cells in the pulmonary alveolus leads to respiratory dysfunction.

Mouse Model of Influenza A Infection (IAV)

Kohei Tatsumi

- Influenza A is a ssRNA virus.
- Influenza A/Puerto Rico/8/1934 H1N1.
- Mouse adapted and highly pathologic.
- Infected intranasally.

Silvio Antoniak and Kohei Tatsumi

Mouse Model of Influenza A Infection

H1N1 (0.02 HAU, 50μL i.n.)

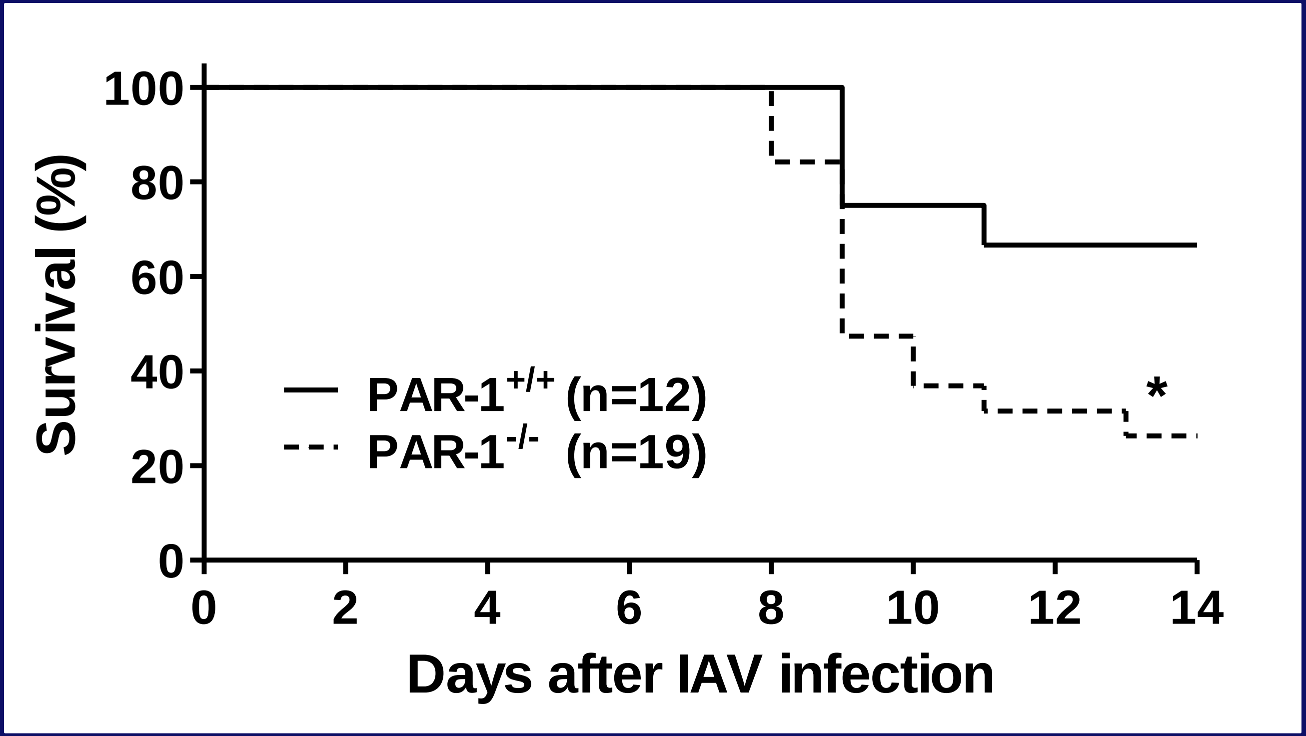
0d — **3-7d** — **14d** time points

3-7d: Inflammation, viral load and vascular permeability

14d: Survival and weight loss

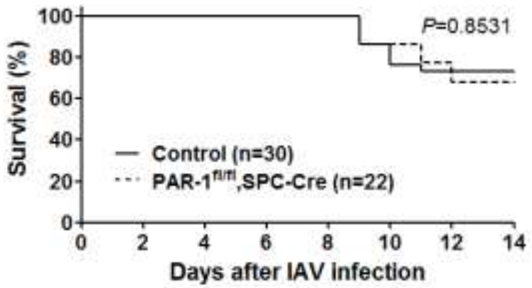
Model leads to ~20% mortality

PAR-1 Deficiency is Associated with Increased Mortality after Influenza A Infection

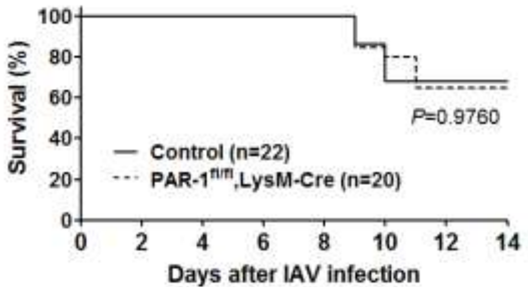


Tatsumi K et al. unpublished data

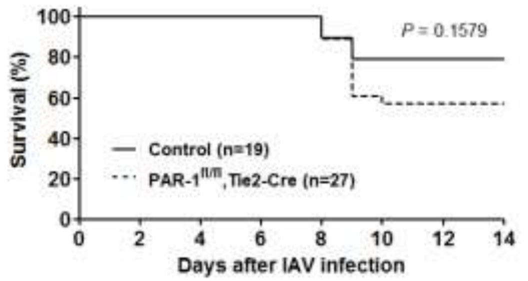
Effect of Deletion of PAR-1 in Different Cell Types on IAV Infection



PAR-1 deletion in lung epithelial cells



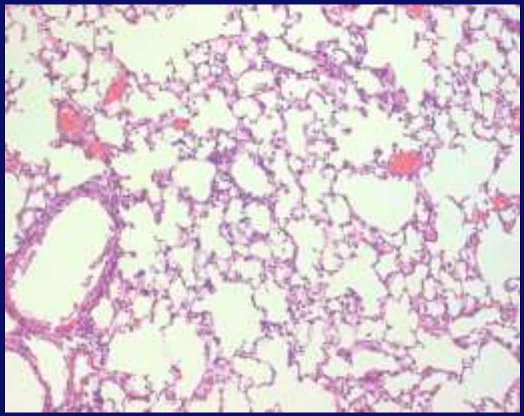
PAR-1 deletion in myeloid cells



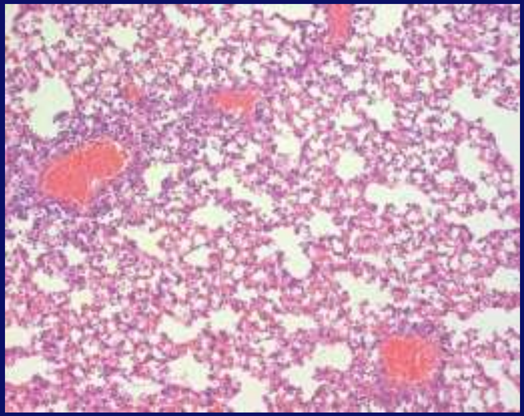
PAR-1 deletion in ECs and hematopoietic cells

PAR-1 Deficiency is Associated with Increased Levels of Parenchymal Cellularity after Influenza A Infection

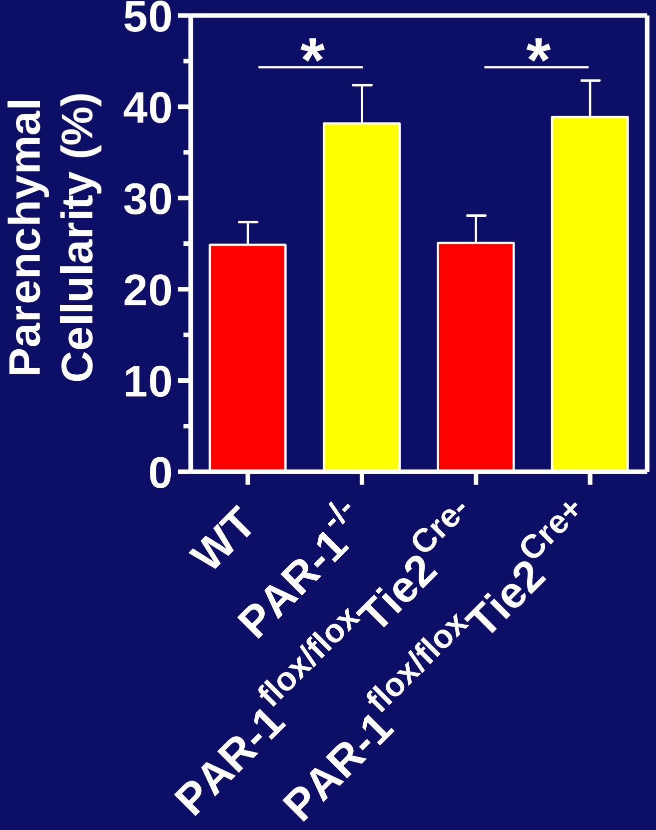
WT



PAR-1$^{-/-}$



4 days

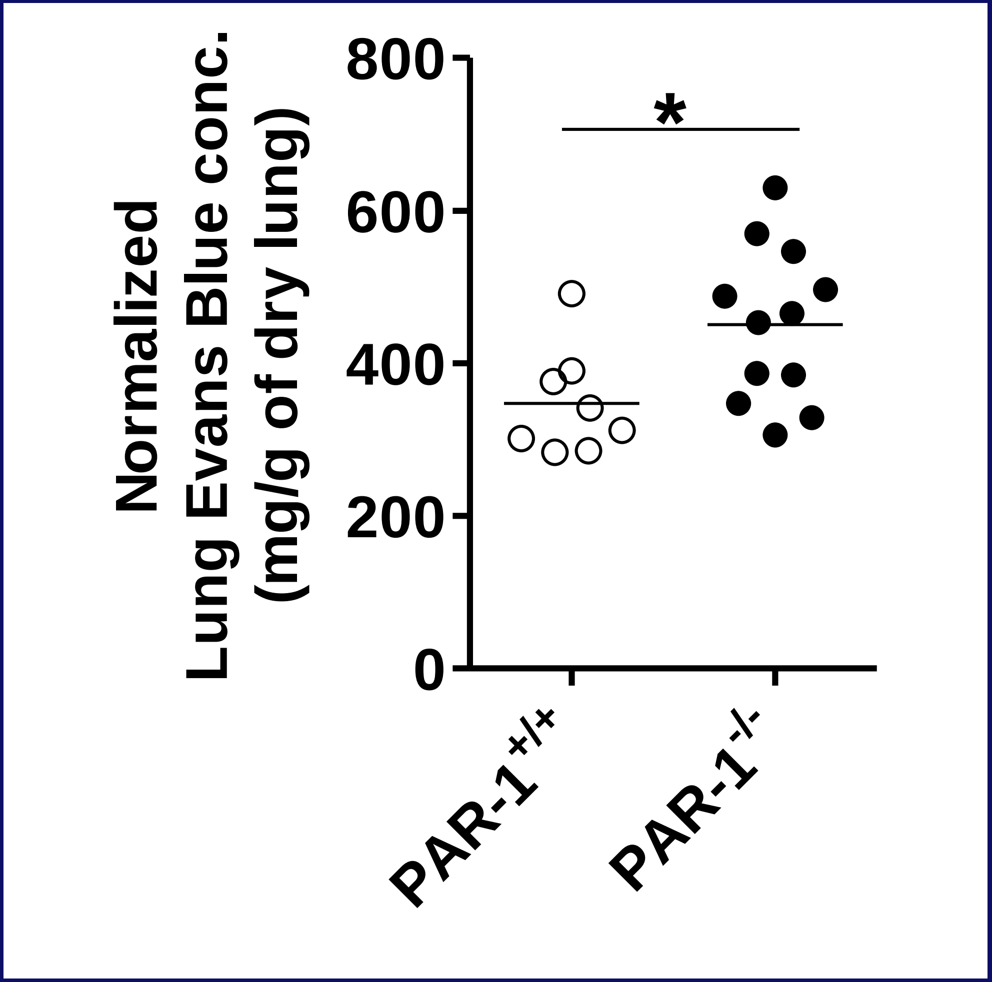


J. Palumbo unpublished data

Hypothesis

PAR-1 expression on the endothelium is barrier protective during influenza A infection.

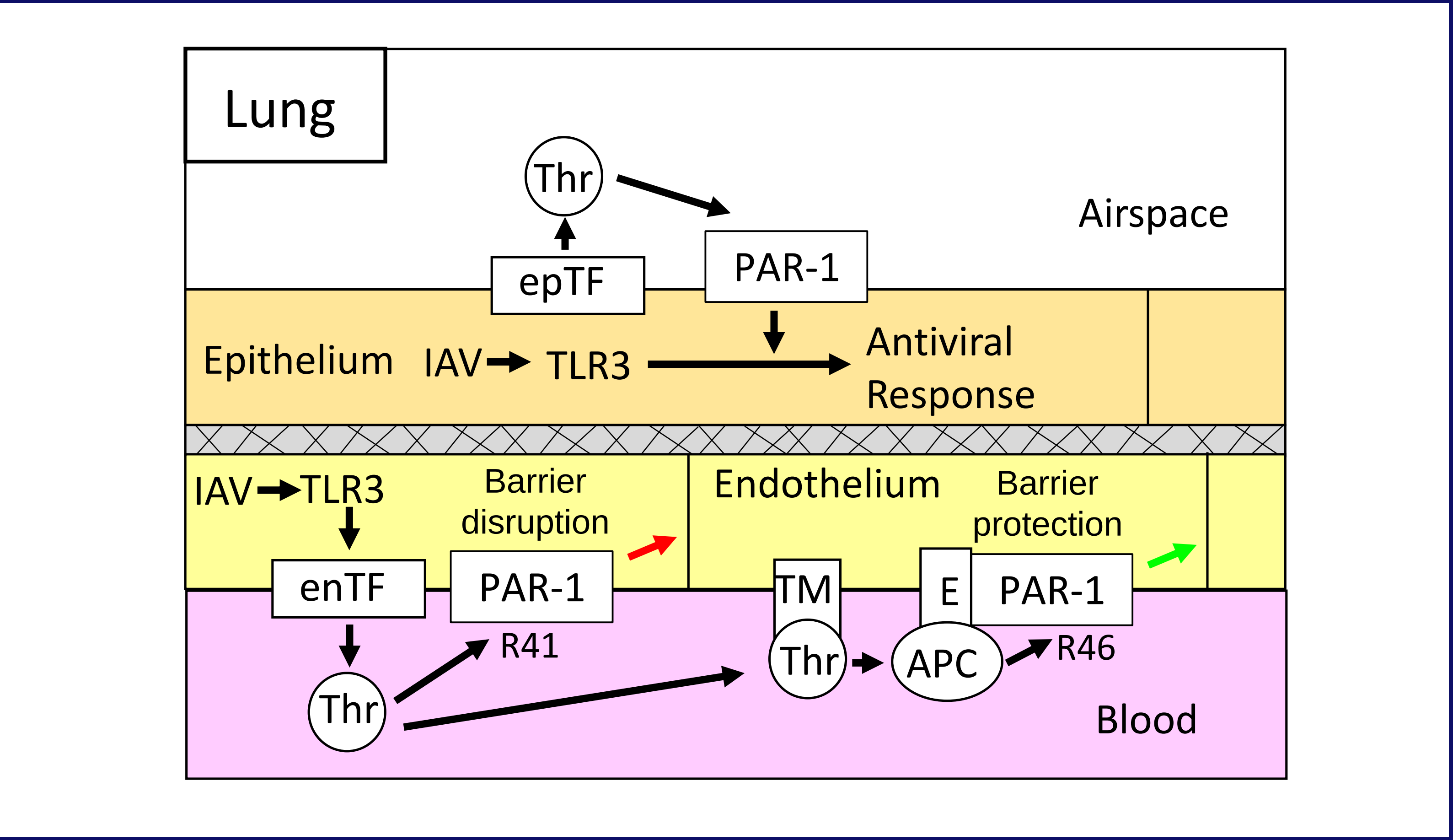
Role of PAR-1 in Vascular Permeability in the Lung after Influenza A Infection



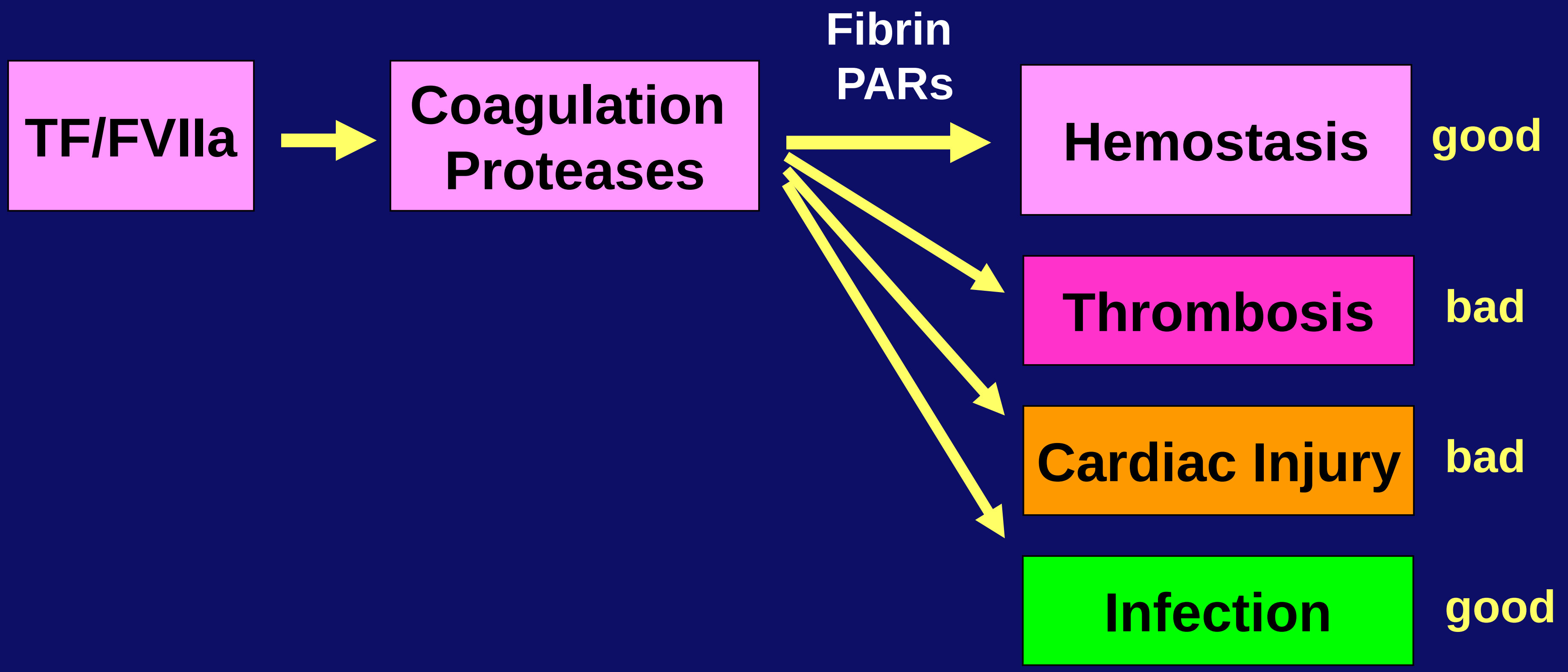
7 days

Tatsumi K et al. unpublished data

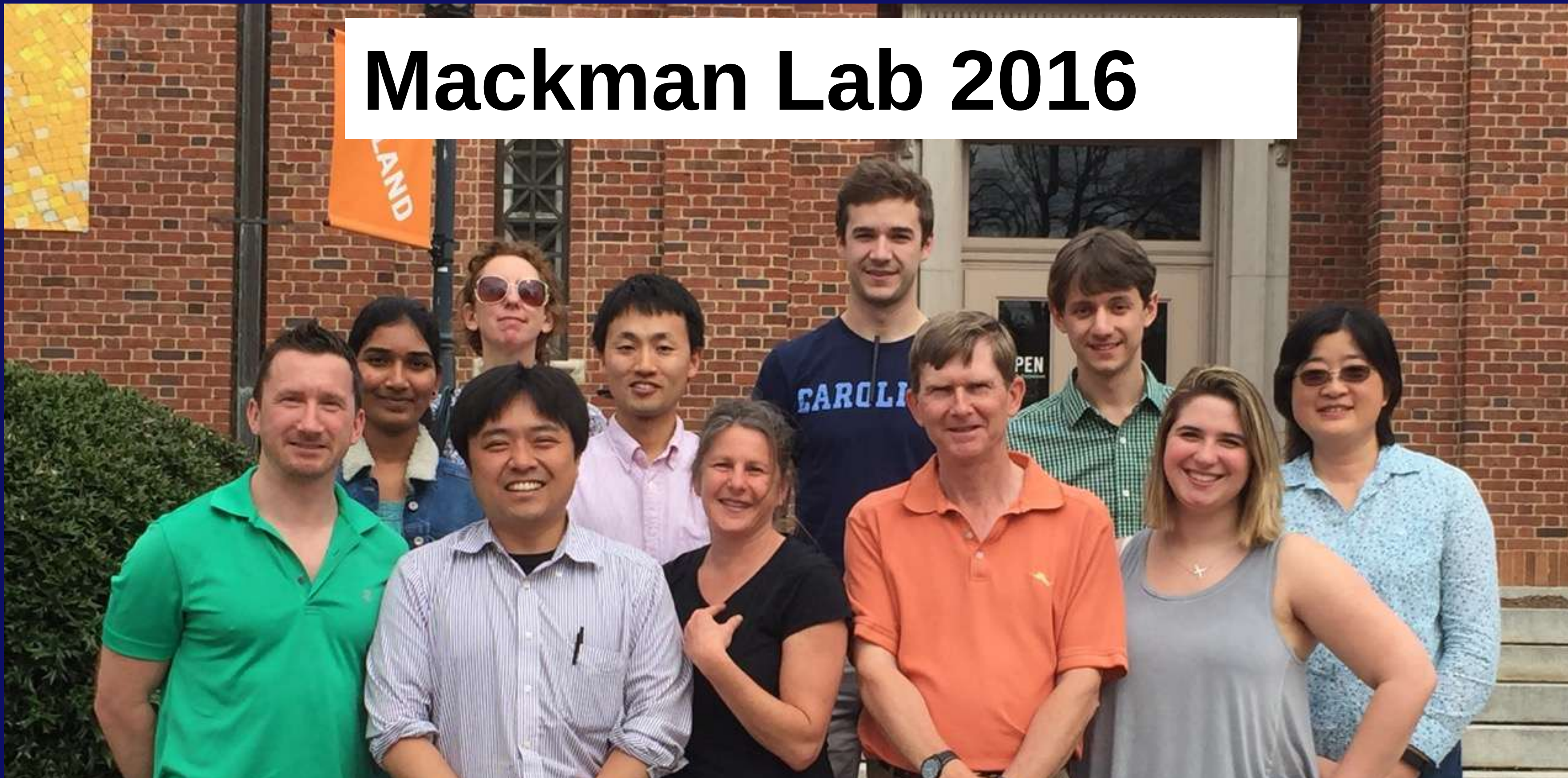
Roles of the TF-Thrombin-PAR-1 Pathways in Influenza A Infection



Tissue Factor, Coagulation Proteases, Fibrin and PARs in Health and Disease



Mackman Lab 2016



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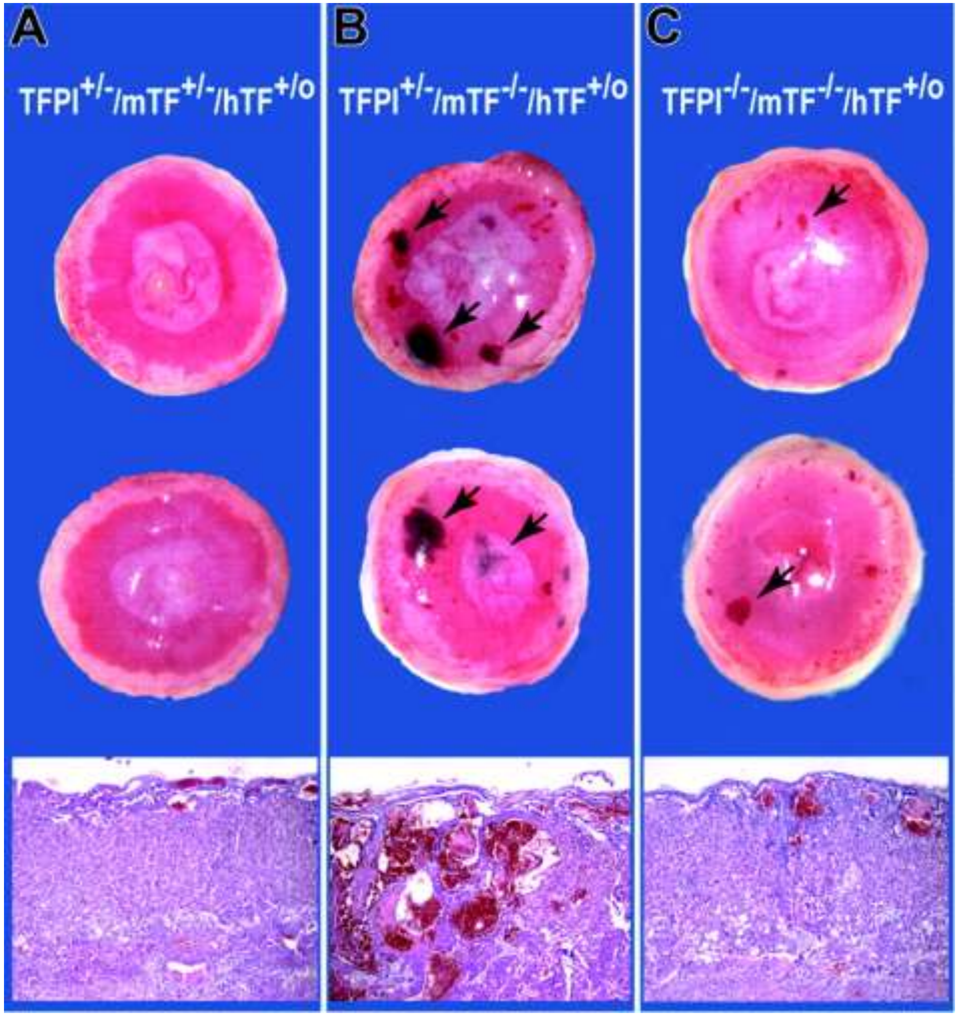
TraCS


ACKLAND
CAROLI
PEN


CAROLINA

Biggs R and Nossel H Tissue extract and the contact reaction in blood coagulation Thromb Diath Haeofrrh 61: 1, 1961

Reduced Blood Pools in "Rescued" Placentas



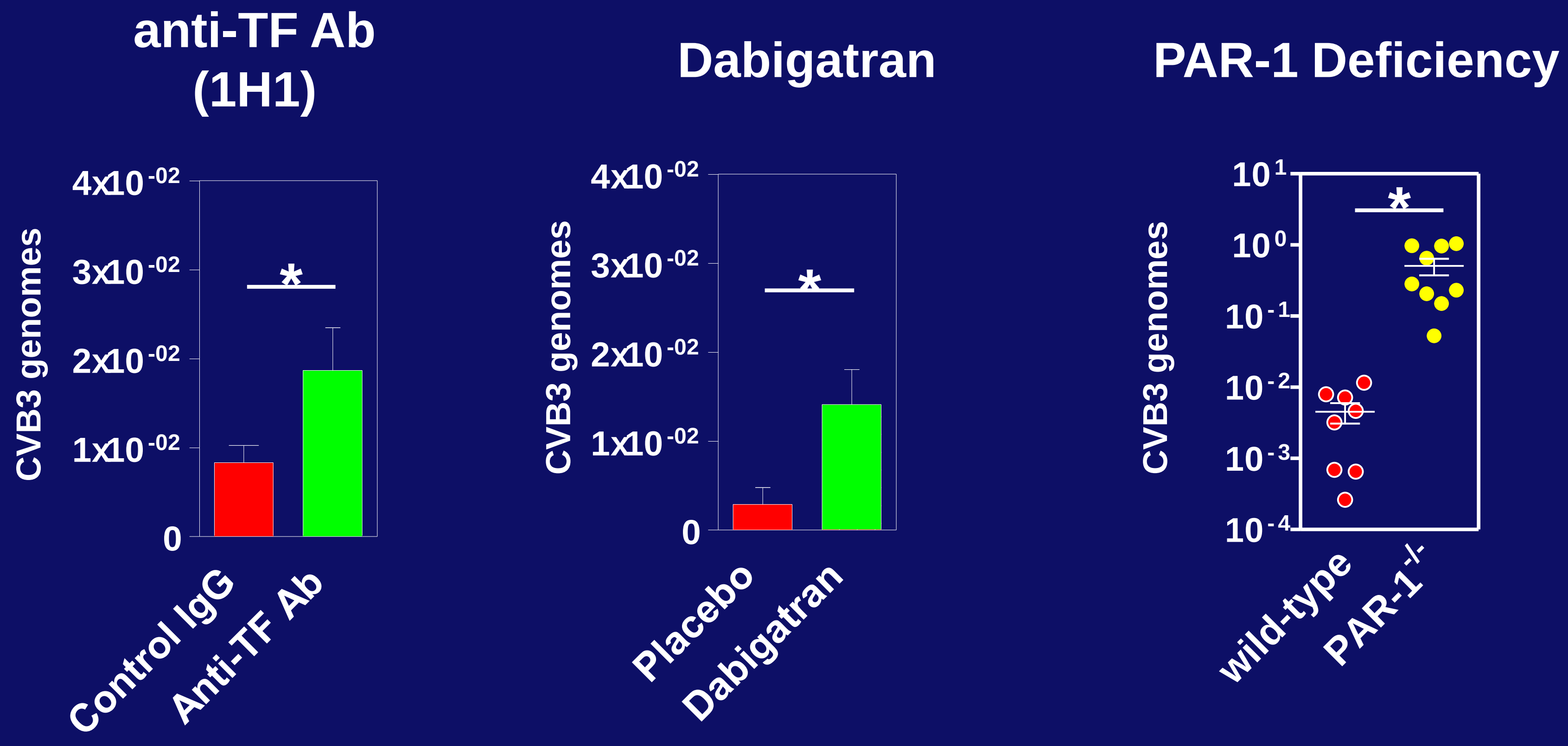
Pedersen B et al Blood 2005

Undergraduates

Janet Yan


CAROLINA

Role of the TF-thrombin-PAR-1 Pathway in CVB3-induced Myocarditis



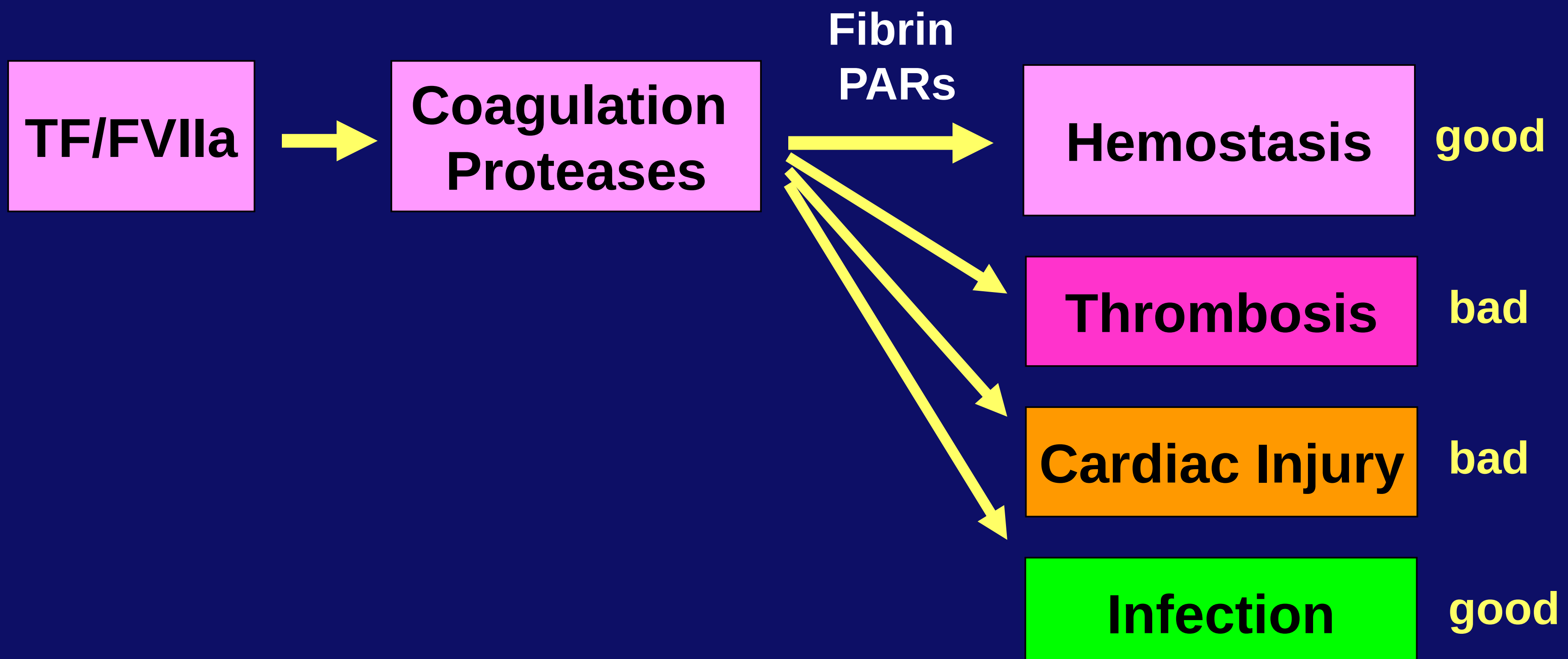
Antoniak S et al JCI 2013

Microvesicles

Microvesicles (MVs) were originally defined as small (0.1-1 µm) membrane vesicles that are released from activated or apoptotic cells

MVs are also referred to as microparticles (MPs) or extracellular vesicles (EVs) (= any type of vesicle released from a cell)

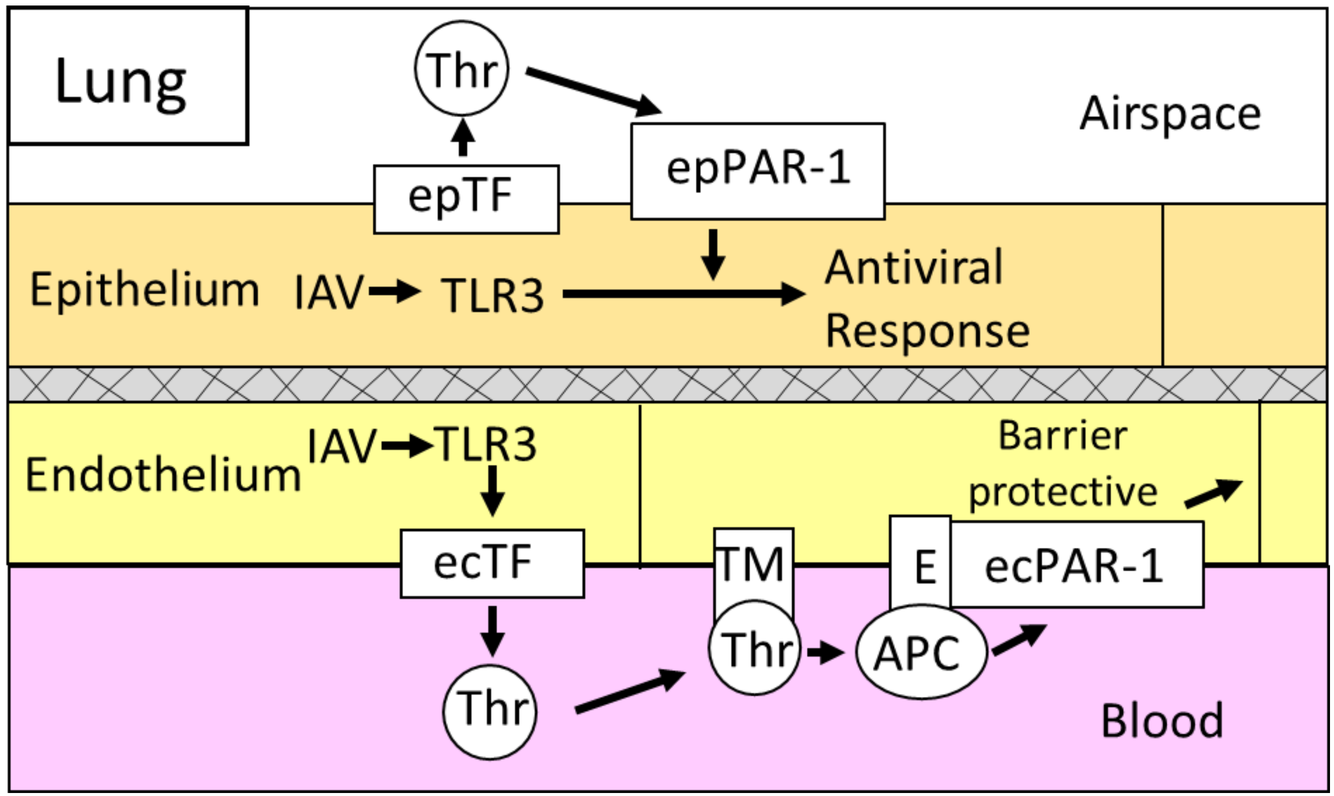
Tissue Factor, Coagulation Proteases, Fibrin and PARs in Health and Disease



Conclusions

- PAR-1 deficiency increases IAV-induced mortality.
- PAR-1 deficiency increases vascular permeability in the lung after IAV infection.
- PAR-1 also contributes to the immune response to IAV infection.

