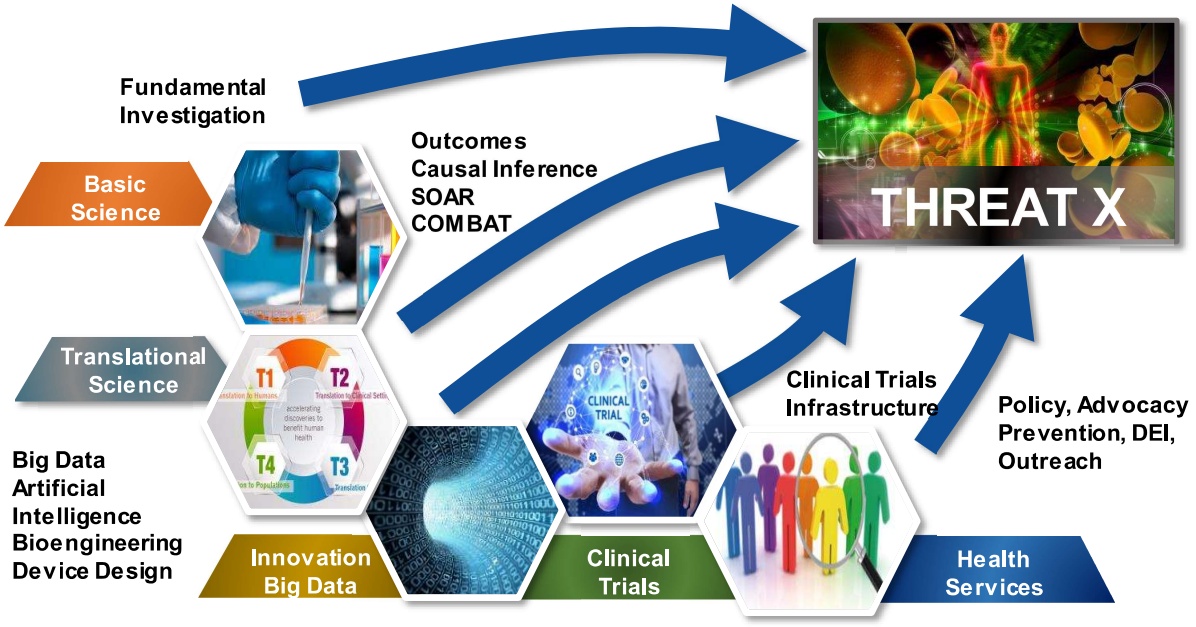
Translational Approaches to Thromoinflammation: Addressing Threat X With Big Science:

Mitchell Jay Cohen, MD, FACS
Professor and Vice Chair of Surgery
University of Colorado School of Medicine

University of Colorado
Anschutz Medical Campus

THREAT X:
COMMON BIOLOGY UNDERLYING AGNOSTIC THREAT

Threat X research needs to examine the common biology and solutions that drive critical illness and injury.



Critical Illness and Injury Research Center

The leading and most comprehensive program for illness and injury research

Scientific Program Pillars	Basic Science	Translational Science	Innovation/Big Data	Clinical Trials	Health Services
Summary and Funding	NIH, DOD, BARDA funded basic labs.	NIH, DOD, PCORI funding. Patient enrollment sample collection and translation. SOAR	NIH, DOD, NSF funding Analytics and outcomes. Data and Model based modeling.	NIH, DOD, PCORI funded Trauma/Burn/Sepsis/ARDS trials. Implementation and outcomes research	Prevention DEI Health Services Research Outreach
Programs	Burn, Alcohol, and Aging Thrombo-inflammation Endothelial biology Coagulation Biology Small molecule biology	Integrated organ response to disease Cellular behavior and dynamics in disease Transcriptomics, proteomics, and metabolomics of disease Genetic and epigenetic basis of disease (population-based)	Molecule Bioengineering Pathogen-host response Physiologic immune response Metabolism and function of pathogens	Trauma Resuscitation Trials Burn Resuscitation Trials Post Partum Hemorrhage Biorepository	Disparities in Surgical Access Health Equity Late Presentation of Surgical Disease Violence Intervention
Examples	Organ response Cellular response Drug binding	Immune Function Coagulation Sepsis	Big Data/AI Bioengineering Device Innovation	Trials Outcomes Implementation	Prevention Advocacy Policy

length-scale

Anschutz SURGERY

COMMUNITY PARTNERSHIPS IN INJURY PREVENTION

CU Center for COmbat Medicine and BATtlefield (COMBAT) Research

University of Colorado Anschutz Medical Campus

CENTER FOR COMBAT RESEARCH
CU ANSCHUTZ

CU Center for COMBAT Research

50+ DoD Funded Grants and **17** staff

100+ Research Investigators

320+ high impact publications

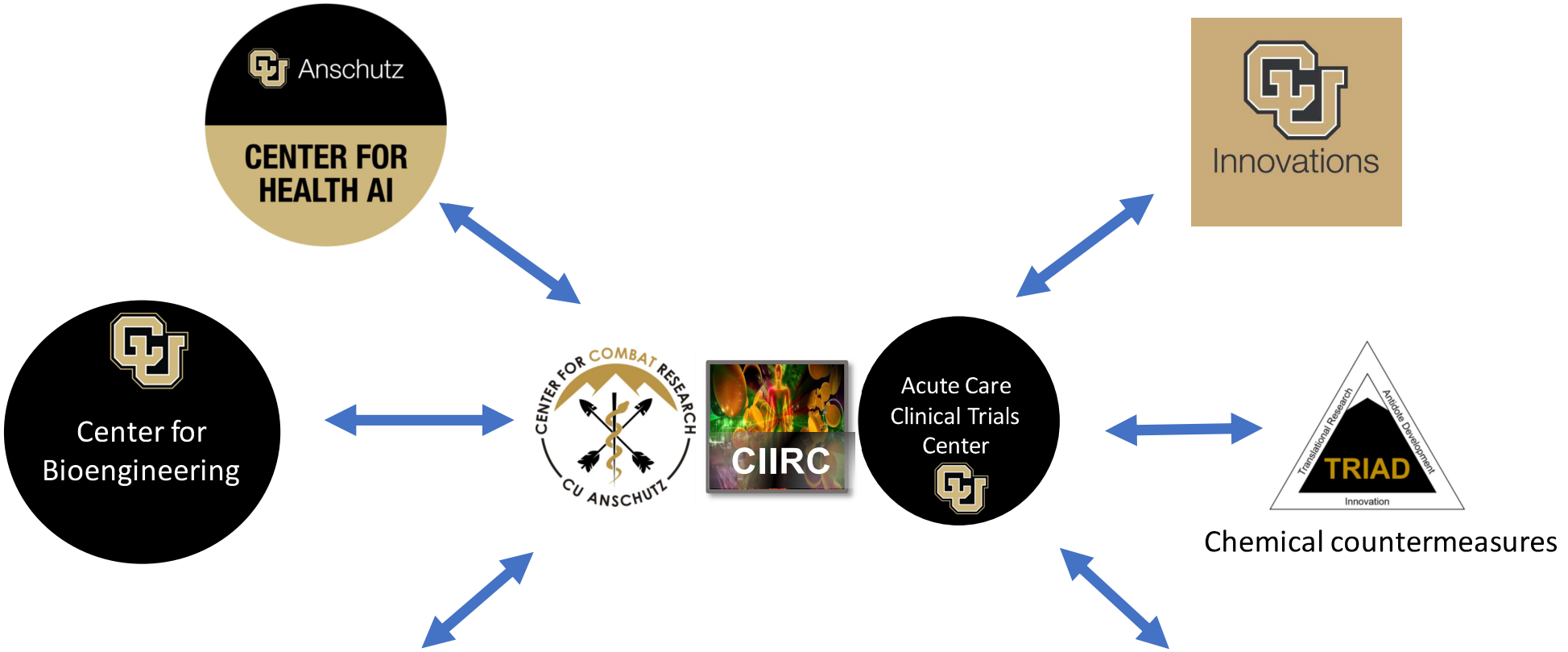
High rate of funding of our research proposals

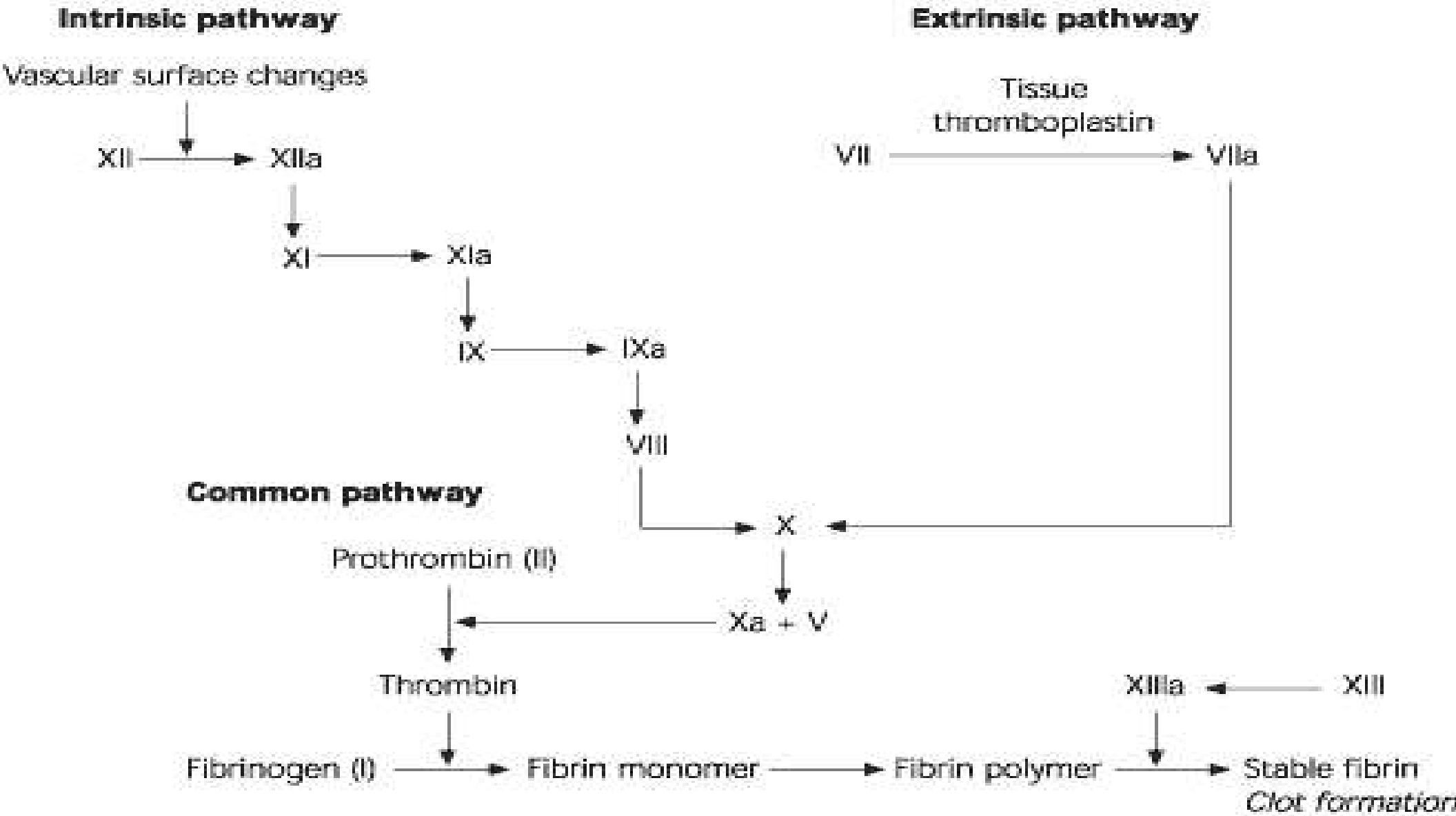
Academic, industry and military collaborators

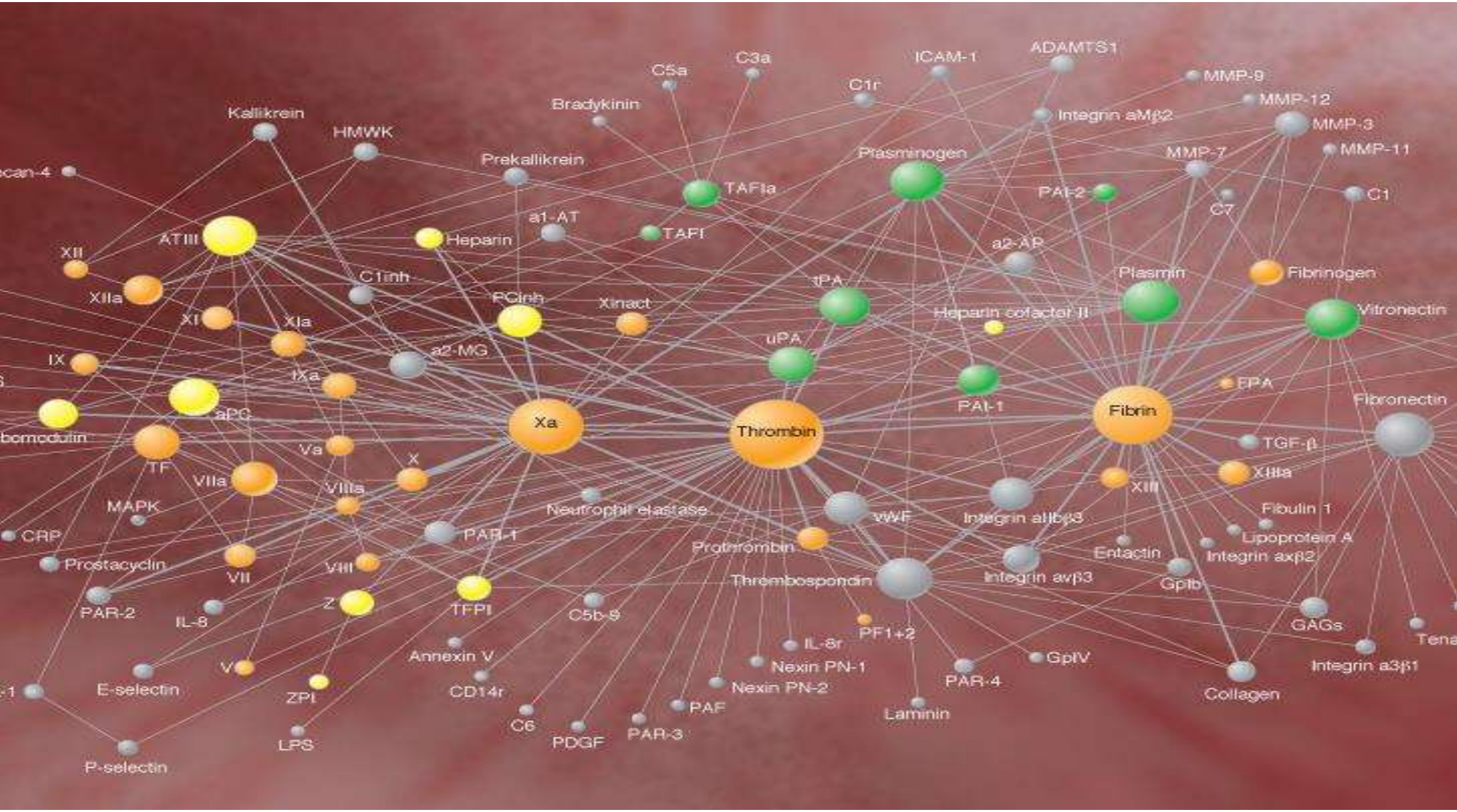
Anschutz SURGERY

Mission - To solve the US military's **toughest** clinical challenges through **innovation**, **research**, and **advanced development** for the future battlespace in combat casualty care