Roles of the TF-Thrombin-PAR-1 Pathways in Influenza A Infection



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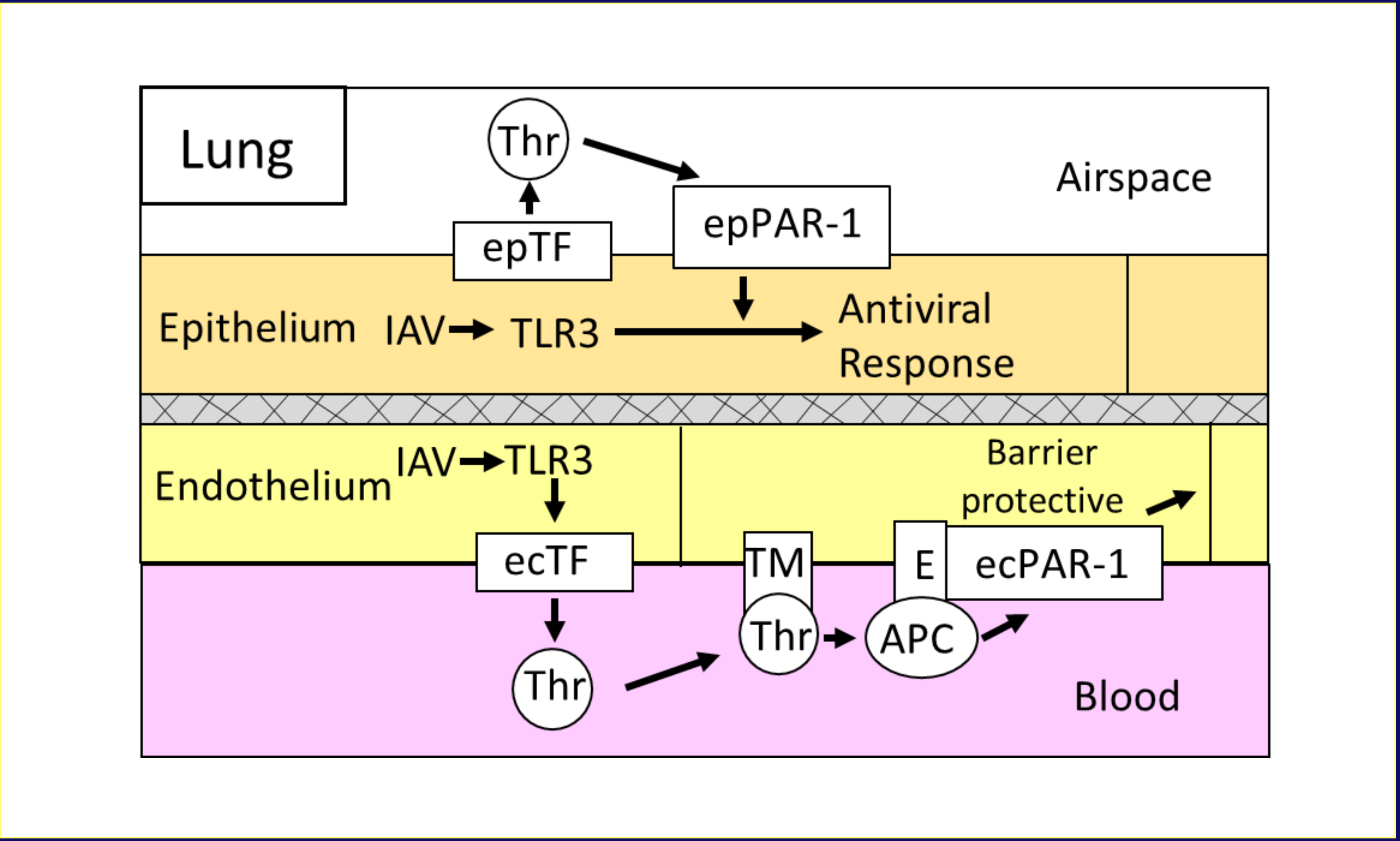
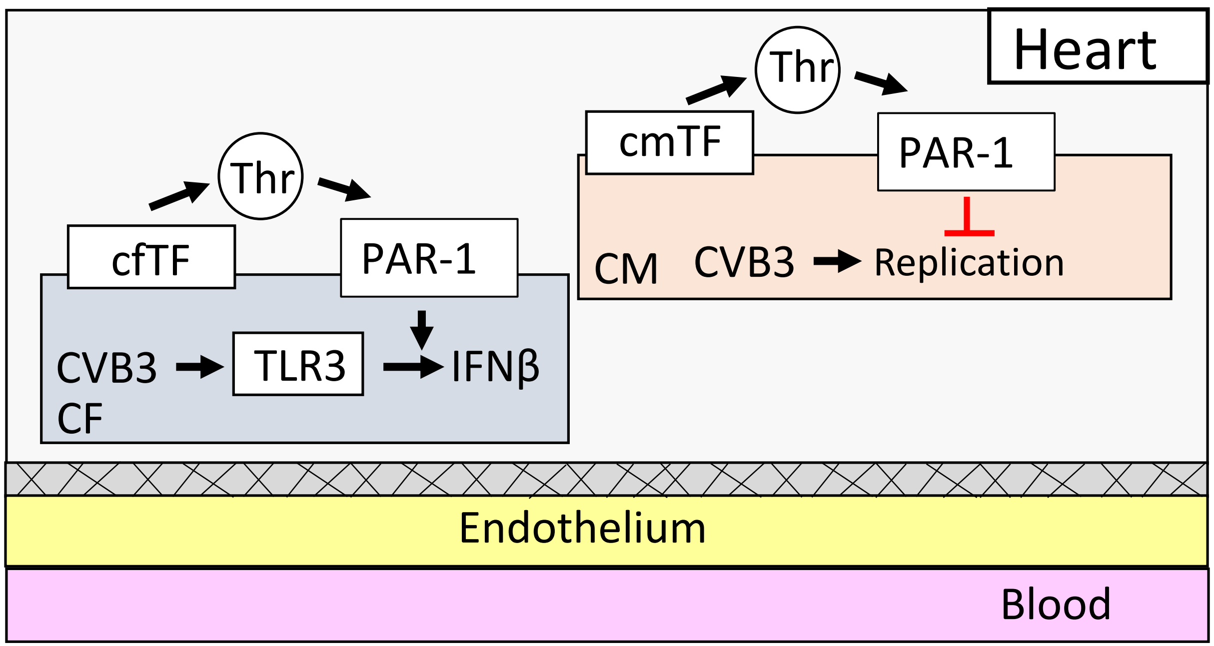


Statins, TF and Thrombosis

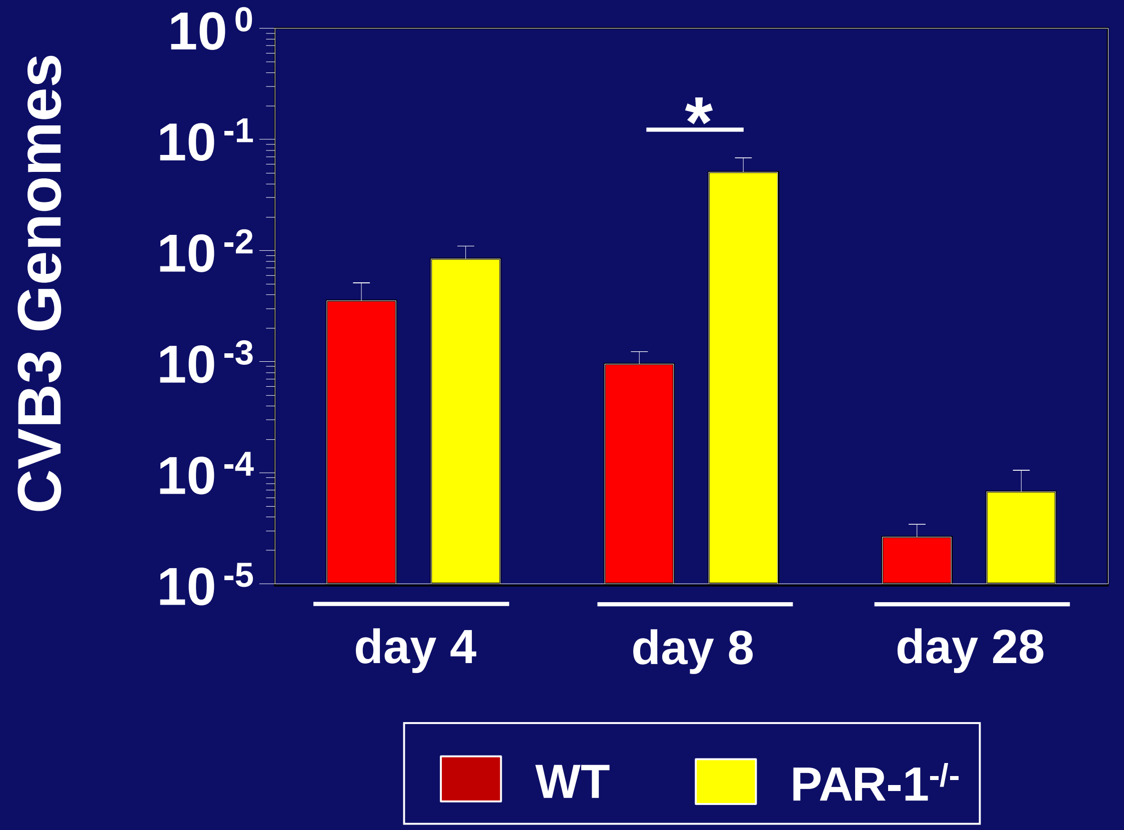
Statins inhibit TF expression in activated monocytes in vitro and in vivo (Colli et al. 1997 ATVB, Ferro et al. Lancet 1997, Ferro et al. 2000 Atherosclerosis, Nagata et al. 2002 Atherosclerosis, Steiner et al. 2005 Circulation)

Can simvastatin reduce monocyte TF expression sepsis and other diseases?

Roles of the TF-Thrombin-PAR-1 Pathways in CVB3-induced Myocarditis



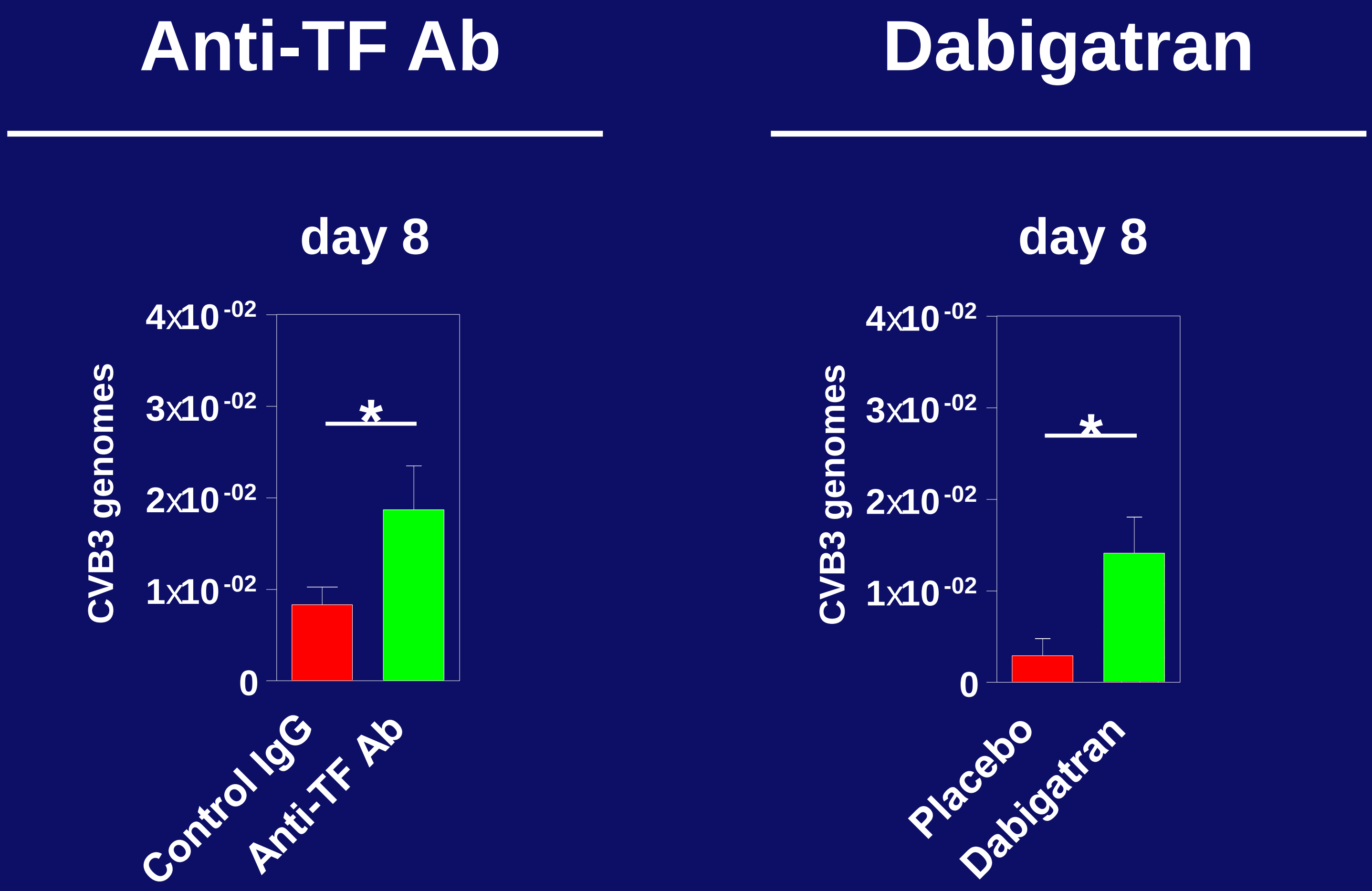
PAR-1 Deficiency is Associated with Increased Viral Load in the Heart after CVB3 infection



*P<0.05
Two-Way ANOVA

Antoniak S et al, JCI 2013

Effect of Inhibition of Tissue Factor or Thrombin on CVB3-induced Myocarditis



Antoniak S et al, JCI 2013

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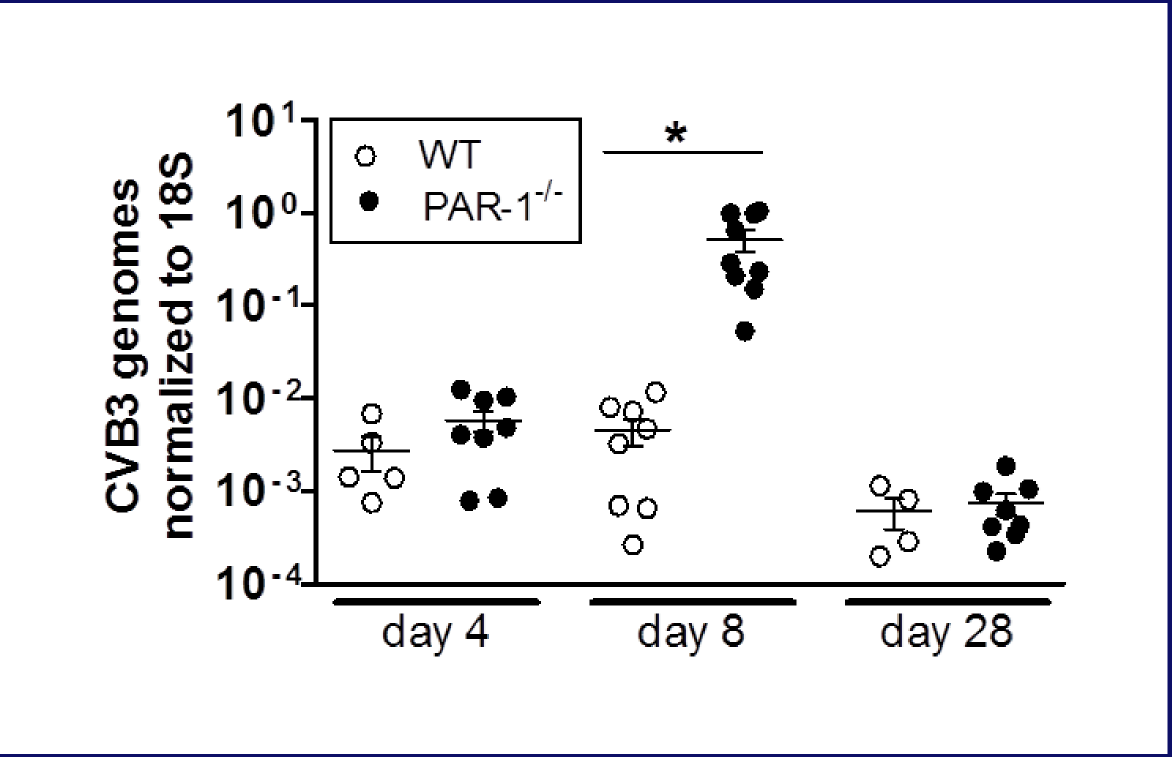


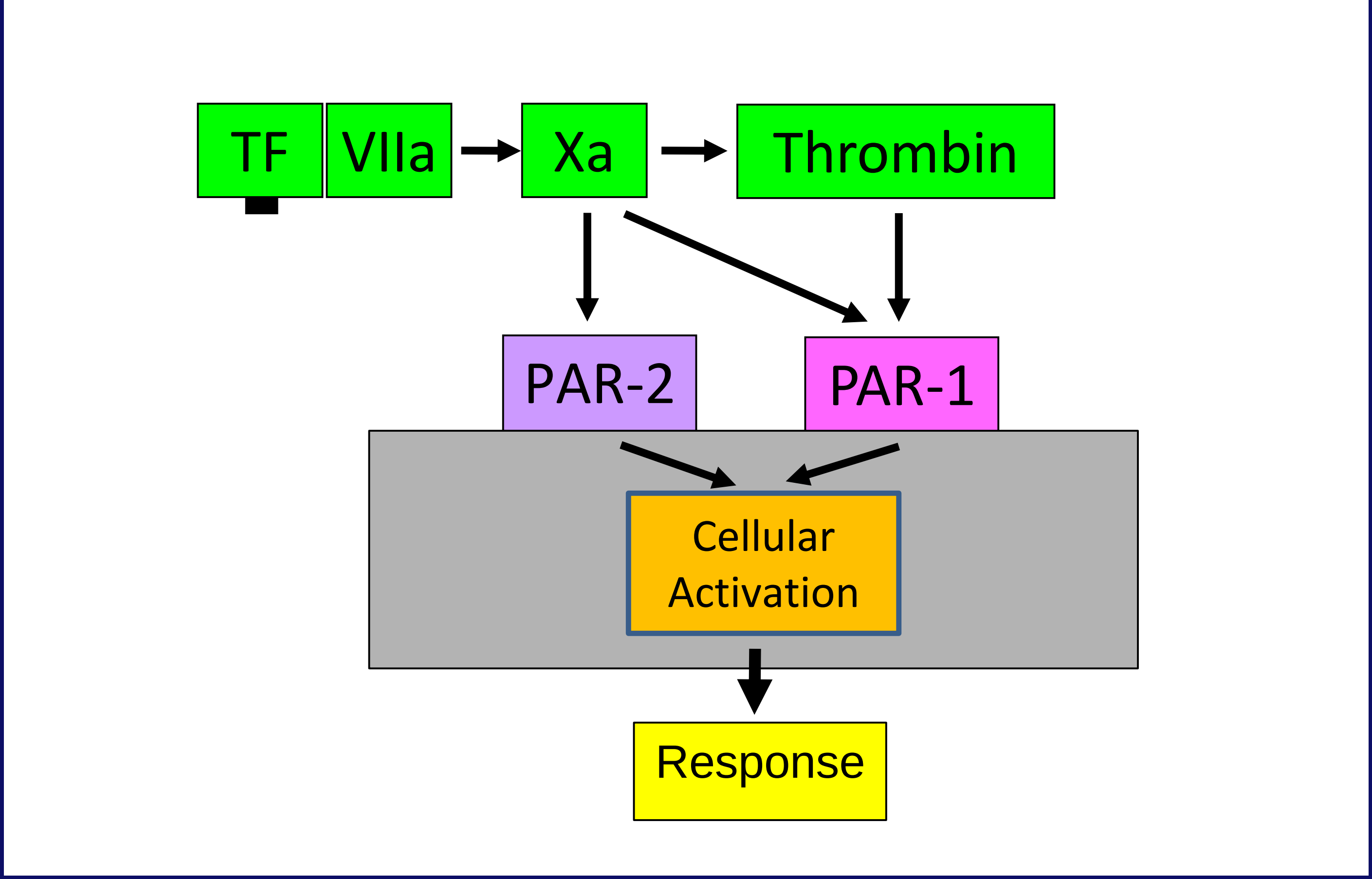
*P<0.05
Two-Way ANOVA

Antoniak et al, JCI 2013

Outline

- Coxsackievirus B3 virus model
- Influenza A virus model

Role of Protease-activated Receptors as Sensors of the Extracellular Environment

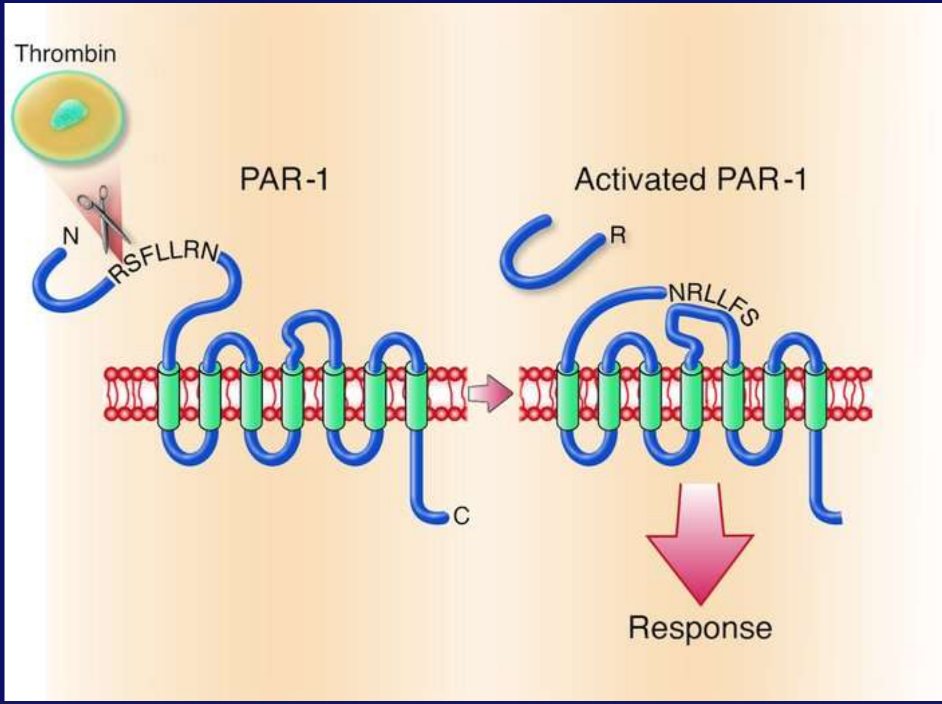


The Protease-Activated Receptor Family

	PAR-1	PAR-2	PAR-3	PAR-4
Primary activating proteases (EC_{50})	Thrombin (50 pM)	Trypsin (1 nM) Tryptase (1 nM)	Thrombin (0.2 nM)	Thrombin (5 nM) Trypsin (5 nM)
Secondary activating proteases	Granzyme A FXa Trypsin Plasmin MMP-1	FVIIa FXa MT-SP1		Cathepsin G
PAR-APs	SFLLRN TFLLRN	SLIGKV SLIGIRL SFLLRN		GYPGKF AYPGKF

Adapted from Major et al., 2003 *Arterioscler.Thromb.Vasc.Biol.*

Thrombin Activation of PAR-1

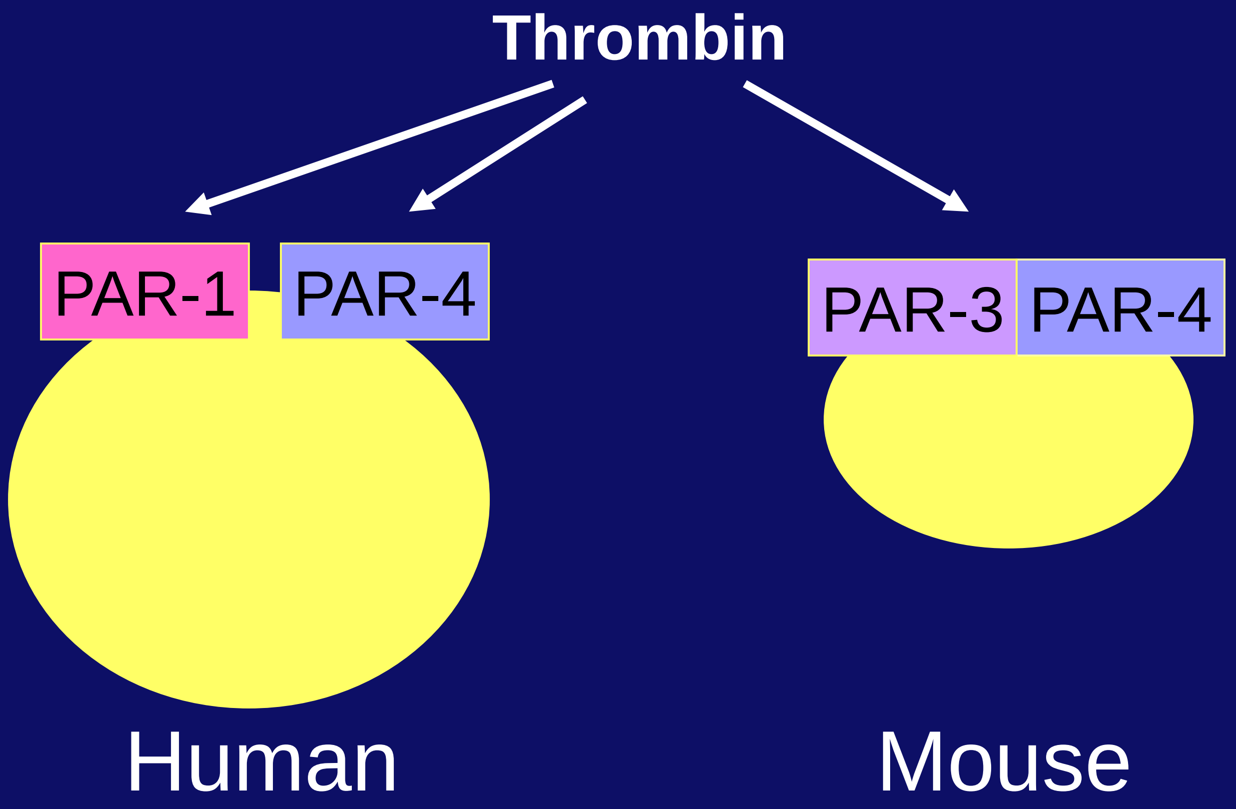


PAR-1 is a unique GPCR because it is activated by proteolytic cleavage. It is the major thrombin receptor on human platelets.

Vorapaxar (Zontivity) was approved by the FDA in 2014 for use with daily aspirin and/or clopidogrel.

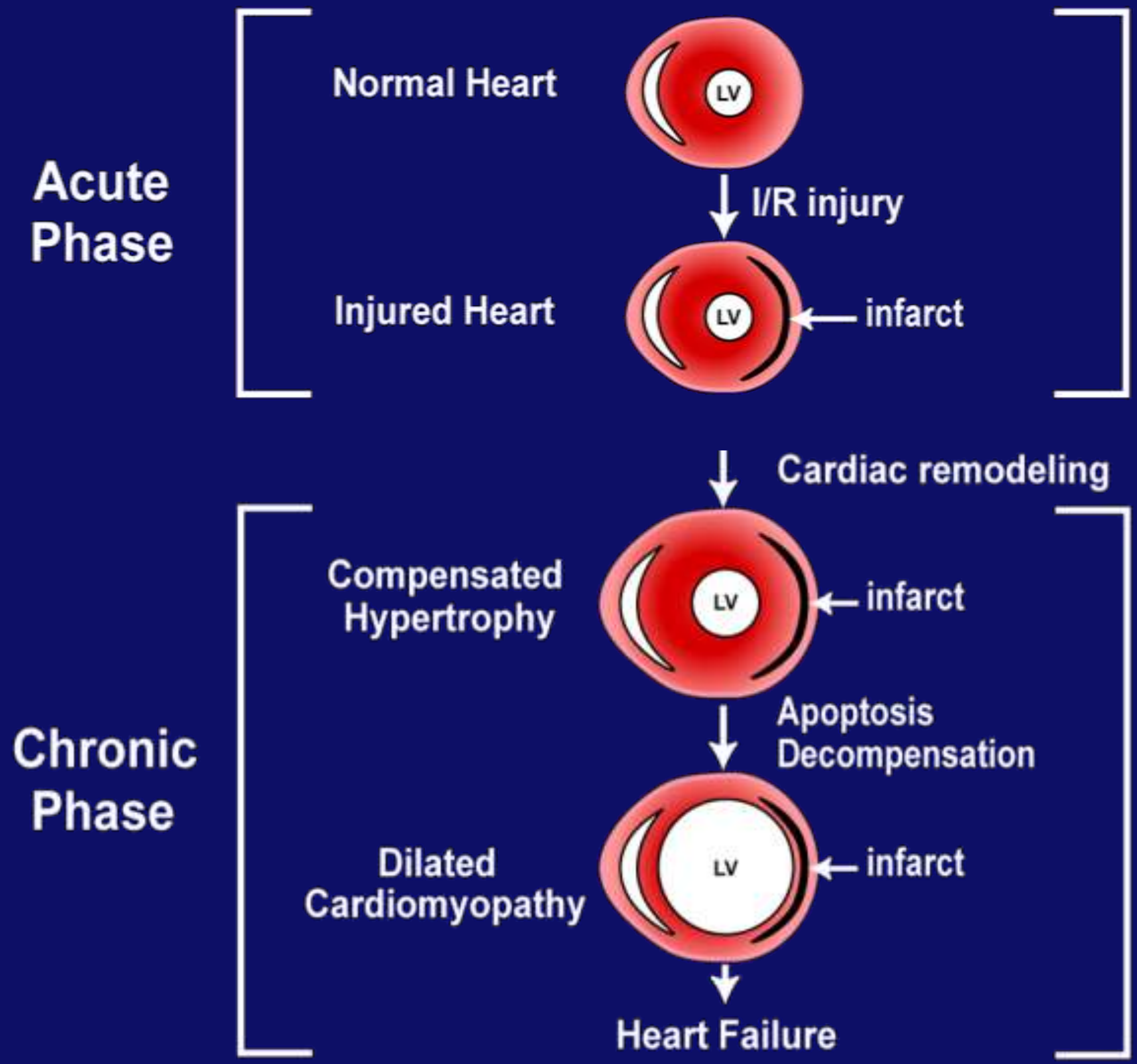
Camerer and Coughlin JCI 2003

PAR Expression on Human and Mouse Platelets



Any phenotype in PAR-1$^{-/-}$ mice cannot be due to a defect in thrombin activation of platelets

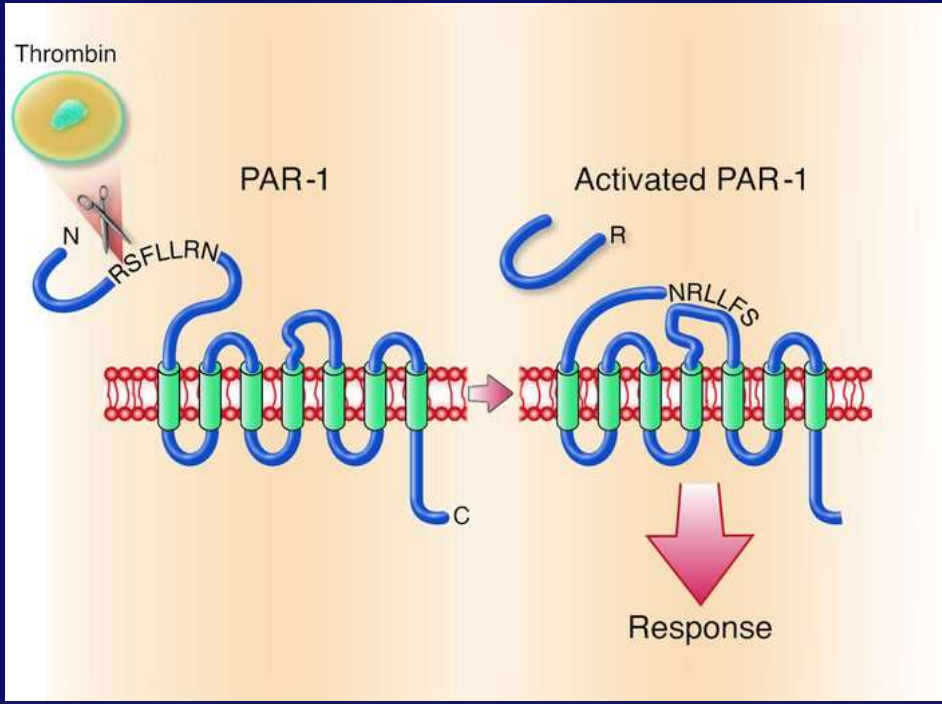
Cardiac I/R Injury Induces Infarction and Remodeling



Viral Infections and the Clotting Cascade

- Acute viral infections lead to strong activation of the clotting cascade and secondary hemorrhage (i.e. Ebola hemorrhagic fever).
- Inhibition of TF reduced death in a monkey model of Ebola.
- Chronic viral infections lead to a low level activation of the clotting cascade that increases cardiovascular disease (i.e. HIV).
- Two ongoing clinical trials are targeting FXa and PAR-1 in HIV patients.

Thrombin Activation of PAR-1



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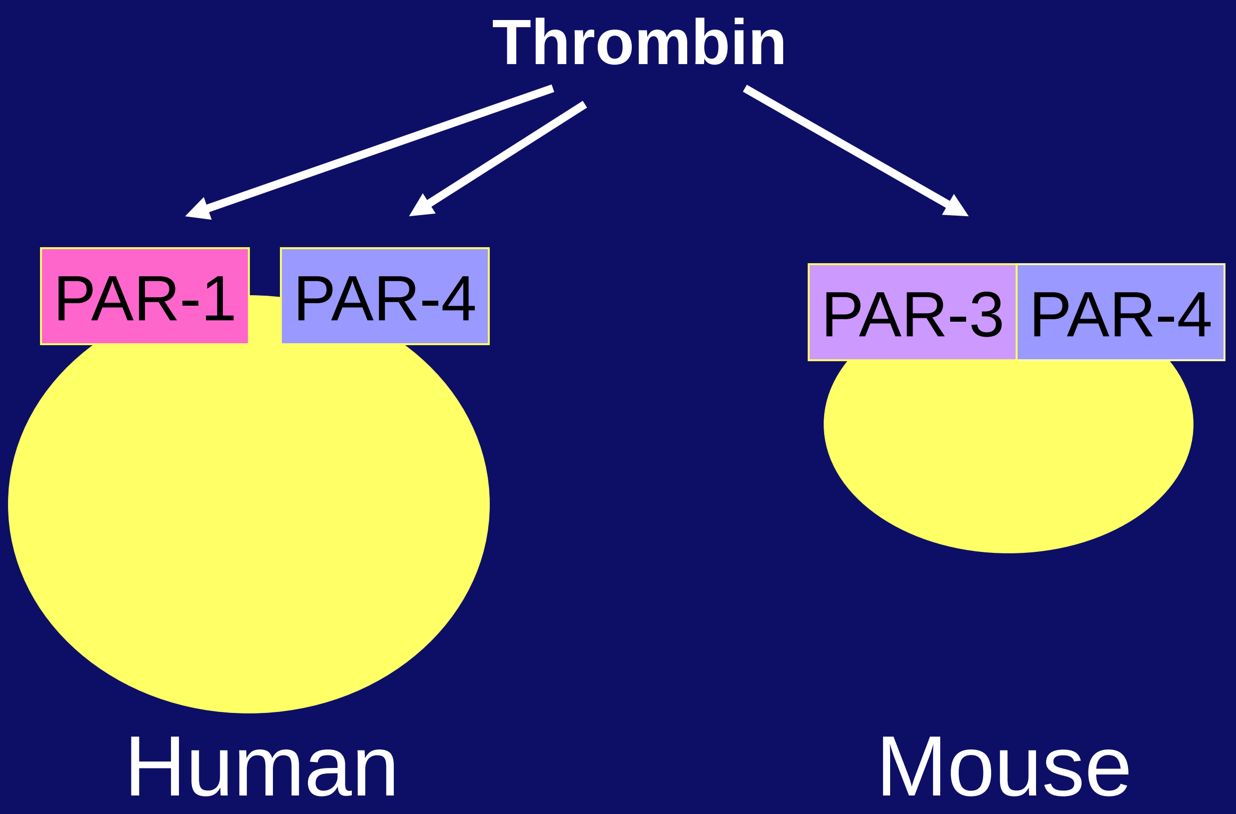
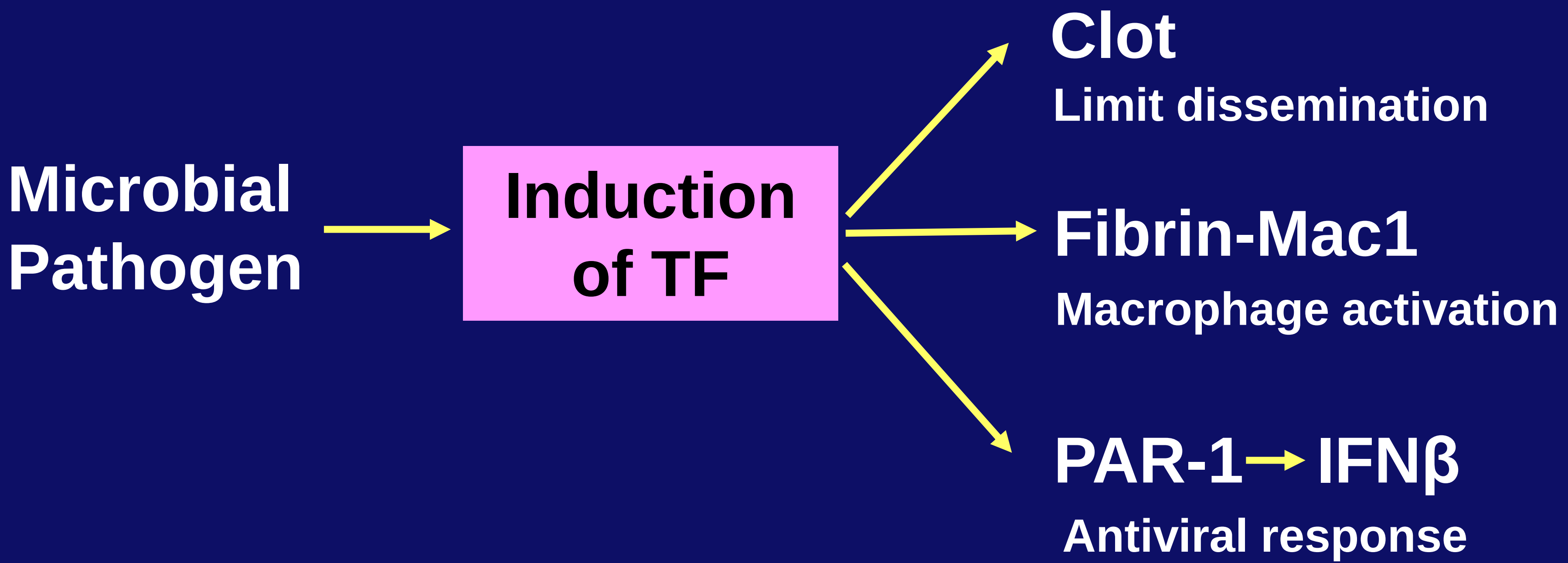
Camerer and Coughlin JCI 2003

PAR Expression on Human and Mouse Platelets

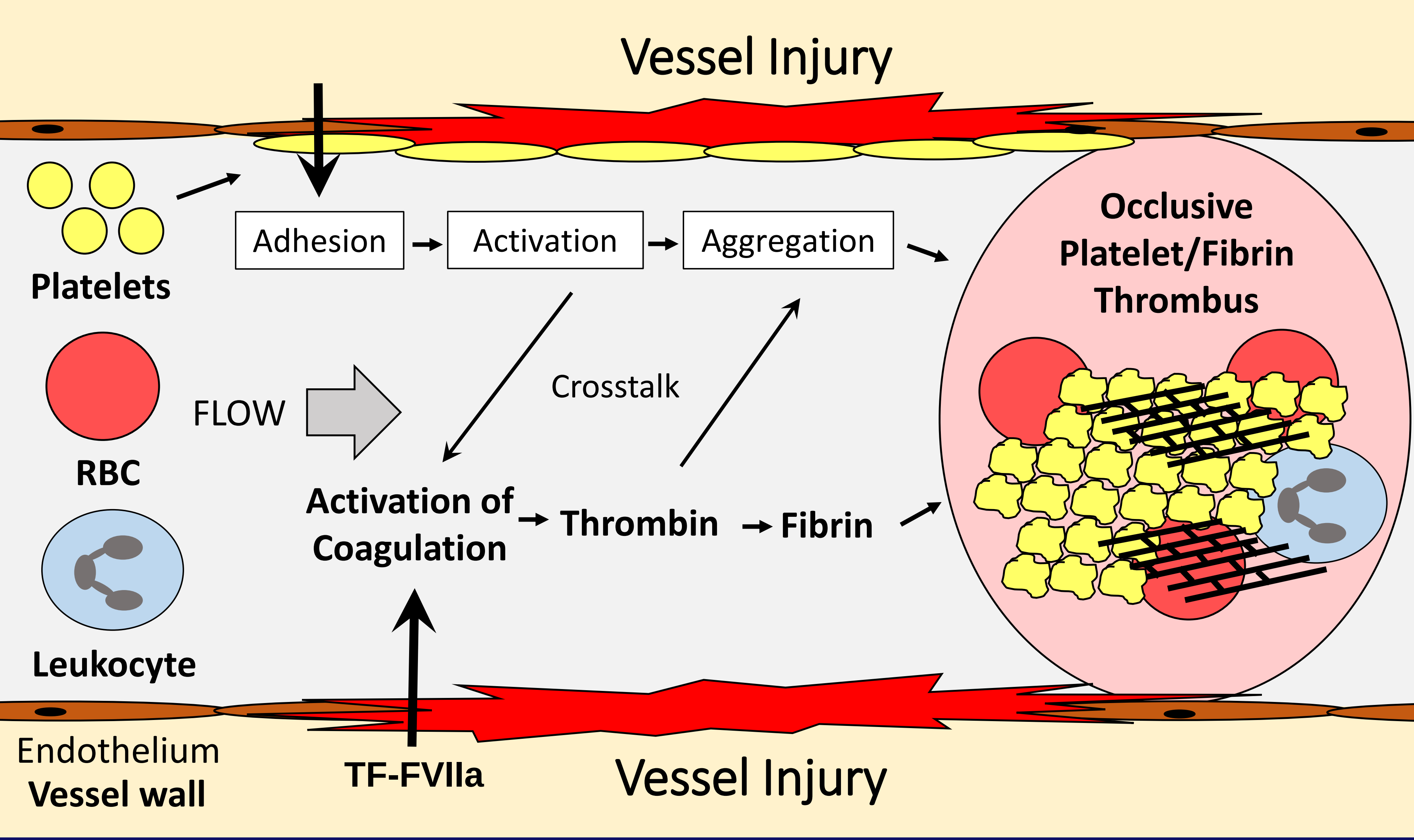


Any phenotype in PAR-1$^{-/-}$ mice cannot be due to a defect in thrombin activation of platelets

The Clotting Cascade and Host Defense



Formation of an Occlusive Thrombus



Jagadeeswaran P et al.. Mackman Circ Res 2016

Ischemia-Reperfusion Injury

Ischemia	Reperfusion
Hypoxic Damage	Inflammatory Damage
Apoptosis/necrosis	Chemokines, cytokines, oxidants
	Leukocyte recruitment and activation
	Coagulation and fibrin deposition
	Complement activation

Conclusions

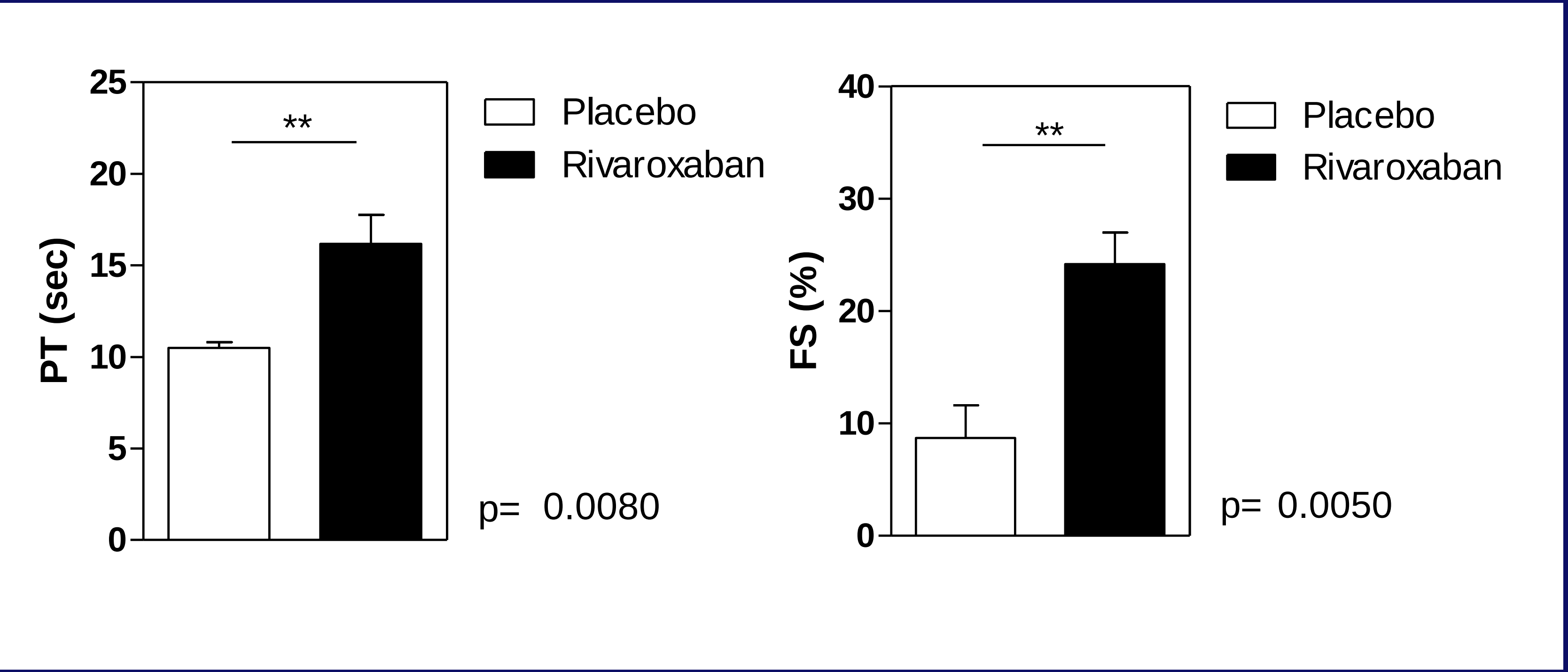
- PAR-1 contributes to the antiviral response to CVB3 infection.
- PAR-1 enhances poly I:C induction of IFNβ expression in cardiac fibroblasts.
- PAR-1 inhibits CVB3 replication in cardiac myocytes.

I/R

Conclusion

Inhibition of TF, coagulation proteases or PAR-1 may reduce cardiac remodeling and heart failure in MI patients

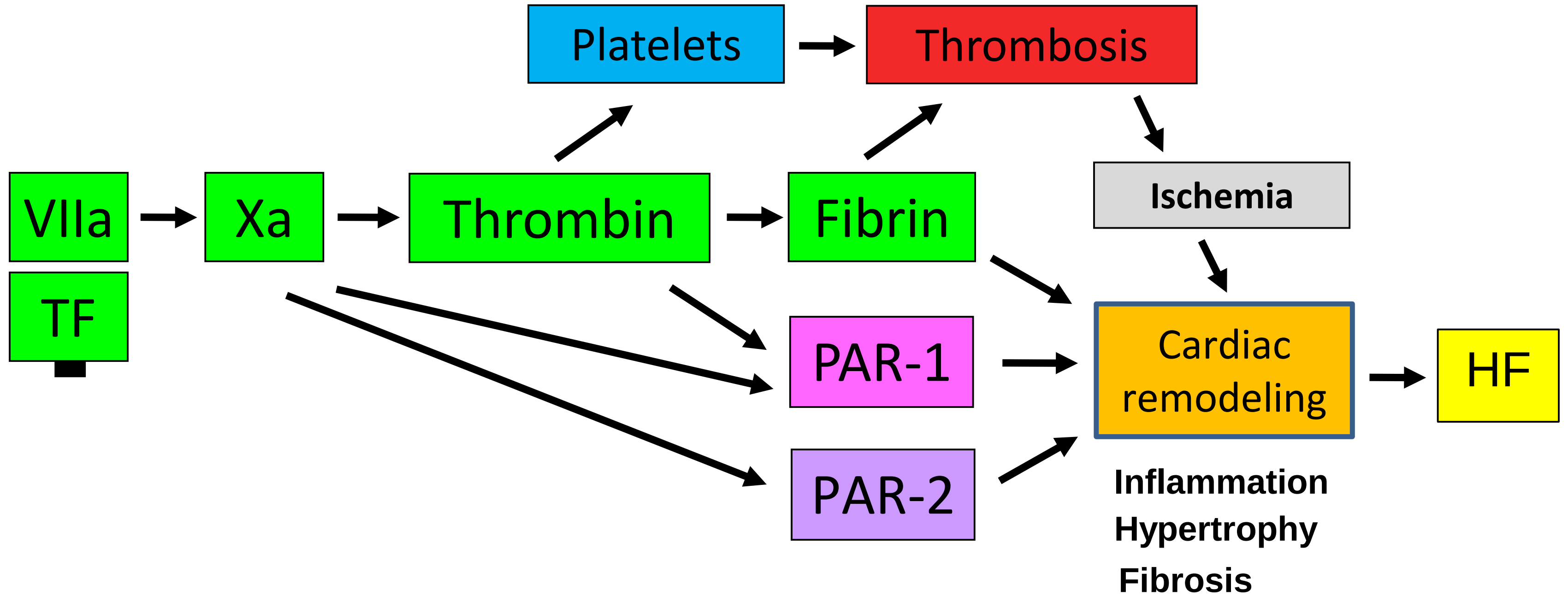
Effect of Rivaroxaban on Cardiac Remodeling and Heart Failure in a Mouse Model of LAD Ligation



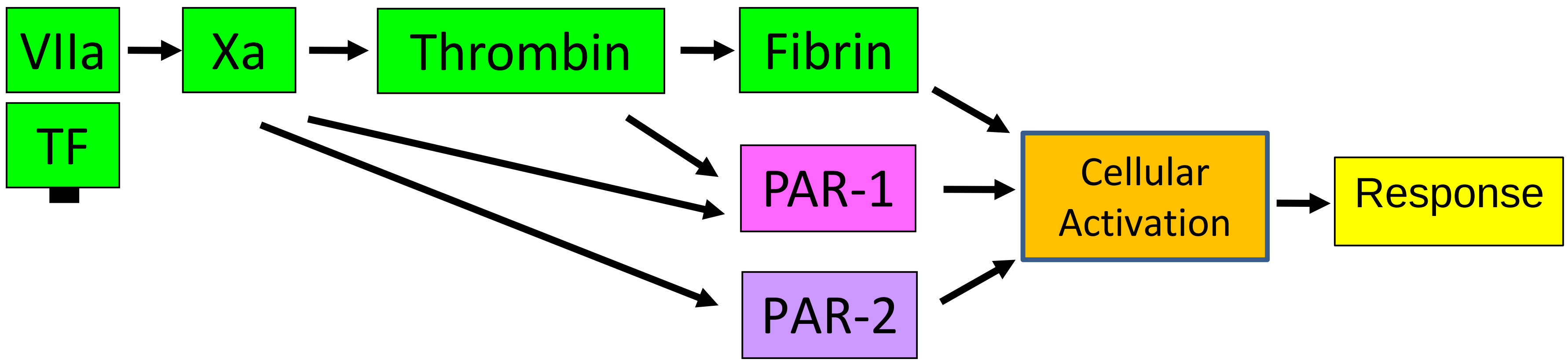
28 days after LAD ligation

Bode M unpublished results

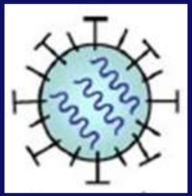
Coagulation Proteases and PARs in Cardiac Remodeling and Heart Failure



Role of Protease-activated Receptors as Sensors of the Extracellular Environment

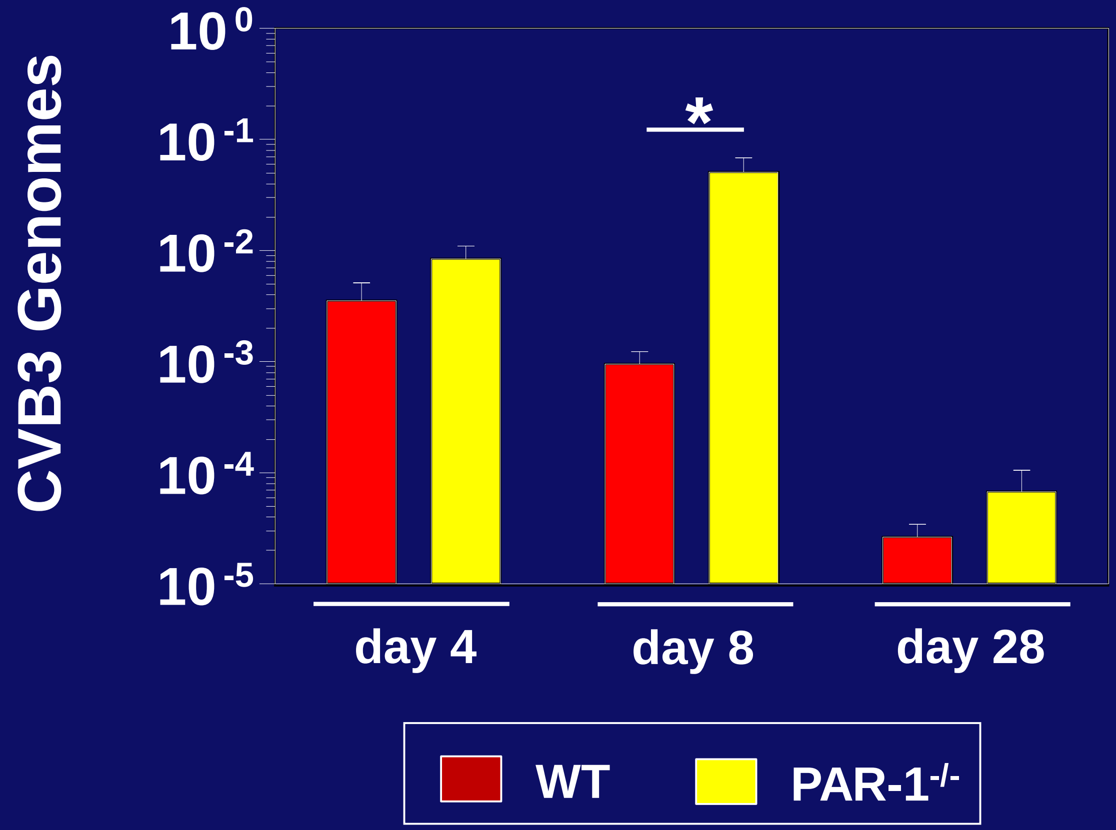


The Innate Immune Response to Coxsackievirus B3 Infection



CVB3 → **TLR3** (Resident cells and immune cells) → **IFNβ** → (STAT-1) **CXCL10** → NK cells → Virus killing

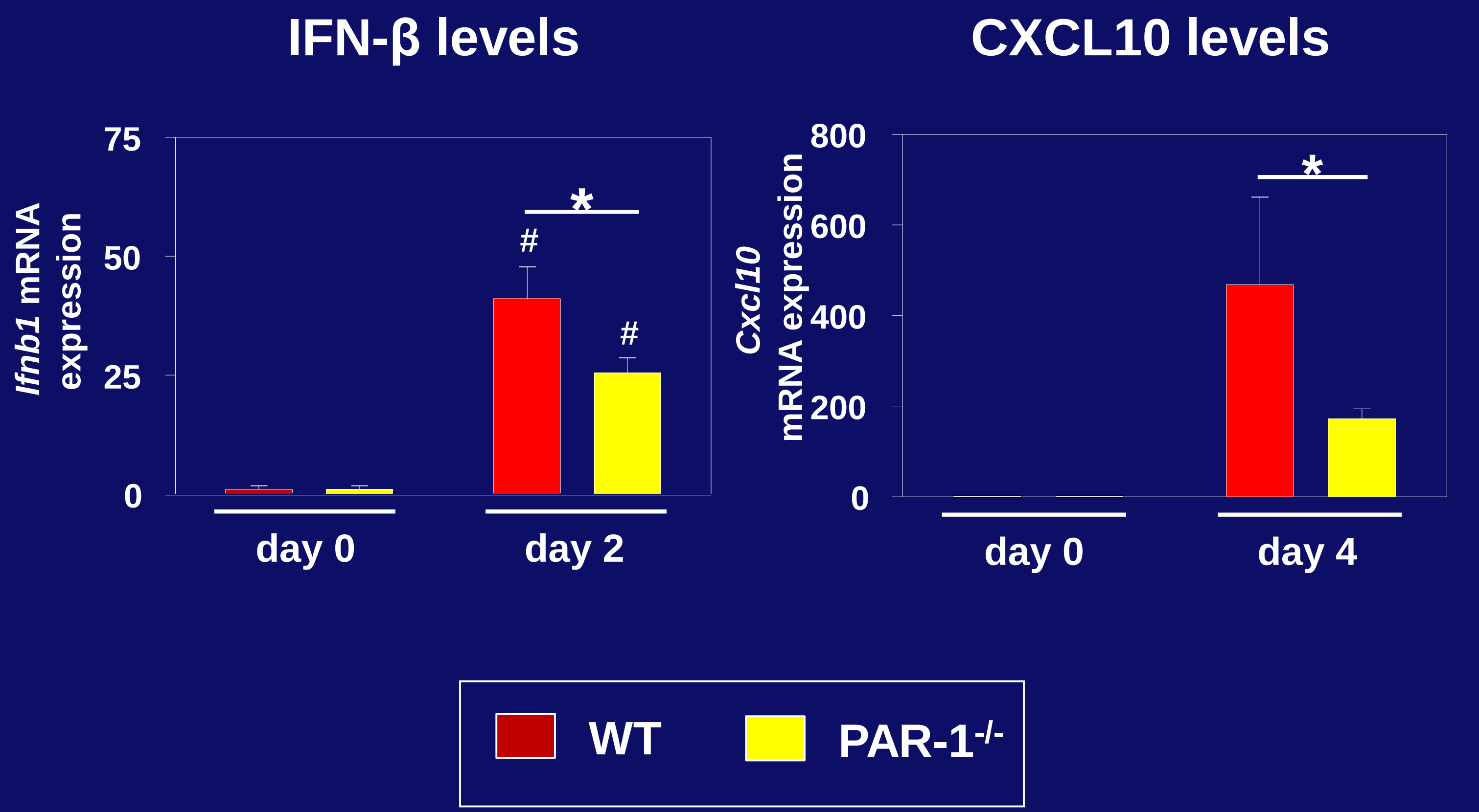
PAR-1 Deficiency is Associated with Increased Viral Load in the Heart after CVB3 infection



*P<0.05
Two-Way ANOVA

Antoniak et al, JCI 2013

PAR-1 Deficiency is Associated with Reduced IFN-β and CXCL10 expression After CVB3 Infection

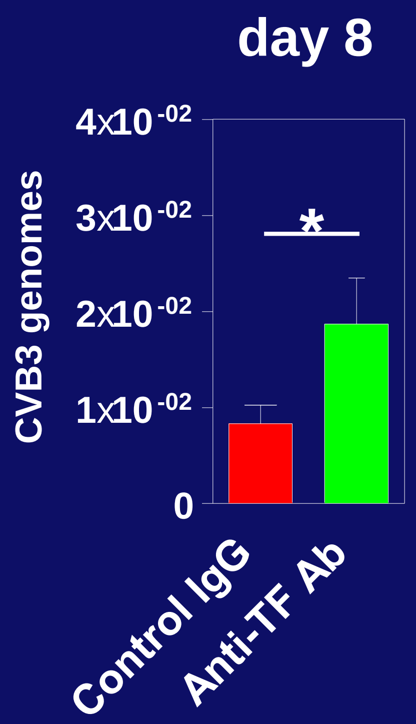


*P<0.05; #P<0.05 vs. day 0
Two-Way ANOVA

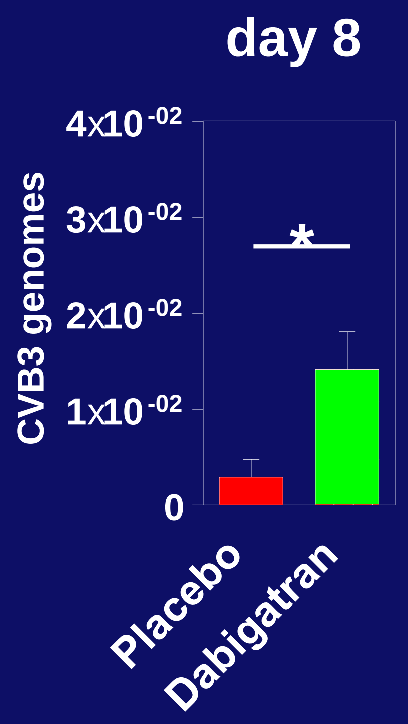
Antoniak et al, JCI 2013

Effect of Inhibition of Tissue Factor or Thrombin on CVB3-induced Myocarditis

Anti-TF Ab



Dabigatran



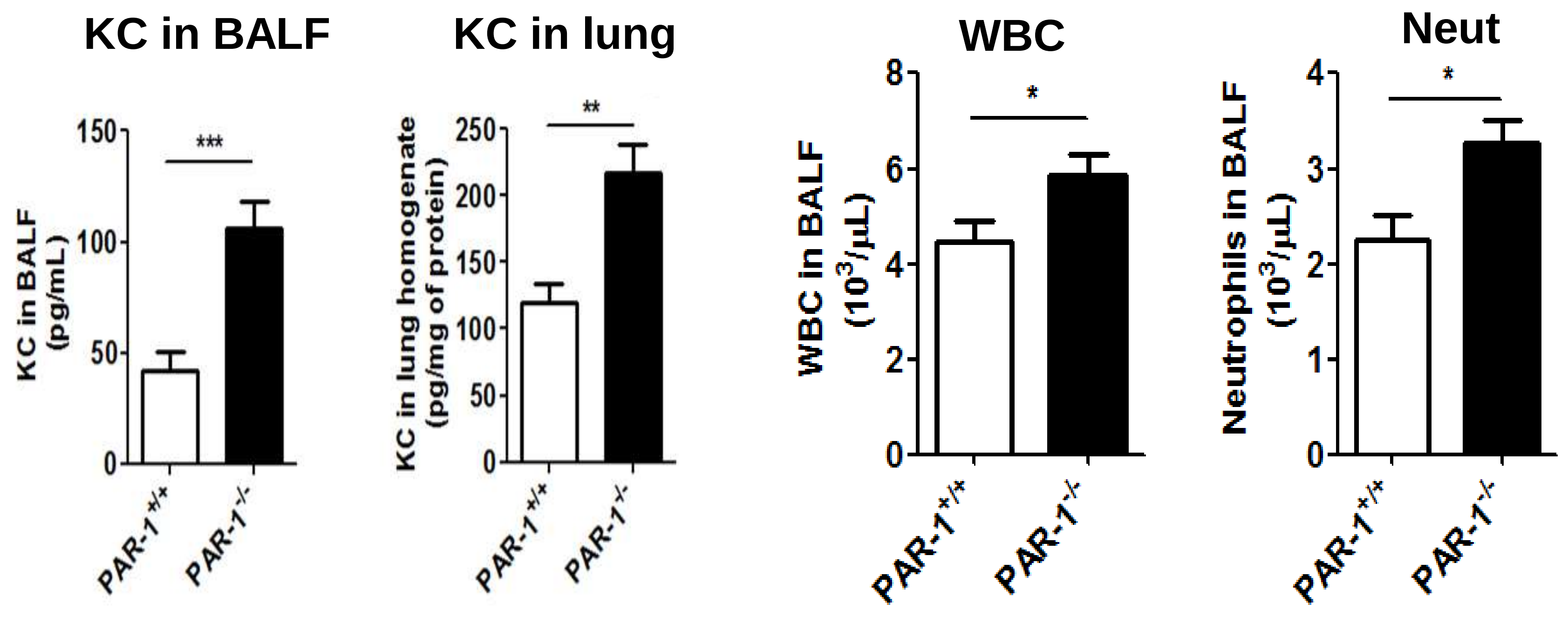
Antoniak S et al, JCI 2013

Ongoing Studies

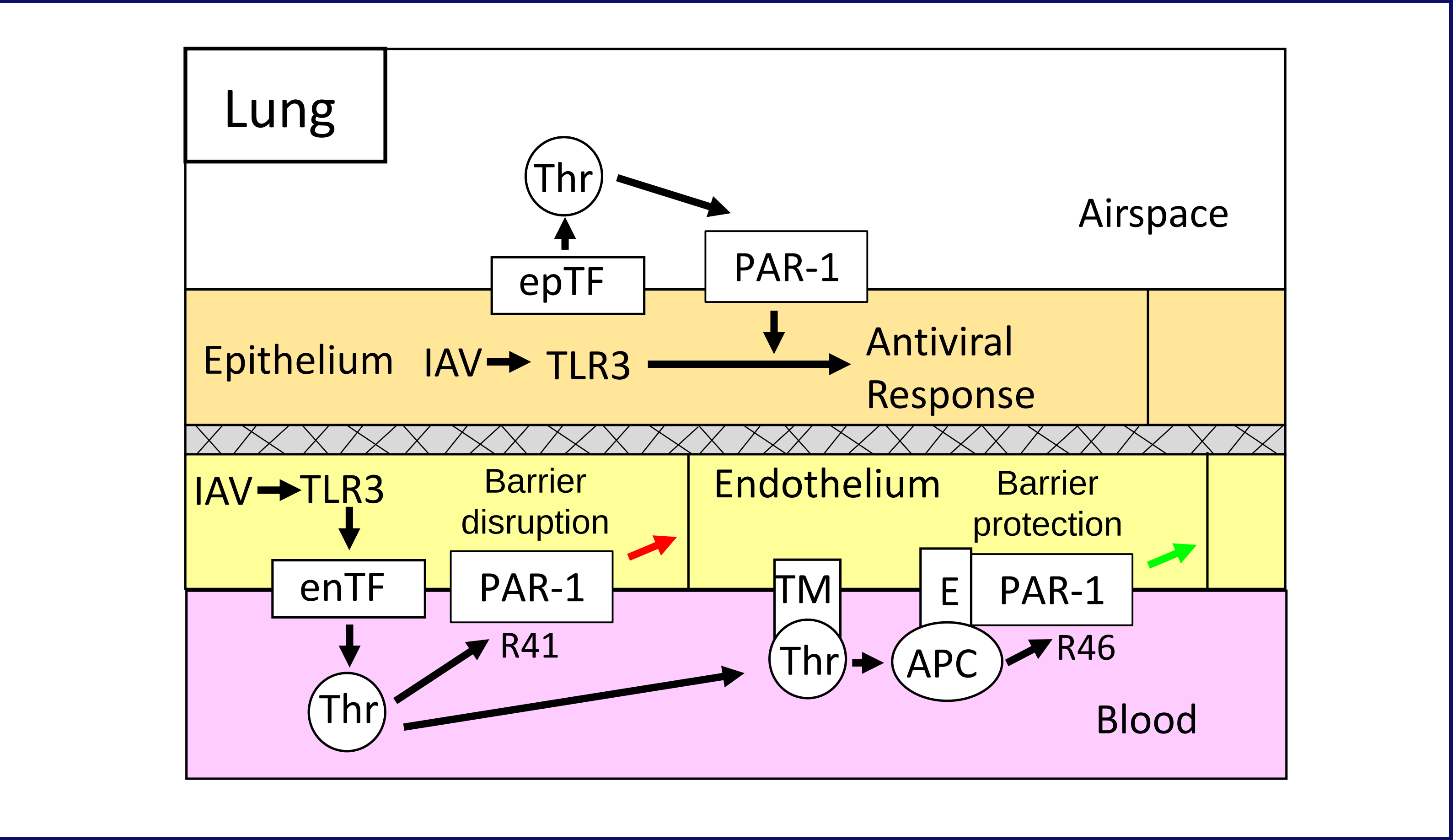
Determine the effect of the following on IAV-induced vascular permeability in the lung:

(i) deletion of PAR-1 in endothelial cells.
(ii) EPCR deficiency.
(iii) mutation of the APC cleavage site in PAR-1 (R46Q).

Effect of PAR-1 Deficiency on KC Expression and Leukocyte Recruitment after IAV Infection



Roles of the TF-Thrombin-PAR-1 Pathways in Influenza A Infection

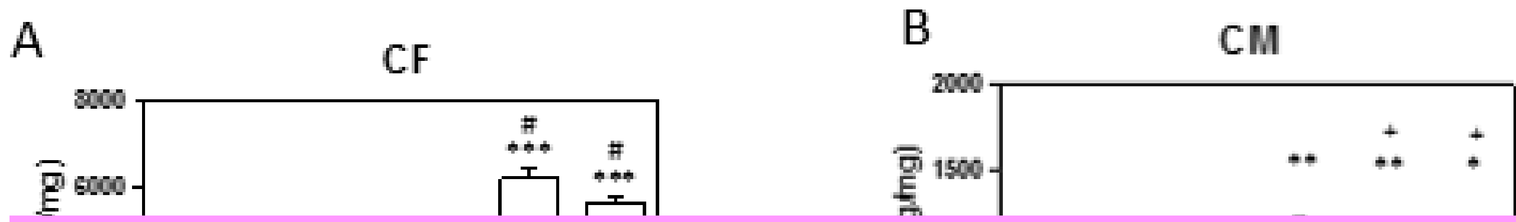


Ongoing Studies

Determine the effect of deletion of PAR-1 in cardiac myocytes or cardiac fibroblasts on CVB3-induced myocarditis.

- **PAR-1$^{fl/fl}$,Mlc2v^{Cre} (CMs)- 72% reduction in PAR-1 expression. Increased CVB3-induced myocarditis.**
- **PAR-1$^{fl/fl}$,TCF21$^{Cre\text{-}ERT2}$ (CFs)**

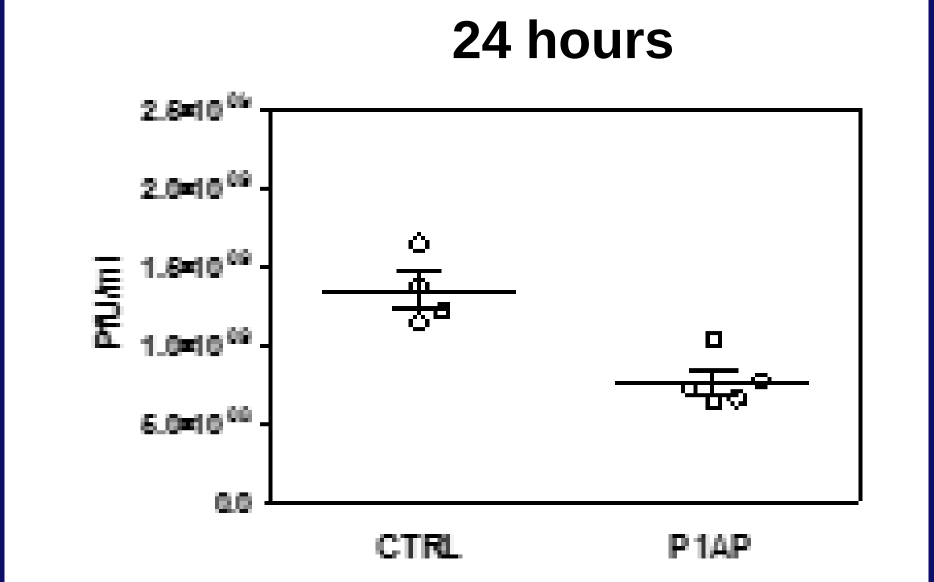
Effect of PAR-1 Activation on Poly I:C Induction of IFNβ Expression in Cardiac Fibroblasts and Cardiac Myocytes



How is CM PAR-1 protecting mice from CVB3-induced myocarditis?

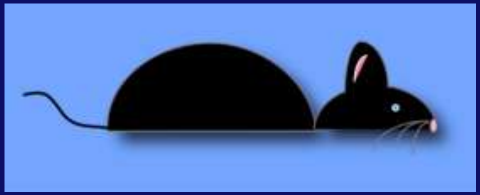
Bode M et al unpublished data

PAR-1 Activation Inhibits CVB3 Replication in Cardiac Myocytes



Bode M et al unpublished data

Mouse Models



PAR-1$^{fl/fl}$ mice

Cell type specific deletion of PAR-1 (crossed with Cre^{+})

Palumbo J unpublished data

Transgenic Mouse Lines

Table 1 Transgenic mouse lines

Mouse line	Description
PAR-1$^{-/-}$	Δ All cells
PAR-1$^{fl/fl}$,Mlc2v^{cre}	Δ CMs
PAR-1$^{fl/fl}$,TCF21$^{cre-ERT2}$	Δ CFs
PAR-1$^{fl/fl}$,LysM^{cre}	Δ Myeloid cells
PAR-1$^{fl/fl}$,Alb^{cre}	Δ Hepatocytes
PAR-1^{R41Q}	No thrombin activation
PAR-1^{R46Q}	No APC activation
PAR-1$^{fl/fl}$,Spc^{cre}	Δ Lung epithelial cells
PAR-1$^{fl/fl}$,VE-cad$^{cre-ERT2}$	Δ EC*
Procr^{Lox}	Δ EPCR in all cells

* Currently being generated.

Generation of Transgenic Mice Expressing PAR-1 Mutants

Thrombin ↓ APC ↓

WT PAR-1 DPR SFLLR N

41 46

APC ↓

PAR-1^{R41Q} DPQ SFLLR N

Thrombin ↓

PAR-1^{R46Q} DPR SFLLQ N

John Griffin

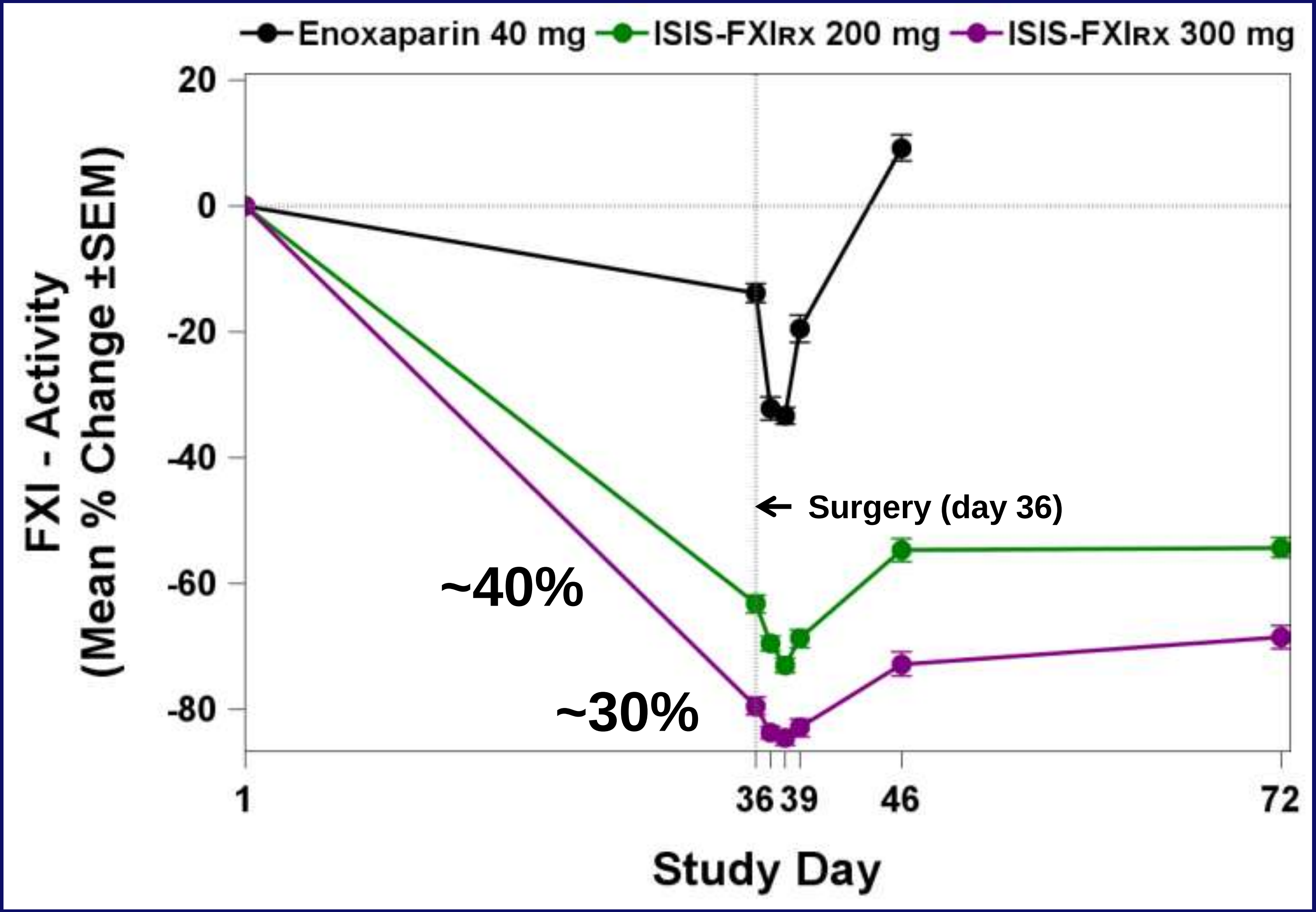
Clinical Characteristics

	Enoxaparin 40 mg (n =72)	ISIS-FXI$_{Rx}$ 200 mg (n = 144)	ISIS-FXI$_{Rx}$ 300 mg (n = 77)
Mean age – yr	64 ± 9	63 ± 9	63 ± 8
Female – no. (%)	60 (83%)	118 (82%)	60 (78%)
Mean Weight, kg (range)	87 (52, 132)	89 (52,124)	90 (52,130)
Creatinine clearance (ml/min)	111 ± 30	112 ± 31	116 ± 30
Mean factor XI activity (units/ml)	1.23 ± 0.21	1.20 ± 0.20	1.16 ± 0.22

ISIS-FXI$_{Rx}$ – FXI ASO

Buller H et al N Engl J Med 2015

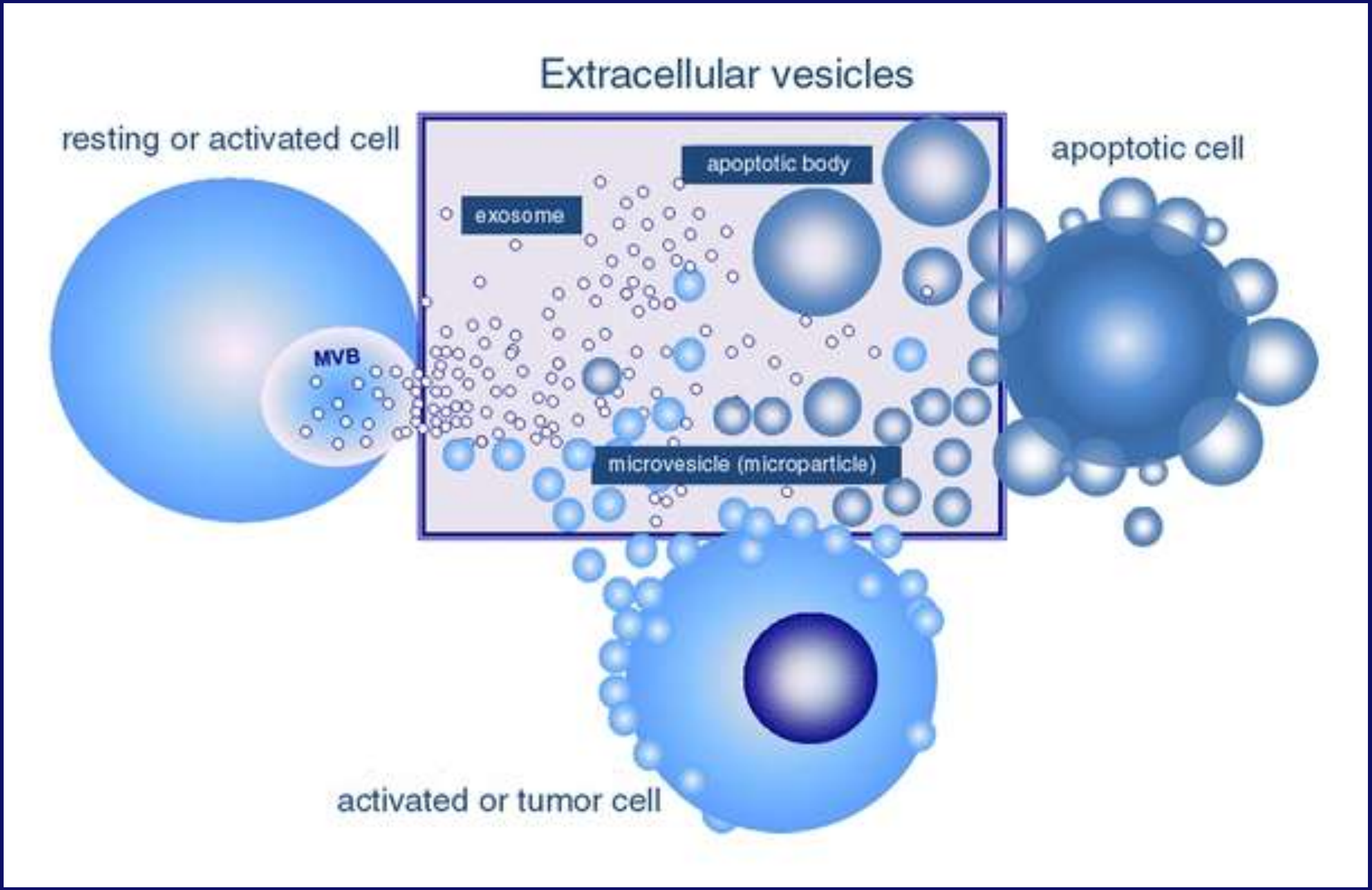
Treatment With Ionis-FXI$_{Rx}$ Produced a Dose-dependent and Sustained Decrease in Factor XI Activity



Buller H et al N Engl J Med 2015

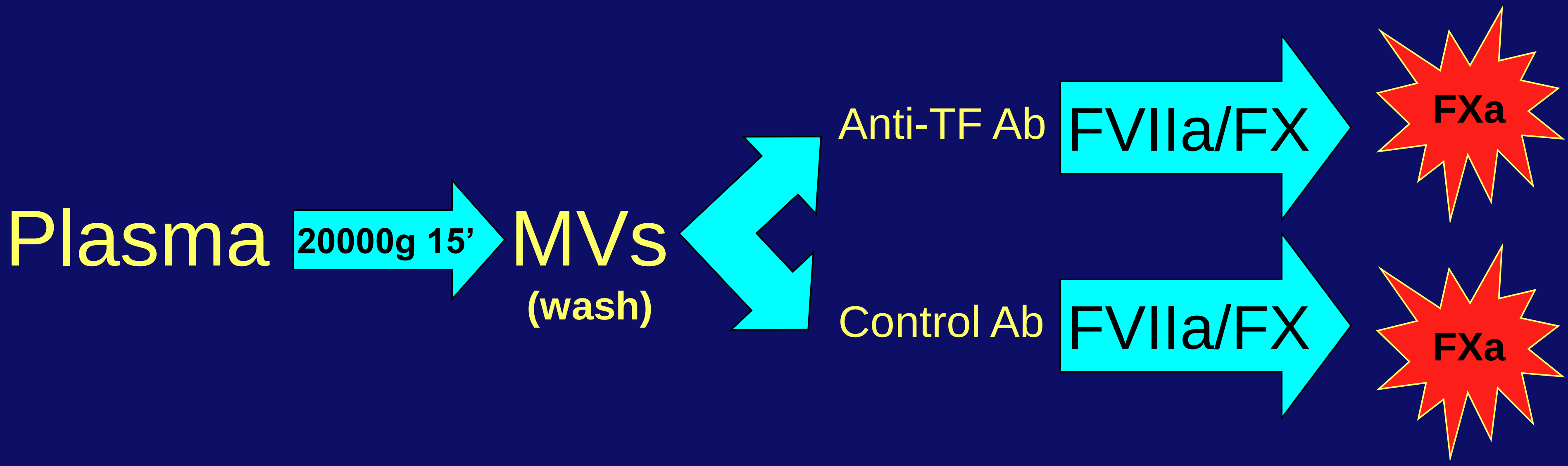
WORLD THROMBOSIS DAY
13 OCTOBER

Extracellular Vesicles



Gyorgy B et al Cell Mol Life Sci 2011

Development of a New Assay to Measure Levels of MV TF Activity in Plasma



TF-dependent and TF-independent factor Xa activity

Khorona A et al JTH 2008, Wang J-G et al JTH 2009
Tesselaar M et al JTH 2007

Healthy individuals do not have TF^{+} MVs in the circulation

TF is expressed in a tissue-specific manner (e.g. high in heart and low in skeletal muscle)

Hypothesis:
TF provides additional hemostatic protection to select tissues

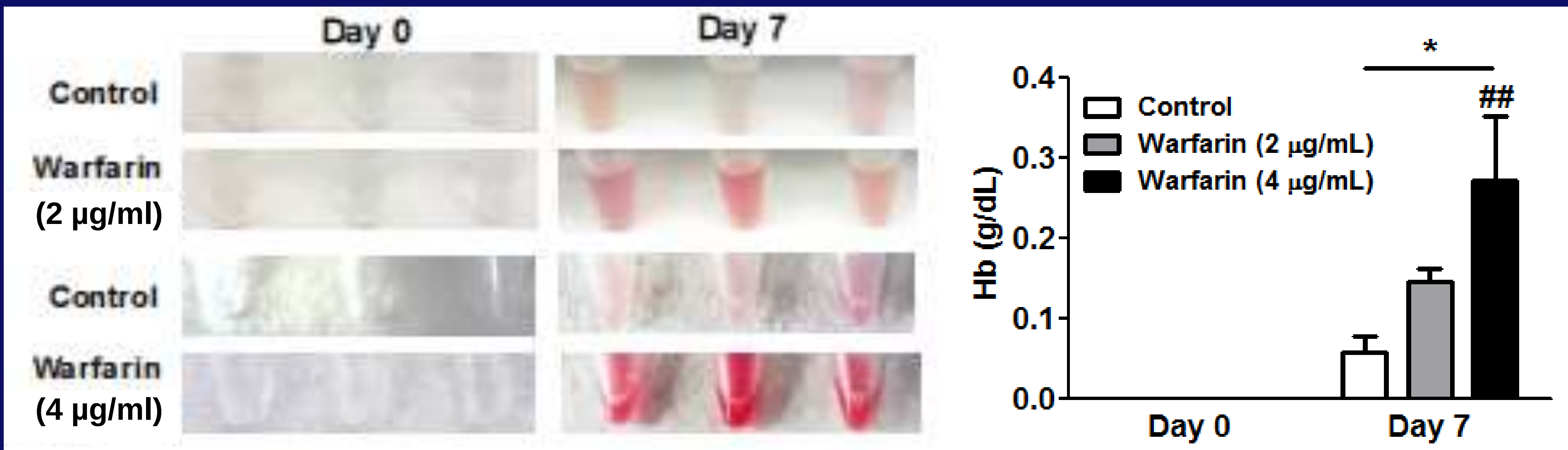
Analyze the hemostatic defects in low TF mice

Spontaneous Hemostatic Defects in Low TF Mice

Uterus	Fatal hemorrhage post-partum	Erlich PNAS 1999
Placenta	Blood pools	Erlich PNAS 1999
Heart	Hemosiderin, fibrosis	Pawlinski PNAS 2002
Lung	Hemosiderin, fatal hemorrhages	Pedersen Blood 2005
Testis	Hemorrhage, calcification	Mackman JTH 2007
Brain	Occasional brain hemorrhage	Pawlinski T&H 2004

Blood vessels in the heart and lung may be more prone to mechanical damage

Warfarin Increases Lung Hemorrhage After IAV Infection



Tatsumi K et al In prep

Conclusion

Anticoagulants may increase alveolar hemorrhage and morbidity in patients infected with influenza A