CU Anschutz Ecosystem




Intrinsic pathway
Extrinsic pathway
Vascular surface changes
Tissue thromboplastin
XII
XIIa
VII
VIIa
XI
XIa
IX
IXa
VIII
Common pathway
X
Prothrombin (II)
Xa + V
Thrombin
XIIIa
XIII
Fibrinogen (I)
Fibrin monomer
Fibrin polymer
Stable fibrin
Clot formation


ADAMTS1
ICAM-1
C5a
C3a
C1r
MMP-9
MMP-12
Bradykinin
Integrin aMβ2
MMP-3
Kallikrein
HMWK
Prekallikrein
Plasminogen
MMP-7
MMP-11
TAFIa
PAI-2
C7
C1
a1-AT
ATIII
Heparin
TAFI
a2-AP
XII
C1inh
tPA
Plasmin
Fibrinogen
XIIa
PCInh
Xinact
Heparin cofactor II
Vitronectin
XI
XIa
uPA
a2-MG
IX
IXa
EPA
PAI-1
Fibronectin
aPC
Xa
Thrombin
Fibrin
TF
Va
X
TGF-β
VIIa
VIIIa
XIIIa
XIII
MAPK
Neutrophil elastase
vWF
Integrin αIIbβ3
Fibulin 1
CRP
PAR-1
Lipoprotein A
Prothrombin
Entactin
Integrin axβ2
Prostacyclin
VII
VIII
Thrombospondin
Integrin avβ3
GpIb
PAR-2
Z
TFPI
C5b-9
GAGs
IL-8
PF1+2
Annexin V
IL-8r
GpIV
Nexin PN-1
V
Integrin a3β1
E-selectin
Nexin PN-2
PAR-4
ZPI
CD14r
Collagen
PAF
Laminin
C6
PDGF
PAR-3
LPS
P-selectin

Threat X

- Agnostic multiple threats.
- A common immunothromboinflammatoendotheliopathy response?
- A individualized immunothromboinflammatoendotheliopathy response?

We have pretty good clinical insight of how and why Threat X patients die.

- Anatomic injury
- Coagulopathy
- Endotheliopathy
- Perturbed inflammation/immune response
- Infection
- Clinical experience of similar patients and underlying data should not be discounted.

Models of Threat X

- There are no current relevant models of threat X.
- There are good trauma models but…
- There are good radiation models but…
- There are good chemical exposure models but…

- All of these models suffer from limitations, inadequate development and the lack of combined injury models.
- However this can be fixed and we are starting.

Clinical models

- Get as much data as possible (trans omics).
- Trauma is pretty good.
- Radiation is fortunately sparse but not unheard of and there is much we can bidirectionally extrapolate.
- Chem is fortunately sparse but not unheard of
- Threat X will likely be coming and we need to be ready to collect that data (and treat).

Clinical Insight

- Requires a common data structure and ontology.
- Fortunately limited data in radiation but some.
- Lots of trauma data which is less integrated and difficult to fund.
- Some chemical data.
- Pandemic and infectious and other inflammatory data is pervasive but not standardized.
- There is a place for synthetic data.

So how can we address this?

- We have to better understand the phenotypes of injury across clinical, in vitro, in vivo and in silico models.
- In the era of big data and personalized medicine experimentation is key including broad diverse data and synthetic data..
- This can then be supervised by clinicians and experts to drive inference.

Coagulopathy and Inflammation after Trauma:
(and Burn, Chem, Radiation, Infection…)
are linked by Protein C

PROTEIN C

```
-42                MWQLTSLLLLFVATWGISGTPAPLDSVFSSSERAHQVLRIRKR  -1
  1  ANSFLEELRHSSLEREDCIEEICDFEEAKEIFQNVDDTLAFWSKHVDGDQC   50
 51  LVLPLEHPCASLCCGHGTCIDGIGSFSCDCRSGWEGRFCQREVSFLNCSL  100
101  DNGGCTHYCLEEVGWRRCSCAPGYKLGDDLLQCHPAVKFPCGRPWKRMEK  150
151  KRSHLKR↓DTEDQEDQVDPRLIDGKMTRRGDSPWQVVLLDSKKKLACGAVL  200
201  IHPSWVLTAAHCMDESKKLLVRLGEYDLRRWEKWELDLDIKEVFVHPNYS  250
251  KSTTDNDIALLHLAQPATLSQTIVPICLPDSGLAERELNQAGQETLVTGW  300
301  GYHSSREKEAKRNRTFVLNFIKIPVVPHNECSEVMSNMVSENMLCAGILG  350
351  DRQDACEGDSGGPMVASFHGTWFLVGLVSWGEGCGLLHNYGVYTKVSRYL  400
401  DWIHGHIRDKEAPQKSWAP                                  419
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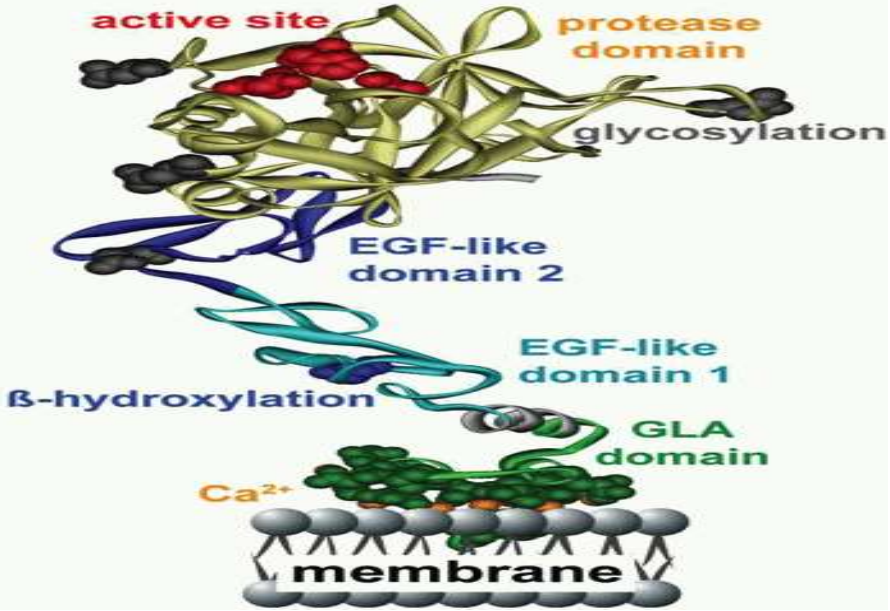
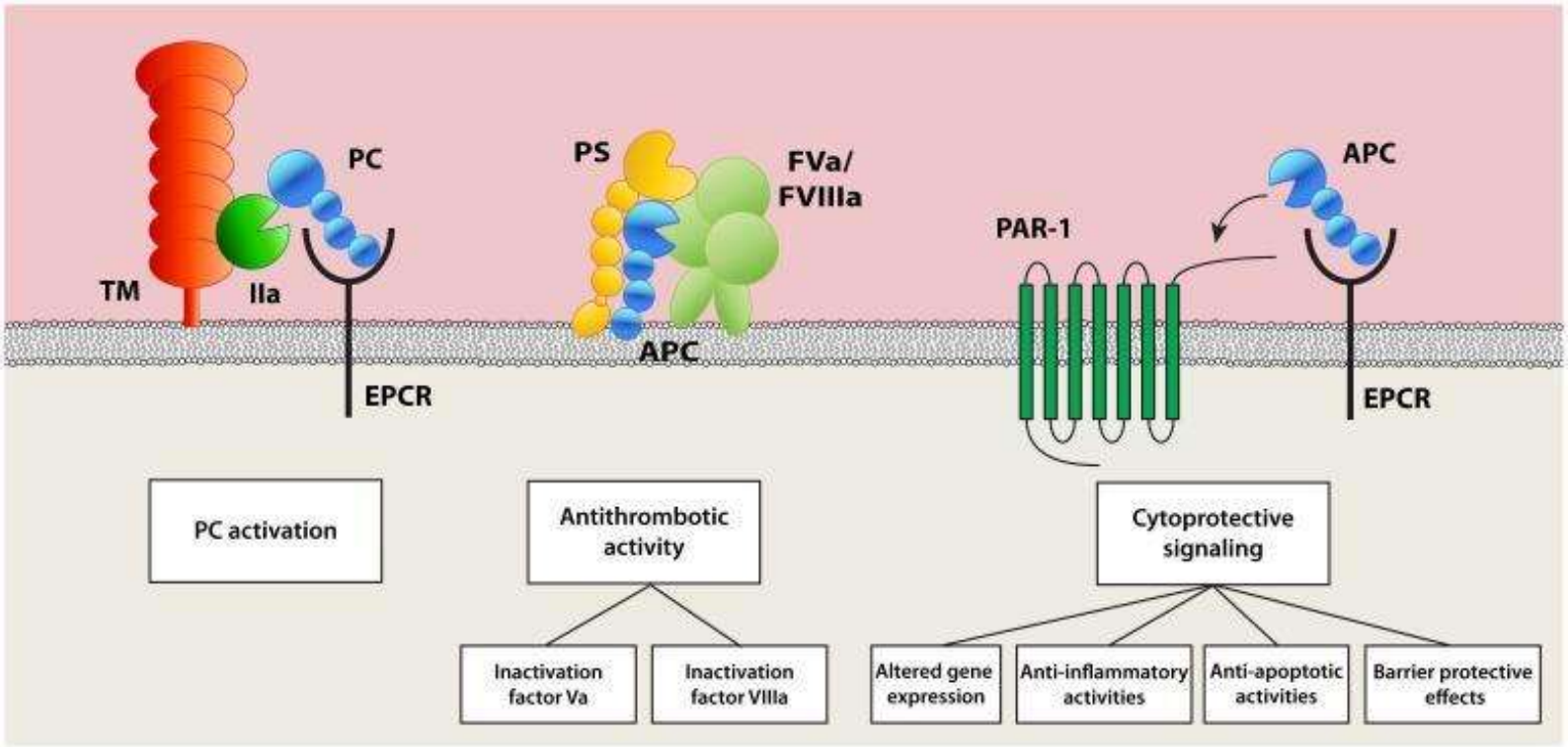
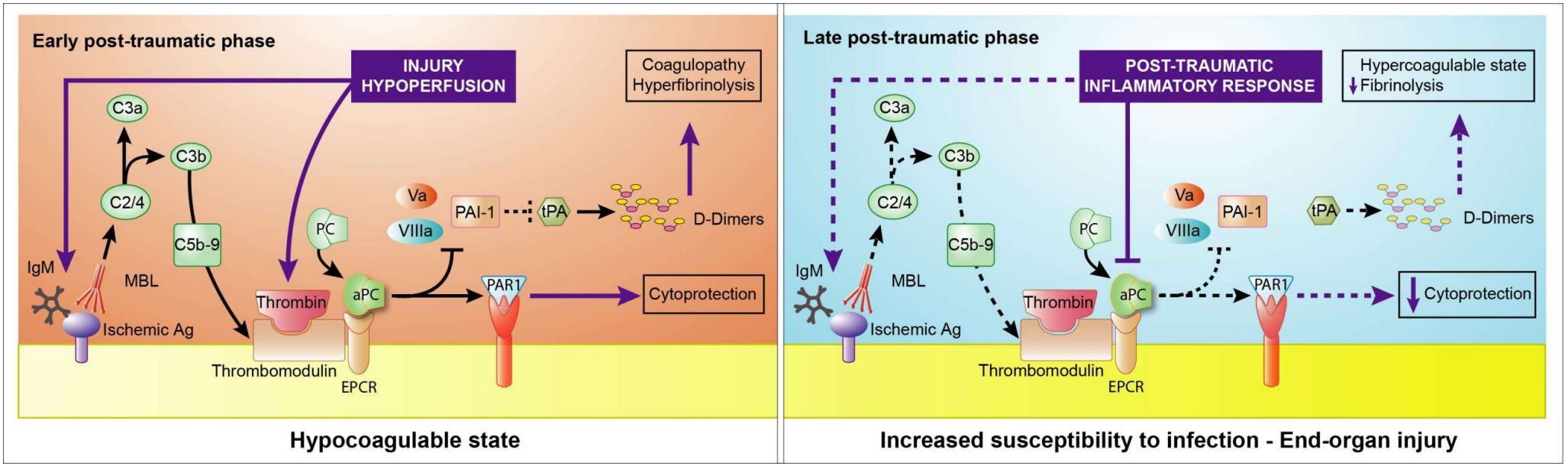


aPC Activation and Function



Bouwens EA, Stavenuiter F, Mosnier LO. Mechanisms of anticoagulant and cytoprotective actions of the protein C pathway. *J Thromb Haemost*. 2013;11 Suppl 1(0 1):242-253.

Maladaptive response to trauma. Early coagulopathy, later hypercoagulable state and loss of cytoprotectivity.



aPC Increases After Trauma and Correlates with TIC

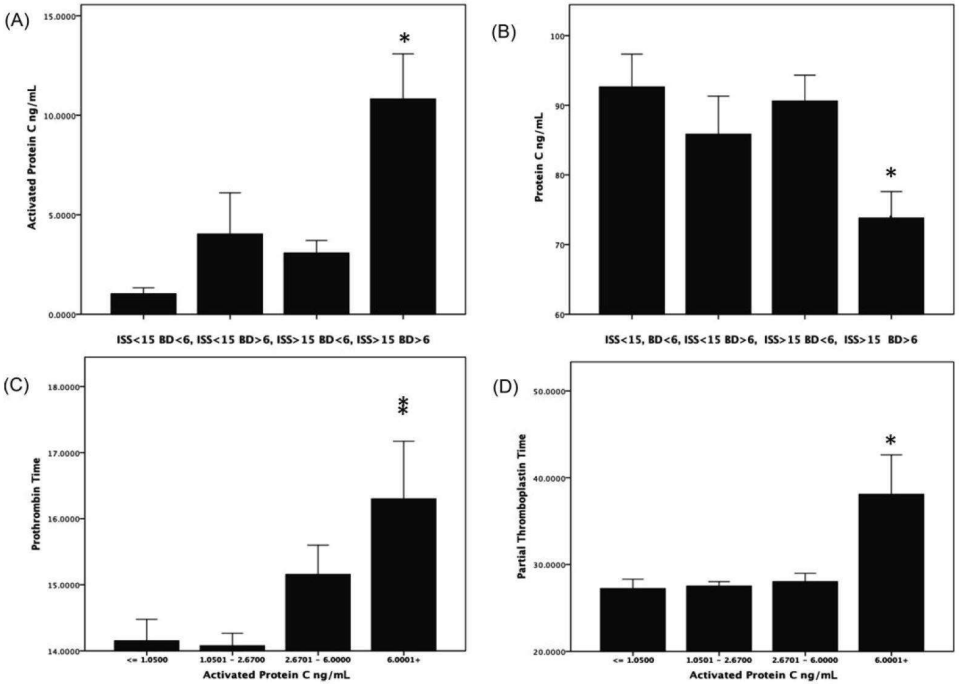
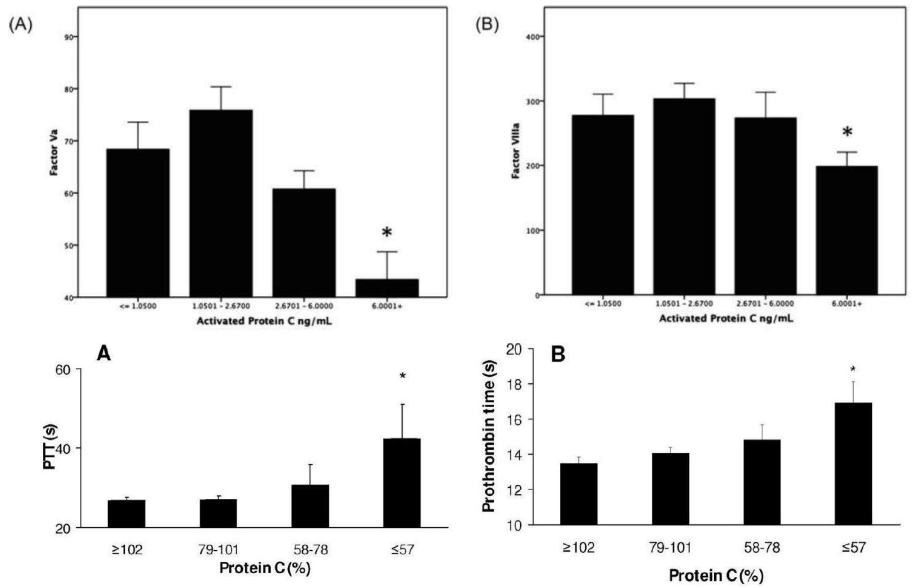


FIGURE 2. Tissue injury and shock result in a systemic activation of protein C pathway associated with coagulopathy in trauma



1.Cohen MJ, Call M, Nelson M, et al. Critical role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. *Ann Surg*. 2012;255(2):379-385.

2.Brohi K, Cohen MJ, Ganter MT, Matthay MA, Mackersie RC, Pittet JF. Acute traumatic coagulopathy: initiated by hypoperfusion: modulated through the protein C pathway? Ann Surg. 2007 May;245(5):812-8.

Group 1: No Injury, No Shock

ID	Activity (%)*	Intact FV +, -, n.d.** (% of 10 donor)	Fragments +,- (identity)	αTAT, nM***	PAP, nM	TFPI, nM
134	104	+ (103)	+	0	2.6	1.54
148	48	+ (78)	-	0	9.2	1.15
173	78	+ (178)	-	0	7.5	1.70
1619	46	+ (106)	-	0	3.3	1.14
1693	n.a.	+ (123)	-	0	2.2	1.50
474	60	+ (88)	-	0	1.7	1.29
589	57	+ (149)	+ (HC)	0	5.6	0.88
1379	n.a.	+ (229)	-	1.3	5.3	1.53
1067	40	+ (105)	-	0	2.0	1.19
1890	15	+ (229)	-	0	2.9	2.06
1783	34	+ (113)	-	0	1.9	1.26
881	34	- (32)	+	9.4	2.7	1.48
879	91	+ (178)	-	0	2.0	1.68
1760	n.a.	- (57)	-	0	1.8	1.37
1338	98	- (44)	+	11.4	6.0	2.39
75	33	+ (60)	-	0	6.4	1.19
76	n.a.	- (56)	-	5.9	4.8	1.66
81	41	- (49)	+ (HC)	8.0	52.5	1.20
513	32	+ (76)	+ (HC)	8.4	40.9	2.37
865	102	+ (94)	+ (HC)	0	2.1	1.86

Group 2: No Injury, Shock

ID	Activity (%)*	Intact FV +, -, n.d.** (% of 10 donor)	Fragments +,- (identity)	αTAT, nM***	PAP, nM	TFPI, nM
899	n.a.	-, n.d	+	23.1	3.6	1.56
864	40	-, n.d	+	11.8	44.1	2.37
1729	n.a.	+ (80)	-	0	1.4	1.86
2013	116	+ (211)	+	0	2.9	1.72
2204	n.a.	+ (84)	-	0	2.9	1.50
2205	n.a.	+ (91)	-	0	9.6	0.99
2256	24	+ (108)	-	0	2.2	0.41
2157	87	+ (158)	-	0	2.8	1.56
2224	162	+ (262)	-	0	2.3	1.42
2117	32	+ (63)	-	0	3.2	0.41
2146	43	- (52)	+	0	31.6	1.04
1427	n.a.	- (53)	+ (HC)	1.3	35.8	1.70
2086	57	+ (73)	+	0	2.8	1.37
2050	94	- (47)	+	0	2.2	1.33
164	n.a.	- (50)	+	0	4.0	1.29
2175	64	+ (104)	+	0	2.6	1.34
219	73	+ (85)	+	0	3.8	1.35
1977	38	+ (69)	+ (HC)	0	6.8	0.96
2194	49	+ (140)	+	0	10.3	1.60
1964	45	+ (65)	-	0	1.7	1.32

Group 3: Injury, No Shock

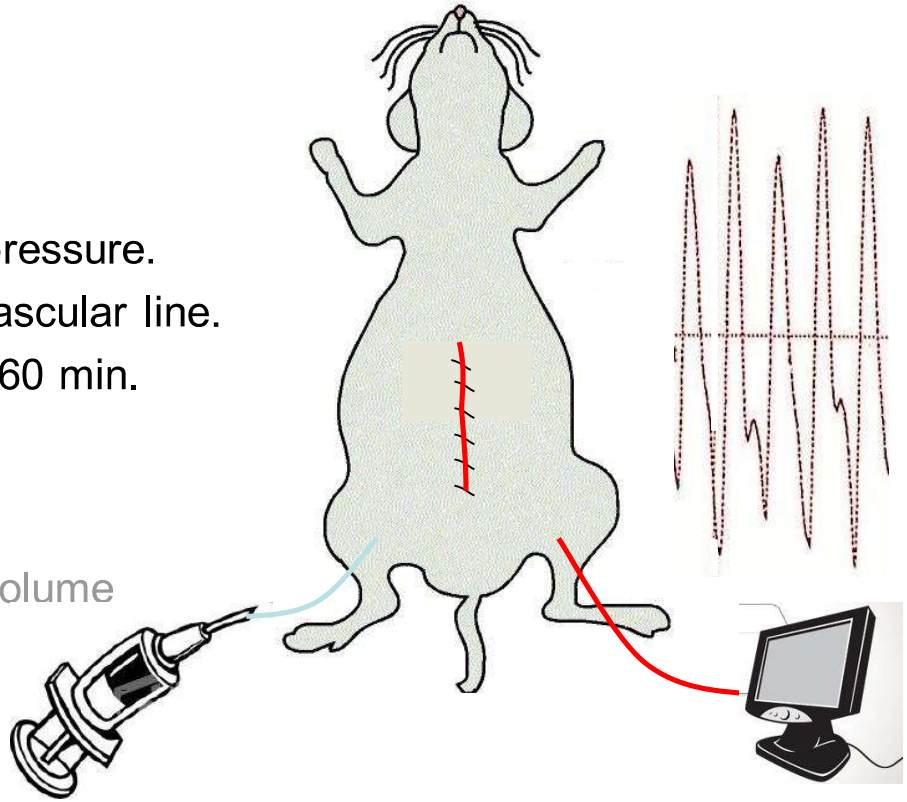
ID	Activity (%)*	Intact FV +, -, n.d.** (% of 10 donor)	Fragments +,- (identity)	αTAT, nM***	PAP, nM	TFPI, nM
252	n.a.	+ (153)	+ (HC)	5.3	31.7	2.83
1063	46	- (40)	-	4.8	25.0	1.20
920	58	- (49)	+	14.5	7.0	1.97
1046	30	+ (93)	+ (HC)	1.5	43.7	1.98
854	49	+ (96)	+ (HC)	9.7	44.9	1.94
1000	29	n.d.	+ (30 KD)	29.7	49.0	2.75
447	33	+ (78)	+ (30 KD)	2.3	5.6	1.55
204	87	+ (148)	+	4.3	11.9	1.49
361	68	+ (111)	+	4.7	13.7	1.21
955	68	n.d.	+	34.2	18.0	1.42
992	46	+ (79)	+ (HC)	5.4	28.9	2.38
676	62	+ (130)	+ (HC)	0	7.9	0.93
951	21	n.d.	+ (HC, 30KD)	27.3	44.9	3.10
685	116	+ (205)	-	1.0	6.3	1.76
163	59	+ (77)	-	0	15.4	1.20
761	101	+ (139)	+	2.7	4.3	2.34
1008	26	+ (126)	-	2.5	19.0	1.69
11	51	+ (68)	-	4.1	32.2	2.02
21	21	+ (83)	+ (HC)	0	19.7	0.82
3	83	+ (63)	+ (HC)	0	15.6	1.39

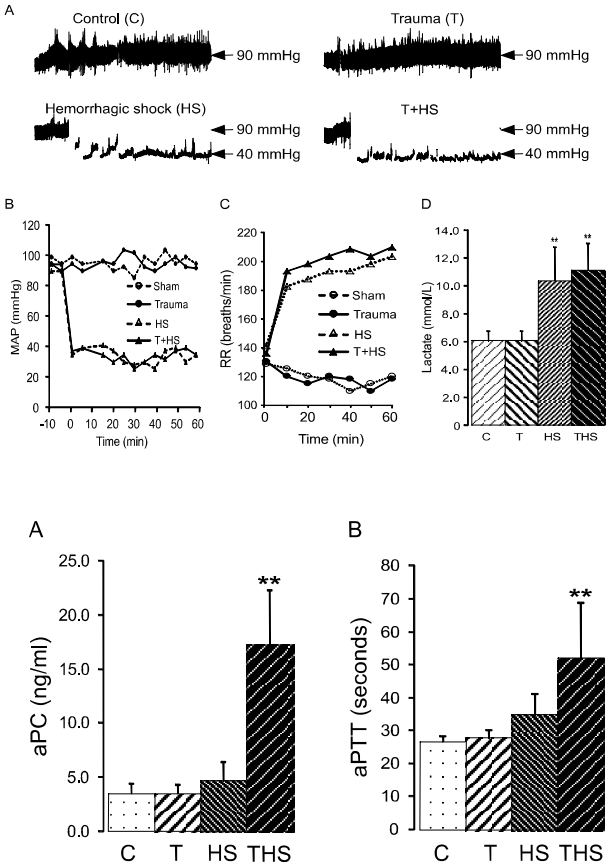
Group 4: Injury and Shock

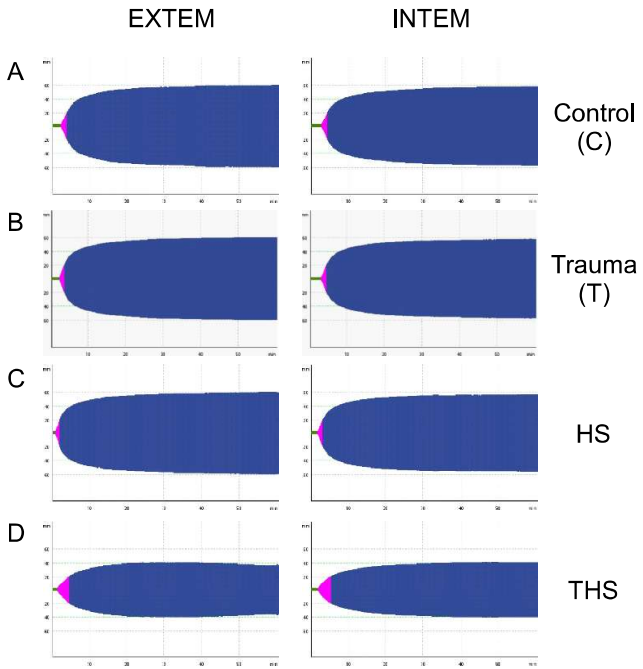
ID	Activity (%)*	Intact FV +, -, n.d.** (% of 10 donor)	Fragments +,- (identity)	αTAT, nM***	PAP, nM	TFPI, nM
1891	15	- (25)	+ (30KD)	5.1	49.5	0.58
2134	n.a.	- (21)	+ (HC, 30KD)	0	40.4	0.82
2060	44	- (26)	+ (HC, 30KD)	11.7	49.1	0.95
1888	10	- (25)	+ (HC, 30KD)	10.5	59.5	1.48
1586	n.a.	- (45)	-	0	1.9	1.38
1747	17	+ (67)	+ (HC)	8.9	43.2	2.52
1827	7	- (13)	+ (30KD)	22.3	59.5	1.03
1991	46	- (59)	+ (HC)	4.9	14.4	1.25
2031	32	- (43)	-	0	11.3	0.77
1612	n.a.	- (51)	-	0	1.9	1.13
1637	35	- (28)	-	0	4.8	3.00
2226	21	- (38)	+ (HC)	6.5	44.9	3.15
2231	n.a.	+ (71)	-	0	21.5	0.90
1771	n.a.	+ (99)	+	0	19.1	1.02
2140	46	+ (139)	+ (HC)	14.4	41.0	2.27
1848	n.a.	- (49)	+ (HC, 30KD)	5.7	40.7	1.46
1839	n.a.	- (30)	+ (HC, 30KD)	15.4	44.7	1.94
1966	64	- (44)	+ (HC, 30KD)	1.7	21.2	1.39
1643	4	n.d.	+ (30KD)	49.0	44.9	2.06
1535	19	- (22)	+ (30KD)	5.4	46.5	0.79

Animal Model: Traumatic Coagulopathy

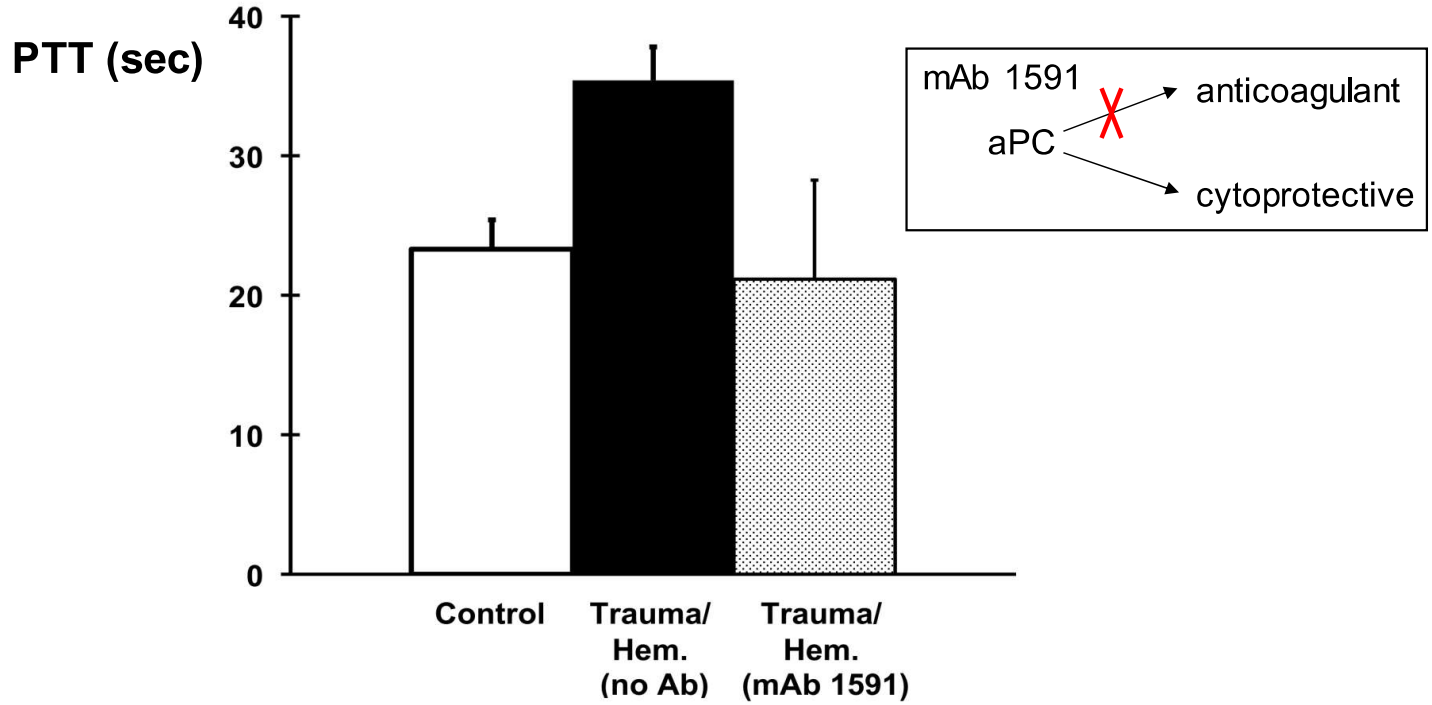
- Tissue Injury
- Hemorrhagic shock:
  - Non-ventilated, fixed-pressure.
  - Blood withdrawn via vascular line.
  - MAP 35 +/- 5mmHg x 60 min.
- Resuscitation:
  - LR @ 2x shed blood volume
    + shed blood



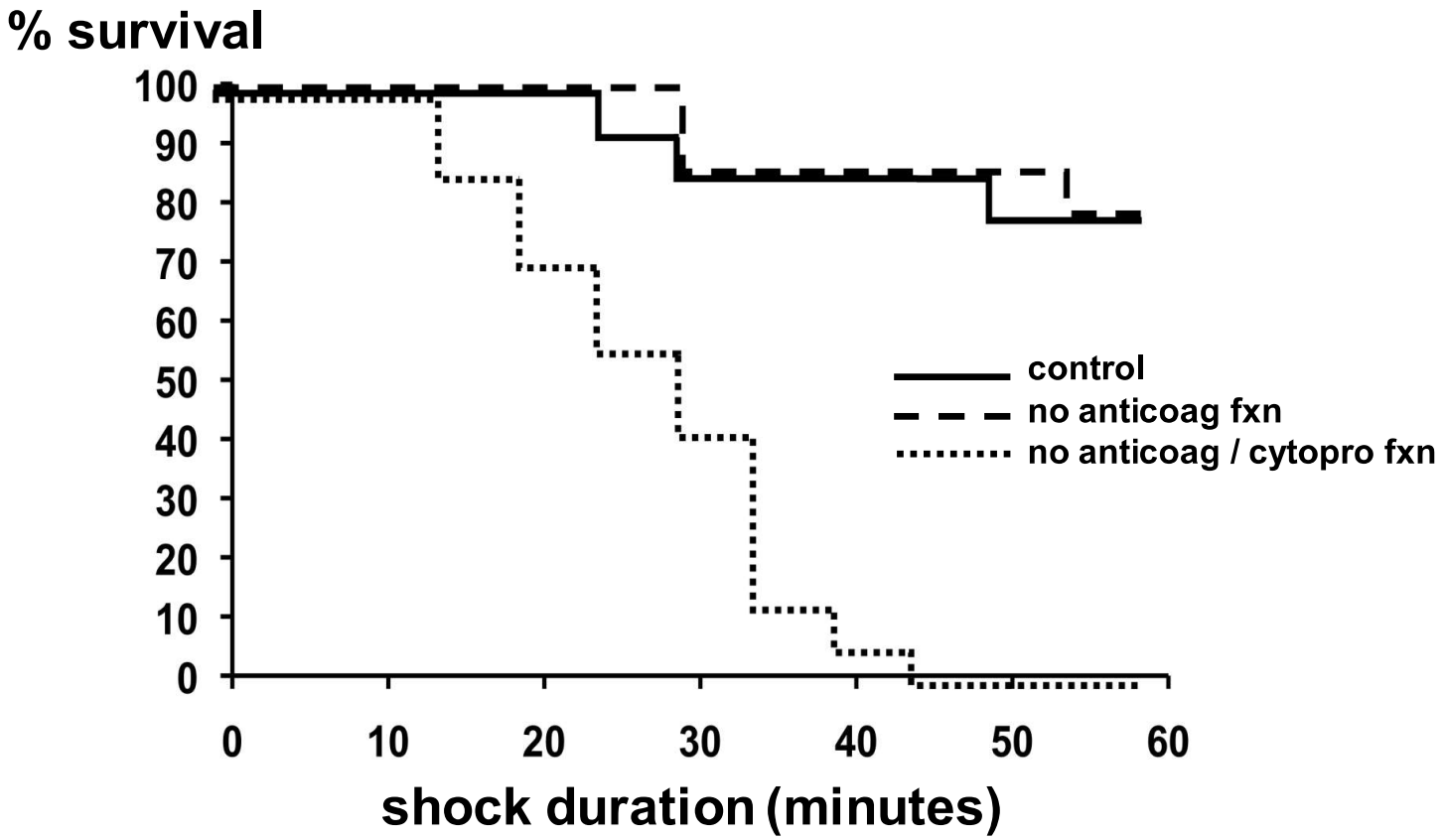

A
Control (C)
90 mmHg
Trauma (T)
90 mmHg
Hemorrhagic shock (HS)
90 mmHg
40 mmHg
T+HS
90 mmHg
40 mmHg
B
MAP (mmHg)
Sham
Trauma
HS
T+HS
Time (min)
C
RR (breaths/min)
Sham
Trauma
HS
T+HS