Cancer Cells and Procoagulant MVs

Tumor Shedding and Coagulation

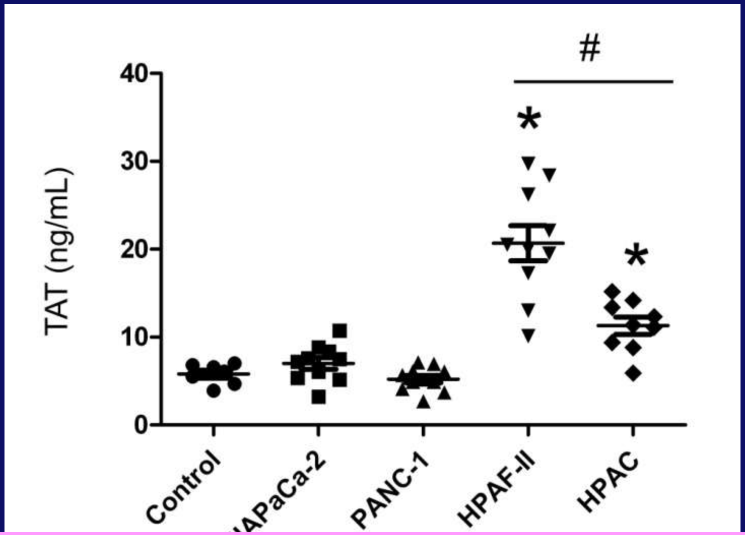
Dvorak HF et al Science 1981

Tumor cells shed plasma membrane vesicles when cultured in vitro. Shed vesicles carrying procoagulant activity may account for activation of the clotting system associated with malignancy

The procoagulant activity of tumor-derived MVs is due to TF

Dvorak et al Cancer Res 1983; Bastida et al Blood 1984;Yu and Rak JTH 2004

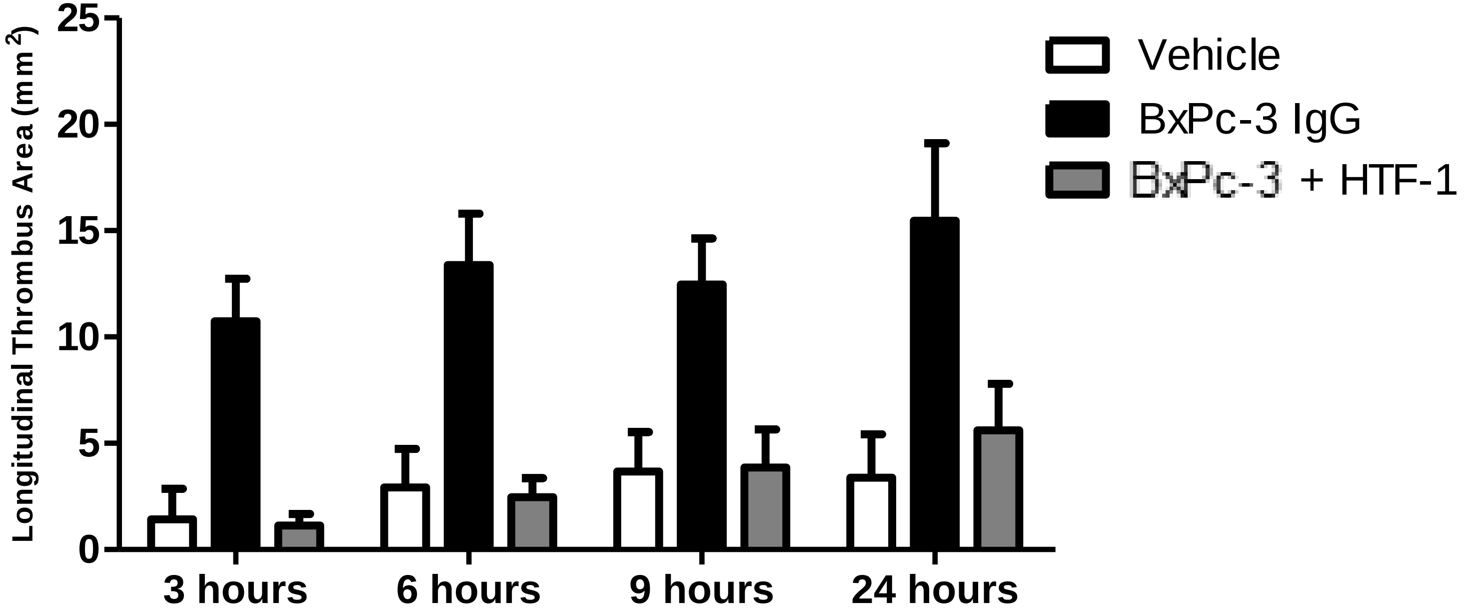
Activation of Coagulation in Nude Mice containing Human Pancreatic Tumors



An anti-human TF Ab reduces the activation of coagulation

Wang JG et al Blood 2012

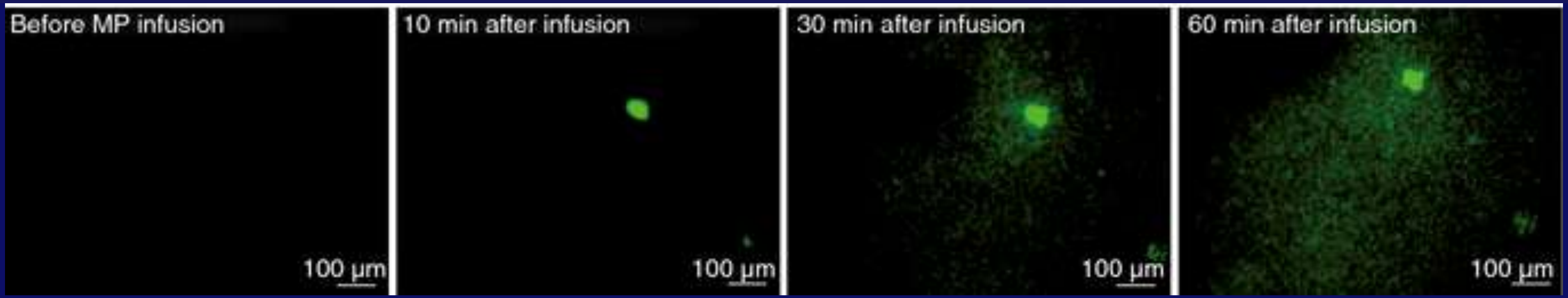
Exogenous TF^{+} Tumor MVs Enhance Thrombosis in the IVC Stenosis Model



TF on tumor MV is required for the enhancement of thrombosis

Geddings J et al JTH 2015

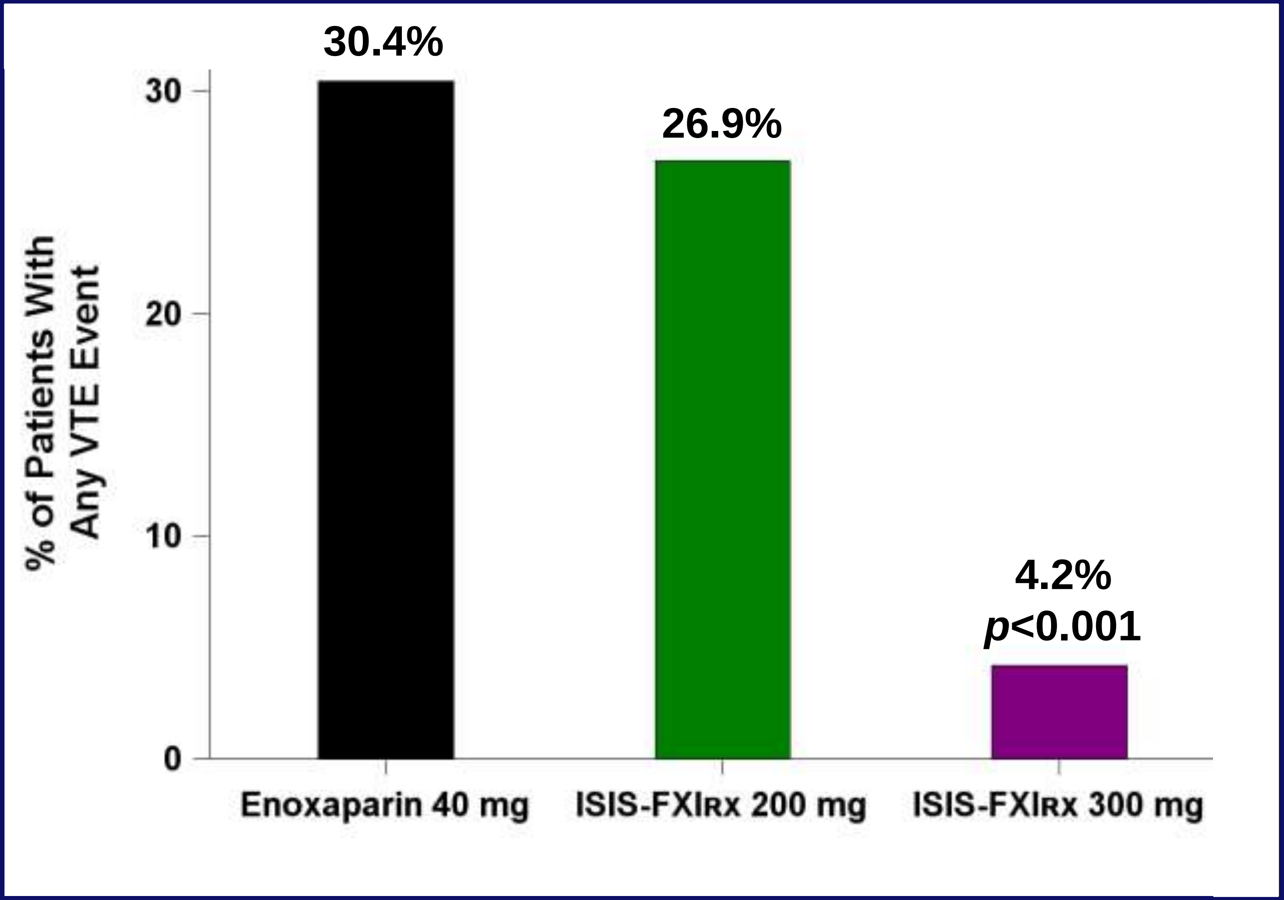
Injected Tumor MVs Localize to the Site of Thrombosis in an IVC Stenosis Model



Panc02 = mouse pancreatic cancer cell line

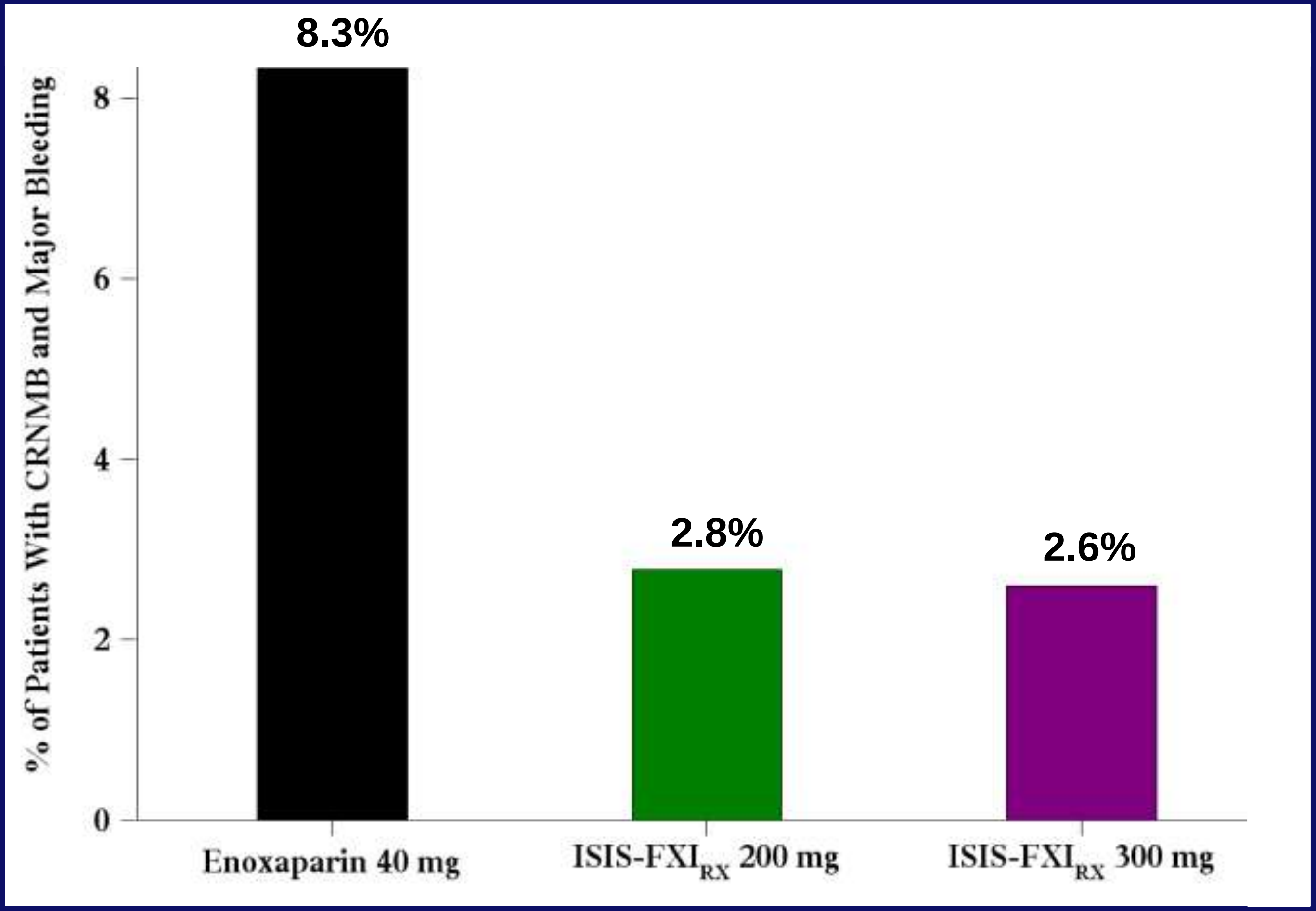
Thomas G et al JTH 2015

Primary Efficacy Outcome: Reduction in Incidence of VTE with Ionis-FXI_{Rx} Compared with Enoxaparin



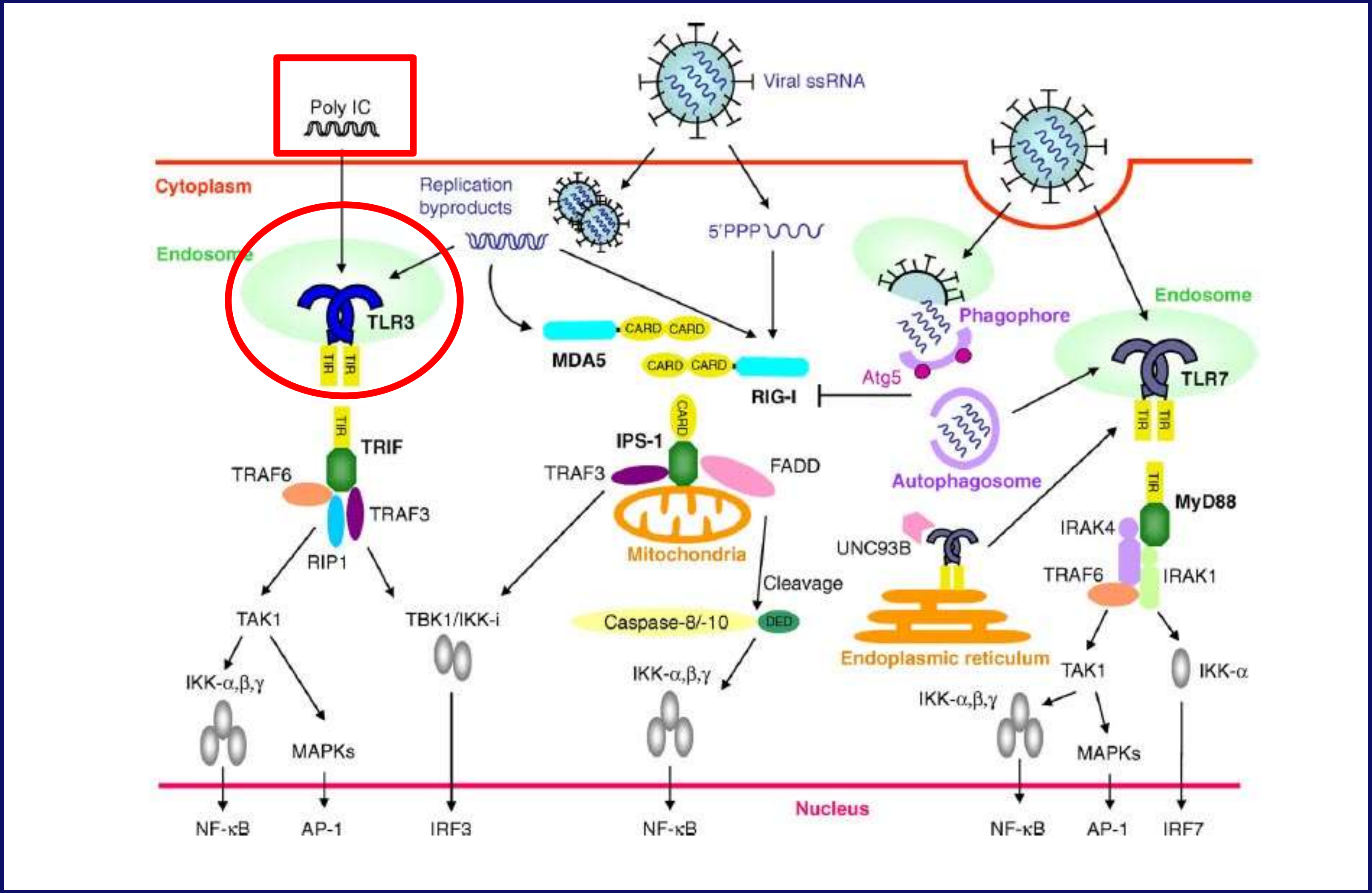
Buller H et al N Engl J Med 2015

Clinically Relevant Bleeding Events with Ionis-FXI_{Rx} or Enoxaparin



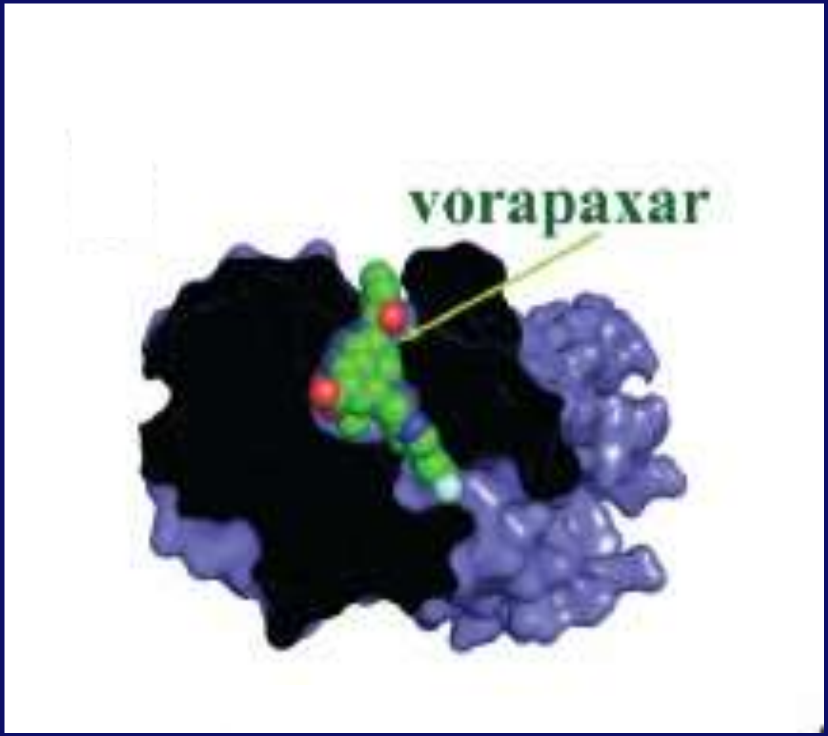
Buller H et al N Engl J Med 2015

Detection of ssRNA Virus Infections by the Immune System



Koyama S et al Cytokine 2009

Development of a New Antiplatelet Drug that Targets PAR-1



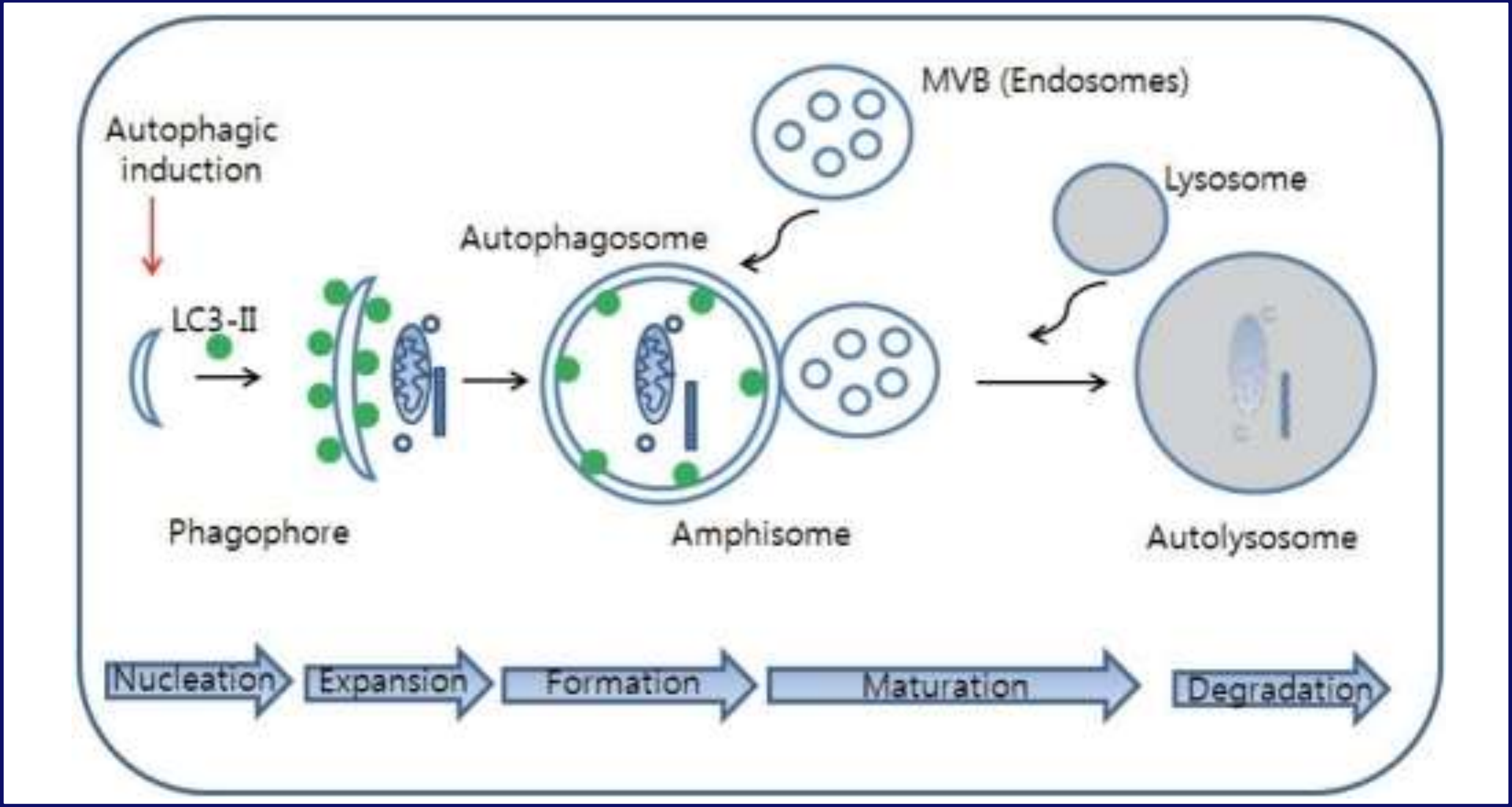
Vorapaxar (Zontivity) was approved by the FDA in 2014 for use with daily aspirin and/or clopidogrel

Yang C et al Nature 2012

Virus Infection and Autophagy

- Autophagy is usually used to destroy viruses.
- However, CVB3 is different because it utilizes autophagy to increase its replication.

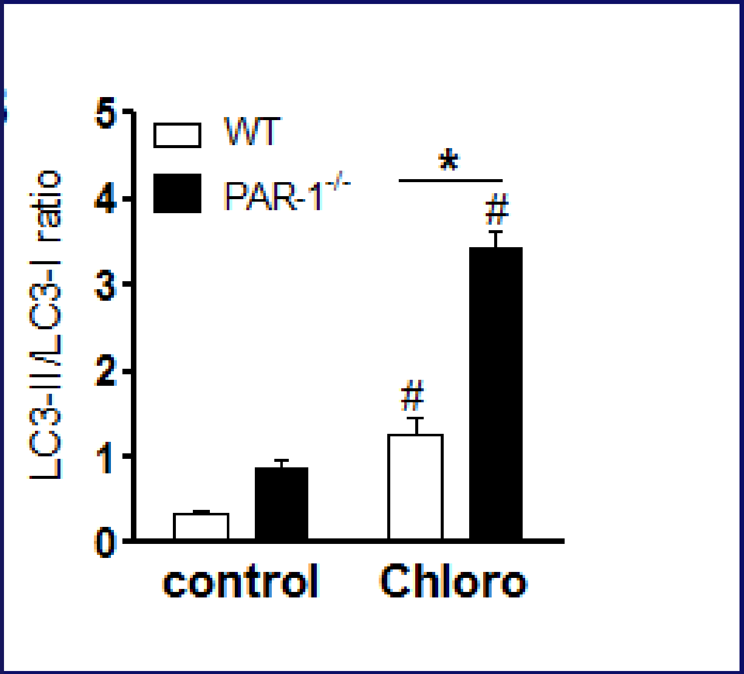
Autophagy



Dr. Y. Ohsumi received the

Hypothesis: PAR-1 reduces CVB3 replication by inhibiting autophagy

Effect of PAR-1 Deficiency on Autophagy



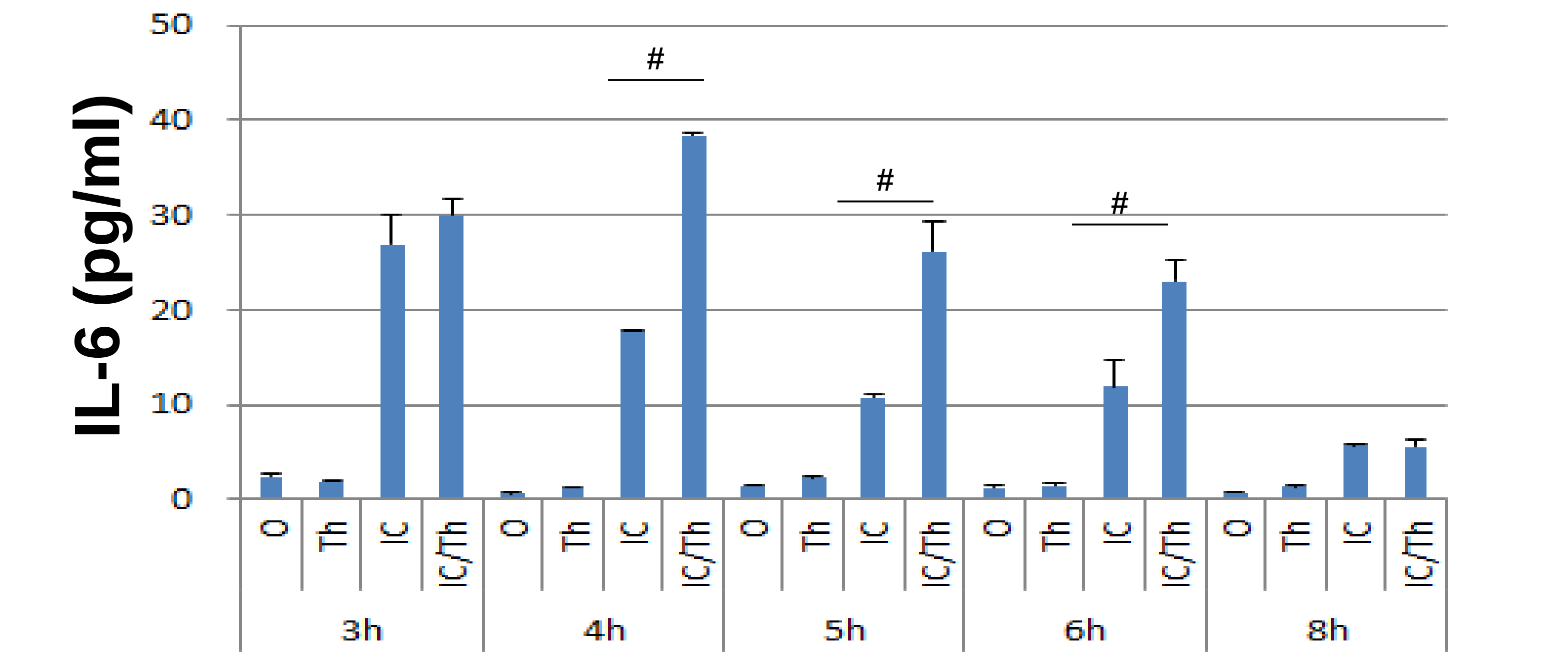
Cloroquine inhibits autophagy and increases the accumulation of LC3-II and LC3-I

Vanja S et al. unpublished data

Conclusion

- PAR-1 reduces CVB3 replication in CMs.
- Activation of autophagy increases CVB3 replication.
- PAR-1 deficiency is associated with increased autophagy.

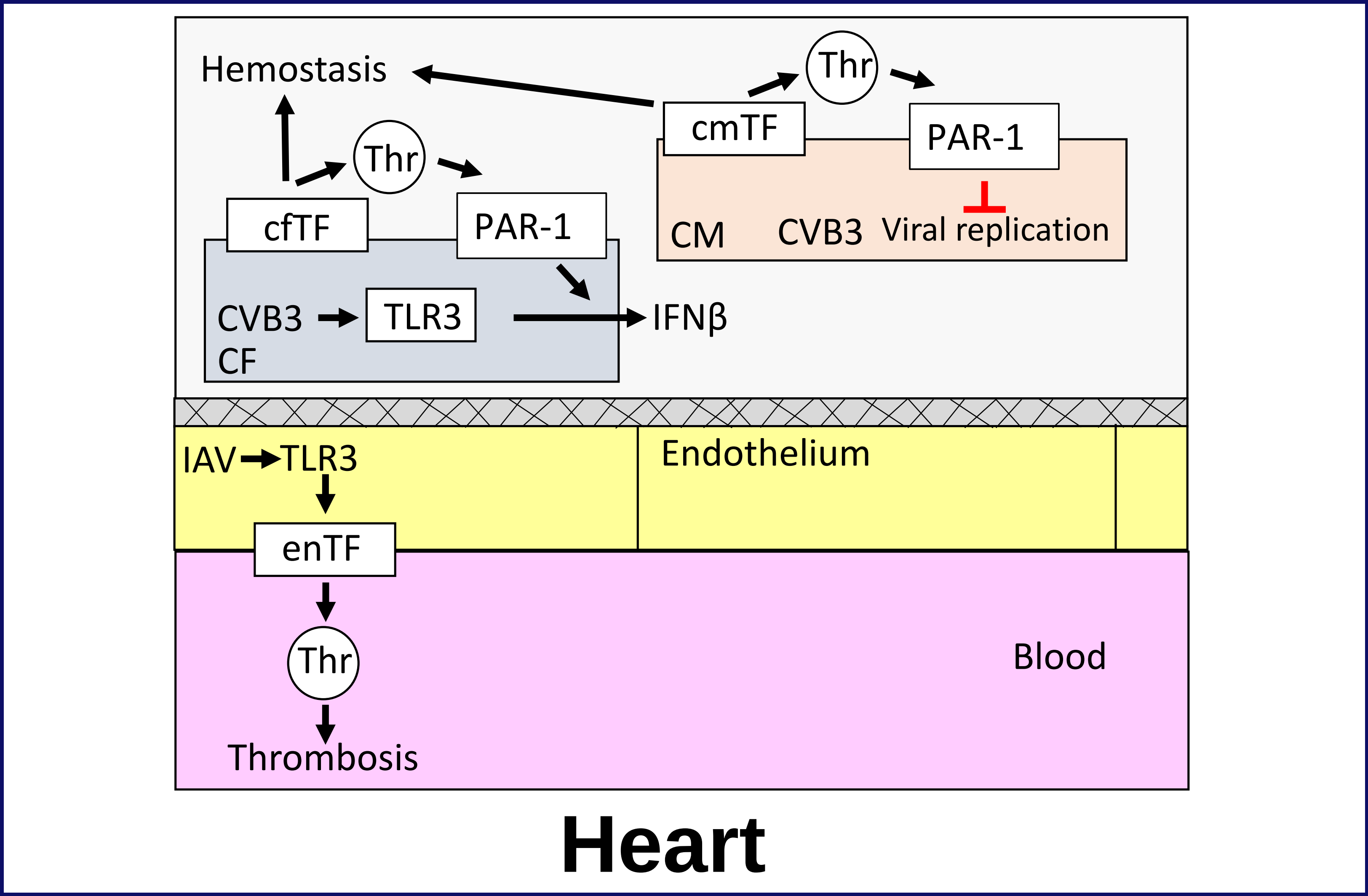
PAR-1 Enhances Poly I:C Induction of IL-6 Expression in Primary Human Lung Epithelial Cells



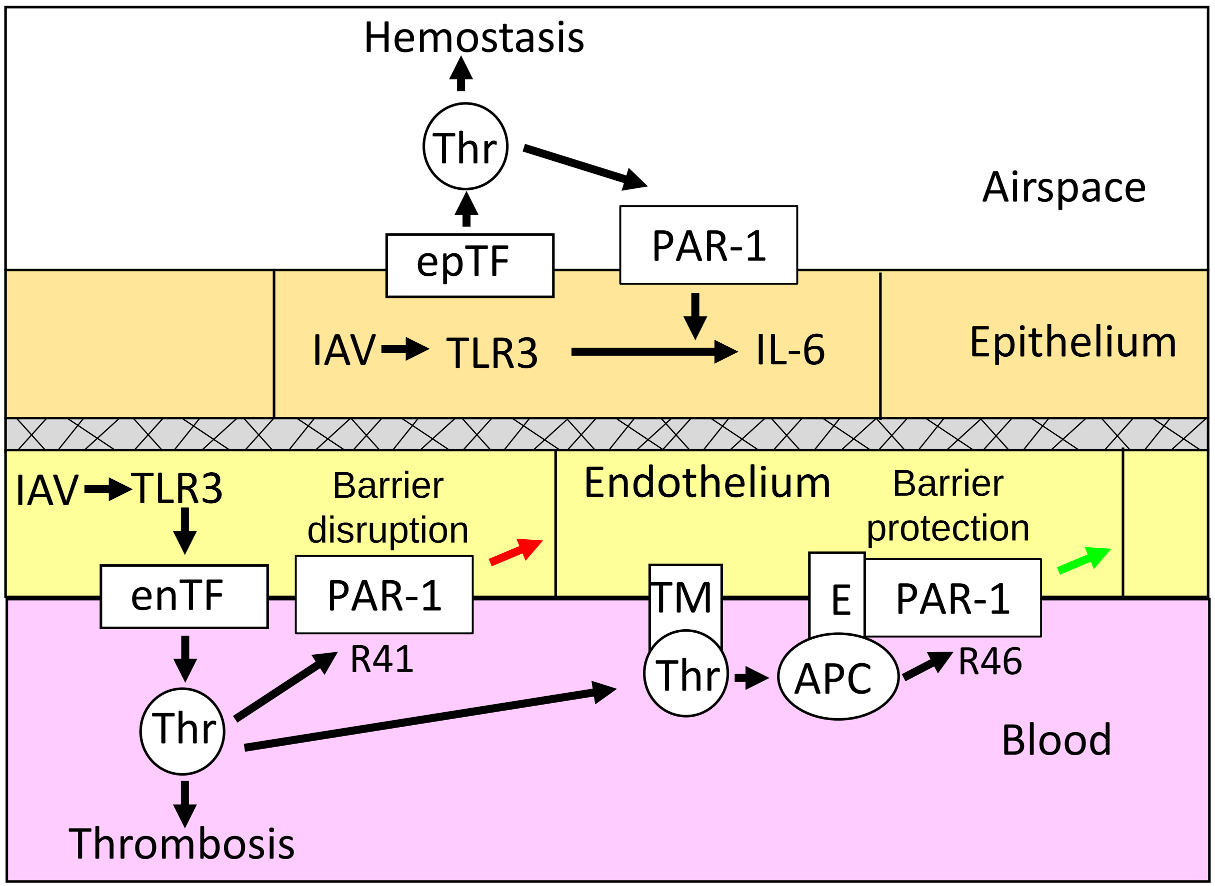
No differences in poly I:C induction of IFNβ, CXCL10, OAS1, TNFα and MCP-1

Tatsumi K et al. unpublished data

Roles of the TF-Thrombin-PAR-1 Pathways in CVB3-induced Myocarditis



Roles of the TF-Thrombin-PAR-1 Pathways in Influenza A Infection



Lung

Viral Infections and the Clotting Cascade

- Acute viral infections lead to strong activation of the clotting cascade and secondary hemorrhage (i.e. Ebola hemorrhagic fever).
- Inhibition of TF reduced death in a monkey model of Ebola.
- Chronic viral infections lead to a low level activation of the clotting cascade that increases cardiovascular disease (i.e. HIV).
- Two ongoing clinical trials are targeting FXa and PAR-1 in HIV patients.

Coagulation and the Innate Immune Response to Pathogens

- Formation of a clot reduces dissemination of pathogens
- Fibrin enhances activation of macrophages and cytokine expression by binding to Mac-1 (CD11b/CD18)
- Thrombin activation of PAR-1 enhances IFN-β and CXCL10 expression

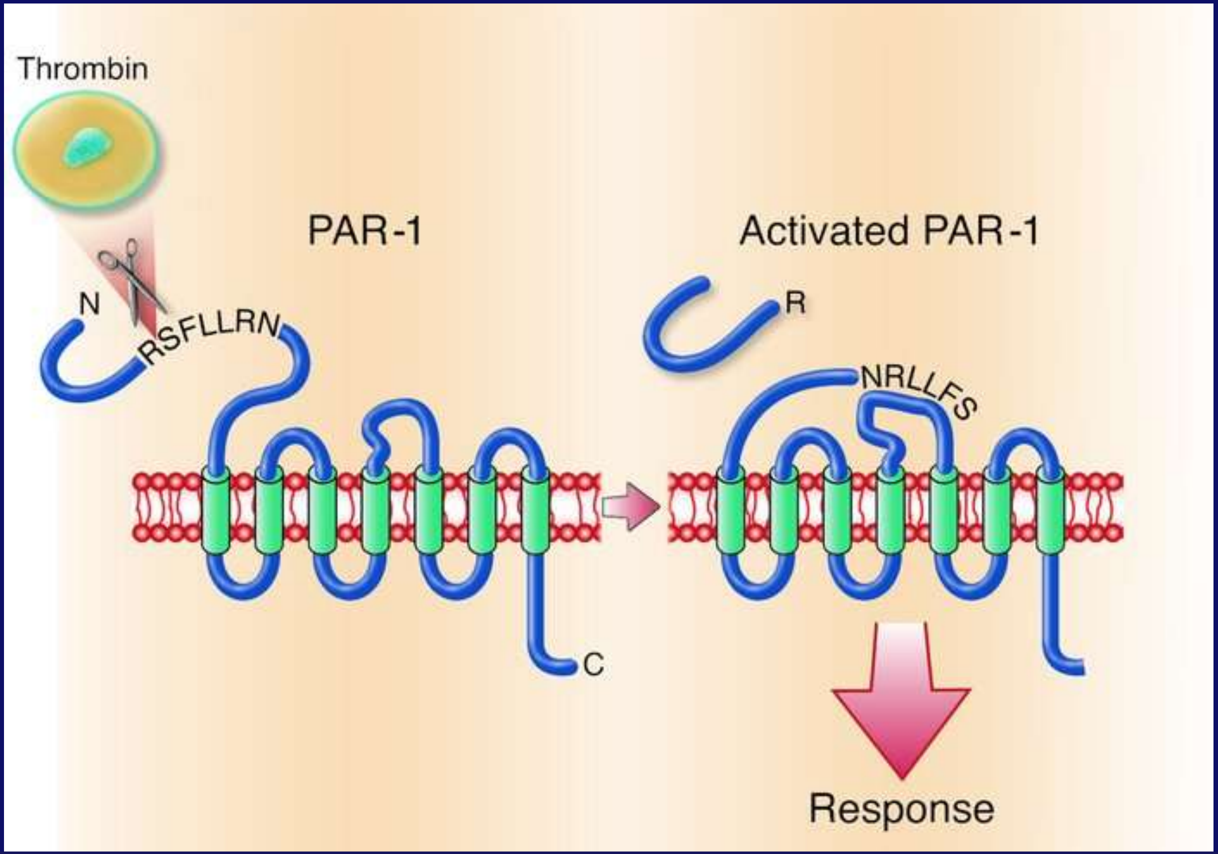
General Hypothesis:
The thrombin-PAR-1 pathway contributes to innate antiviral immunity

The Protease-Activated Receptor Family

	PAR-1	PAR-2	PAR-3	PAR-4
Primary activating proteases (EC_{50})	Thrombin (50 pM)	Trypsin (1 nM) Tryptase (1 nM)	Thrombin (0.2 nM)	Thrombin (5 nM) Trypsin (5 nM)
Secondary activating proteases	Granzyme A FXa Trypsin Plasmin MMP-1	FVIIa FXa MT-SP1		Cathepsin G
PAR-APs	SFLLRN TFLLRN	SLIGKV SLIGIRL SFLLRN		GYPGKF AYPGKF

Adapted from Major et al., 2003 *Arterioscler.Thromb.Vasc.Biol.*

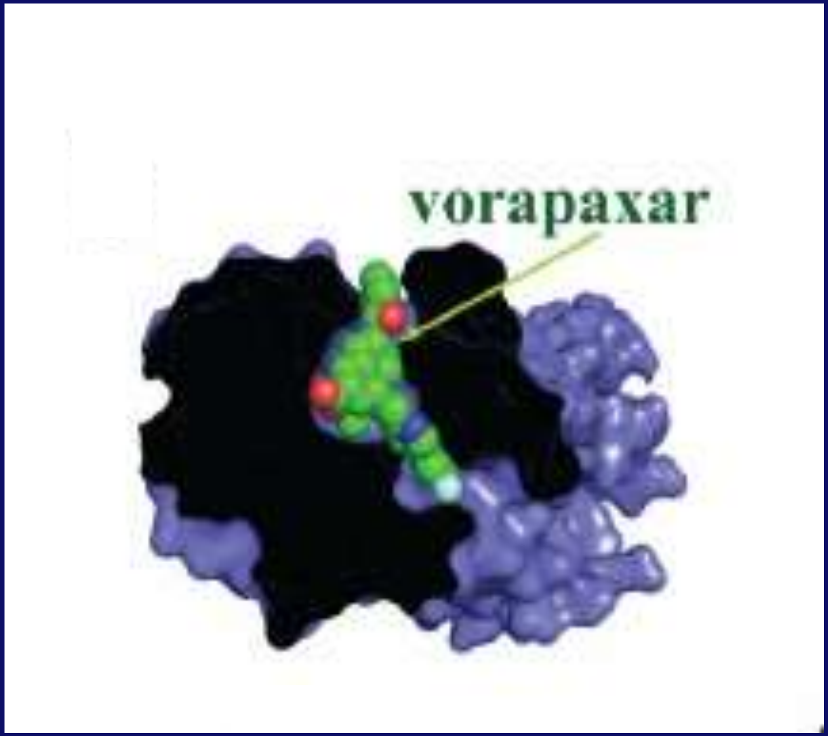
Thrombin Activation of PAR-1



PAR-1 is a unique GPCR because it is activated by proteolytic cleavage. It is the major thrombin receptor on human platelets.