Time (min)
D
Lactate (mmol/L)
C
T
HS
THS
A
aPC (ng/ml)
**
C
T
HS
THS
B
aPTT (seconds)
**
C
T
HS
THS


EXTEM
INTEM
A
Control
(C)
B
Trauma
(T)
C
HS
D
THS

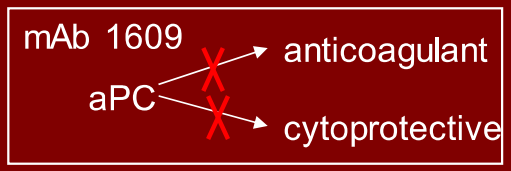
Acute Traumatic Coagulopathy: mediated by aPC *anticoagulant* function



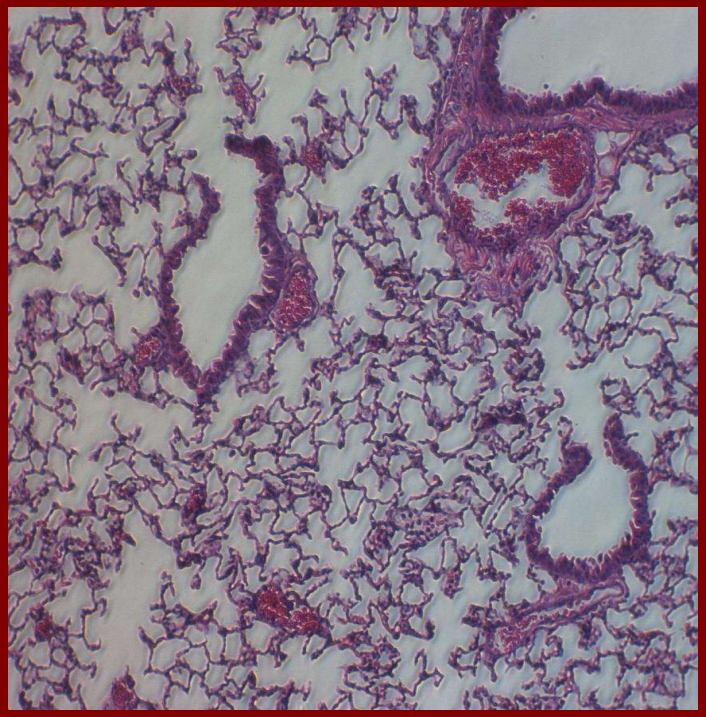
aPC is required for survival of Trauma/Hemorrhage



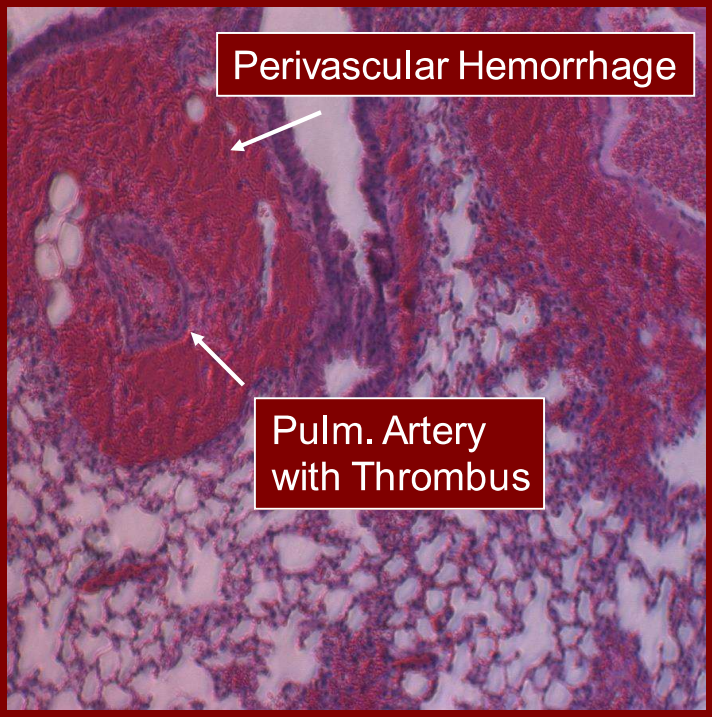
COMPLETE inhibition of Protein C causes diffuse intravascular coagulation & pulmonary injury.



A. Control mAb



B. mAb 1609



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RESEARCH ARTICLE

Inducing Acute Traumatic Coagulopathy *In Vitro*: The Effects of Activated Protein C on Healthy Human Whole Blood

Benjamin M. Howard[1]*, Lucy Z. Kornblith[1], Christopher K. Cheung[1], Matthew E. Kutcher[2], Byron Y. Miyazawa[1], Ryan F. Vilardi[1], Mitchell J. Cohen[1]

1 Department of Surgery, University of California San Francisco and San Francisco General Hospital, San Francisco, California, United States of America, 2 Department of Surgery, University of Pittsburgh Medical Center and Presbyterian University Hospital, Pittsburgh, Pennsylvania, United States of America

* benjamin.howard@ucsf.edu

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

Introduction

Acute traumatic coagulopathy has been associated with shock and tissue injury, and may be mediated via activation of the protein C pathway. Patients with acute traumatic coagulopathy have prolonged PT and PTT, and decreased activity of factors V and VIII; they are also hypocoagulable by thromboelastometry (ROTEM) and other viscoelastic assays. To test the etiology of this phenomenon, we hypothesized that such coagulopathy could be induced *in vitro* in healthy human blood with the addition of activated protein C (aPC).

Methods

Whole blood was collected from 20 healthy human subjects, and was "spiked" with increasing concentrations of purified human aPC (control, 75, 300, 2000 ng/mL). PT/PTT, factor activity assays, and ROTEM were performed on each sample. Mixed effect regression modeling was performed to assess the association of aPC concentration with PT/PTT, factor activity, and ROTEM parameters.

Results

In all subjects, increasing concentrations of aPC produced ROTEM tracings consistent with traumatic coagulopathy. ROTEM EXTEM parameters differed significantly by aPC concentration, with stepwise prolongation of clotting time (CT) and clot formation time (CFT), decreased alpha angle (α), impaired early clot formation (a10 and a20), and reduced maximum clot firmness (MCF). PT and PTT were significantly prolonged at higher aPC concentrations, with corresponding significant decreases in factor V and VIII activity.

Conclusion

A phenotype of acute traumatic coagulopathy can be induced in healthy blood by the *in vitro* addition of aPC alone, as evidenced by viscoelastic measures and confirmed by

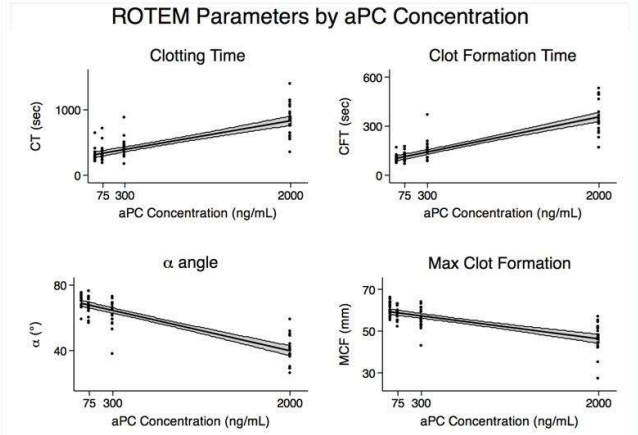
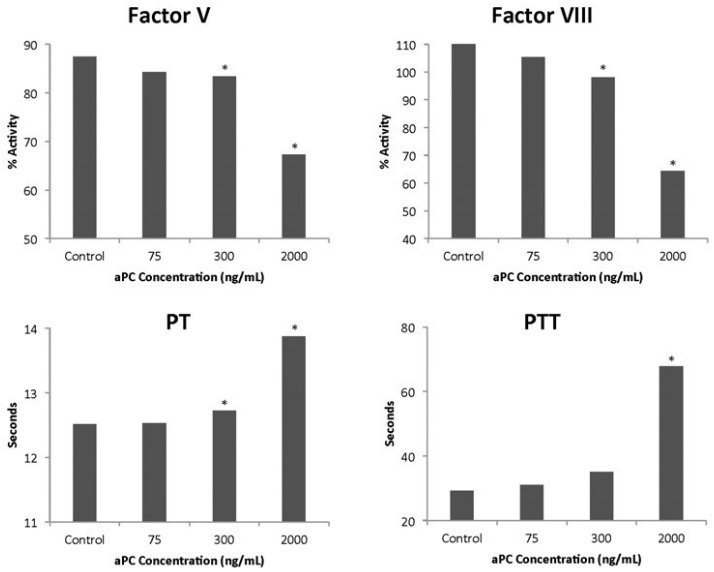


Fig 2. Linear regression analysis of ROTEM parameters. ROTEM EXTEM parameters changed significantly by aPC concentration, with strong linear correlation between aPC concentration and prolonged clotting time (CT) and clot formation time (CFT), decreased alpha angle (α), and reduced maximum clot firmness (MCF). The coefficients of these changes are delineated in Table 1.

doi:10.1371/journal.pone.0150930.g002



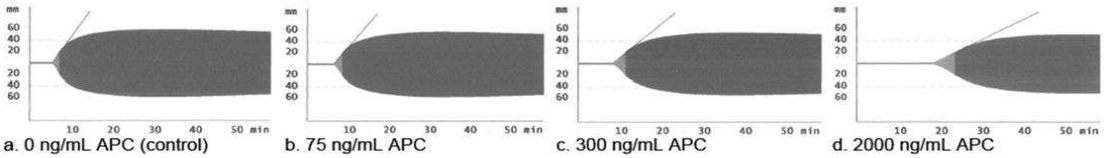


Fig 1. Characteristic ROTEM EXTEM tracings from a study subject. In every single one of the 20 subjects, as depicted here, increasing concentration of aPC produced ROTEM tracings consistent with worsening acute traumatic coagulopathy.

doi:10.1371/journal.pone.0150930.g001

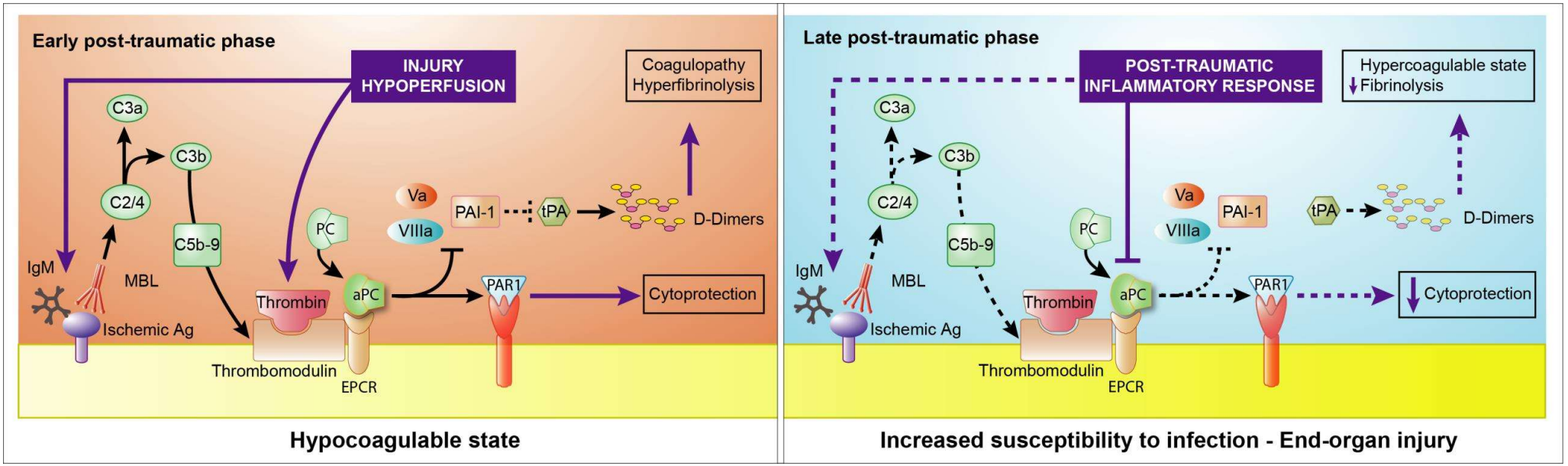
Activation of Protein C mechanistically dries TIC

- Elevated aPC is associated with TIC shortly after ED arrival
- Later depletion of PC is associated with infection and organ failure
- In mice, coagulopathy in T/HS mice was blocked with Ab blocking the anticoagulant activity of aPC
- Blocking global aPC function was 100% fatal, indicating that the cytoprotective function of aPC is essential to survival of initial T/HS

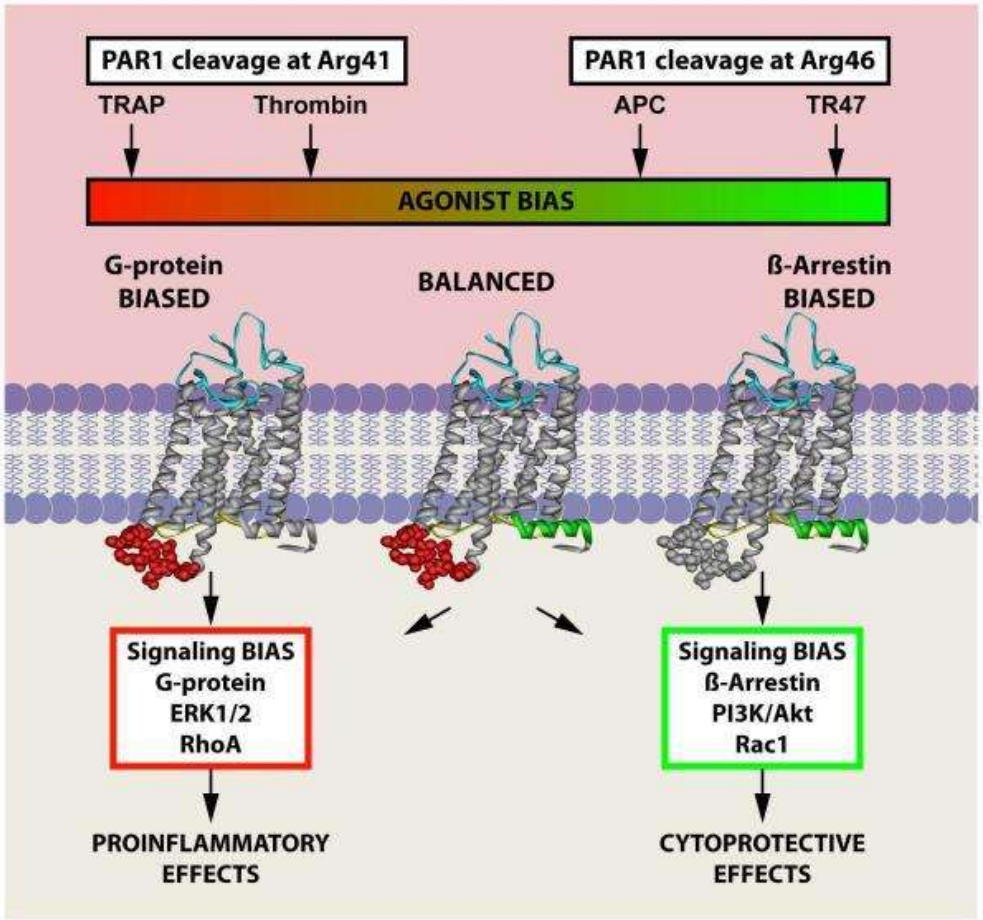
Cohen MJ, Call M, Nelson M, et al. Critical role of activated protein C in early coagulopathy and later organ failure, infection and death in trauma patients. *Ann Surg*. 2012;255(2):379-385.
Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, Frith D, Pittet JF, Cohen MJ. Increase in activated protein C mediates acute traumatic coagulopathy in mice. Shock. 2009 Dec;32(6):659-65.

Early activation and later depletion of PC results in early TIC and later inflammatory complications and is associated with a unique proteomic signature

Maladaptive response to trauma. Early coagulopathy, later hypercoagulable state and loss of cytoprotectivity.



The PAR1 Paradox: aPC beneficial signaling

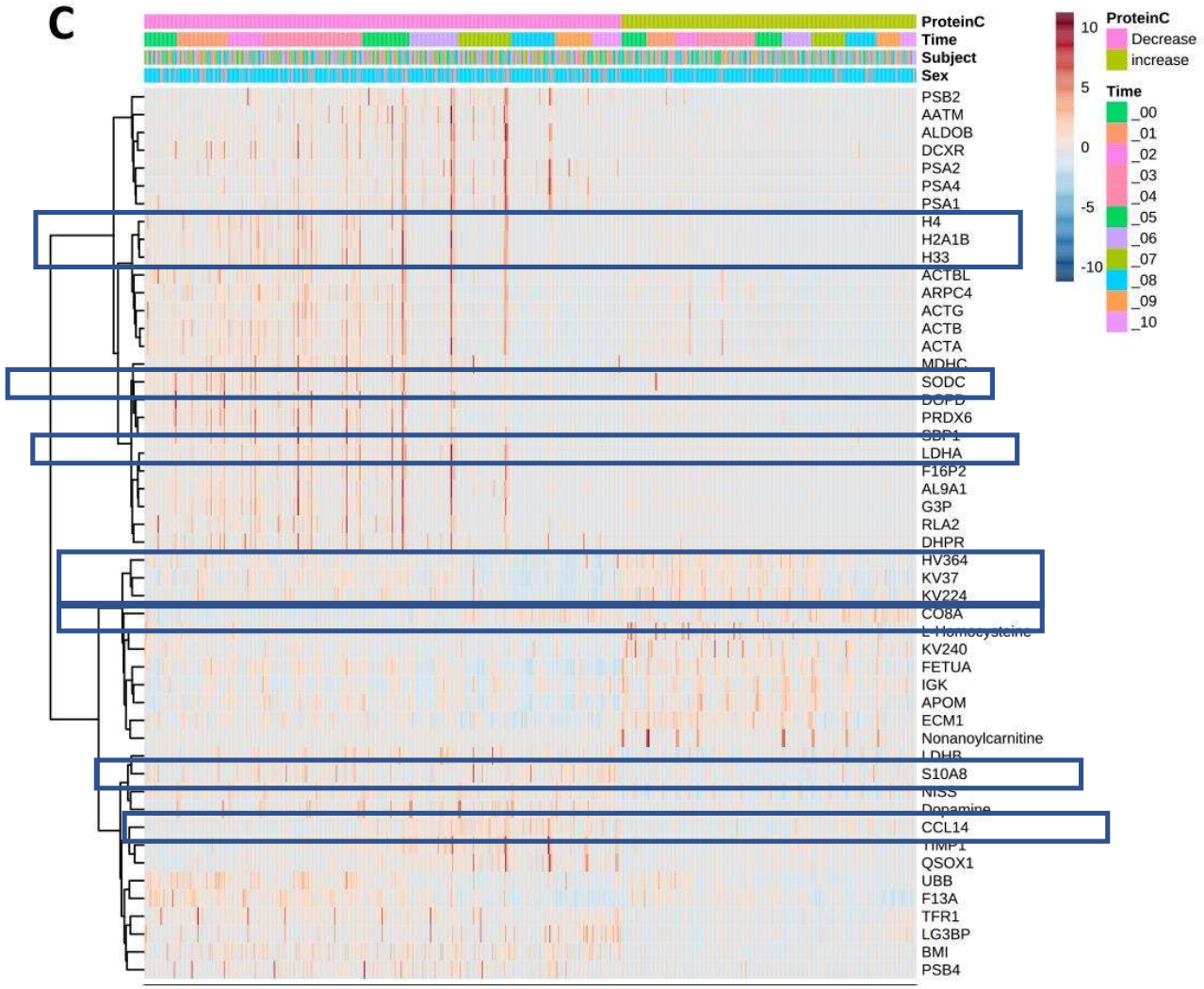


- aPC and Thrombin signal through PAR1 with different downstream effectors and opposing effects
- Thrombin-cleaved PAR1 is rapidly internalized
- aPC-activated PAR1 tends to stay membrane bound and requires "critical mass" for signaling
  - May explain why bolus dosing is more effective

Bouwens EA, Stavenuiter F, Mosnier LO. Mechanisms of anticoagulant and cytoprotective actions of the protein C pathway. *J Thromb Haemost*. 2013;11 Suppl 1(0 1):242-253.

Proteomics of aPC

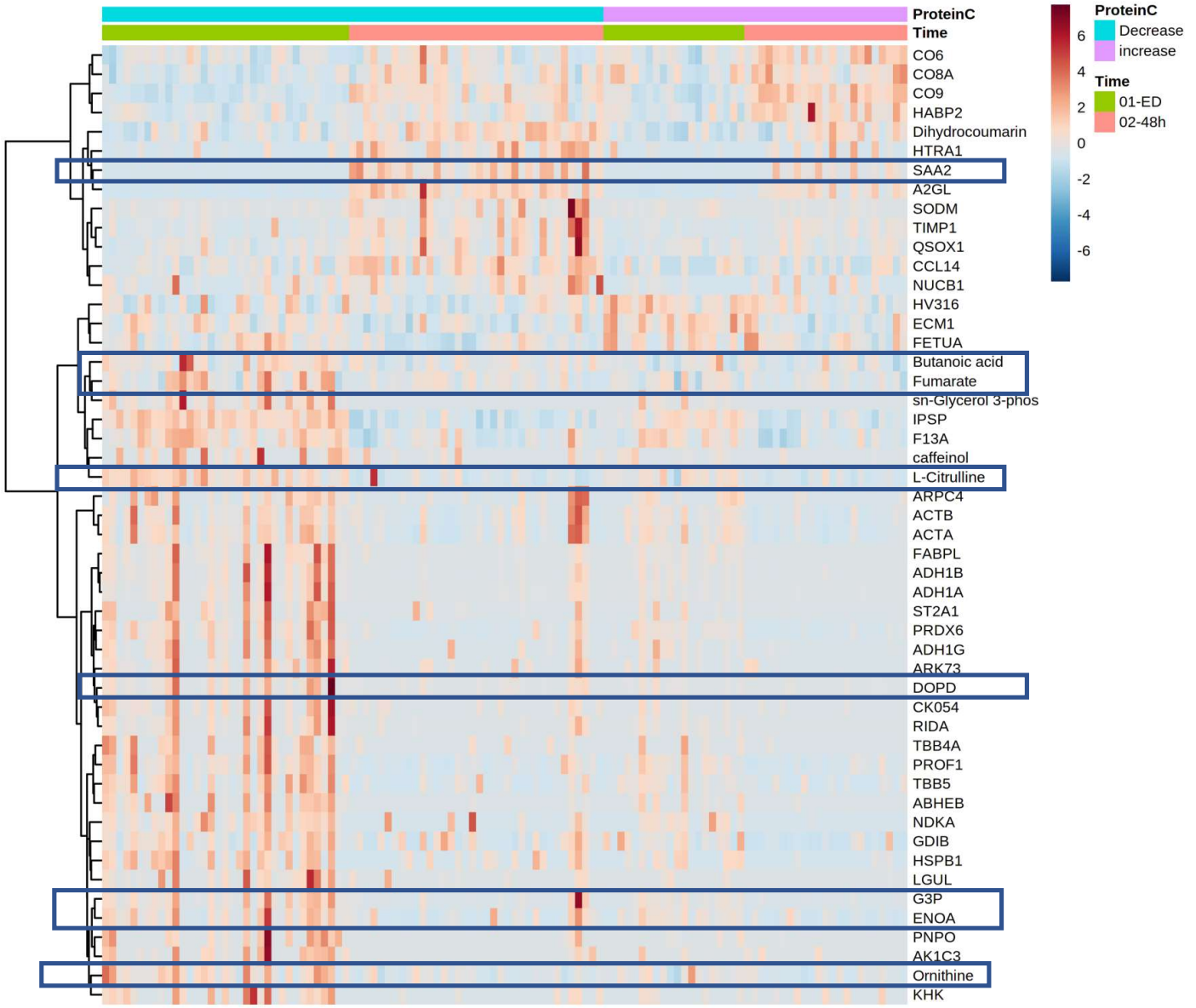
- Histones