Camerer and Coughlin JCI 2003

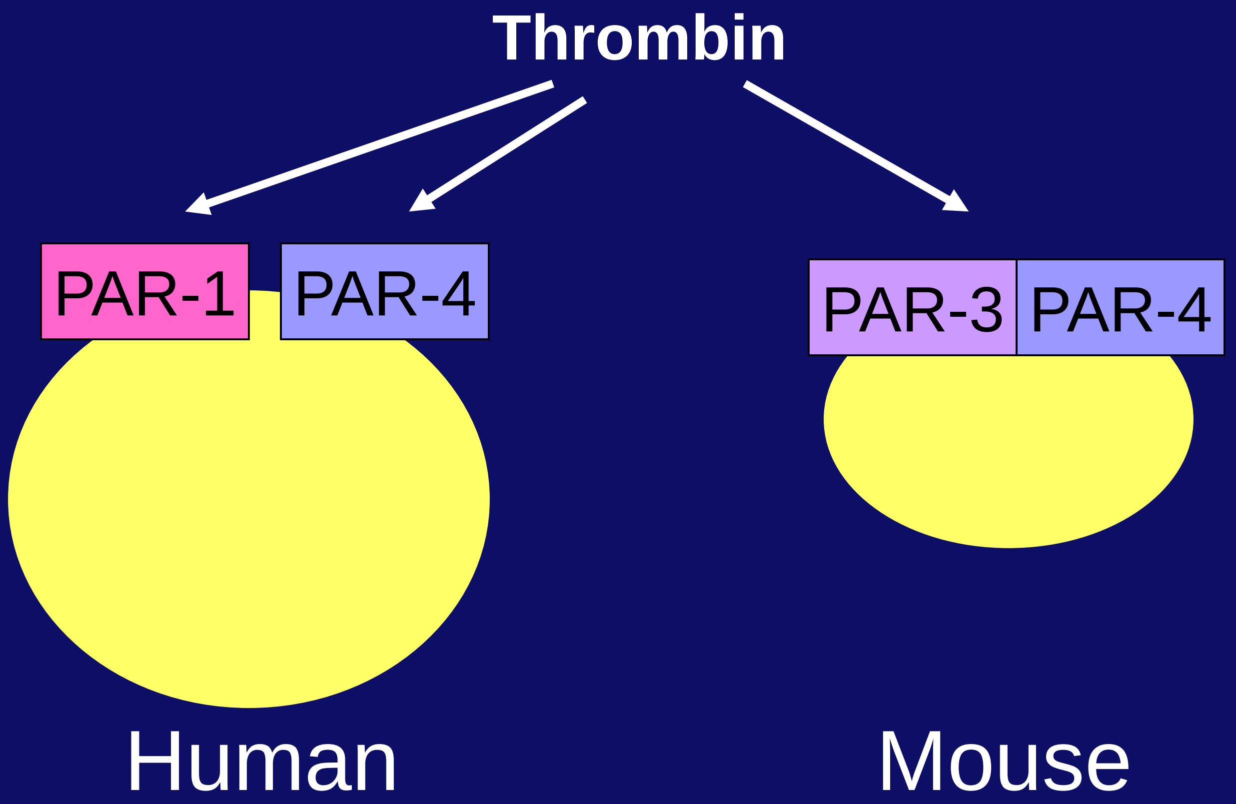
Development of a New Antiplatelet Drug that Targets PAR-1



Vorapaxar (Zontivity) was approved by the FDA in 2014 for use with daily aspirin and/or clopidogrel

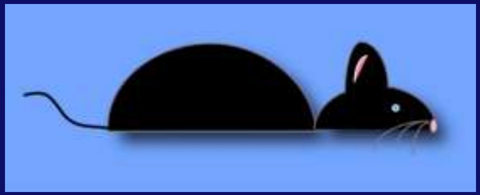
Yang C et al Nature 2012

PAR Expression on Human and Mouse Platelets



Any phenotype in PAR-1$^{-/-}$ mice cannot be due to a defect in thrombin activation of platelets

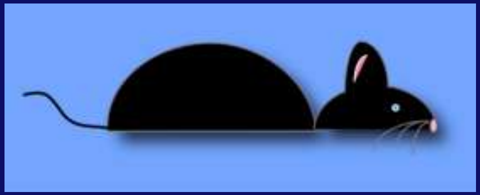
Mouse Models



PAR-1$^{-/-}$ mice

Cell type specific deletion of PAR-1 (crossed with Cre^{+})

Darrow A et al
Thromb & Hemost
1996



PAR-1$^{fl/fl}$ mice

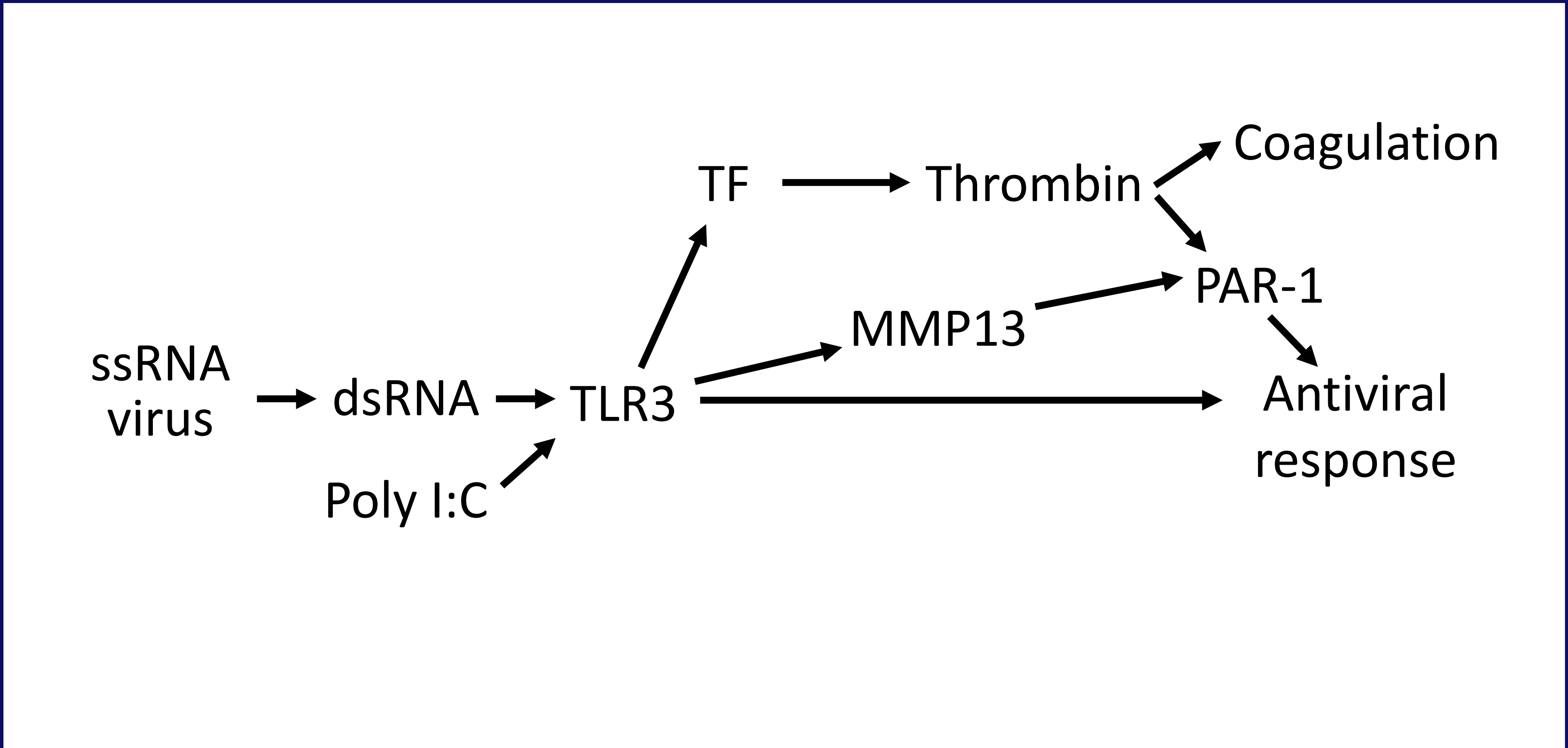
Cell type specific deletion of PAR-1 (crossed with Cre^{+})

Palumbo J
unpublished data

Viral Models

- **Coxsackievirus B3**
  (liver, spleen, pancreas and heart)
- **Influenza A virus**
  (lung)

Role of the Thrombin-PAR-1 Pathway in Innate Antiviral Immunity



Outline

- Poly I:C studies
- Coxsackievirus B3
- Influenza A virus

Poly I:C as a mimetic of an antiviral response

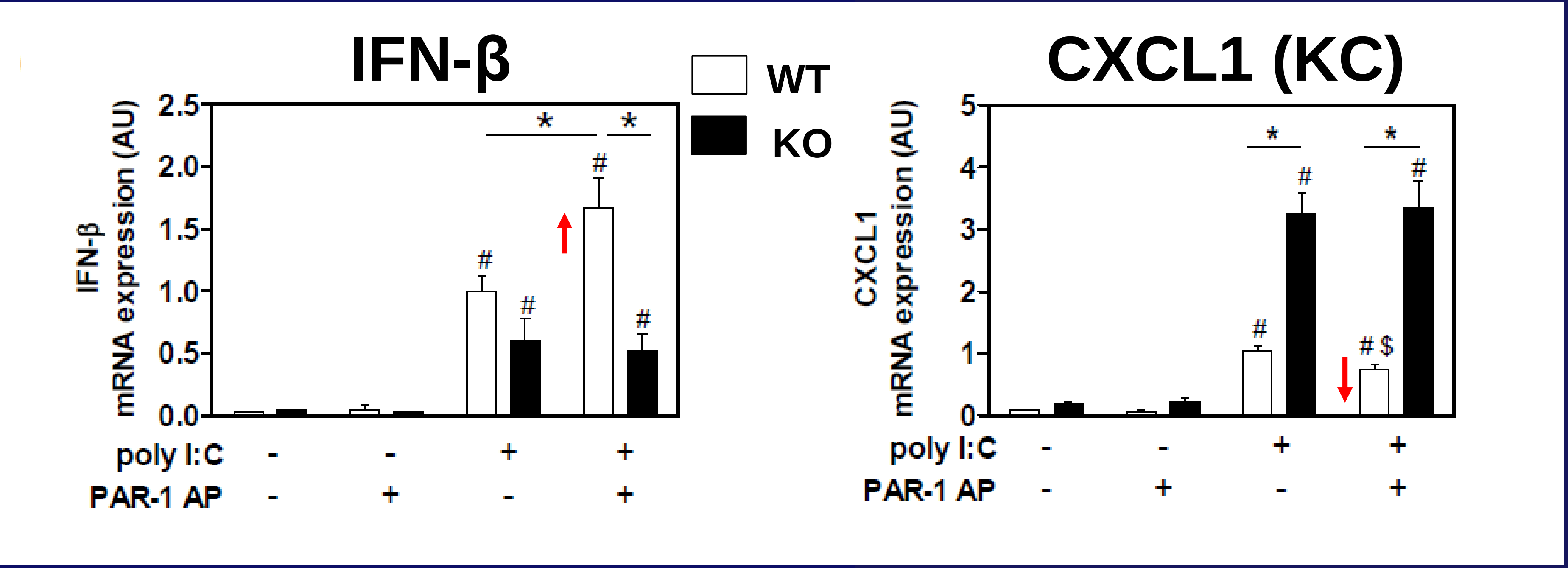
Role of PAR-1 in Poly I:C induction of IFNβ, CXCL10 and CXCL1 in Macrophages and Splenocytes

Poly I:C was used to stimulate TLR3

PAR-1 agonist peptide (AP) was used to stimulate PAR-1

Use mouse bone marrow-derived macrophages (BMDM) and splenocytes

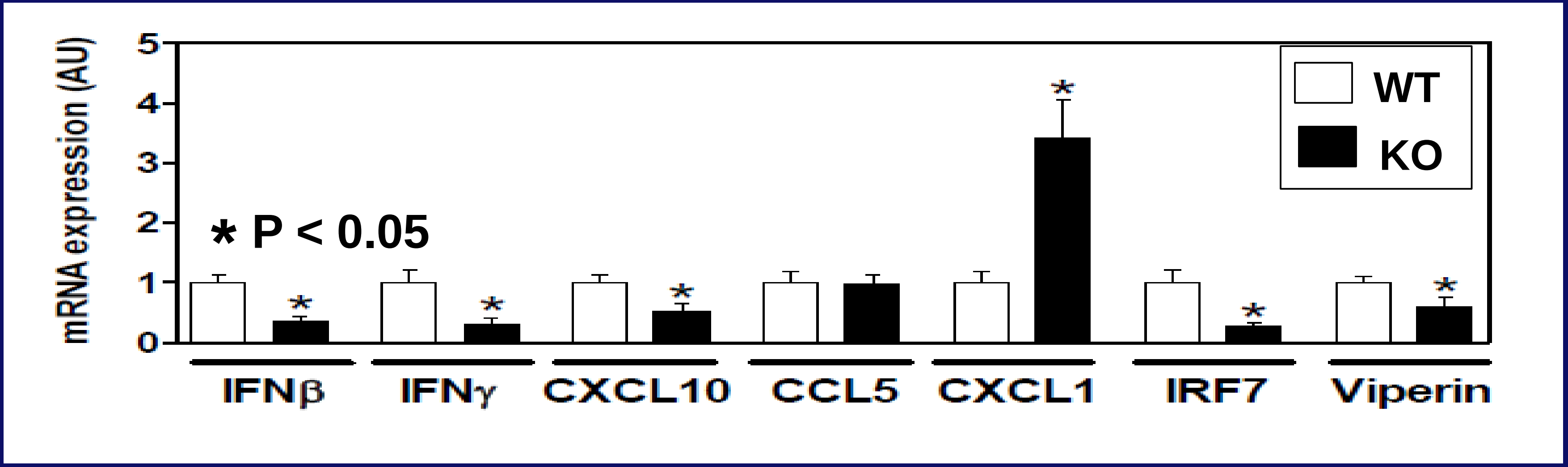
Role of PAR-1 in Poly I:C Induction of Antiviral Genes in Splenocytes

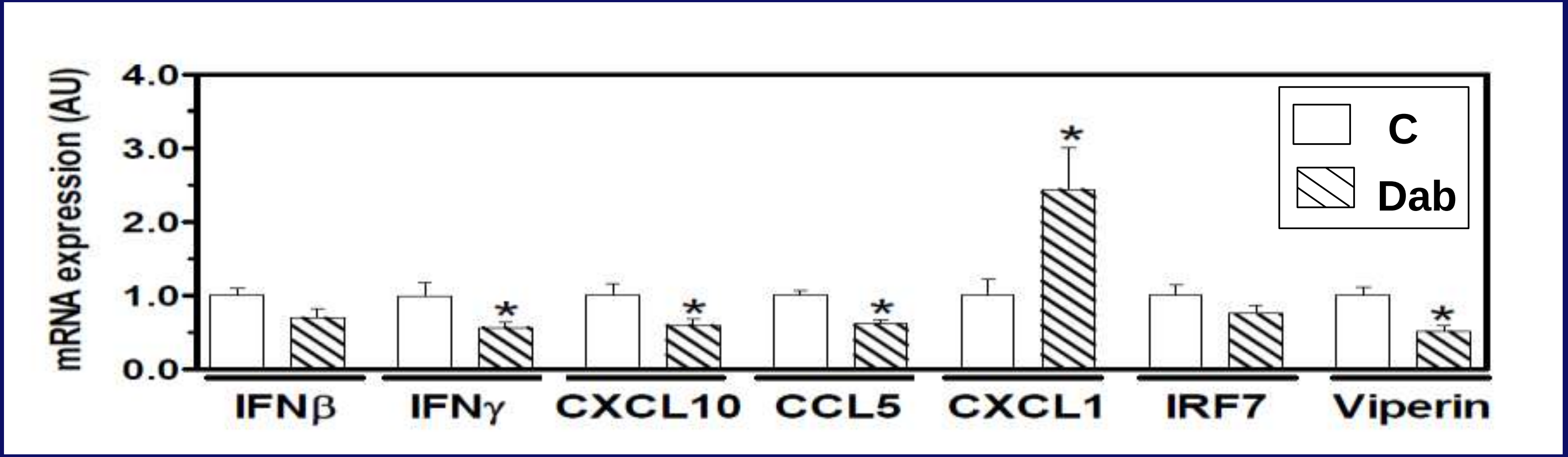


Antoniak S et al unpublished data

Effect of PAR-1 deficiency and thrombin inhibition in WT mice on poly I:C induction of the innate immune response in mice

Effect of PAR-1 Deficiency and Thrombin Inhibition in WT Mice on Poly I:C Induction of Antiviral Genes in the Spleen





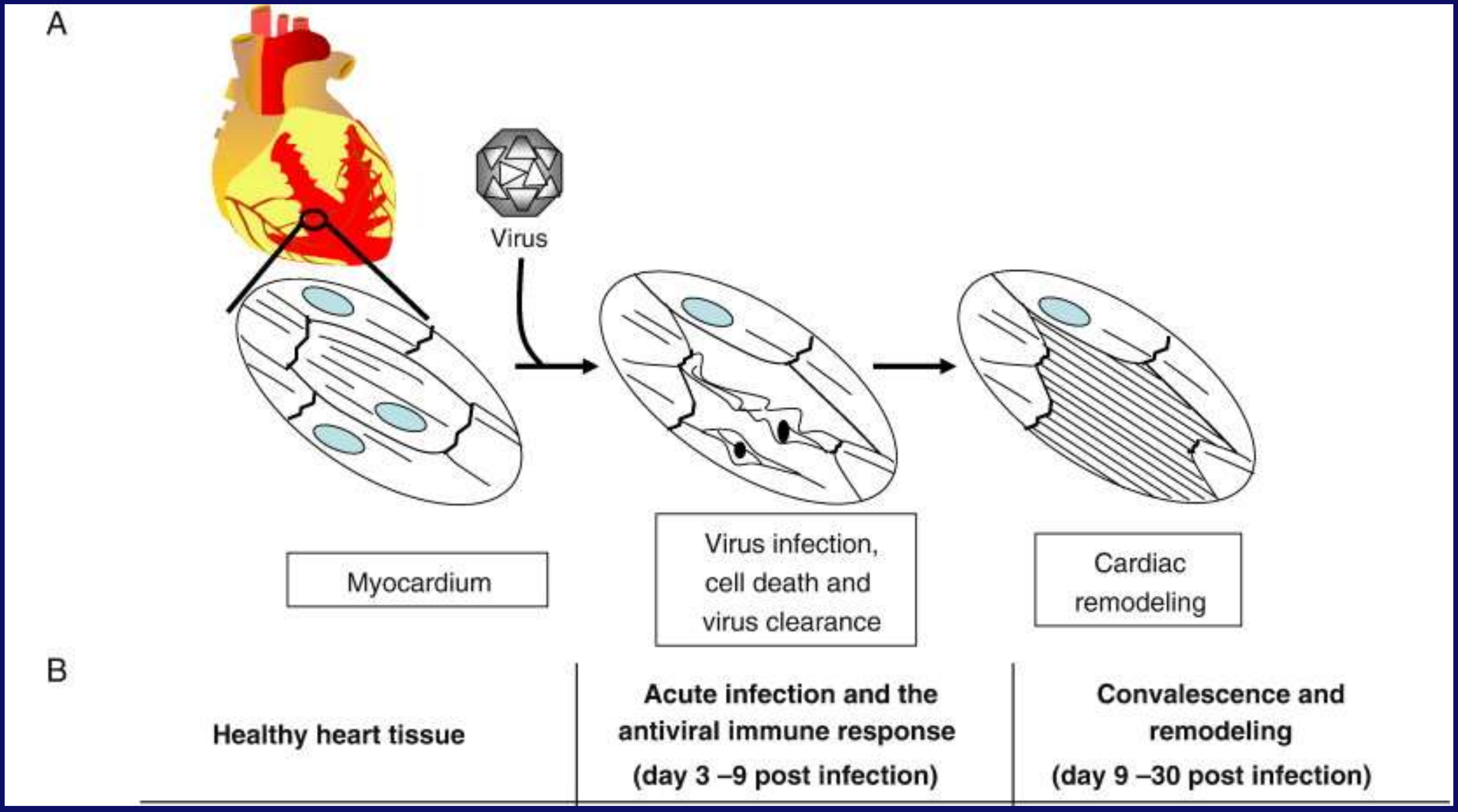
Antoniak S et al unpublished data

Conclusion

PAR-1 deficient mice and WT mice treated with dabigatran have decreased levels of various proteins, such as CXCL10, and increased levels of CXCL1 compared with controls after poly I:C injection

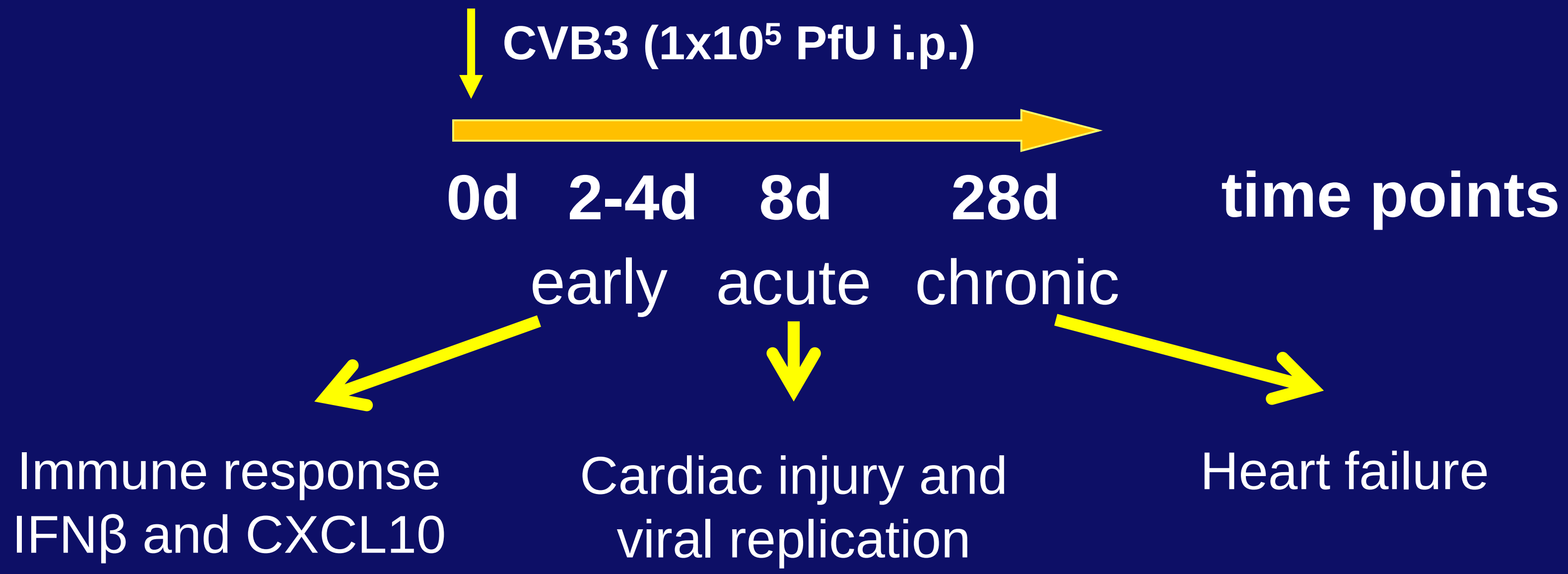
Viral Myocarditis

Accounts for up to 20% of sudden cardiac death among young people (<40 years of age)



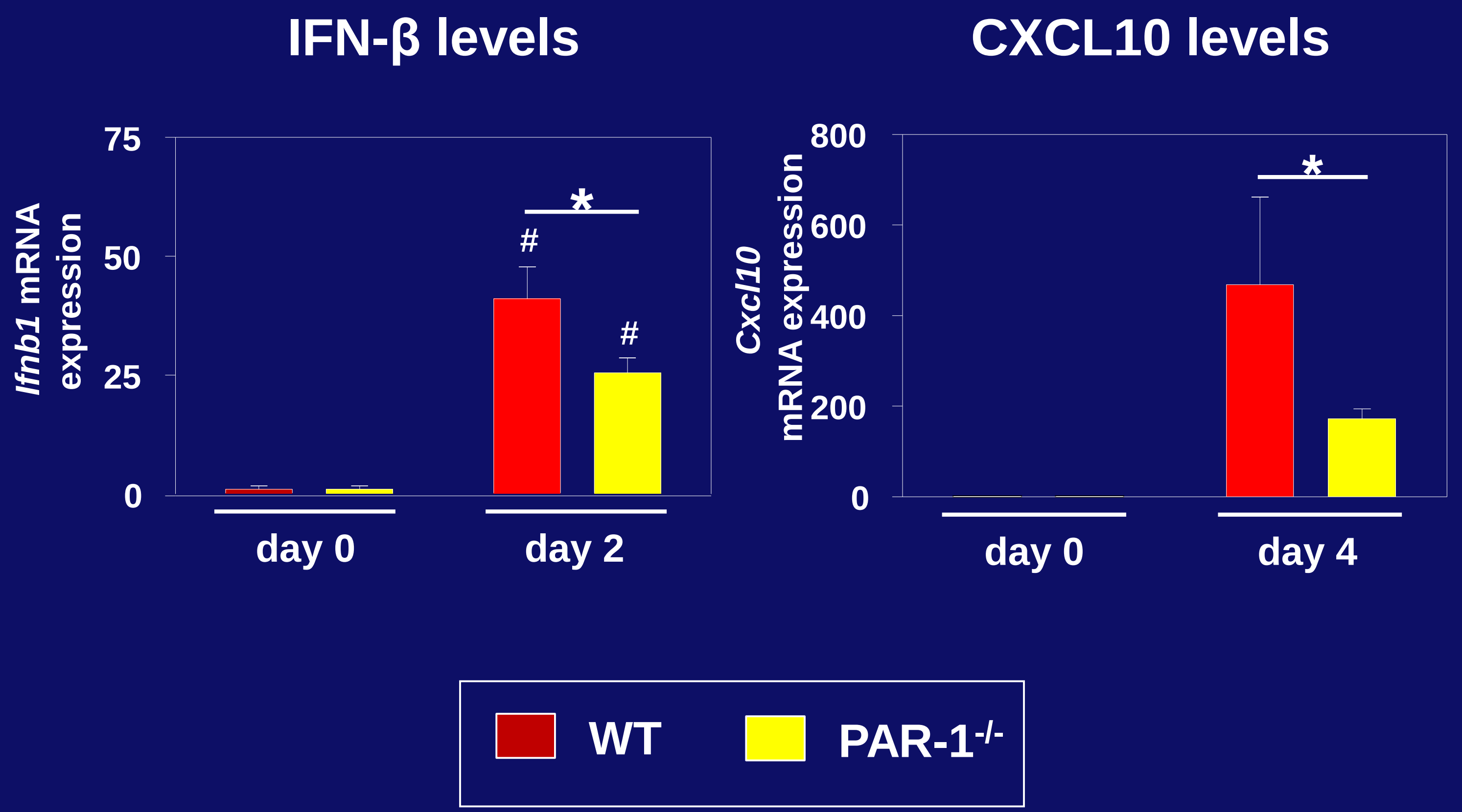
Mouse Model of Coxsackievirus B3 Myocarditis

- Coxsackievirus B3 (CVB3) = cardiotrophic ssRNA(+) virus



Silvio Antoniak

PAR-1 Deficiency is Associated with Reduced IFN-β and CXCL10 expression After CVB3 Infection



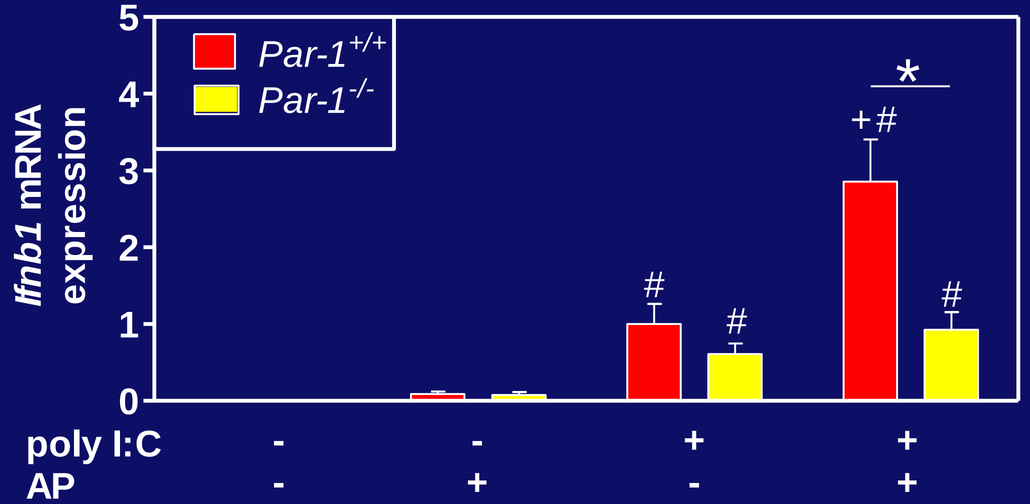
*P<0.05; #P<0.05 vs. day 0
Two-Way ANOVA

Antoniak et al, JCI 2013

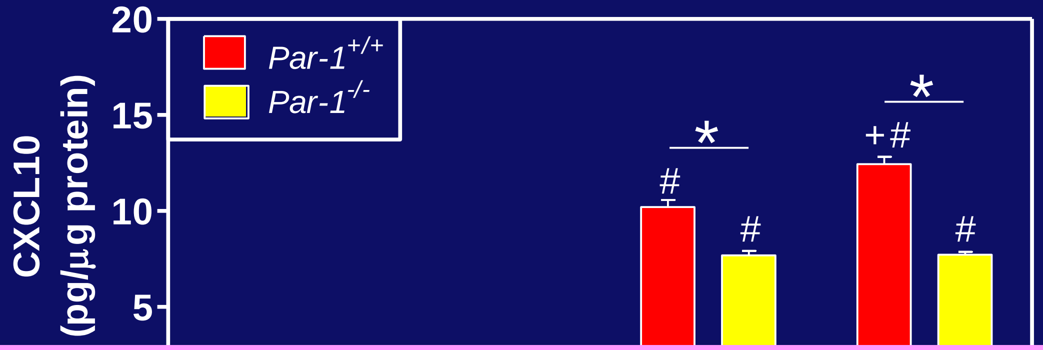
BMT studies indicated that PAR-1 on both hematopoietic and non-hematopoietic cells contributed to the protection from CVB3 infection

PAR-1 Activation Enhances TLR3-dependent IFN-β and CXCL10 Expression in Cardiac Fibroblasts

IFN-β mRNA



CXCL10 Protein

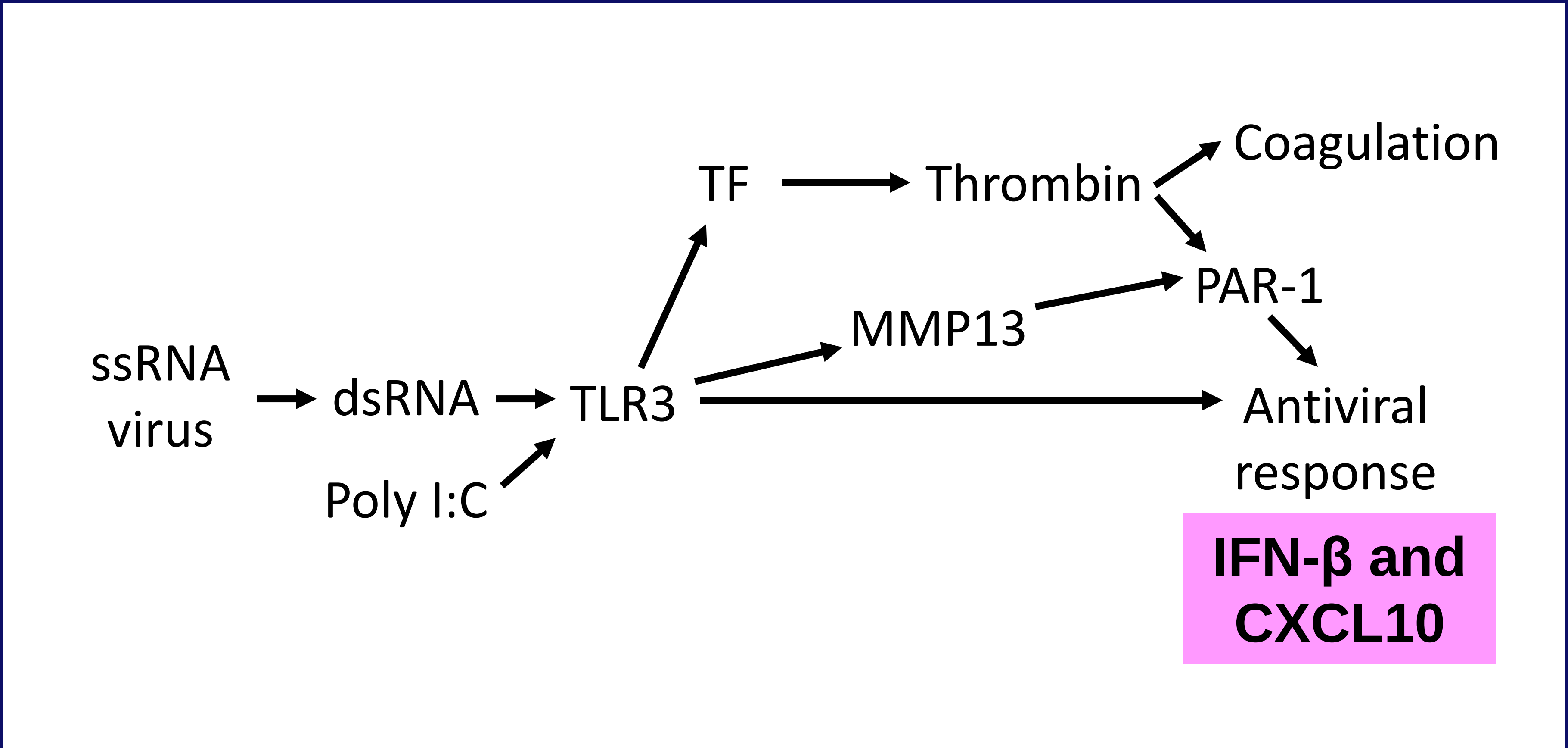


Poly I:C induces MMP13 expression that activates PAR-1

Ongoing Studies: Effect of Deletion of PAR-1 in Myeloid Cells, Cardiac Fibroblasts or Cardiac Myocytes on CVB3-induced Myocarditis

- PAR-1$^{fl/fl}$,LysM-Cre (myeloid cells)
- PAR-1$^{fl/fl}$,Mlc2v-Cre (CMs)
- PAR-1$^{fl/fl}$,TCF21-iCre (CFs)

Role of the Thrombin-PAR-1 Pathway in Innate Antiviral Immunity



Influenza A Infection

- Influenza A (IAV) is a respiratory pathogen
- IAV causes acute infection of the upper respiratory tract
- Severe IAV infection can cause infection of the lower respiratory tract, resulting in viral pneumonia
- Damage to the barrier formed by epithelial and endothelial cells in the pulmonary alveolus leads to respiratory dysfunction

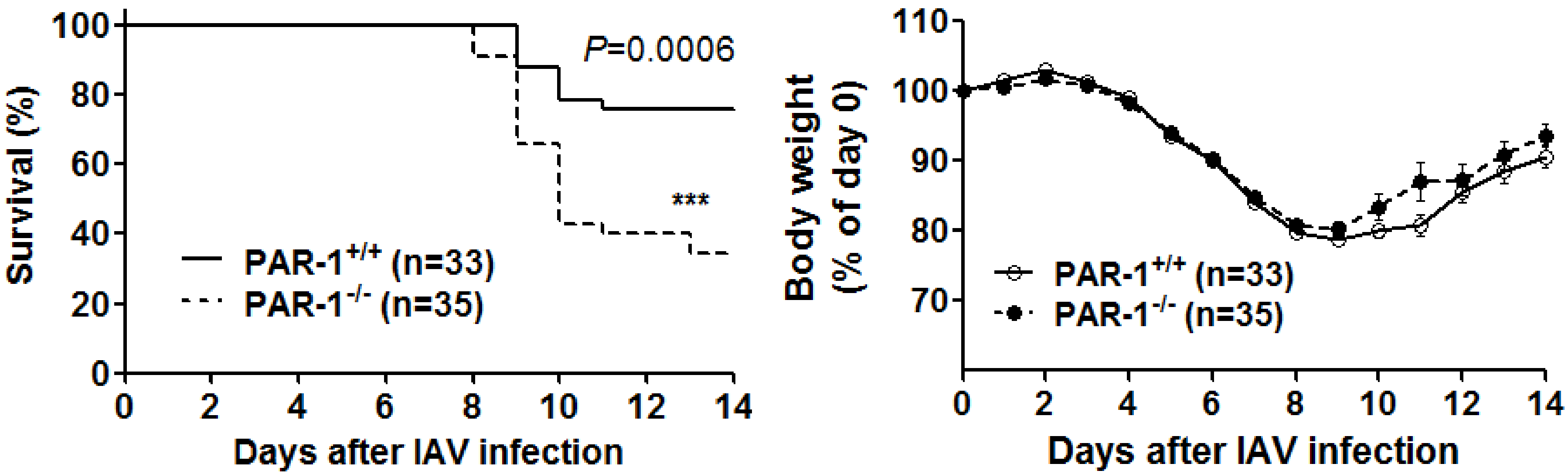
Mouse Model of Influenza A Infection (IAV)

- Influenza A is a ssRNA virus
- Influenza A/Puerto Rico/8/1934 H1N1
- Mouse adapted and highly pathologic
- Infected intranasally

Silvio Antoniak and Kohei Tatsumi

Mouse Model of Influenza A Infection

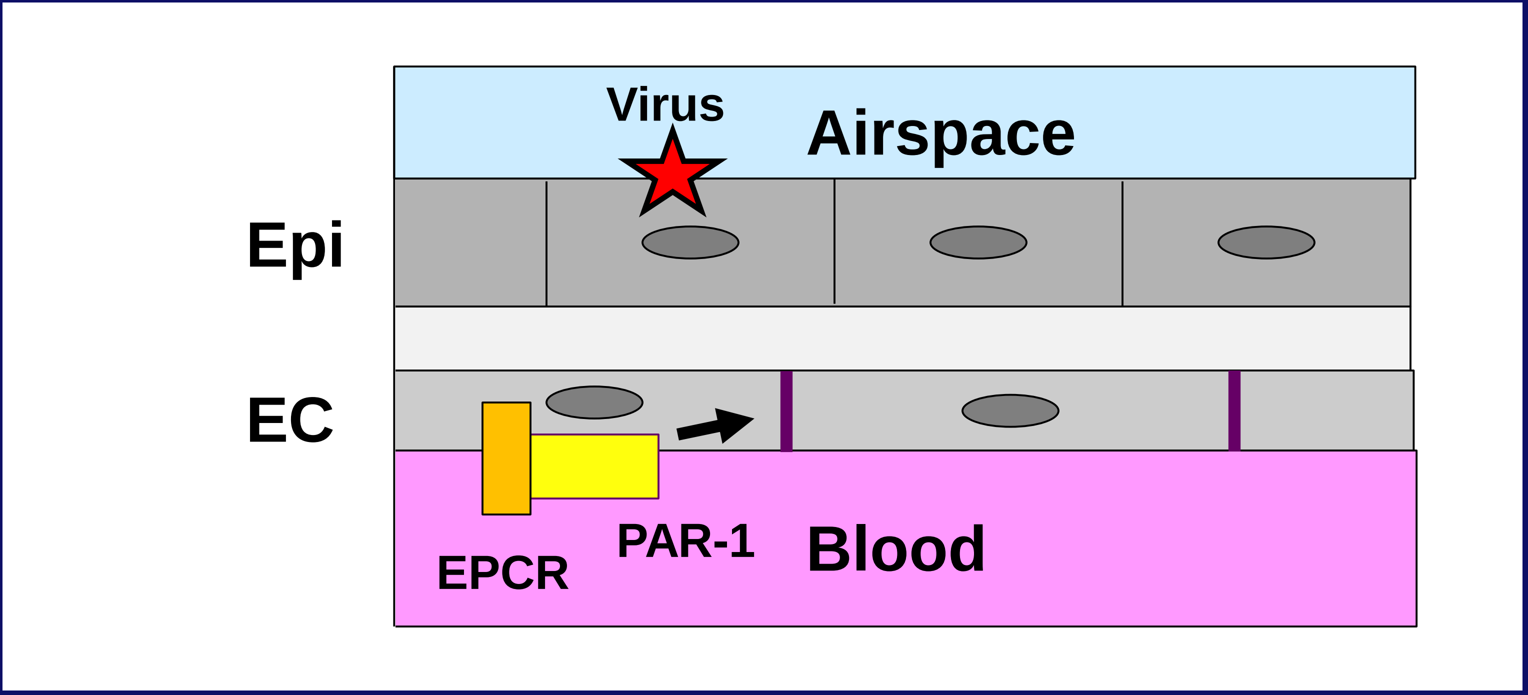
PAR-1 Deficiency is Associated with Increased Mortality after Influenza A Infection