- Superoxide dismutase
- Lactate dehydrogenase
- Immunoglobulins
- Complement
- S100
- Chemokines



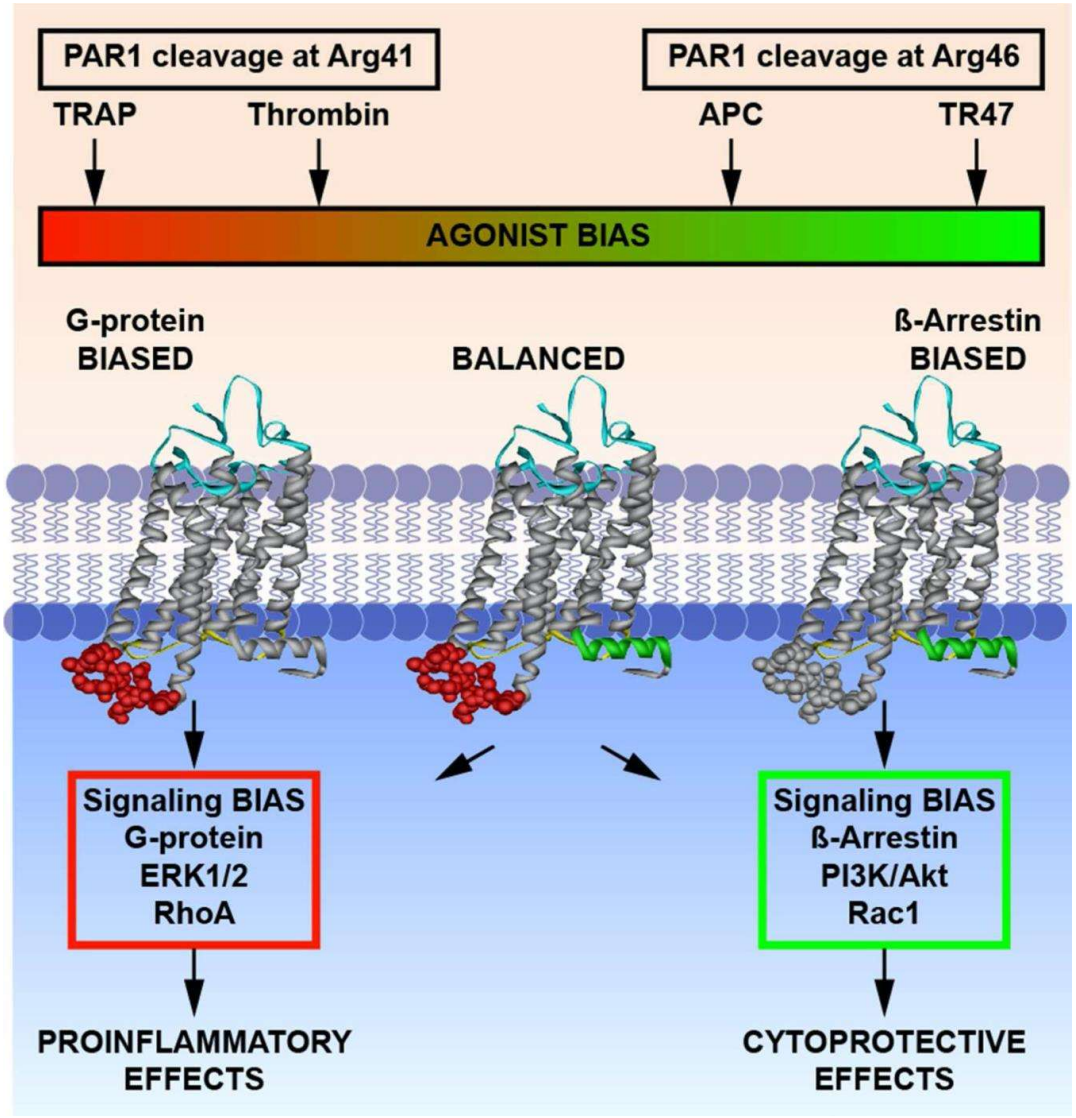
Proteomics

Protein C Depletion associated with:

- nitric oxide metabolism and endothelial dysfunction
  - Citrulline
  - ornithine
- catecholamine metabolism
  - DOPD
- mitochondrial dysfunction
  - Fumarate
  - butanoate
- glycolytic enzymes from hemolysis
  - G3P
  - ENOA



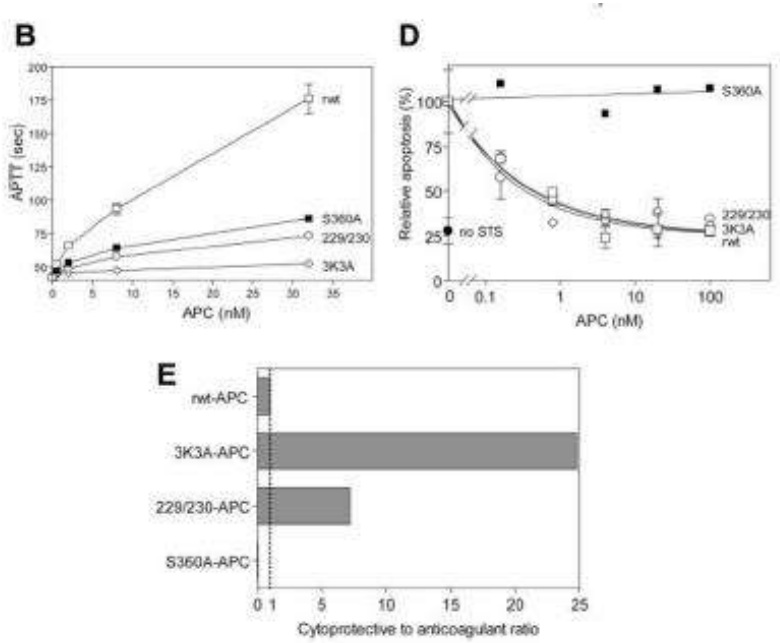
Protein C Brain Injury, Stroke and Neuroprotection



Mosnier et al.

aPC mutants

- 4-20% of anticoagulant activity
- Preserved cytoprotective activity
- 3K3A-APC in phase 2 stroke trials



Mosnier LO, Gale AJ, Yegneswaran S, Griffin JH. Activated protein C variants with normal cytoprotective but reduced anticoagulant activity. Blood. 2004 Sep 15;104(6):1740-4. doi: 10.1182/blood-2004-01-0110. Epub 2004 Jun 3. PMID: 15178575.

JEM Journal of Experimental Medicine

ARTICLE

Protection of ischemic white matter and oligodendrocytes in mice by 3K3A-activated protein C

Mikko T. Huuskonen[1,2*], Yaoming Wang[1,2*], Angeliki Maria Nikolakopoulou[1,2*], Axel Montagne[1,2*], Zhonghua Dai[1,2], Divna Lazic[1,2], Abhay P. Sagare[1,2], Zhen Zhao[1,2], Jose A. Fernandez[3], John H. Griffin[3,4], and Berislav V. Zlokovic[2]

Subcortical white matter (WM) stroke accounts for 25% of all strokes and is the second leading cause of dementia. Despite such clinical importance, we still do not have an effective treatment for ischemic WM stroke, and the mechanisms of WM postischemic neuroprotection remain elusive. 3K3A-activated protein C (APC) is a signaling-selective analogue of endogenous blood protease APC that is currently in development as a neuroprotectant for ischemic stroke patients. Here, we show that 3K3A-APC protects WM tracts and oligodendrocytes from ischemic injury in the corpus callosum in middle-aged mice by activating protease-activated receptor 1 (PAR1) and PAR3. We show that PAR1 and PAR3 were also required for 3K3A-APC's suppression of post–WM stroke microglia and astrocyte responses and overall improvement in neuropathologic and functional outcomes. Our data provide new insights into the neuroprotective APC pathway in the WM and illustrate 3K3A-APC's potential for treating WM stroke in humans, possibly including multiple WM strokes that result in vascular dementia.

blood Review Series

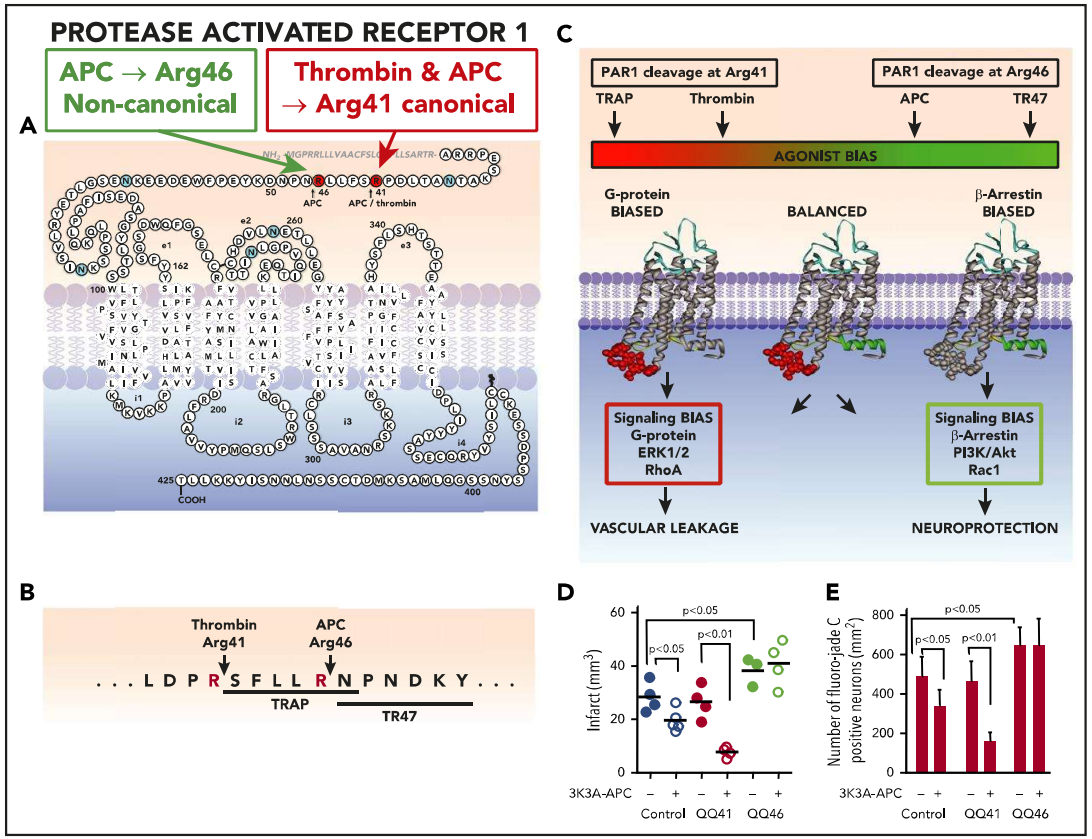
NONTRADITIONAL ROLES FOR THE HEMOSTATIC SYSTEM IN THE VESSEL WALL

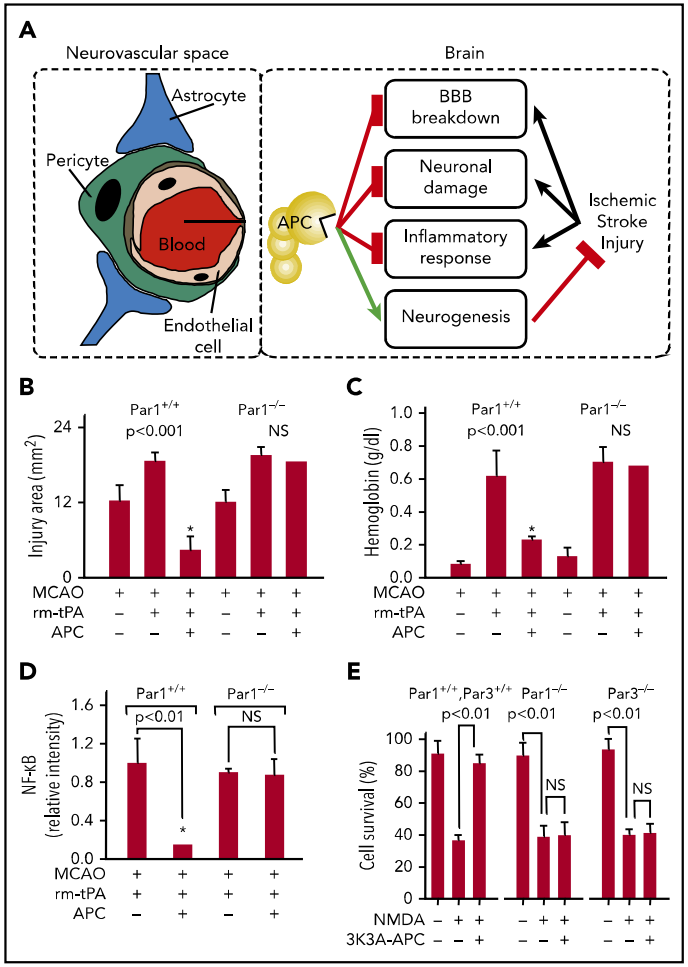
Activated protein C, protease activated receptor 1, and neuroprotection

John H. Griffin,[1,2] Berislav V. Zlokovic,[3] and Laurent O. Mosnier[1]

[1]The Scripps Research Institute, La Jolla, CA; [2]Department of Medicine, University of California, San Diego, CA; and [3]Zilkha Neurogenetic Institute, University of Southern California, Keck School of Medicine, Los Angeles, CA

Protein C is a plasma serine protease zymogen whose active form, activated protein C (APC), exerts potent anticoagulant activity. In addition to its antithrombotic role as a plasma protease, pharmacologic APC is a pleiotropic protease that activates diverse homeostatic cell signaling pathways via multiple receptors on many cells. Engineering of APC by site-directed mutagenesis provided a signaling selective APC mutant with 3 Lys residues replaced by 3 Ala residues, 3K3A-APC, that lacks >90% anticoagulant activity but retains normal cell signaling activities. This 3K3A-APC mutant exerts multiple potent neuroprotective activities, which require the G-protein–coupled receptor, protease activated receptor 1. Potent neuroprotection in murine ischemic stroke models is linked to 3K3A-APC–induced signaling that arises due to APC's cleavage in protease activated receptor 1 at a noncanonical Arg46 site. This cleavage causes biased signaling that provides a major explanation for APC's in vivo mechanism of action for neuroprotective activities. 3K3A-APC appeared to be safe in ischemic stroke patients and reduced bleeding in the brain after tissue plasminogen activator therapy in a recent phase 2 clinical trial. Hence, it merits further clinical testing for its efficacy in ischemic stroke patients. Recent studies using human fetal neural stem and progenitor cells show that 3K3A-APC promotes neurogenesis in vitro as well as in vivo in the murine middle cerebral artery occlusion stroke model. These recent advances should encourage translational research centered on signaling selective APC's for both single-agent therapies and multiagent combination therapies for ischemic stroke and other neuropathologies. (Blood. 2018;132(2):159-169)


PROTEASE ACTIVATED RECEPTOR 1
APC → Arg46
Non-canonical
Thrombin & APC
→ Arg41 canonical
A
APC
APC / thrombin
COOH
B
Thrombin
Arg41
APC
Arg46
. . . L D P R S F L L R N P N D K Y . . .
TRAP
TR47
C
PAR1 cleavage at Arg41
PAR1 cleavage at Arg46
TRAP
Thrombin
APC
TR47
AGONIST BIAS
G-protein
BIASED
BALANCED
β-Arrestin
BIASED
Signaling BIAS
G-protein
ERK1/2
RhoA
Signaling BIAS
β-Arrestin
PI3K/Akt
Rac1
VASCULAR LEAKAGE
NEUROPROTECTION
D
Infarct (mm3)
p<0.05
p<0.05
p<0.01
3K3A-APC
Control
QQ41
QQ46
E
Number of fluoro-jade C positive neurons (mm2)
p<0.05
p<0.05
p<0.01
3K3A-APC
Control
QQ41
QQ46


A
Neurovascular space
Brain
Astrocyte
Pericyte
Blood
Endothelial cell
APC
BBB breakdown
Neuronal damage
Inflammatory response
Neurogenesis
Ischemic Stroke Injury
B
Injury area (mm2)
Par1+/+
p<0.001
Par1−/−
NS
MCAO
rm-tPA
APC
C
Hemoglobin (g/dl)
Par1+/+
p<0.001
Par1−/−
NS
MCAO
rm-tPA
APC
D
NF-κB (relative intensity)
Par1+/+
p<0.01
Par1−/−
NS
MCAO
rm-tPA
APC
E
Cell survival (%)
Par1+/+,Par3+/+
p<0.01
Par1−/−
p<0.01
Par3−/−
p<0.01
NS
NS
NMDA
3K3A-APC

BRAIN RESEARCH 1507 (2013) 97–104

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www.elsevier.com/locate/brainres

Research Report

Activated protein C analog promotes neurogenesis and improves neurological outcome after focal ischemic stroke in mice via protease activated receptor 1

Yaoming Wang[a,b], *Zhen Zhao*[a], *Nienwen Chow*[c], *Tracy Ali*[c], *John H. Griffin*[d], *Berislav V. Zlokovic*[a,*]

[a]*Center for Neurodegeneration and Regeneration, Zilkha Neurogenetic Institute and Department of Physiology and Biophysics, University of Southern California, Keck School of Medicine, Los Angeles, CA 90089, USA*
[b]*Center for Neurodegenerative and Vascular Brain Disorders and Department of Neurosurgery, University of Rochester Medical Center, Rochester, NY 14642, USA*
[c]*ZZ Biotech L.L.C., Rochester, NY 14642, USA*
[d]*Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA*

ARTICLE INFO

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3K3A-APC
Stroke
Neurogenesis
Neuroprotection
Protease activated receptor 1

ABSTRACT

3K3A-APC is a recombinant analog of activated protein C (APC) which is an endogenous protease with multiple functions in the body. Compared to APC, 3K3A-APC has reduced anticoagulant activity but preserved cell signaling activities. In the brain, 3K3A-APC exerts neuroprotective effects after an acute or chronic injury. 3K3A-APC is currently under clinical assessment as a neuroprotective agent following acute ischemic stroke. Whether 3K3A-APC can influence post-ischemic neurogenesis and improve neurological outcome by promoting brain repair remains unknown. Here we show that murine 3K3A-APC 0.8 mg/kg intraperitoneally given at 12 h, 1, 3, 5 and 7 days after permanent distal middle cerebral artery occlusion (dMCAO) in mice compared to vehicle improves significantly sensorimotor and locomotor activity 7 and 14 days after stroke, reduces infarct and edema volumes 7 days after stroke by 43% ($P<0.05$) and 50% ($P<0.05$), respectively, increases the number of newly formed neuroblasts in the subventricular zone, corpus callosum and the peri-infarct area 7 days after stroke by 2.2-fold, 2.3-fold and 2.2-fold ($P<0.05$), respectively, and increases the cortical width index 14 days after stroke by 28% ($P<0.05$). Functional outcome in 3K3A-APC-treated group, but not in vehicle-treated group, correlated inversely with the reductions in the infarct volume, and positively with the number of neuroblasts migrating in the peri-infarct area and the cortical width index. The effects of 3K3A-APC on neuroprotection, neurogenesis and brain repair were lost in protease activated receptor 1 (PAR1) deficient mice. Thus, late therapy with 3K3A-APC is neuroprotective and promotes stroke-induced neurogenesis and repair through PAR1 in mice.

JEM Journal of Experimental Medicine

ARTICLE

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Mikko T. Huuskonen[1,2*], Yaoming Wang[1,2*], Angeliki Maria Nikolakopoulou[1,2*], Axel Montagne[1,2*], Zhonghua Dai[1,2], Divna Lazic[1,2], Abhay P. Sagare[1,2], Zhen Zhao[1,2], Jose A. Fernandez[3], John H. Griffin[3,4], and Berislav V. Zlokovic[2]

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aPC reduces brain damage following TBI

- In mouse controlled cortical impact, 3K3A-APC was neuroprotective
- Decreased lesion volume, increased new blood vessel formation, and promoted neuroblast proliferation
- Improved motor function early, however control mice returned to baseline by day 6

Minhas N, Xue M, Fukudome K, Jackson CJ. Activated protein C utilizes the angiopoietin/Tie2 axis to promote endothelial barrier function. FASEB J. 2010 Mar;24(3):873-81. doi: 10.1096/fj.09-134445. Epub 2009 Oct 26. PMID: 19858095.

The Journal of TRAUMA® Injury, Infection, and Critical Care

Early Coagulopathy After Traumatic Brain Injury: The Role of Hypoperfusion and the Protein C Pathway

Mitchell Jay Cohen, MD, Karim Brohi, FRCS, FRCA, Michael T. Ganter, MD, Geoffrey T. Manley, MD, PhD, Robert C. Mackersie, MD, and Jean-François Pittet, MD

Introduction: Early coagulopathy after traumatic brain injury (TBI) is thought to be the result of injury-mediated local release of tissue factor, although the precise mechanisms that cause hypoperfusion and early systemic coagulopathy in TBI patients are unknown. We have previously reported that early systemic coagulopathy after trauma is present only when tissue injury is associated with severe hypoperfusion leading to the activation of the protein C pathway. However, the role of hypoperfusion as an important mechanism for the development of coagulopathy early after TBI is unclear. The objective of the present study was to determine the importance of hypoperfusion and protein C activation in causing early coagulopathy in TBI patients.

Materials: We performed a prospective cohort study including patients with isolated brain injury admitted to a single trauma center. Blood was drawn on average 32 minutes after injury. Plasma samples were assayed for protein C and thrombomodulin by standard laboratory techniques. Routine coagulation measures (prothrombin time, partial thromboplastin time) and arterial blood gas analysis were performed concurrently. Severe hypoperfusion was evidenced by the presence of an arterial base deficit greater than 6.