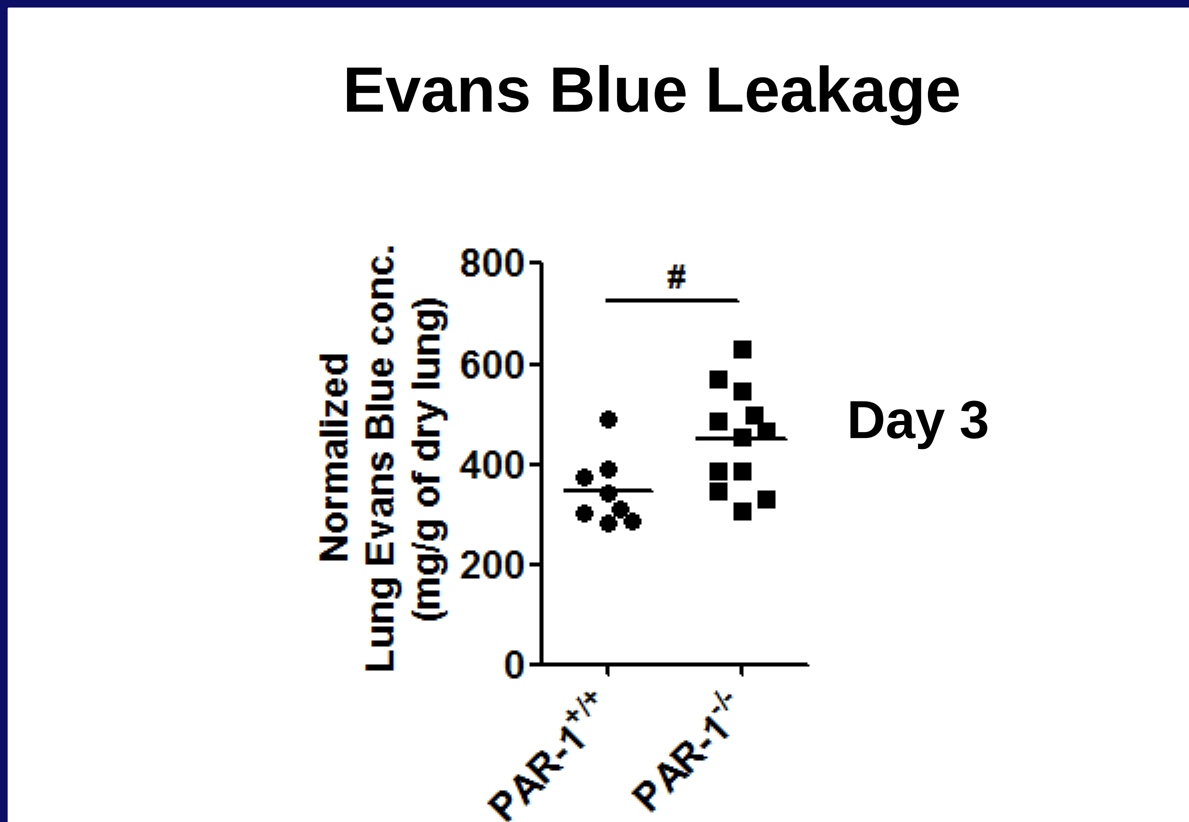
Tatsumi K et al. unpublished data

Hypothesis 1:

APC/EPCR/PAR-1 maintains barrier function during IAV infection



PAR-1 Deficient Mice have Increased Endothelial Permeability after IAV Infection

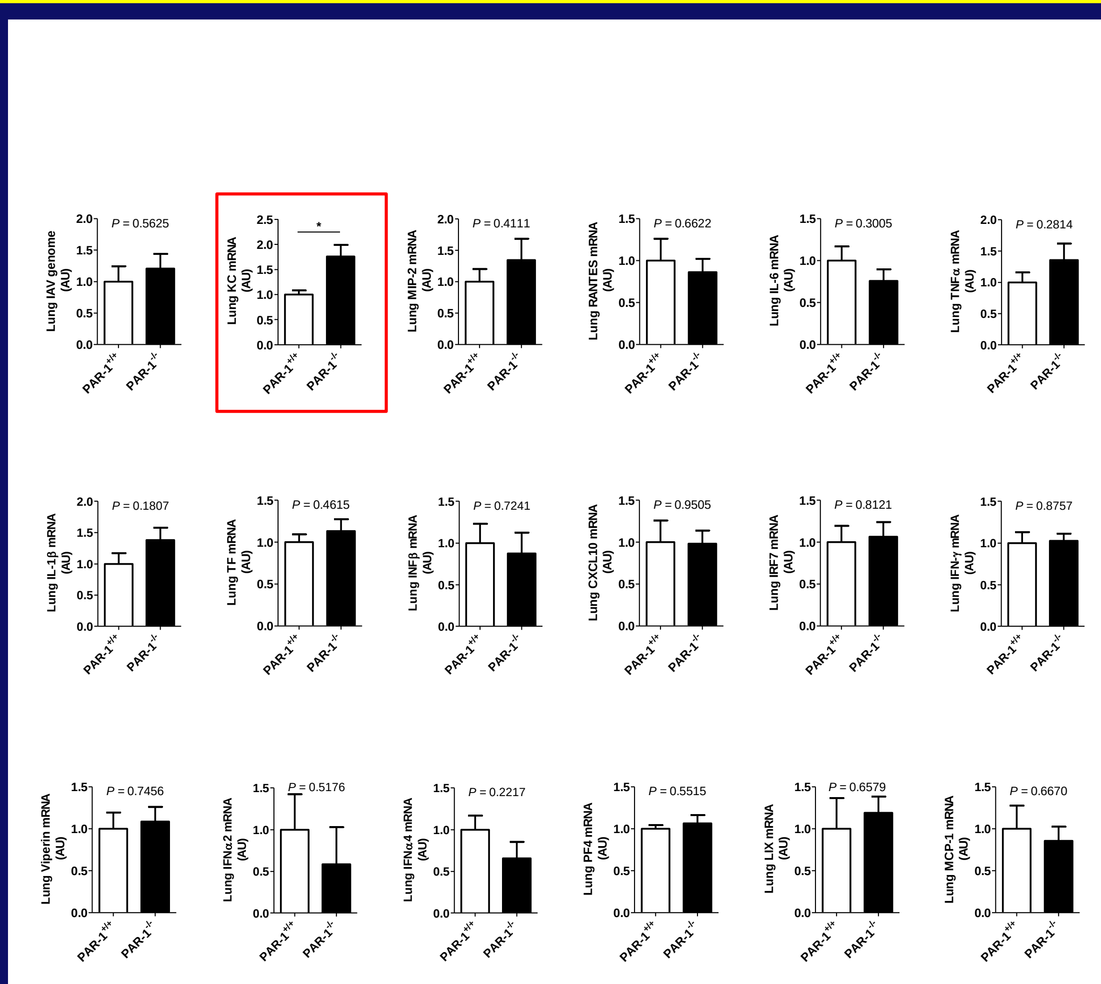


A decrease in barrier protection may contribute to the increased mortality in PAR-1$^{-/-}$ mice

Hypothesis 2:

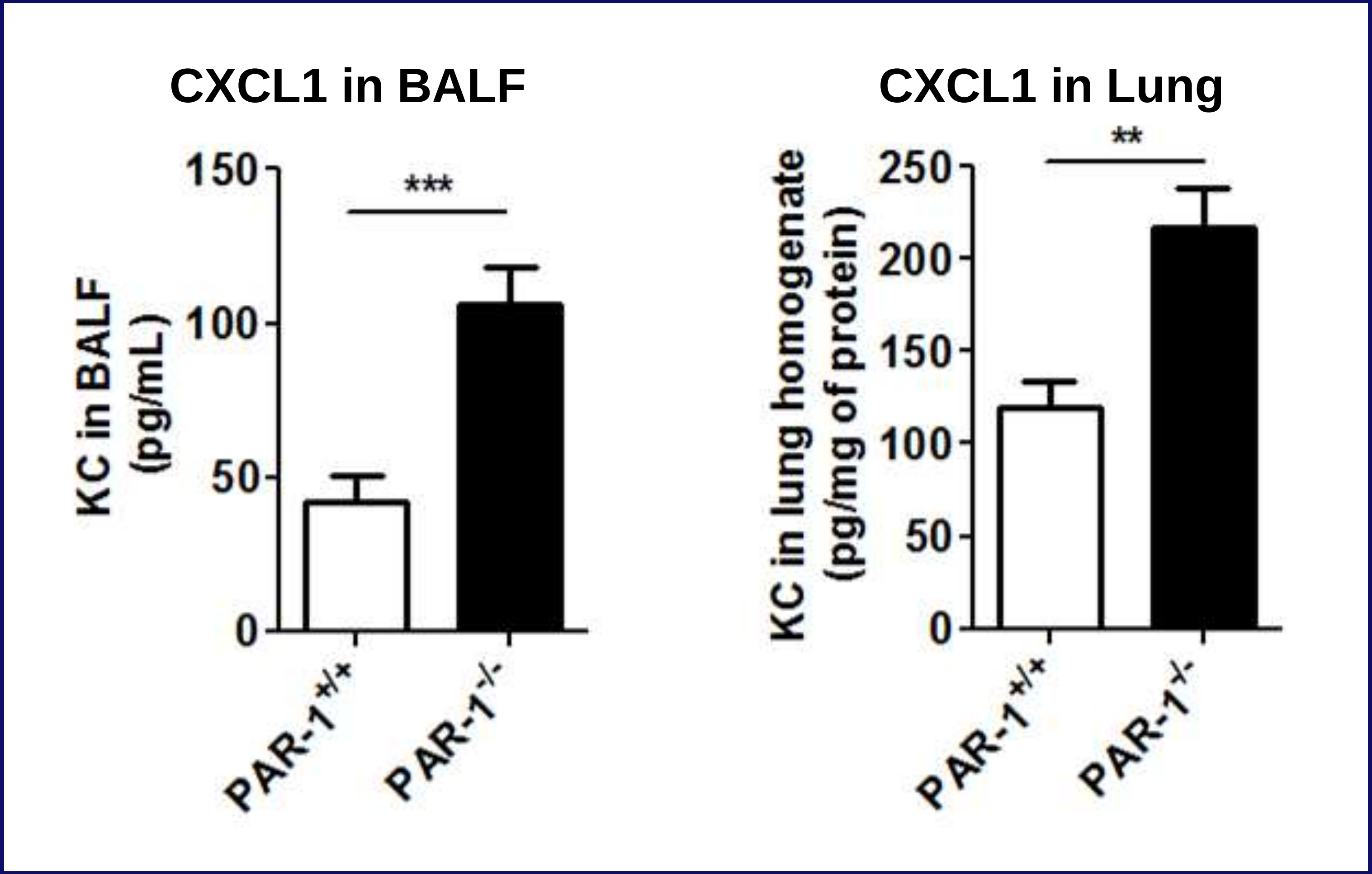
PAR-1 deficiency is associated with a dysregulated inflammatory response after IAV infection

Effect of PAR-1 Deficiency on the Expression of Inflammatory Mediators in the Lung 3 days after IAV Infection



Tatsumi K et al. unpublished data

PAR-1 Deficient Mice have Increased CXCL1 (KC) Expression in the Lungs 3 Days After IAV Infection

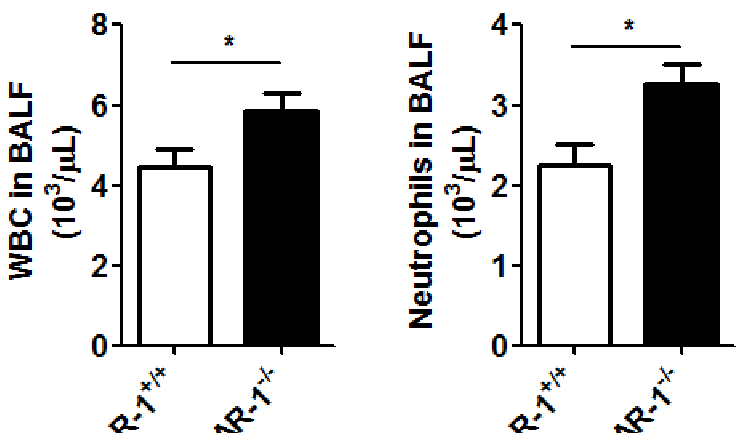


Tatsumi K et al. unpublished data

PAR-1 Deficient Mice Have Increased Numbers of Neutrophils in the BALF 3 Days after IAV Infection

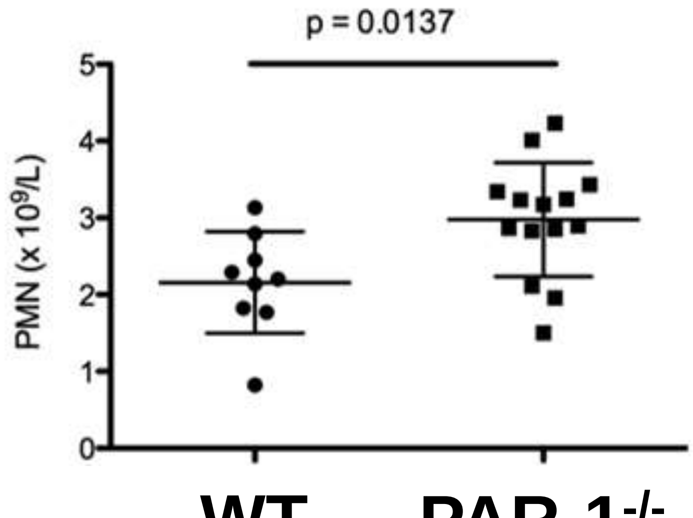
Hemavet

WBC **Neut.**



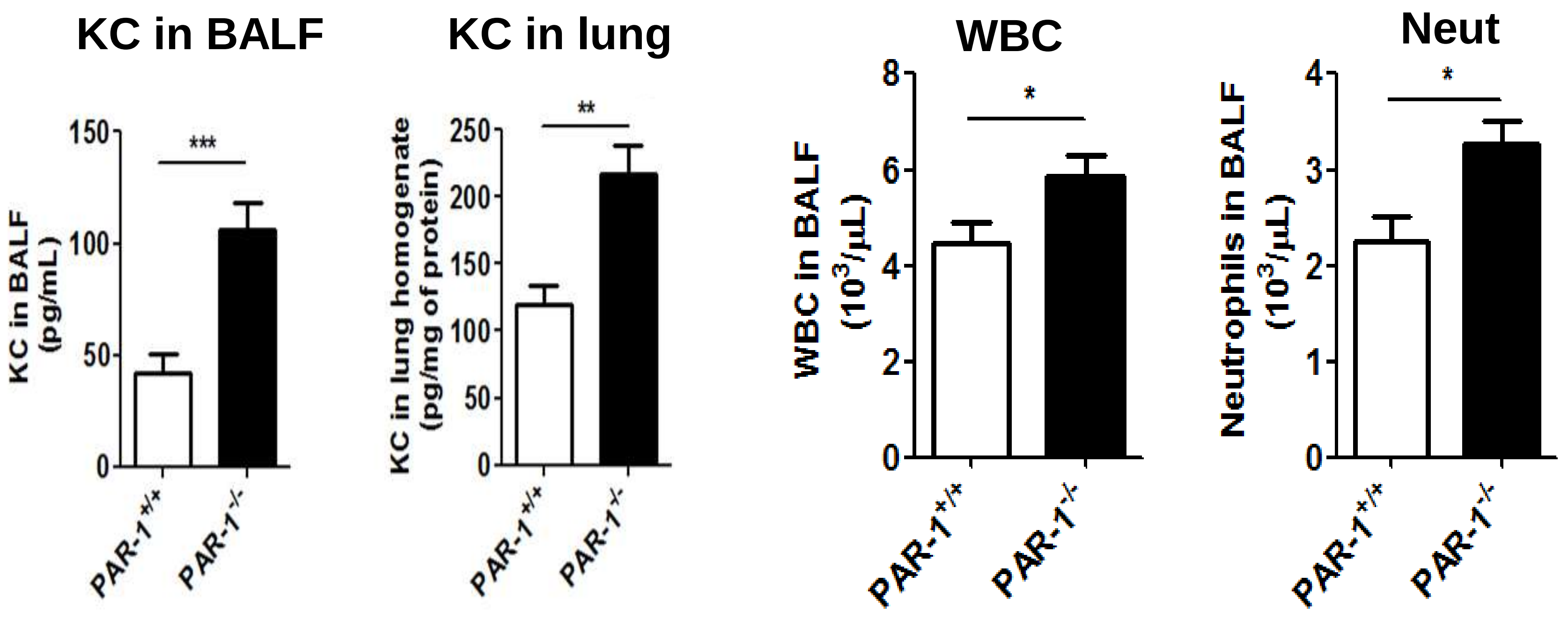
Cytospin

Neutrophils



An increased in neutrophil infiltration ino the lung may contribute to the increased mortality in PAR-1$^{-/-}$ mice

Effect of PAR-1 Deficiency on KC Expression and Leukocyte Recruitment after IAV Infection



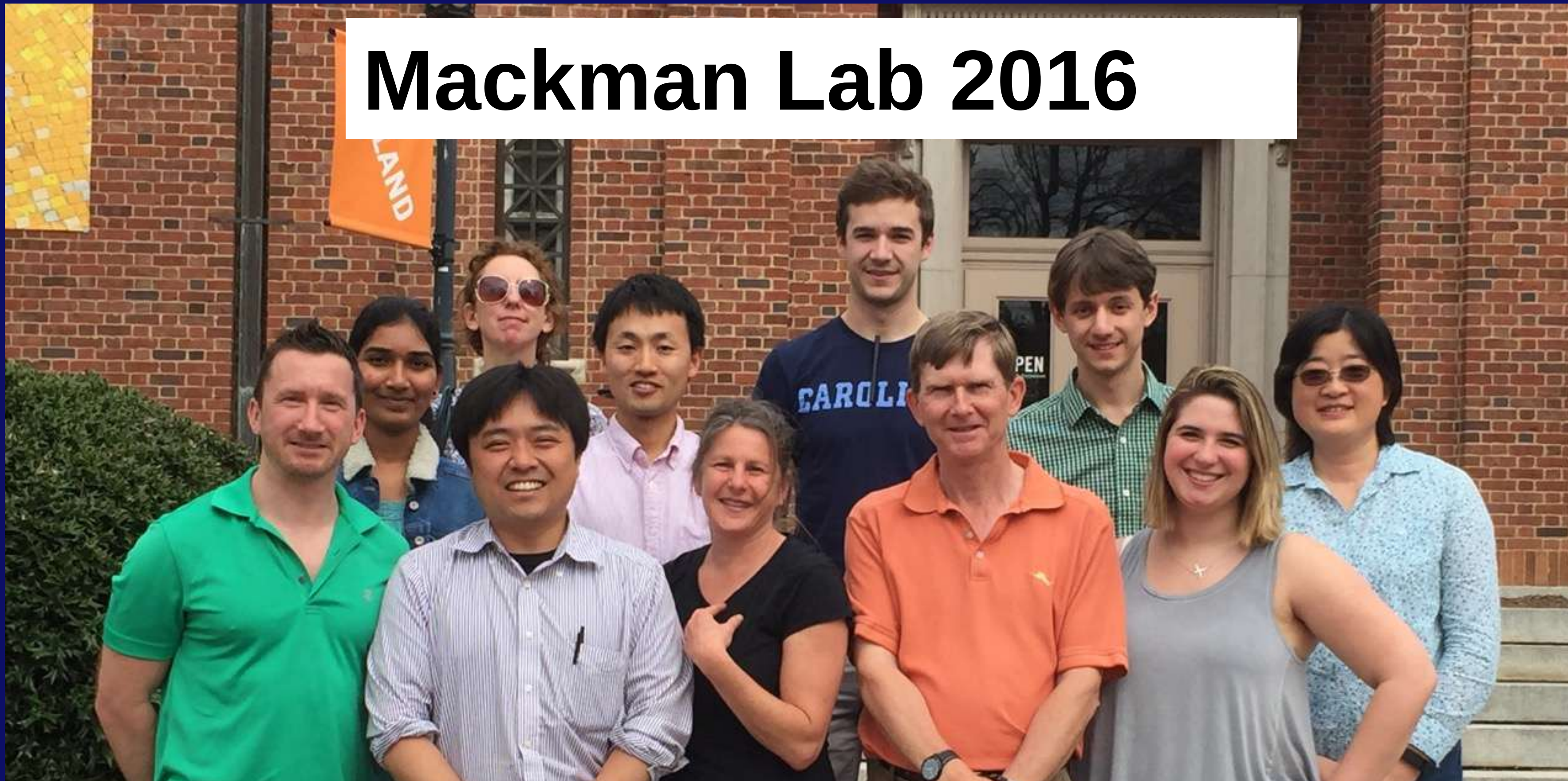
Ongoing Studies: Effect of Deletion of PAR-1 in Different Cell Types on IAV Infection

- PAR-1$^{fl/fl}$,SpcCre- epithelial cell
- PAR-1$^{fl/fl}$,LysMCre- myeloid cell
- PAR-1$^{fl/fl}$,Tie2Cre- EC and hemat cell
- PAR-1$^{fl/fl}$,iVE-cadherin- EC

Conclusion

PAR-1 deficient mice have increased mortality, vascular permeability and a dysregulated immune response after IAV infection

Mackman Lab 2016



NATIONAL INSTITUTES OF HEALTH

American Heart Association.
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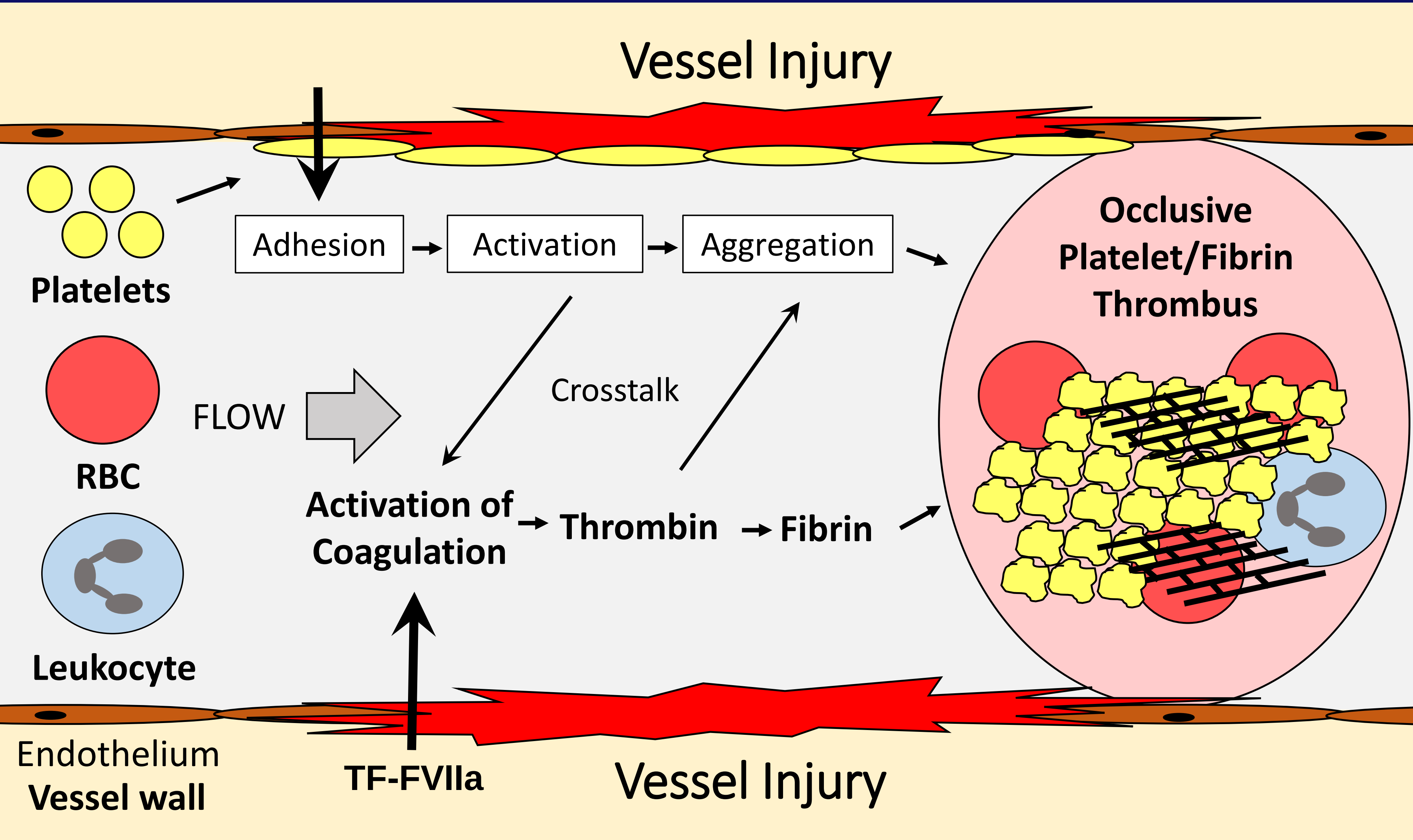
MYOCARDITIS
FOUNDATION

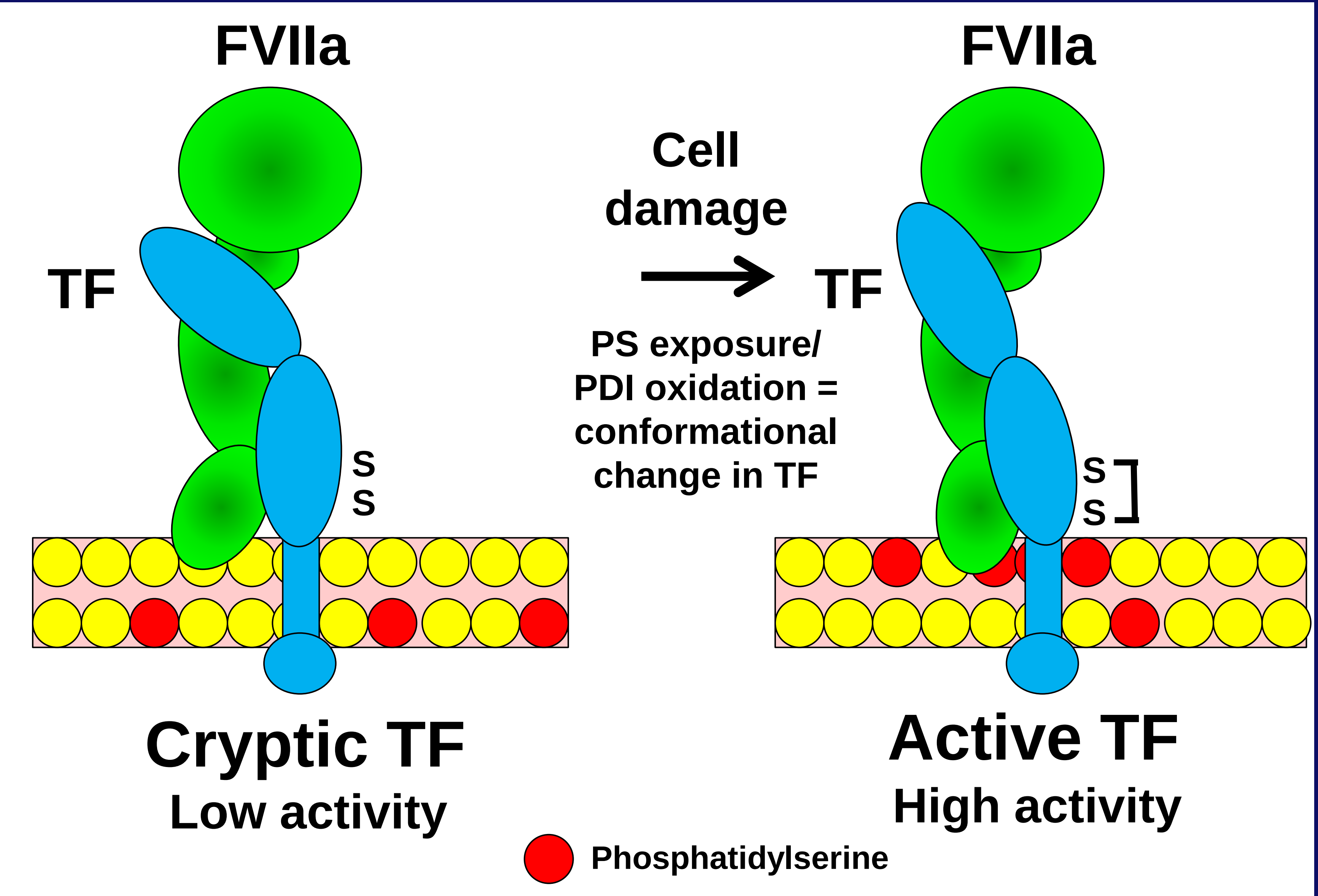
TREC
K.G. JEBSEN THROMBOSIS
RESEARCH AND EXPERTISE CENTER

Formation of an Occlusive Thrombus

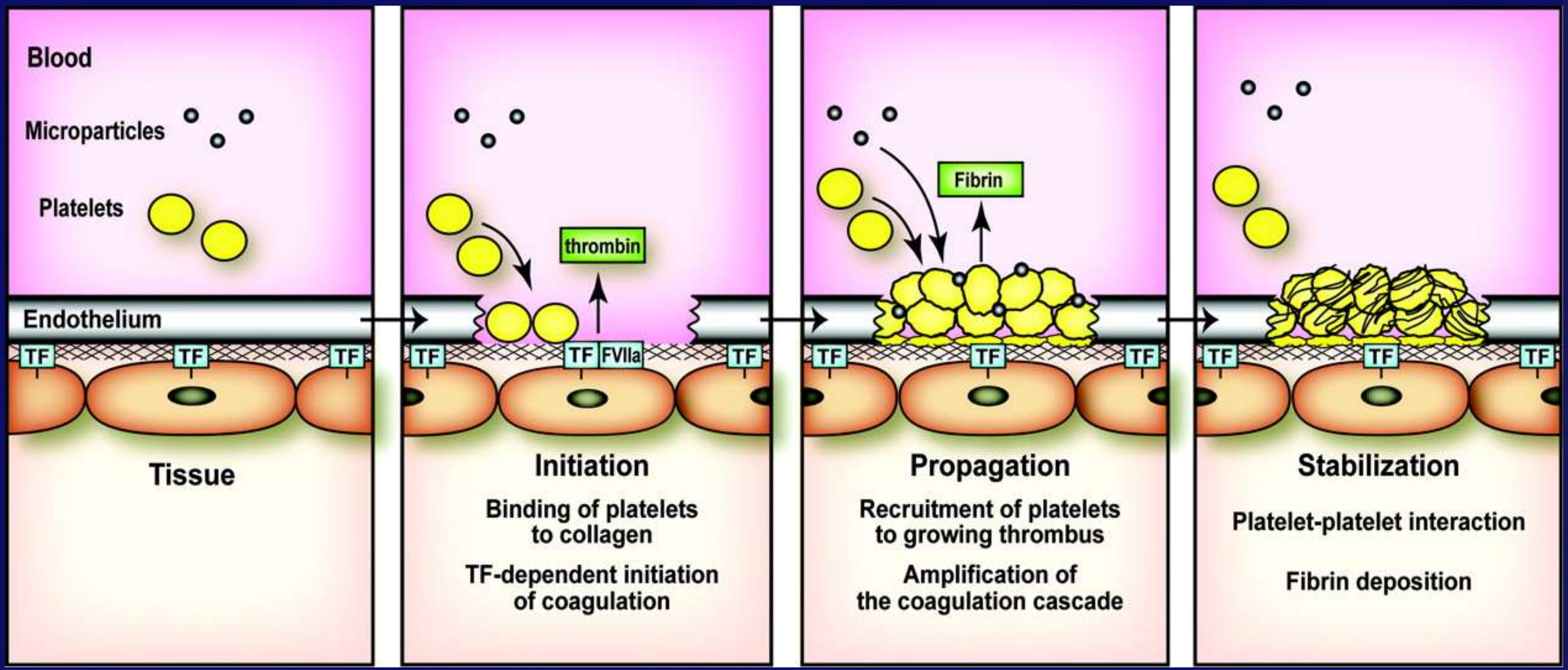


Jagadeeswaran P et al.. Mackman Circ Res 2016

Regulation of TF Activity

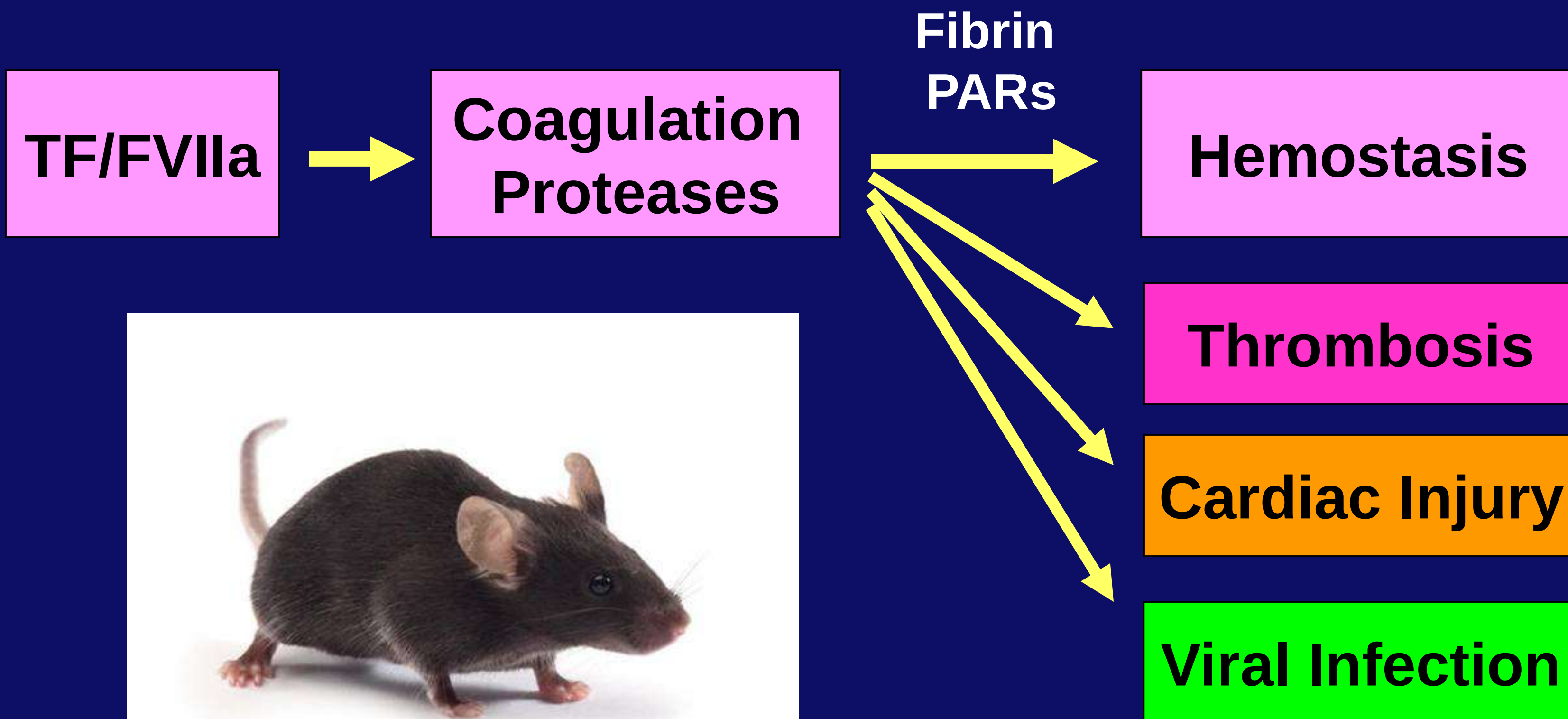


Perivascular TF Maintains Hemostasis After Vessel Injury

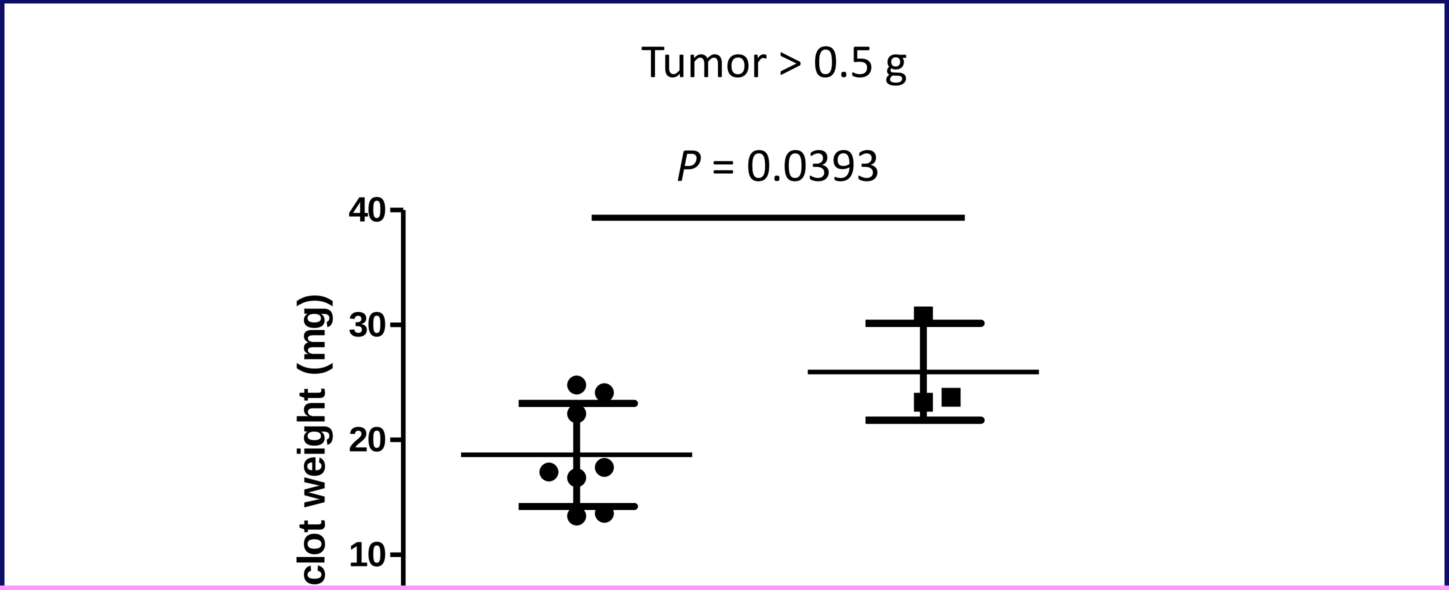


Mackman et al ATVB 2007

Major Projects in the Mackman Lab



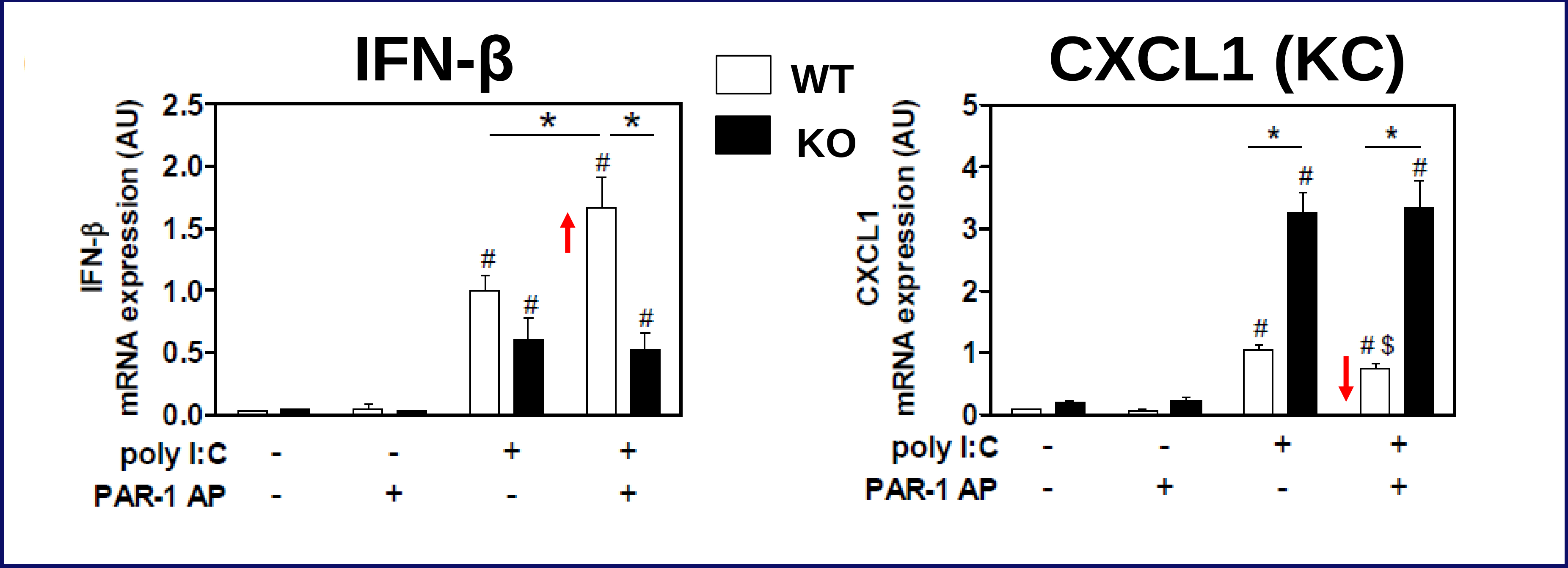
Tumor Bearing Mice have Larger Thrombi in an IVC <u>Full Ligation</u> Model



Determine the effect of inhibiting human TF on the enhanced thrombosis in tumor bearing mice

Hisada Y unpublished data

Role of PAR-1 in Poly I:C Induction of Antiviral Genes in Splenocytes



Antoniak S et al unpublished data

Would inhibition of thrombin or PAR-1 in humans affect viral infections?

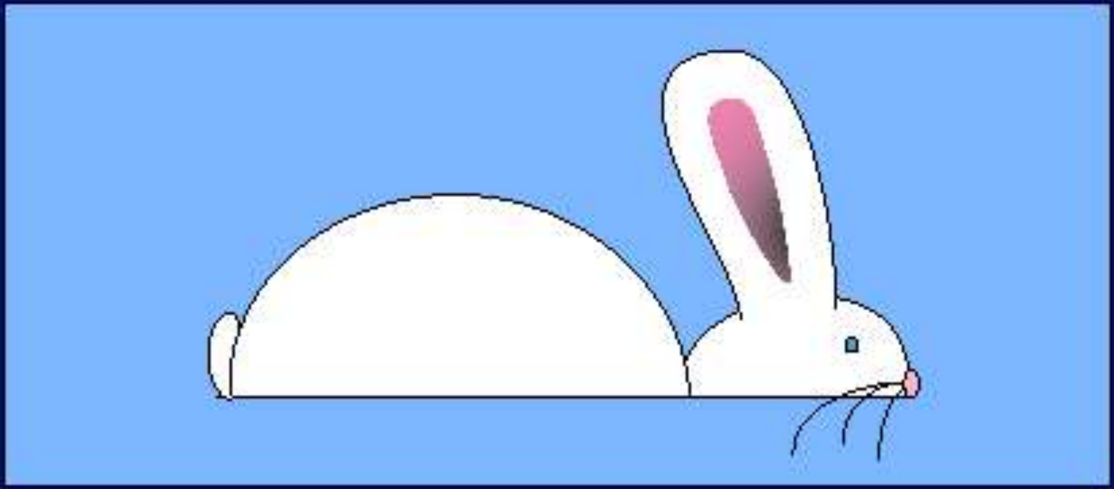
Ischemia-Reperfusion Injury

Ischemia	Reperfusion
Hypoxic Damage	Inflammatory Damage
Apoptosis/necrosis	Chemokines, cytokines, oxidants
	Leukocyte recruitment and activation
	Coagulation and fibrin deposition
	Complement activation

Hypothesis

Inhibition of TF and coagulation proteases will reduce infarct size after cardiac I/R injury in animal models

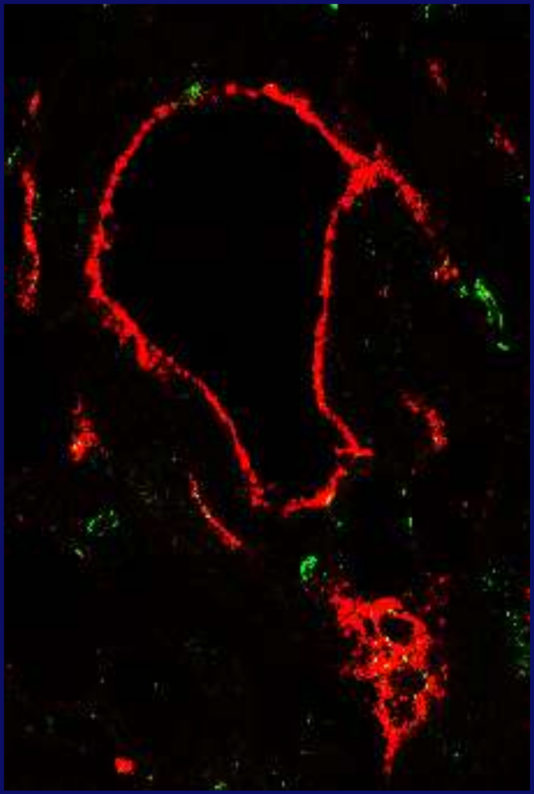
Rabbit Model of Cardiac I/R Injury



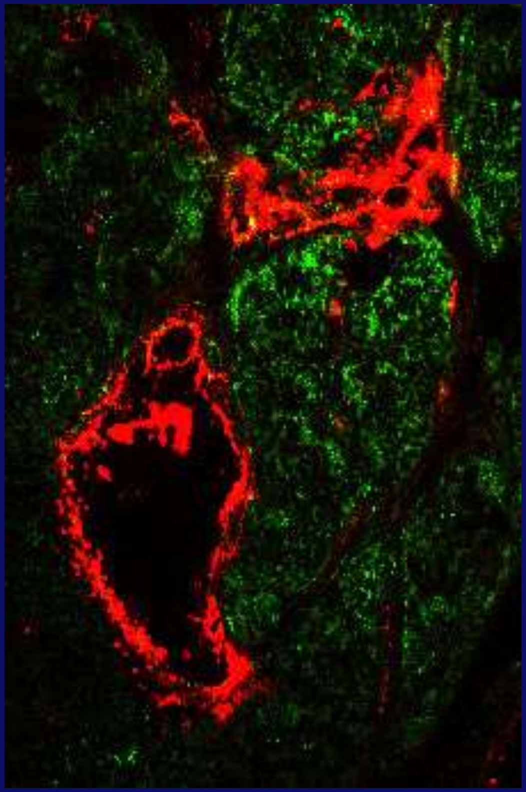
Ischemia	=	45 min
Reperfusion	=	120 min

Cardiac I/R Injury Damages the Endothelial Barrier and Leads to Extravascular Fibrin Deposition

Rabbit model



sham



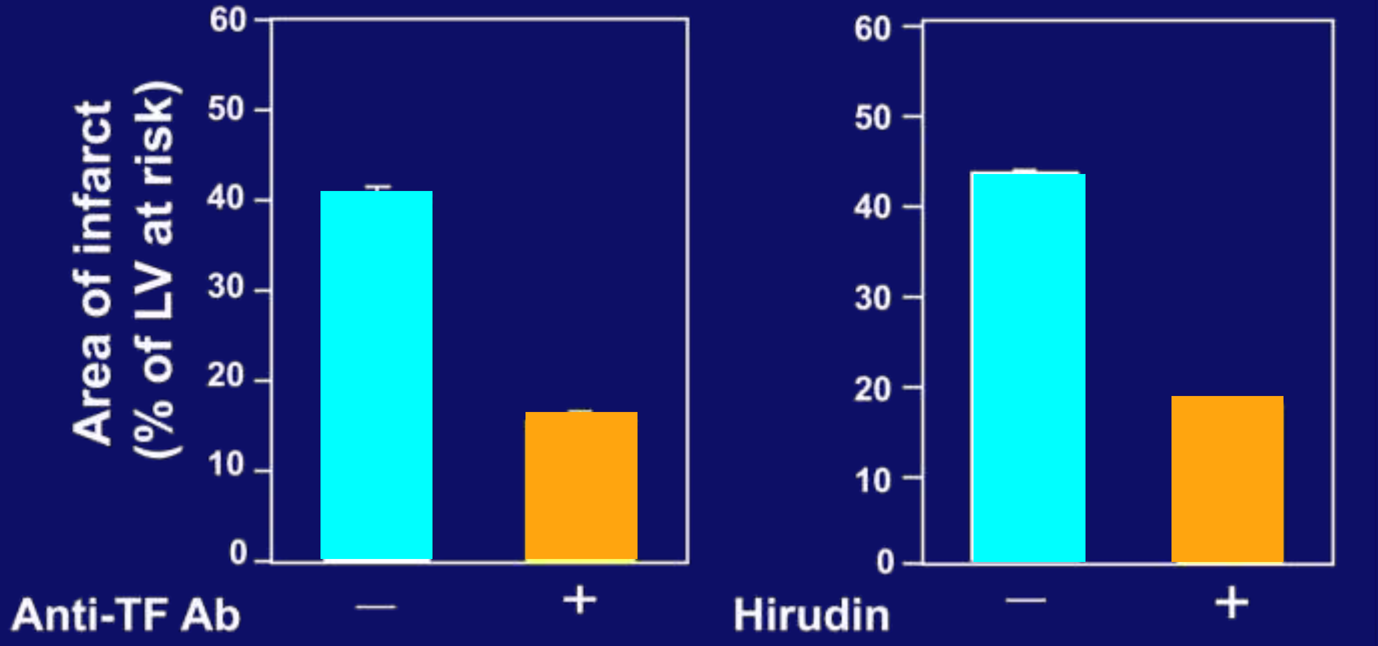
I/R

Red = vWF

Green = fibrin

Erlich et al., *Am J Path* 2000 (Cover)

Effect of Inhibition of TF or Thrombin on Infarct Size in a Rabbit Cardiac I/R Model



Inhibition of TF or thrombin also reduced inflammation

Erlich et al., *Am J Path* 2000

Effect of Inhibition of Thrombin or a Deficiency of PAR-1 or PAR-2 on Infarct Size in a Mouse Cardiac I/R Model

Pawlinski R et al Circ 2007- Thrombin inhibition or PAR-1 deficiency.

Antoniak S et al ATVB 2010- PAR-2 deficiency

Clinical Studies with Rivaroxaban

ATLAS ACS-2-TIMI-51 trial- the factor Xa inhibitor rivaroxaban reduced death due to cardiovascular causes (MI and stroke) in patients with a recent ACS event **Mega J et al NEJM 2012.**

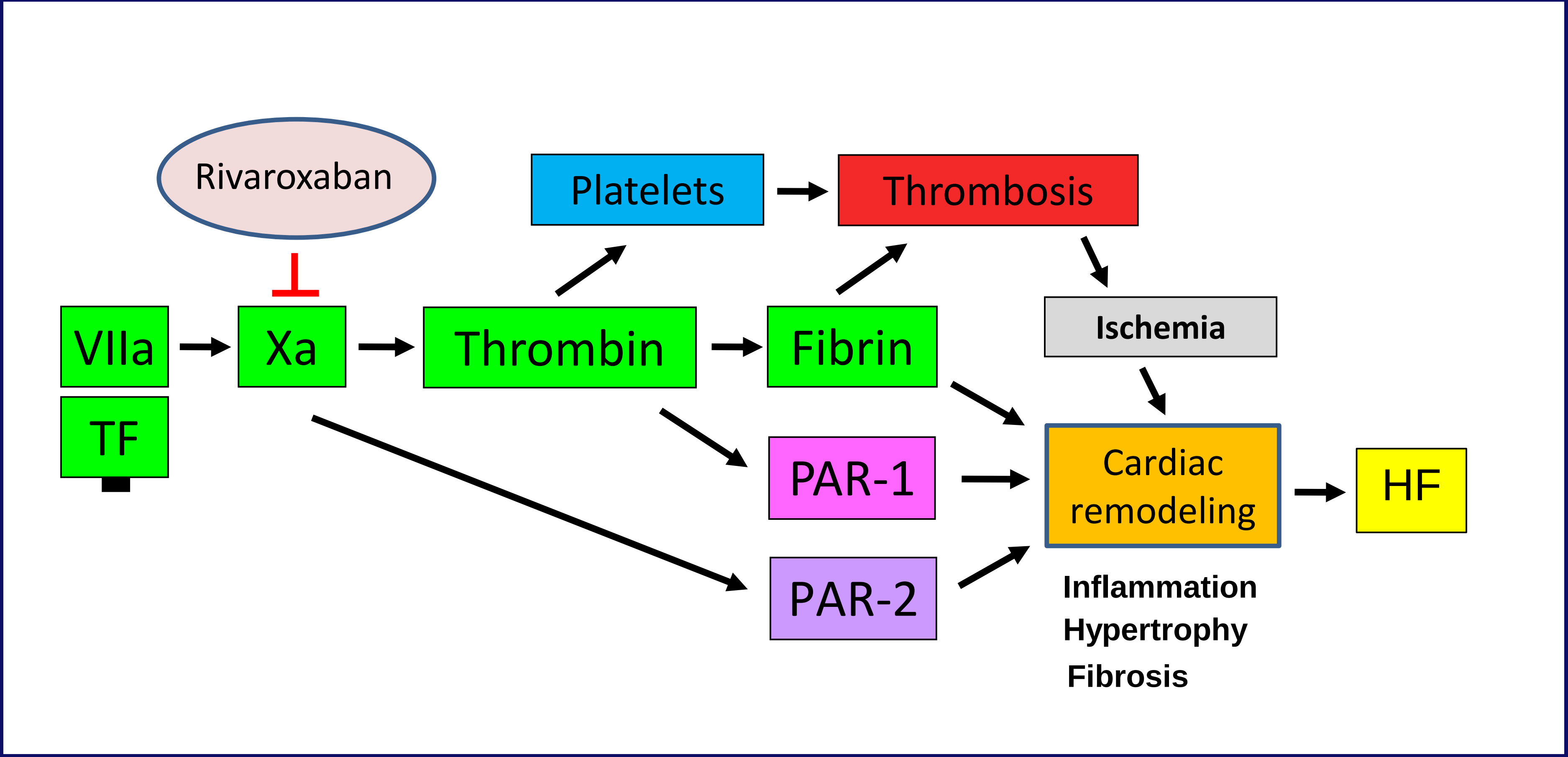
In a subgroup analysis of HF patients, treatment with rivaroxaban 2.5 mg twice daily plus antiplatelet therapy demonstrated decreased rates of cardiovascular events and death compared with antiplatelet therapy alone.

COMMANDER-HF is a phase III clinical trial to evaluate the effect of the new oral anticoagulant rivaroxaban in patients with chronic heart failure.

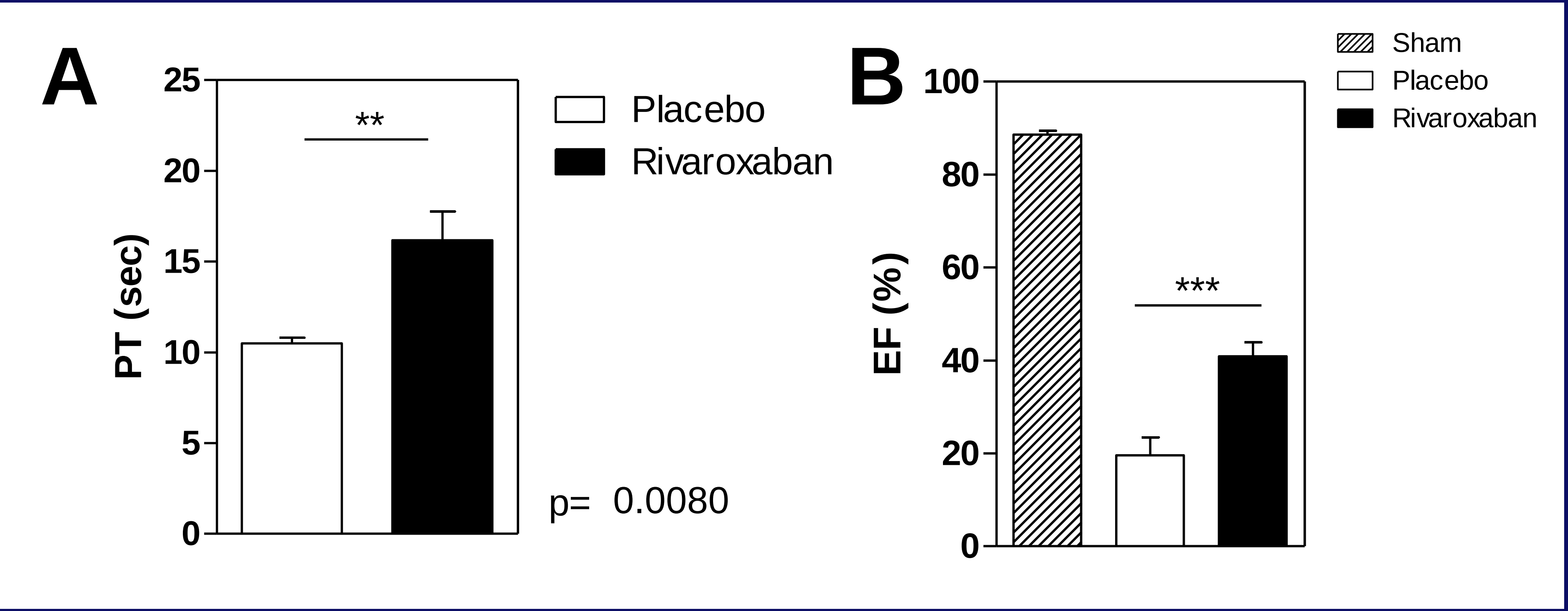
Hypothesis

Inhibition of FXa with rivaroxaban will reduce cardiac remodeling and heart failure in mice

Effect of Rivaroxaban on Cardiac Remodeling and Heart Failure



Effect of Rivaroxaban on Cardiac Remodeling and Heart Failure in a Mouse Model of LAD Ligation



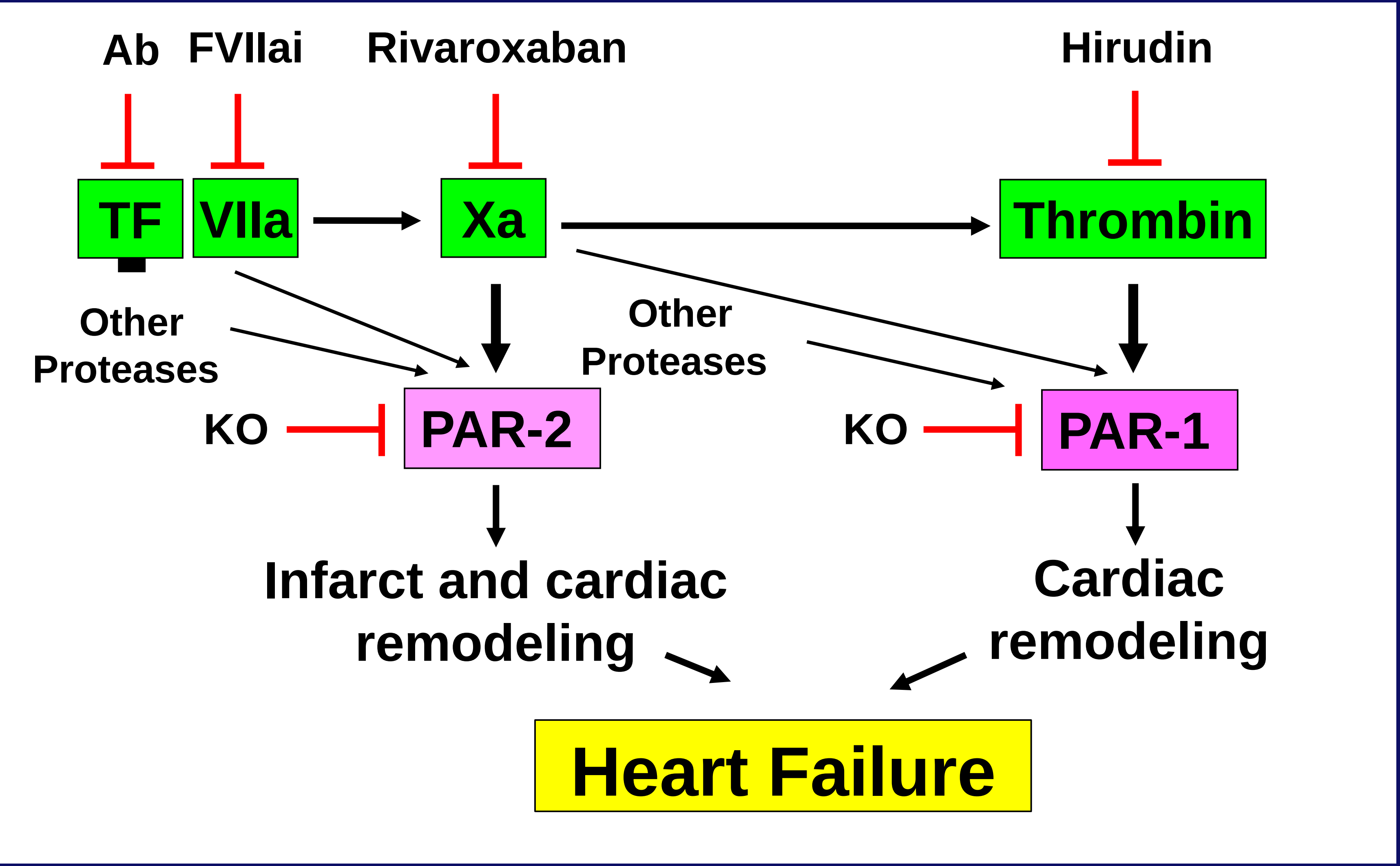
28 days after LAD ligation

Bode M unpublished results

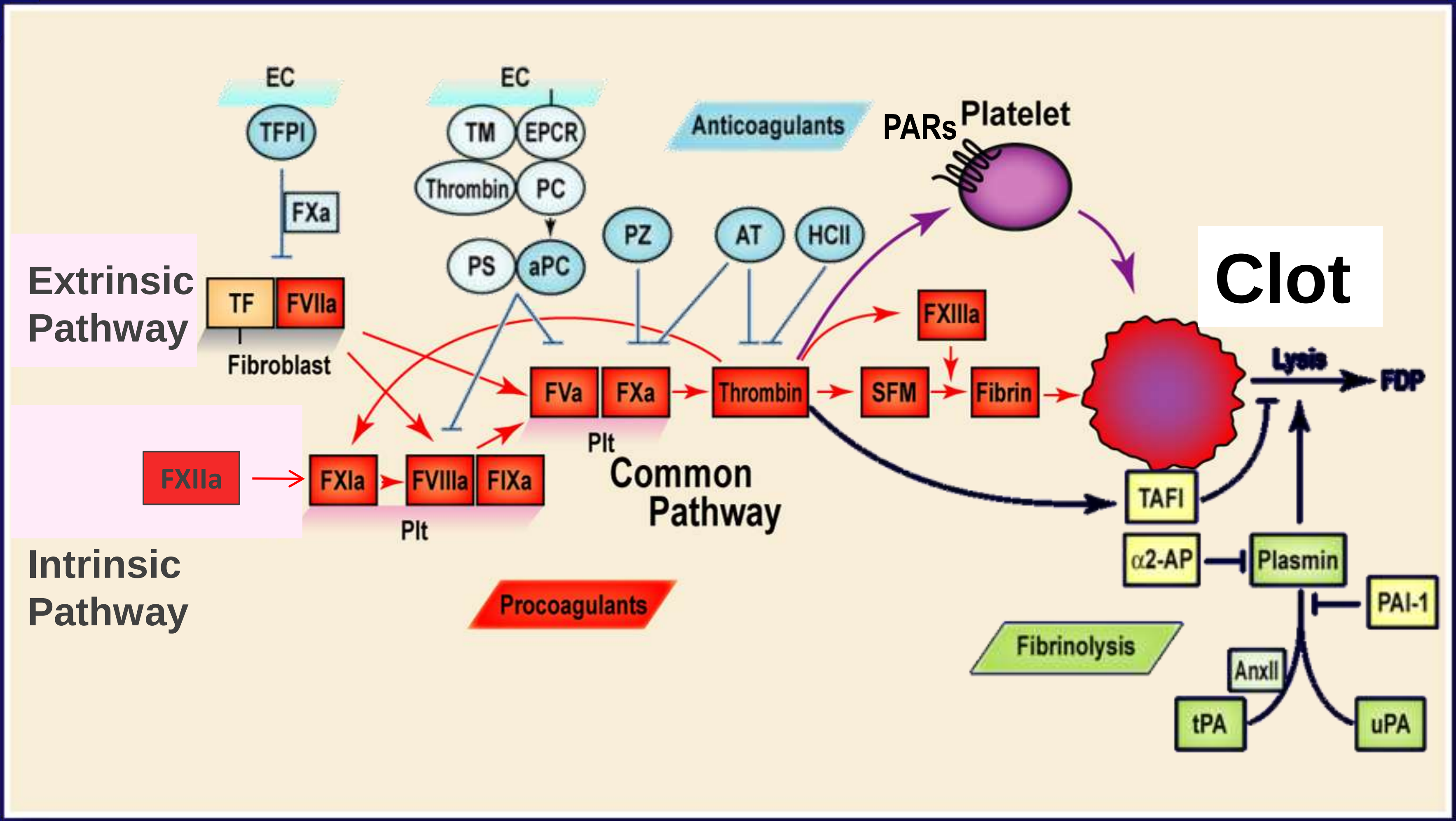
Conclusion

Inhibition of TF, coagulation proteases or PAR-1 may reduce cardiac remodeling and heart failure in MI patients

Role of TF, Coagulation Proteases and PARs in Cardiac I/R Injury

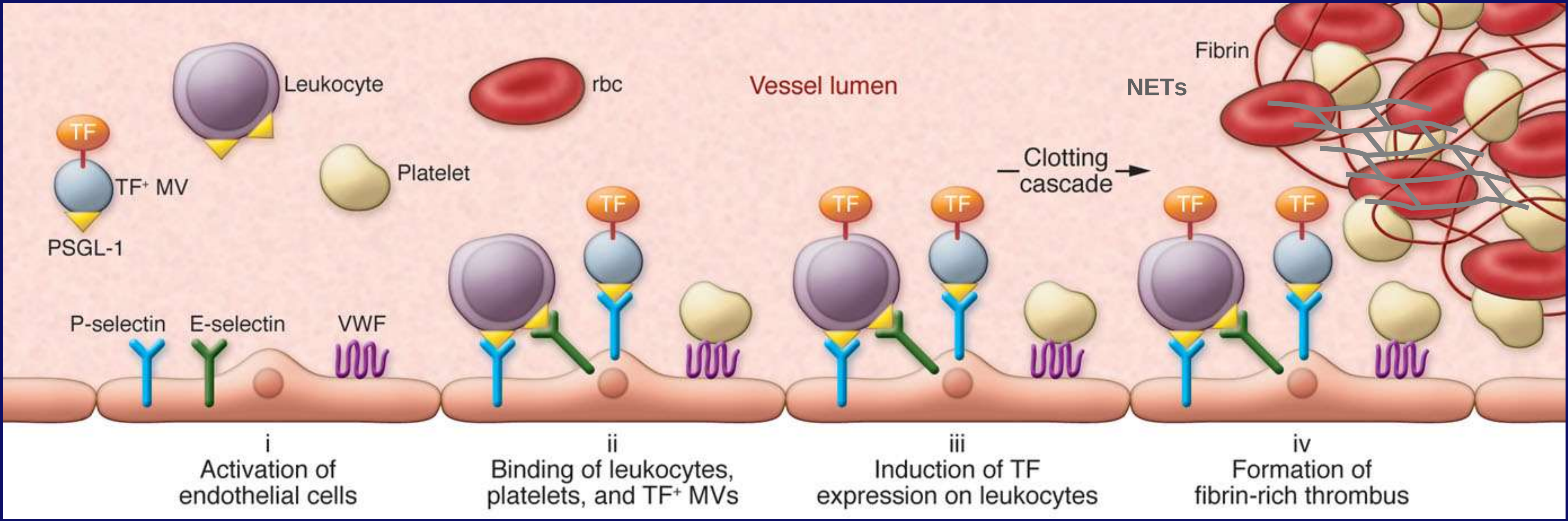


Formation and Lysis of a Clot



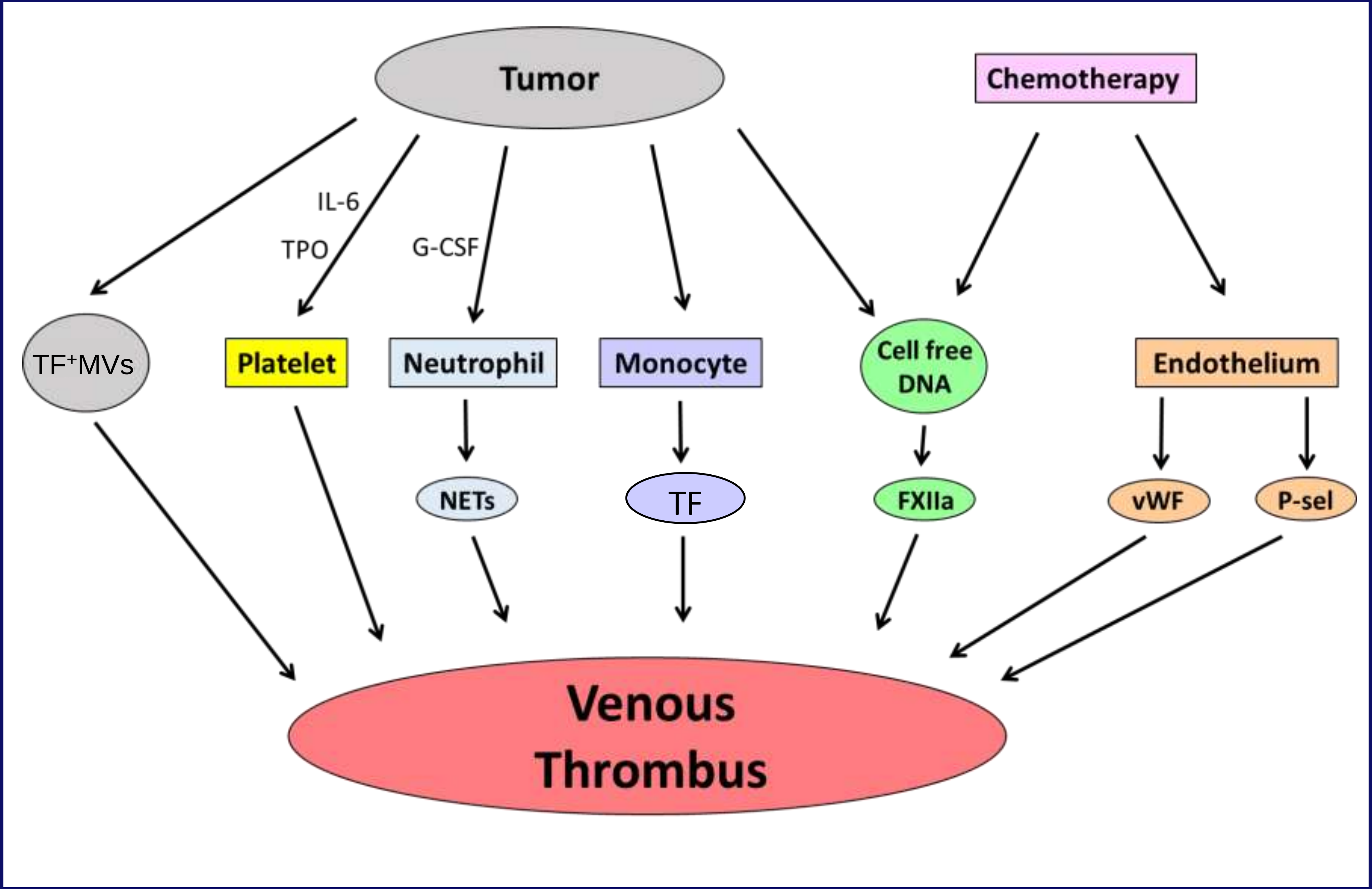
Mackman ATVB 2005

Mechanisms of Venous Thrombosis



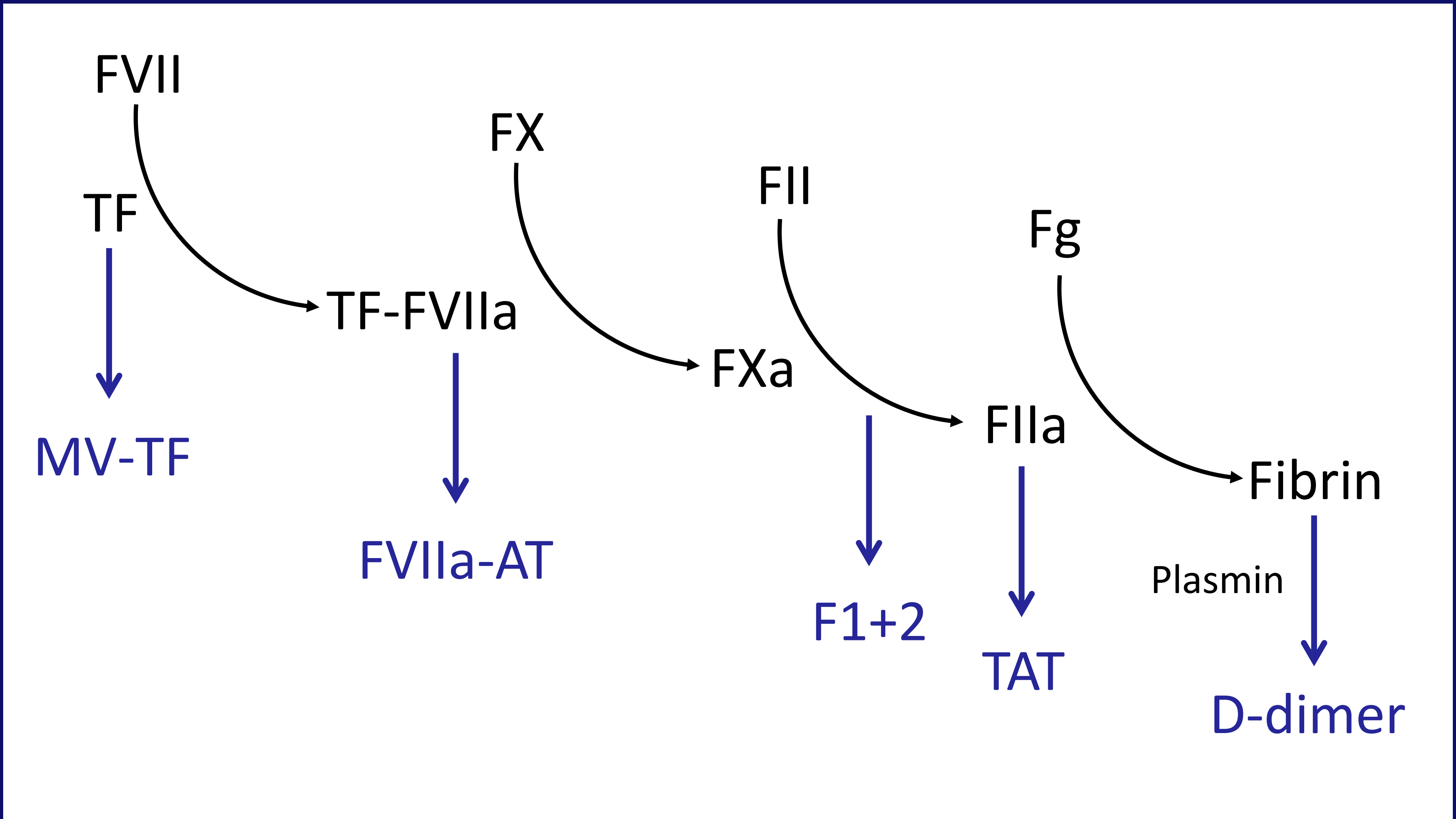
Mackman JCI 2012

Possible Mechanisms of VTE in Cancer Patients



Hisada Y et al JTH 2015

Biomarkers Associated with Activation of the Clotting Cascade



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Factor XI Antisense Oligonucleotide for Prevention of Venous Thrombosis

Harry R. Büller, M.D., Claudette Bethune ,Ph.D., Sanjay Bhanot, M.D., Ph.D, David Gailani, M.D., Brett P. Monia, Ph.D.,Gary E. Raskob, Ph.D., Annelise Segers, M.D., Peter Verhamme, M.D., and Jeffrey I. Weitz, M.D.

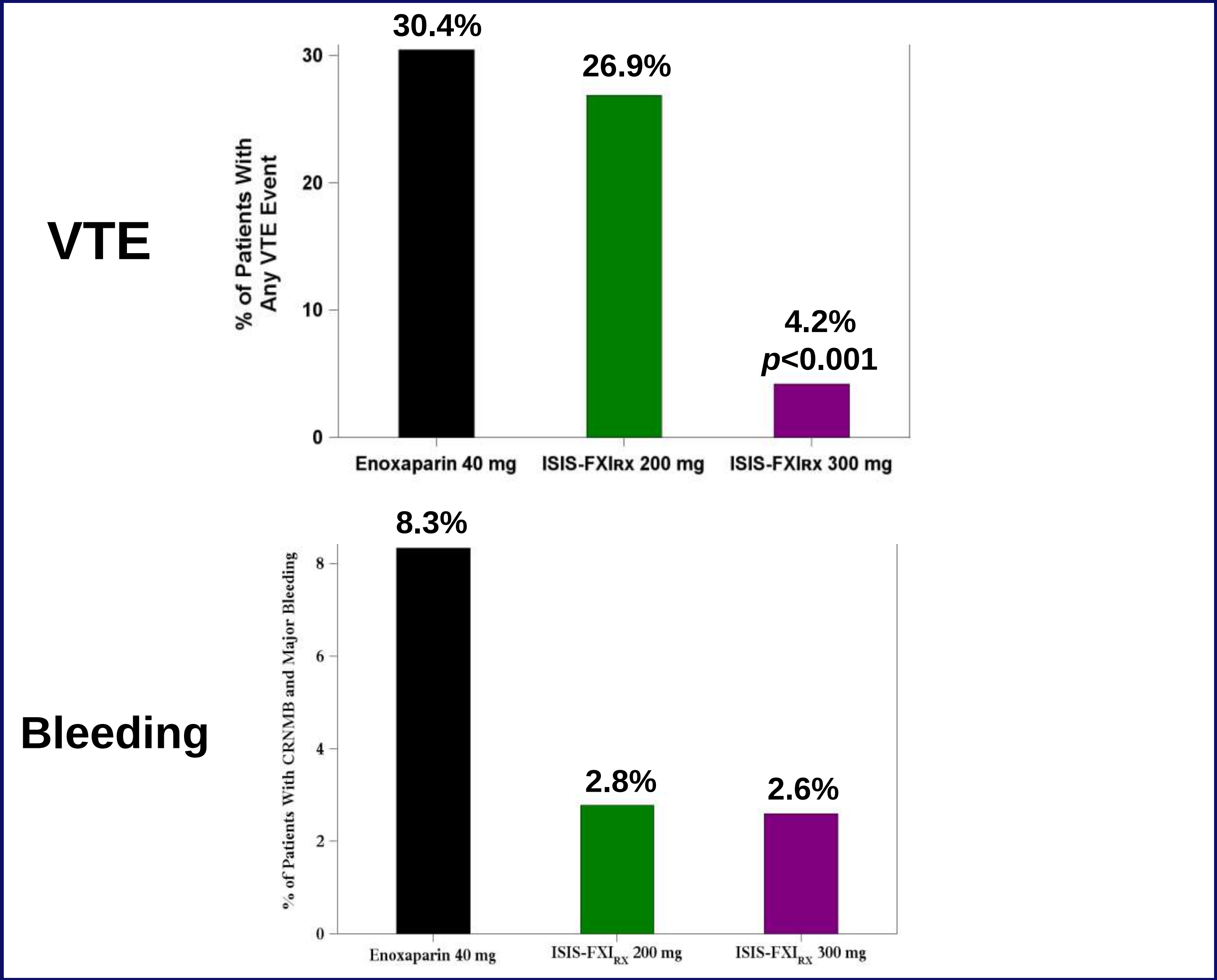
2015;372:232-240

- **Effect of decreased levels of FXI on VTE in pts undergoing total knee replacement**
- **Two doses of a FXI ASO were used: 200 mg ~41% FXI; 300 mg ~22% FXI.**
- **Enoxaparin was used as a control.**

Slides from Dr. J. Weitz

- **Primary efficacy outcome was incidence of venous thromboembolism (VTE) (asymptomatic and symptomatic)**
- **Principal safety outcome was bleeding**

Primary Efficacy and Safety Outcomes



Buller H et al N Engl J Med 2015

Conclusion

FXI appears to be an attractive new target for preventing VTE without increase bleeding

Measurement of TF Antigen and Activity in Whole Blood, Plasma and Microparticles

- Plasma- TF antigen
- MPs- TF antigen flow cytometry
- MPs- TF antigen impedance

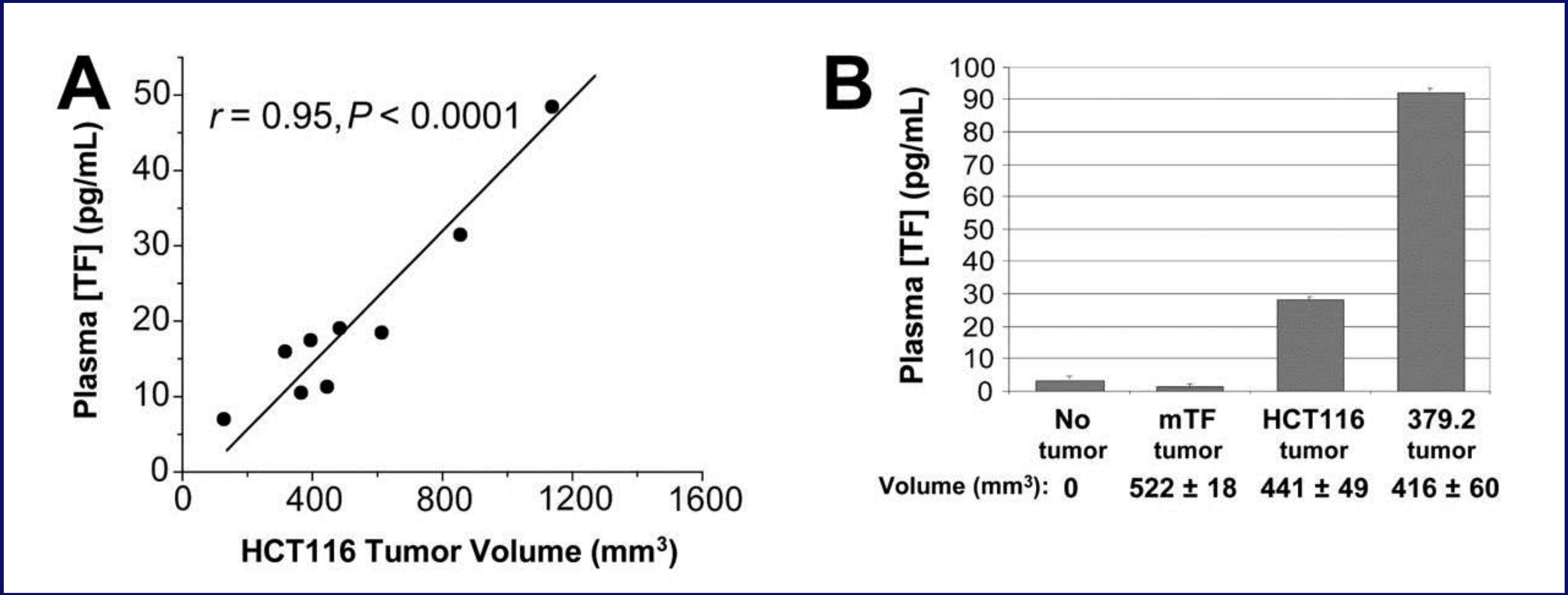
TF antigen

- Whole blood- TF activity
- Plasma- TF activity
- MP (pellet)- TF activity *
- MP (capture)- TF activity
- Fibrin generation