Results: Thirty-nine TBI patients were included in the study during a 15-month period. TBI patients without concurrent hypoperfusion (n = 28) did not develop an early coagulopathy after trauma, no matter the severity of injury. In contrast, patients with TBI who also had severe hypoperfusion (BD >6) (n = 11) were coagulopathic early after injury. Indeed, these patients had higher prothrombin time and partial thromboplastin time, compared with those with TBI and a BD <6 (17.6 ± 3.6 vs. 14.3 ± 2.3, $p < 0.005$; and 43.13 ± 18.3 vs. 27.4 ± 3.8, $p < 0.0001$). Unactivated protein C levels were lower in the TBI group with BD >6 (56 ± 32 vs. 85 ± 35, $p = 0.03$) and thrombomodulin levels were significantly higher (48 ± 26 vs. 35 ± 10, $p = 0.04$). Without hypoperfusion, there was no effect of increasing brain injury on protein C pathway or fibrinolysis pathway mediators.

Conclusions: TBI alone does not cause early coagulopathy, but must be coupled with hypoperfusion to lead to coagulation derangements, associated with the activation of the protein C pathway. This novel finding has significant implications for the treatment of coagulopathy after severe brain injury.

Key Words: Bleeding, Coagulopathy, Protein C, Brain injury.

J Trauma. 2007;63:1254–1262.

PLOS ONE

RESEARCH ARTICLE

Astrocytes and pericytes attenuate severely injured patient plasma mediated expression of tight junction proteins in endothelial cells

Preston Stafford[☯], **Sanchayita Mitra**[☯], **Margot Debot, Patrick Lutz, Arthur Stem, Jamie Hadley, Patrick Hom, Terry R. Schaid, Mitchell J. Cohen***

Division of GITES, Department of Surgery, University of Colorado Anschutz Medical Campus, Aurora, Colorado

☯ These authors contributed equally to this work.
* Mitchell.cohen@cuanschutz.edu

OPEN ACCESS

Citation: Stafford P, Mitra S, Debot M, Lutz P, Stem A, Hadley J, et al. (2022) Astrocytes and pericytes attenuate severely injured patient plasma mediated expression of tight junction proteins in endothelial cells. PLoS ONE 17(7): e0270817. https://doi.org/10.1371/journal.pone.0270817

Editor: Mária A. Deli, Eötvös Loránd Research Network Biological Research Centre, HUNGARY

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Published: July 5, 2022

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: MC Trans-agency Research Consortium for Trauma Induced Coagulopathy (TACTIC UM1-HL120877) from the National Heart, Lung and

Abstract

Blood Brain Barrier (BBB) breakdown is a secondary form of brain injury which has yet to be fully elucidated mechanistically. Existing research suggests that breakdown of tight junction proteins between endothelial cells is a primary driver of increased BBB permeability following injury, and intercellular signaling between primary cells of the neurovascular unit: endothelial cells, astrocytes, and pericytes; contribute to tight junction restoration. To expound upon this body of research, we analyzed the effects of severely injured patient plasma on each of the cell types in monoculture and together in a triculture model for the transcriptional and translational expression of the tight junction proteins Claudins 3 and 5, (CLDN3, CLDN5) and Zona Occludens 1 (ZO-1). Conditioned media transfer studies were performed to illuminate the cell type responsible for differential tight junction expression. Our data show that incubation with 5% human *ex vivo* severely injured patient plasma is sufficient to produce a differential response in endothelial cell tight junction mRNA and protein expression. Endothelial cells in monoculture produced a significant increase of CLDN3 and CLDN5 mRNA expression, (3.98 and 3.51 fold increase vs. control respectively, $p<0.01$) and CLDN5 protein expression, (2.58 fold change vs. control, $p<0.01$), whereas in triculture, this increase was attenuated. Our triculture model and conditioned media experiments suggest that conditioned media from astrocytes and pericytes and a triculture of astrocytes, pericytes and endothelial cells are sufficient in attenuating the transcriptional increases of tight junction proteins CLDN3 and CLDN5 observed in endothelial monocultures following incubation with severely injured trauma plasma. This data suggests that inhibitory molecular signals from astrocytes and pericytes contributes to prolonged BBB breakdown following injury via tight junction transcriptional and translational downregulation of CLDN5.

Blood brain barrier model of cocultured Brain Endothelial cells, Pericytes and Astrocytes.

Ex vivo trauma plasma (both TBI and non TBI results in BBB breakdown and barrier function

Mediated by crosstalk between astrocytes and pericytes with endothelial cells on junctional proteins.

Now inhibited by aPC.

COVID: Infection, Sepsis and the next Pandemic

APC and sepsis

- Recombinant aPC
- Initially showed 6% decrease in mortality in severe sepsis in PROWESS trial
  - 1.5% increase in severe bleeding
- Dosed as low-dose 96h infusion

Efficacy and Safety of Recombinant Human activated protein c for severe sepsis. Bernard, Vincent, Laterre et. Al. Mar 2001. N Engl J Med 2001; 344:699-709

aPC cleaves extracellular histones

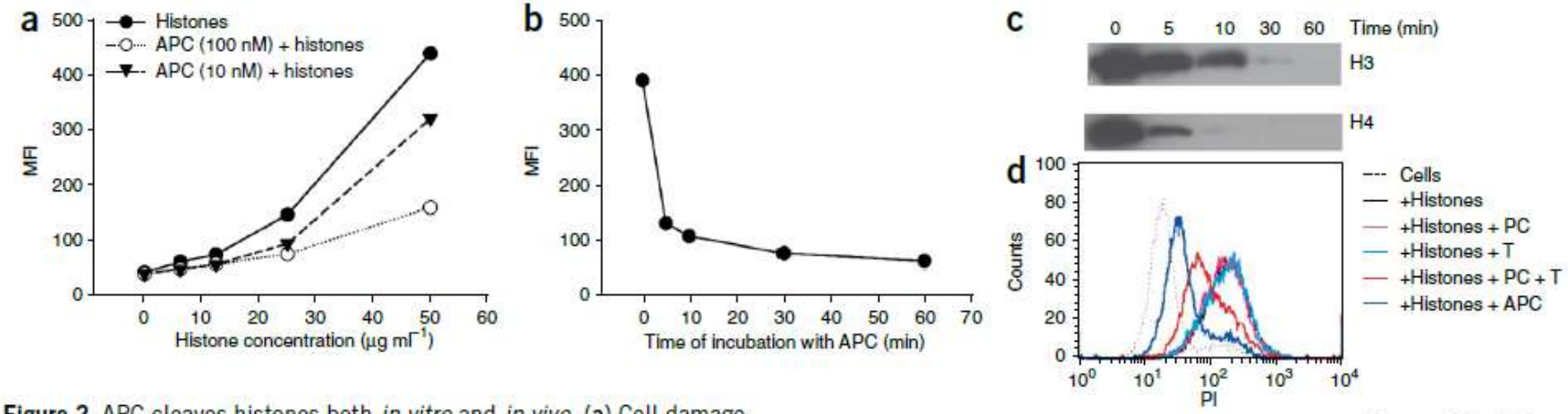
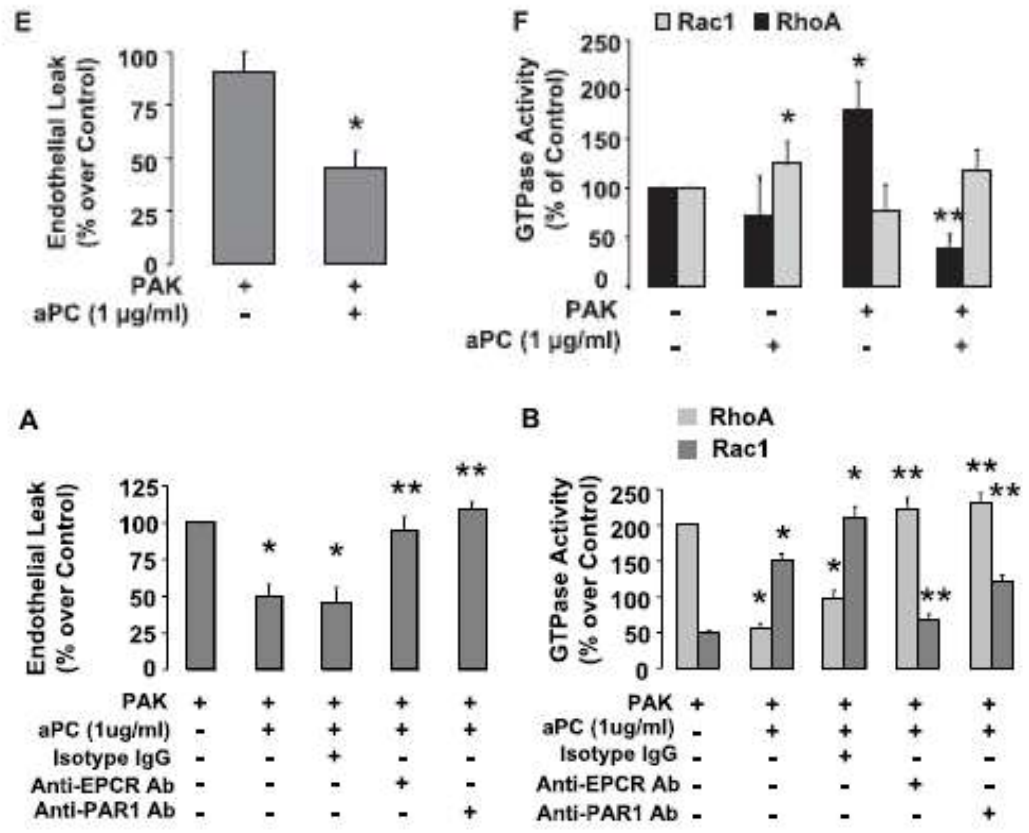