TF activity

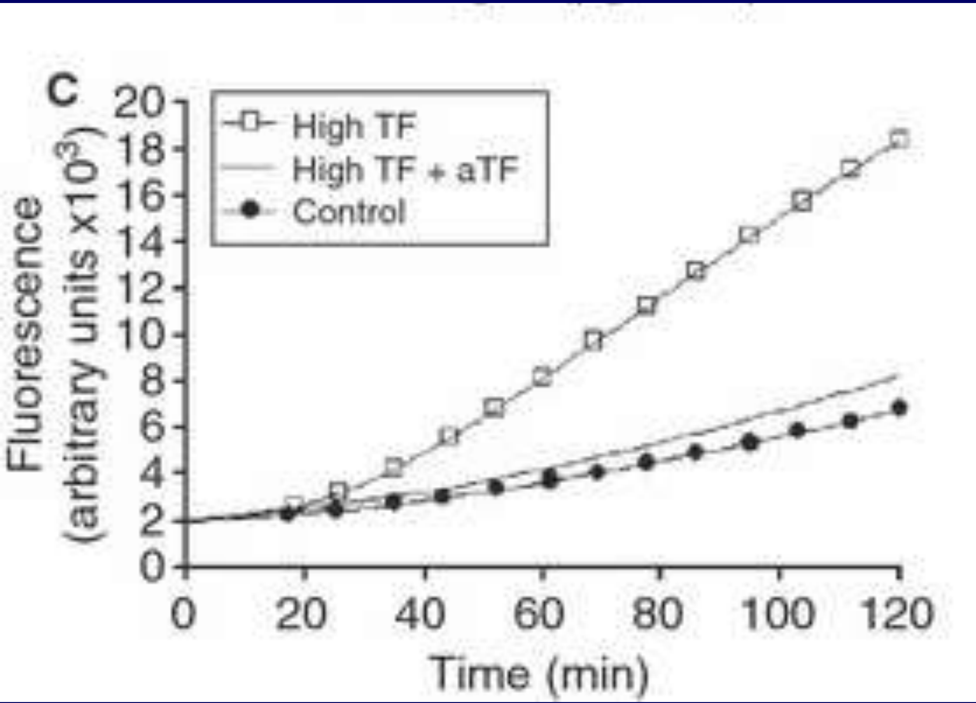
Mouse Studies

Tumors Release Human TF into the Circulation in SCID Mice containing Human Tumors



Yu et al Blood 2005

Tumor-derived Human TF is Functional in an In Vitro Assay

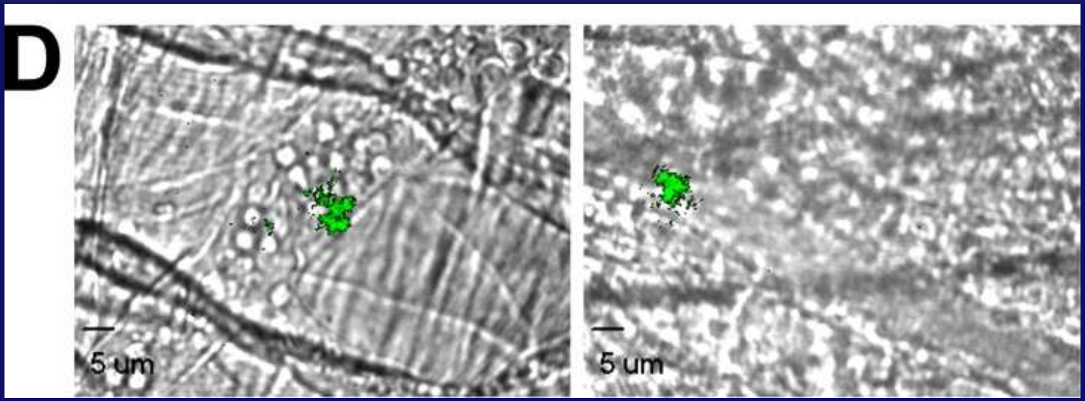


Human pancreatic L3.6pl cells grown orthotopically in nude mice

Davila et al JTH 2008

Tumor-derived MPs Accumulate at the Site of Thrombus Formation In Vivo

Mouse Panc02 pancreatic cells labeled with pEGFP



Venule **Arteriole**

Thomas et al JEM 2009

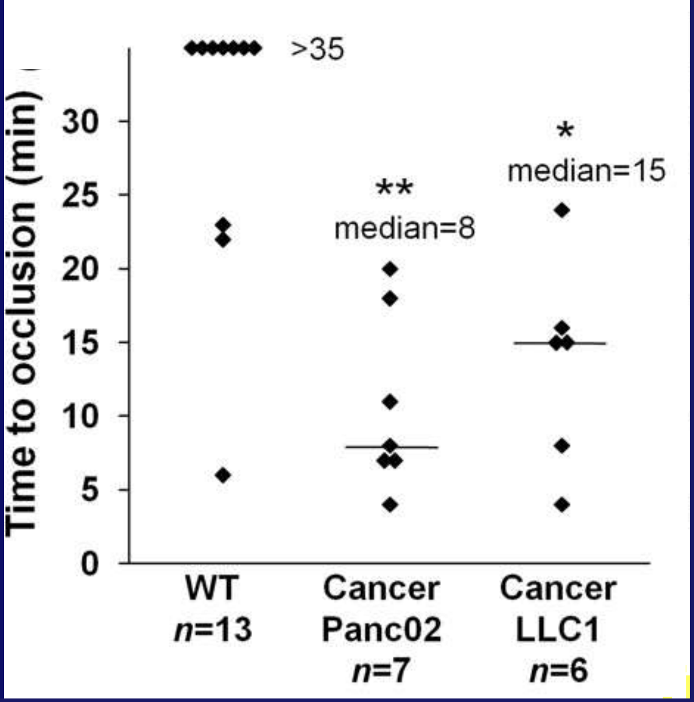
JEM

Tumor-bearing Mice have Larger Thrombi in a Mesenteric Vessel Model

Panc02 = mouse pancreatic carcinoma

LLC = mouse lewis lung carcinoma

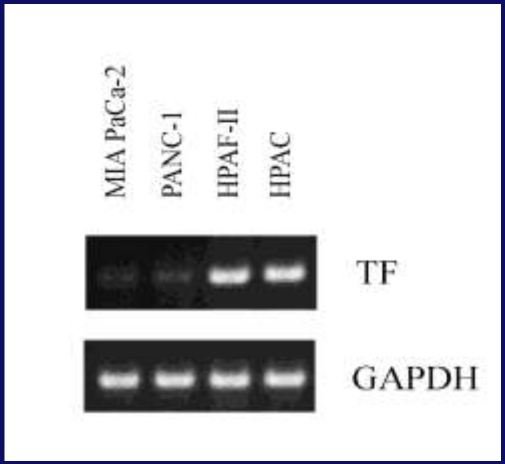
s.c.

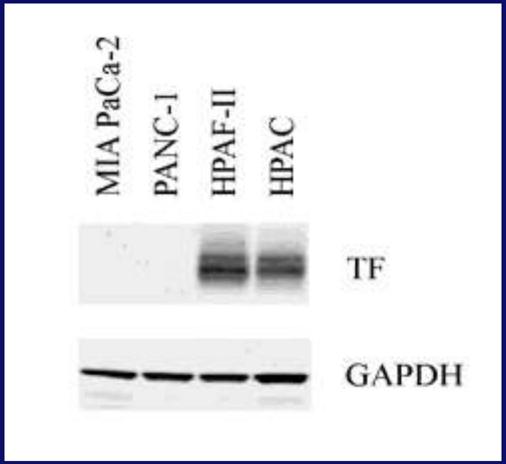


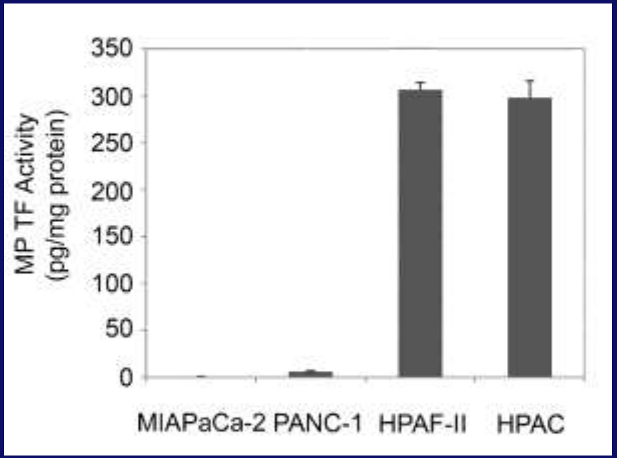
Thomas et al JEM 2009

TF Expression in Various Human Pancreatic Cell Lines In Vitro

TF mRNA | **TF Protein** | **MP TF Activity**





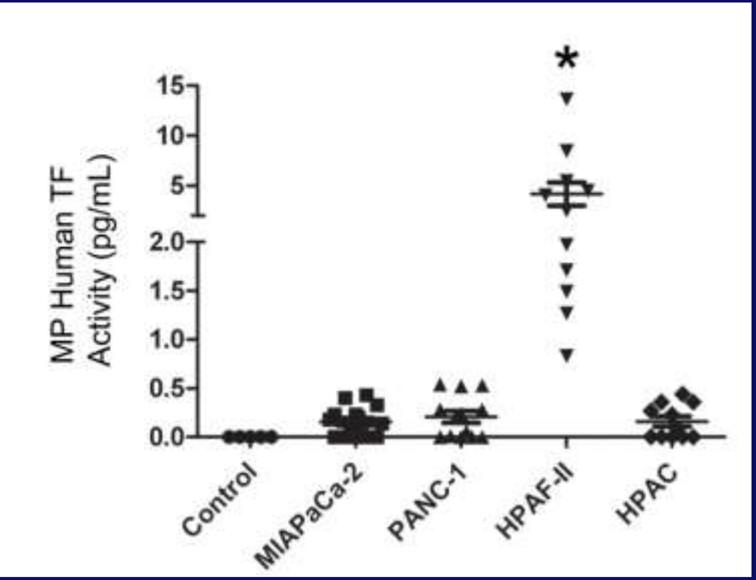


Cells | **Medium**

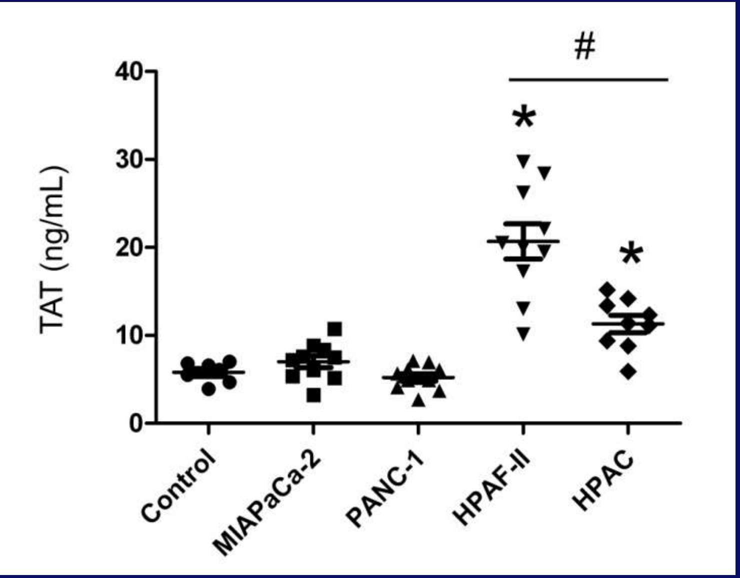
Wang et al unpublished data

MP TF Activity and Activation of Coagulation in Nude Mice containing Human Pancreatic Tumors

MP TF activity



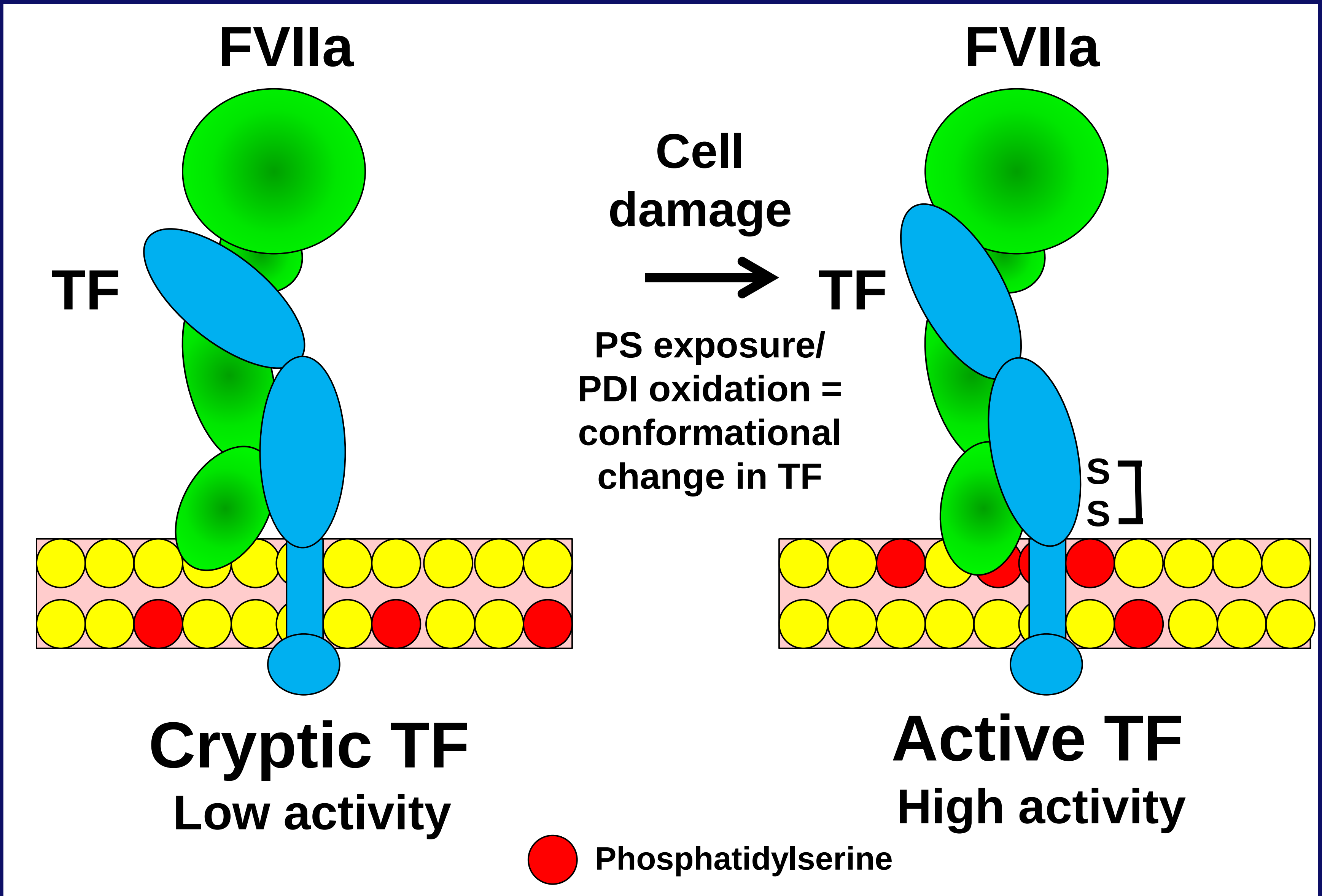
TAT



An anti-human TF Ab reduces the increases in TAT

Wang et al unpublished data

Regulation of TF Activity

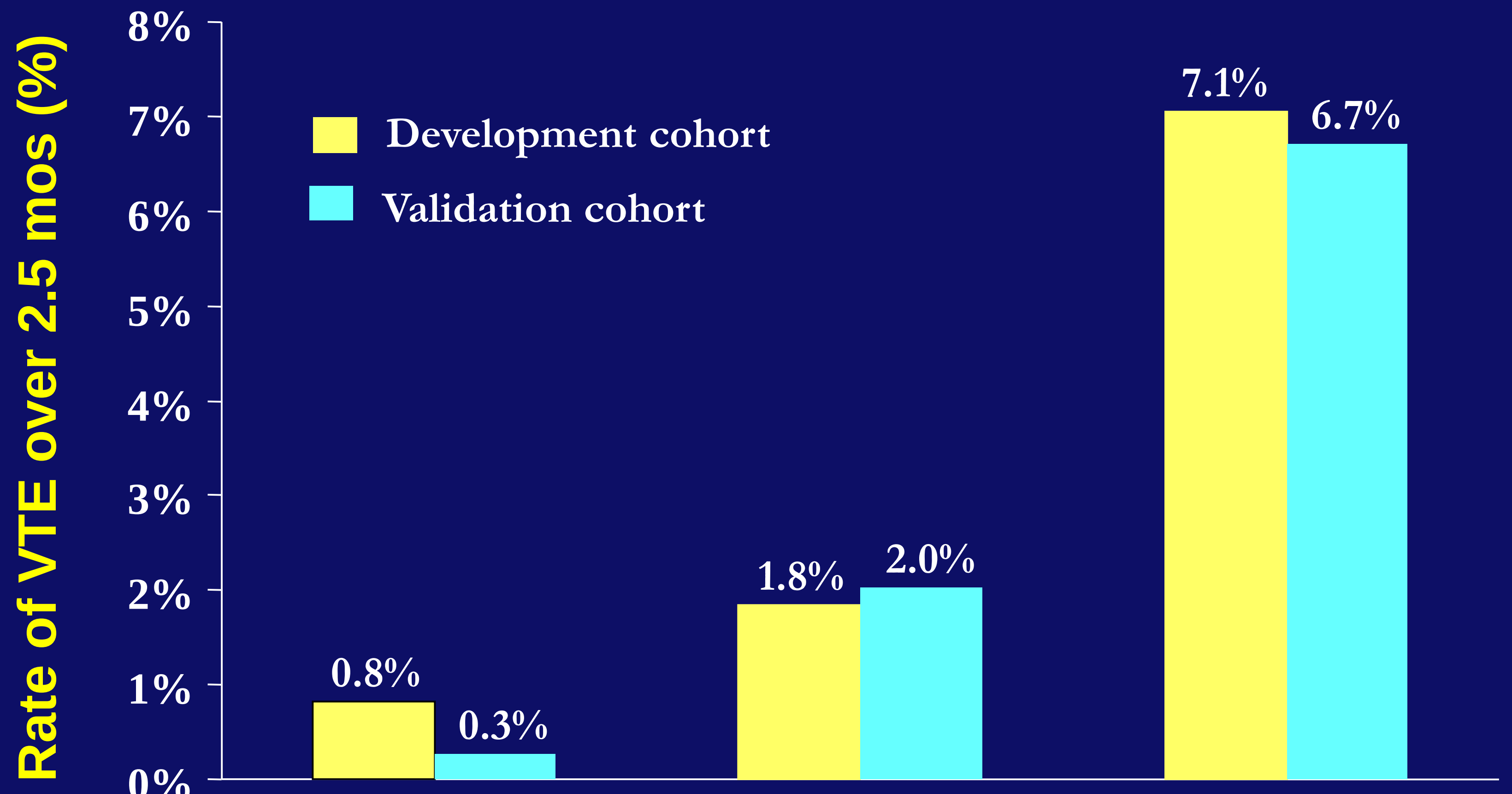


Predictive Model-Khorana Score

Patient Characteristic	Score
Site of Cancer	
Very high risk (stomach, pancreas)	2
High risk (lung, lymphoma, gynecologic, GU excluding prostate)	1
Platelet count $\geq$ 350,000/mm^3	1
Hgb < 10g/dL or use of ESA	1
Leukocyte count > 11,000/mm^3	1
BMI $\geq$ 35	1

Khorana A et al Blood 2008 (Brain not included)

Predictive Model Validation



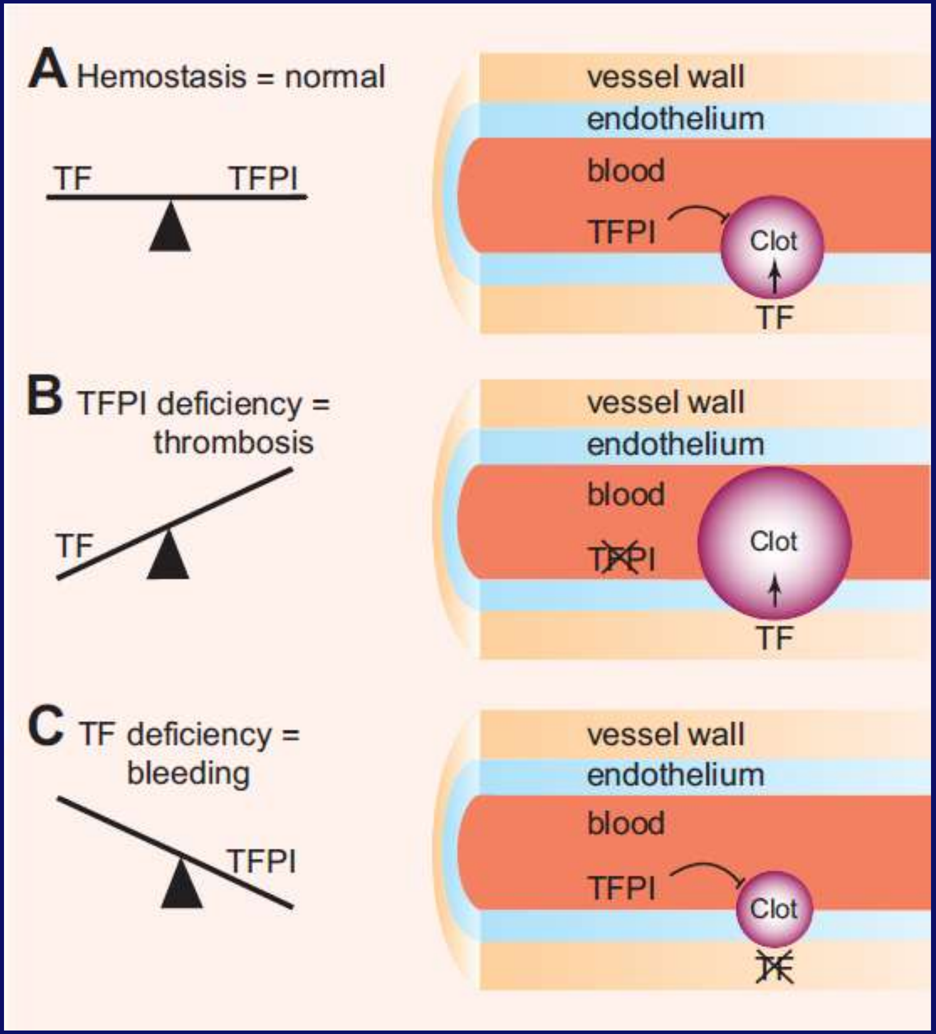
D-dimer and soluble P-selectin have been added- Ay et al Blood 2010

Khorana et al Blood 2008

Biomarkers

A measurable substance in an organism whose presence is indicative of some phenomenon such as disease, infection, or environmental exposure.

Balancing Clotting



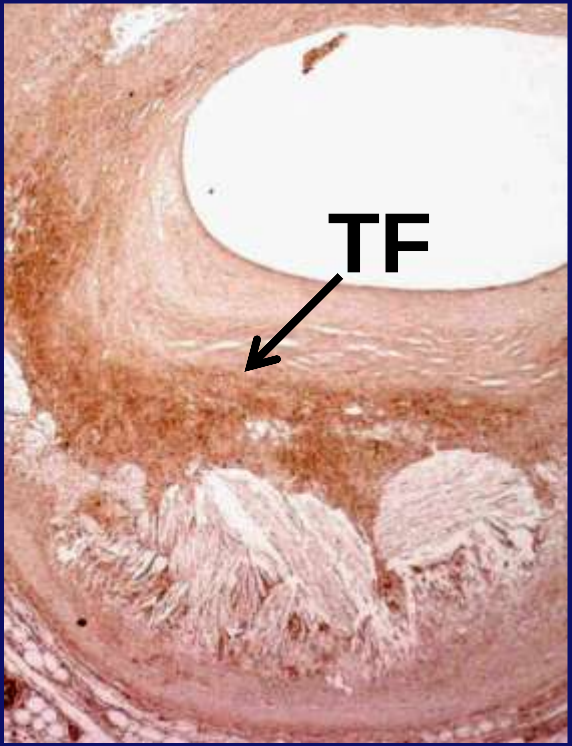
Normal

Thrombosis

Bleeding

Mackman Blood 2010

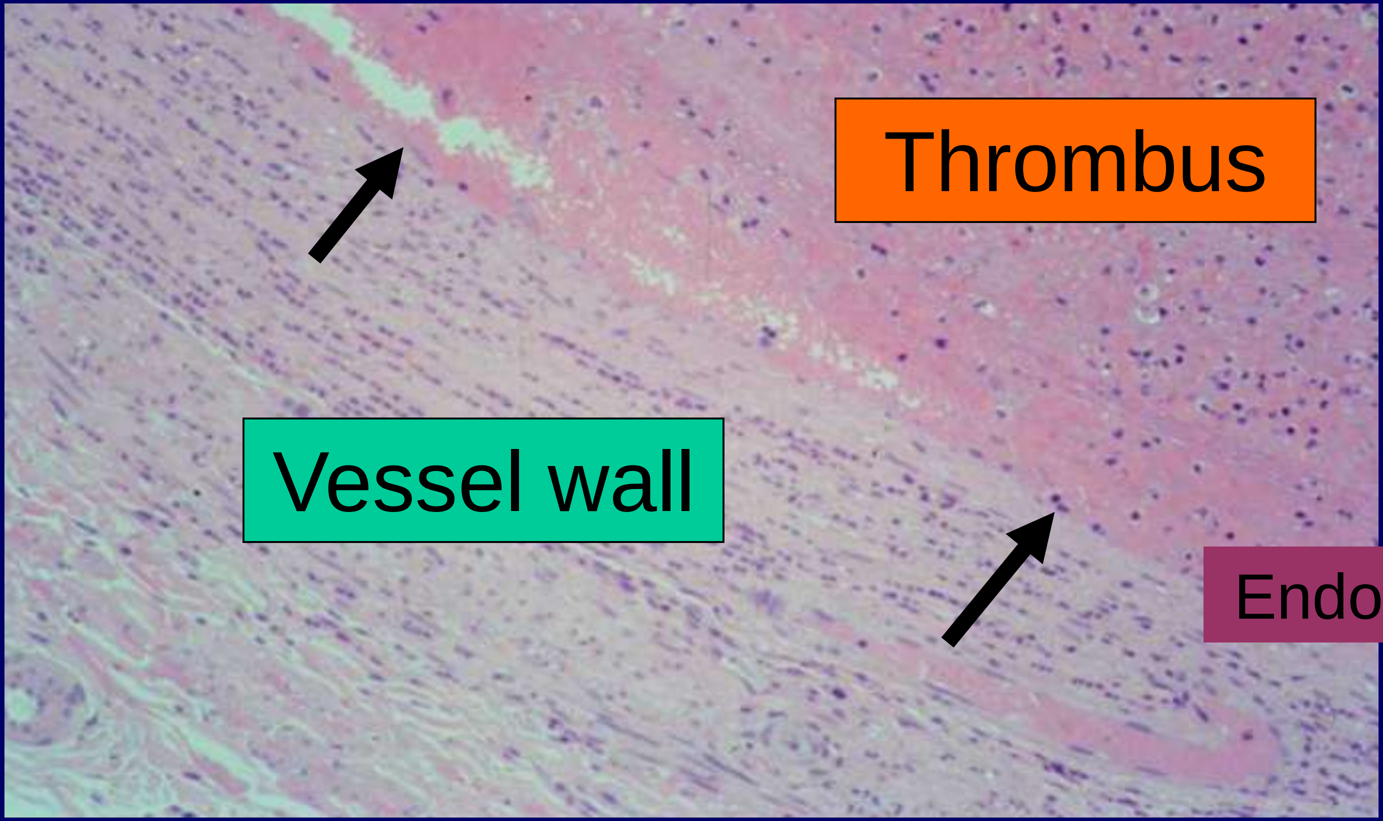
High Levels of TF are Present in Atherosclerotic Plaques



"Thrombotic" TF

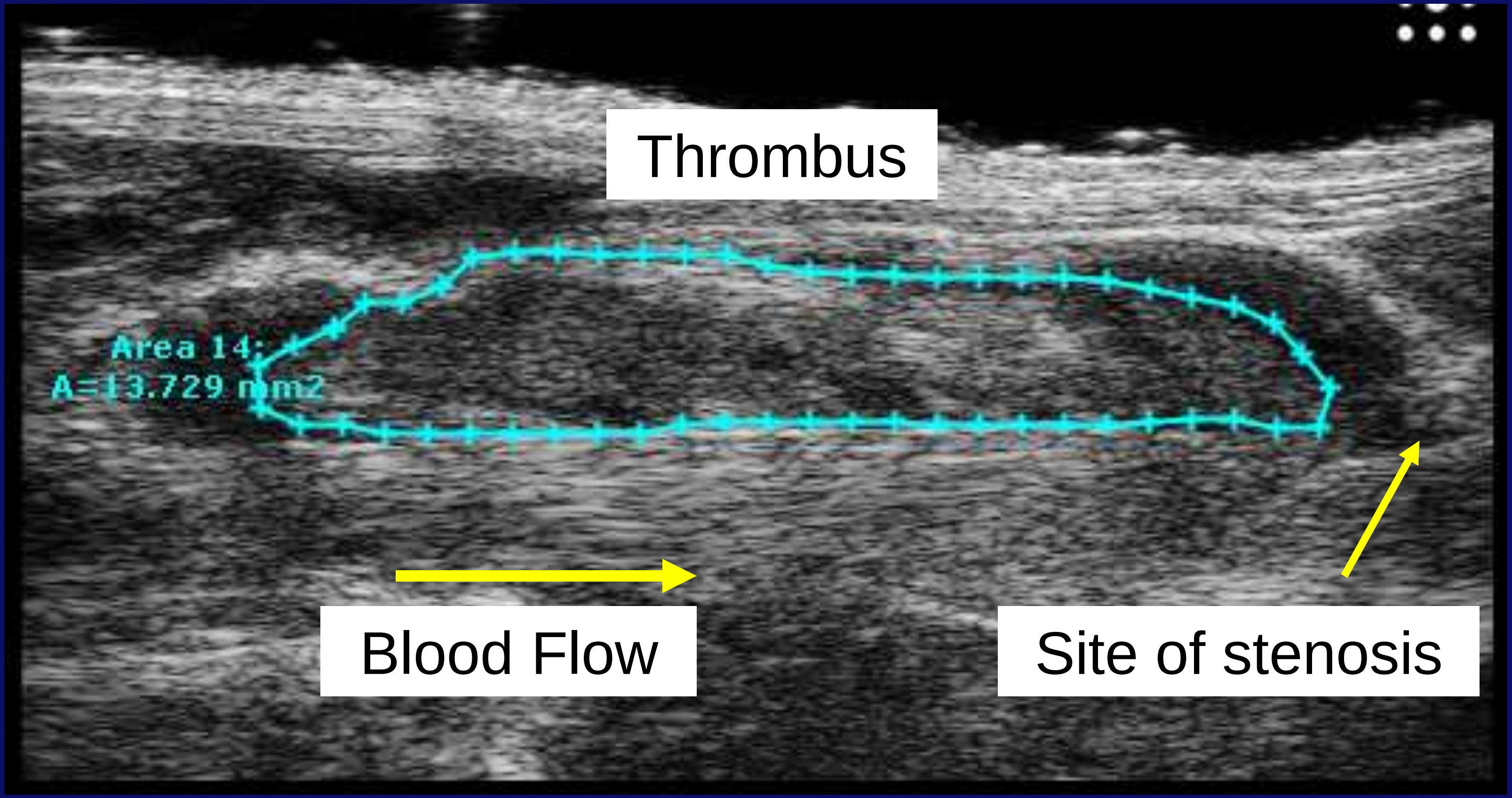
Slide provided by Dr. Y. Asada

Venous Thrombosis



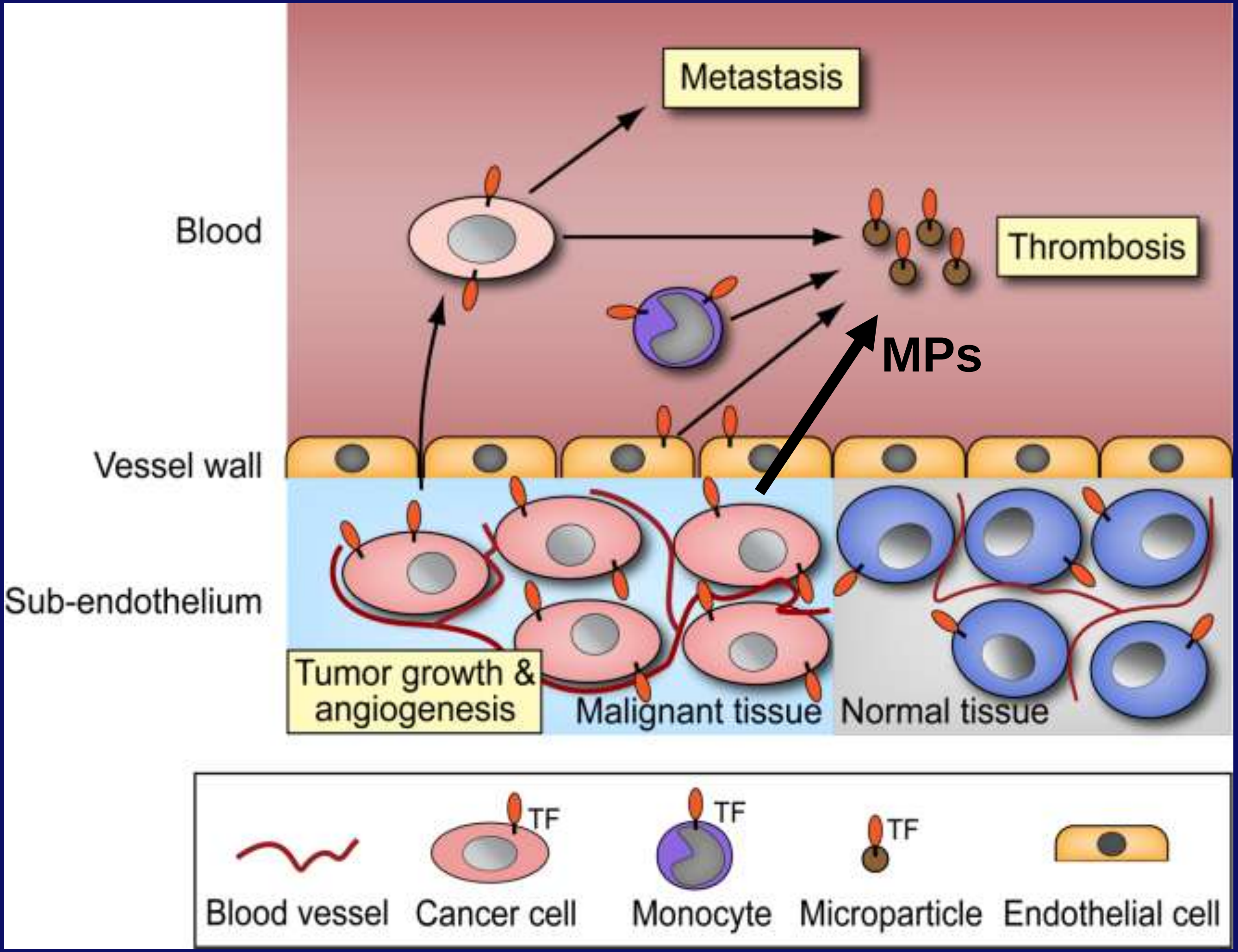
Slide provided by Dr. Wakefield

Measurement of Thrombus Size by Ultrasound



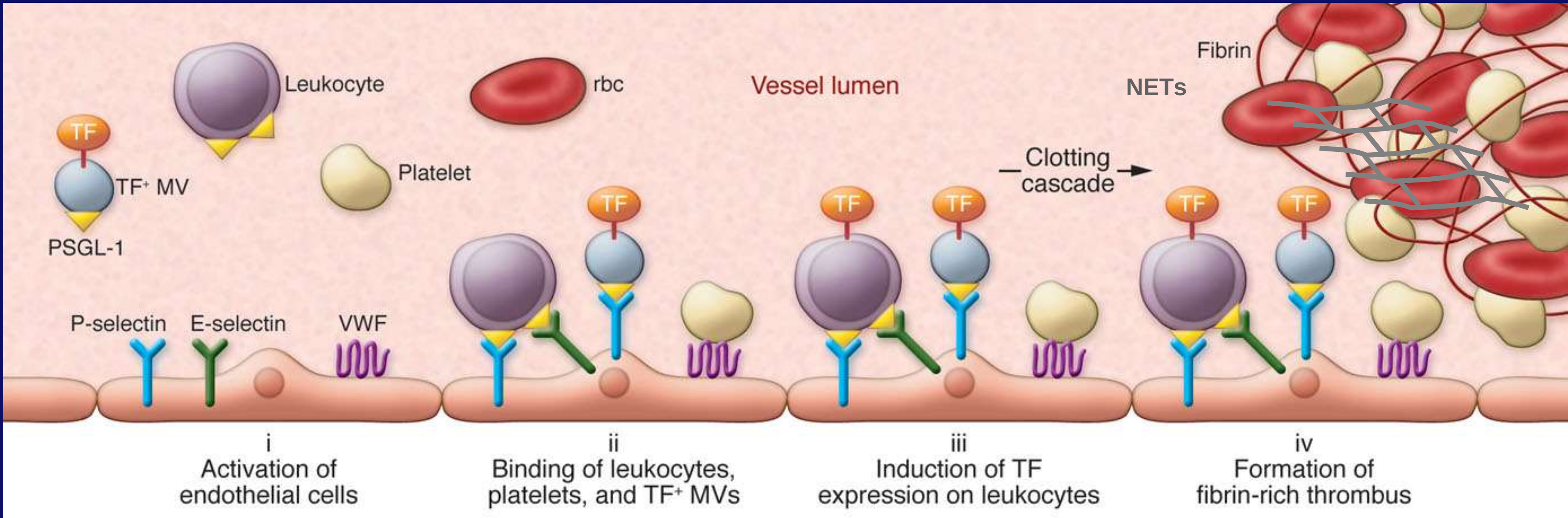
Gedding J unpublished data

Role of Tissue Factor in Cancer

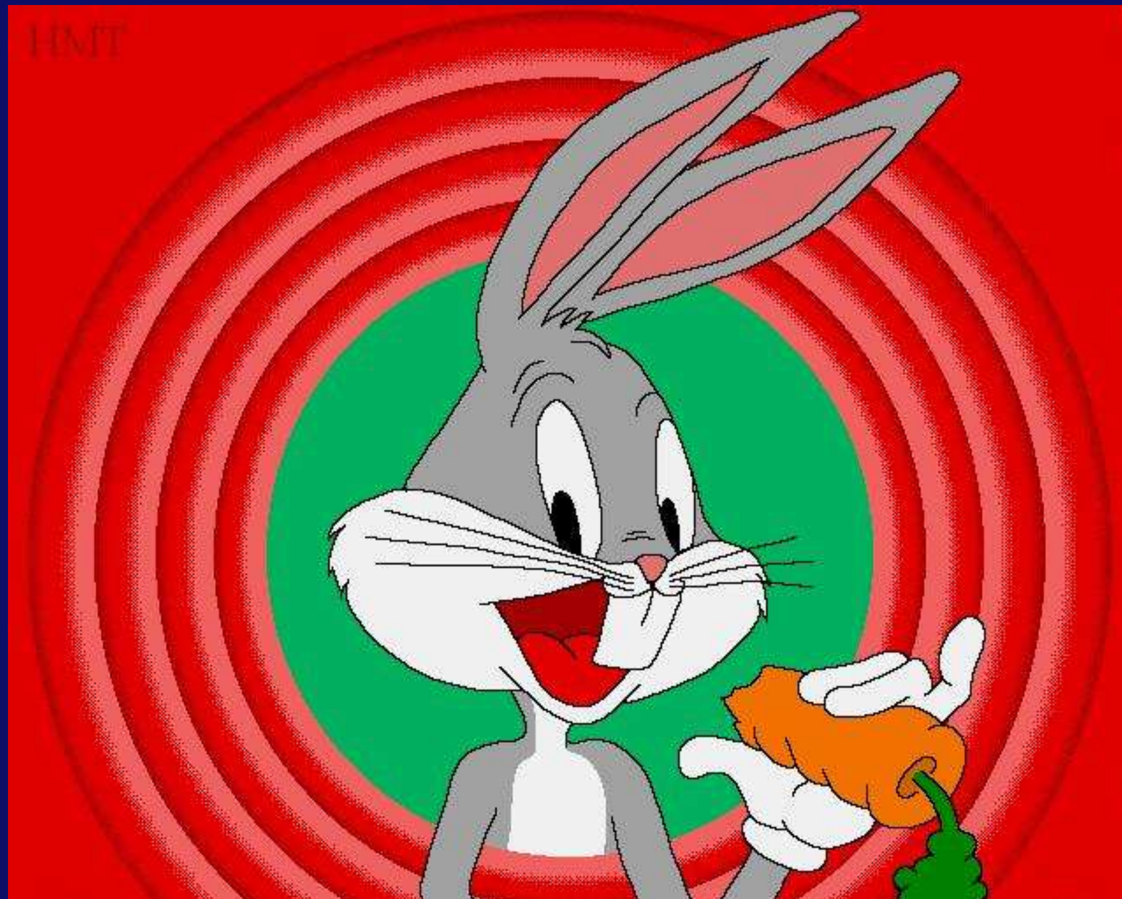


Kasthuri, Taubman and Mackman JCO 2009

Mechanisms of Venous Thrombosis



Mackman JCI 2012



"That's all Folks!"