Figure 2 APC cleaves histones both *in vitro* and *in vivo*. (**a**) Cell damage, as measured by flow cytometry for PI staining and expressed as mean fluorescence index (MFI), in EA.hy926 cells cultured with calf thymus histones in the indicated concentrations in the absence or presence of APC (10 or 100 nM) at 37 °C for 1 h. (**b**,**c**) Cytotoxicity assay (**b**) or western blot analysis for H3 or H4 at the times indicated (**c**) in EA.hy926 cells for 1 h with calf thymus histones (50 μg ml^{-1}) incubated with APC (100 nM) at 37 °C for the indicated time and then mixed with PPACK (10 μM) to inactivate APC. (**d**) Cell damage, as measured by flow cytometry for PI staining, in EA.hy926 cells cultured with calf thymus histones (50 μg ml^{-1}) in the absence or presence of protein C (100 nM), thrombin (T) (10 nM) or APC (100 nM) at 37 °C for 30 min. (**e**) Western blot analysis for H3 of baboon plasma samples at the times indicated after *E. coli* or *E. coli* plus APC challenge. (**f**) Western blot analysis for H3 of plasma samples taken at the times indicated from the start of APC treatment of a human with sepsis. The flow cytometry results are representative of three or more similar experiments, and western blot results are representative of two or more similar experiments.

Xu J, Zhang X, Pelayo R, et al. Extracellular histones are major mediators of death in sepsis. *Nat Med*. 2009;15(11):1318-1321. doi:10.1038/nm.2053

aPC protects against pseudomanas PNA lung leak



Bir N, Lafargue M, Howard M, Goolaerts A, Roux J, Carles M, Cohen MJ, Iles KE, Fernández JA, Griffin JH, Pittet JF. Cytoprotective-selective activated protein C attenuates Pseudomonas aeruginosa-induced lung injury in mice. Am J Respir Cell Mol Biol. 2011 Sep;45(3):632-41. doi: 10.1165/rcmb.2010-0397OC. Epub 2011 Jan 21.

- Decreased lung leak (in vitro and in vivo)
- Improved mortality, but did not increase neutrophils or MPO in airspace
- Improved Rac/Rho ratio
- Protection and Rac/Rho ratio increases lost with EPCR or PAR-1 neutralizing antibody

COVID-19 hypothesis: Activated protein C for therapy of virus-induced pathologic thromboinflammation

John H. Griffin PhD[1,2] | Patrick Lyden MD[3]

[1]Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California

[2]Department of Medicine, University of California, San Diego, California

[3]Department of Neurology, Cedars-Sinai Medical Center, Los Angeles, California

Correspondence
John H. Griffin, Scripps Research Institute, IMM-316, 10550 N. Torrey Pines Rd, La Jolla, CA 92037.
Email: jgriffin@scripps.eduHandling
Handling Editor: Dr Alisa Wolberg.

Abstract

Seriously ill patients with coronavirus disease 2019 (COVID-19) at risk for death exhibit elevated cytokine and chemokine levels and D-dimer, and they often have comorbidities related to vascular dysfunctions. In preclinical studies, activated protein C (APC) provides negative feedback downregulation of excessive inflammation and thrombin generation, attenuates damage caused by ischemia-reperfusion in many organs including lungs, and reduces death caused by bacterial pneumonia. APC exerts both anticoagulant activities and direct cell-signaling activities. Preclinical studies show that its direct cell-signaling actions mediate anti-inflammatory and anti-apoptotic actions, mortality reduction for pneumonia, and beneficial actions for ischemia-reperfusion injury. The APC mutant 3K3A-APC, which was engineered to have diminished anticoagulant activity while retaining cell-signaling actions, was safe in phase 1 and phase 2 human trials. Because of its broad spectrum of homeostatic effects in preclinical studies, we speculate that 3K3A-APC merits consideration for clinical trial studies in appropriately chosen, seriously ill patients with COVID-19.

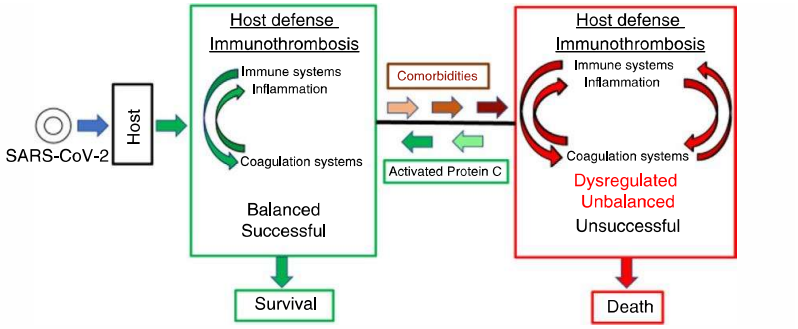
KEYWORDS

activated protein C, coronavirus, COVID-19, cytokine, D-dimer, SARS-CoV-2

Essentials

- Seriously ill patients with coronavirus disease 2019 (COVID-19) exhibit viral pneumonia, cytokine storm, disseminated intravascular coagulation, and comorbidities.
- There are no approved therapies for severely ill patients with COVID-19.
- Activated protein C (APC) reduces inflammation and apoptosis, and it stabilizes endothelial and epithelial barriers.
- The 3K3A-APC mutant merits evaluation for seriously ill, appropriately chosen patients with COVID-19.

Infection with the virus identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can lead to virus-induced pneumonia that is designated coronavirus disease 2019 (COVID-19) by the World Health Organization, and this infection has reached pandemic proportions. The virus is thought to invade a host by attachment to a receptor for the virus spike protein, namely, human angiotensin-converting enzyme 2, which is expressed on epithelial cells throughout the body. However, almost nothing is known about the pathobiology leading to severe illness and death for patients with COVID-19. Peer-reviewed reports from China indicate that seriously ill patients with COVID-19 at risk for death develop extensive elevations in plasma levels of cytokines and chemokines (interleukin [IL]-2, IL-6, IL-7, IL-10, granulocyte colony-stimulating factor, interferon-γ–induced protein 10, monocyte chemoattractant



Medical Hypotheses 149 (2021) 110537

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Old drug, new Trick? The rationale for the treatment of COVID-19 with activated protein C

Steven B. Pestka

Tufts University School of Medicine, Department of Medicine, Newton-Wellesley Hospital, 2014 Washington Street, Newton, MA 02462, USA

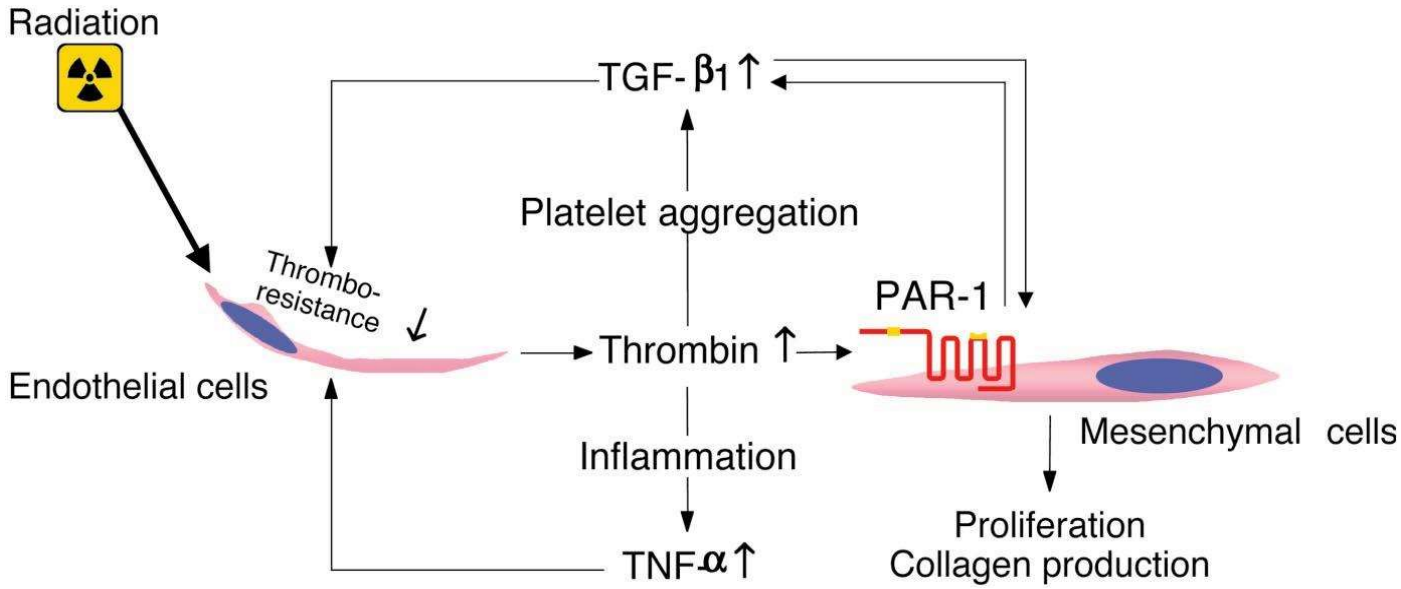
ARTICLE INFO

Keywords:
COVID-19
Activated protein C
COVID-19 therapeutics

ABSTRACT

As the COVID-19 pandemic continues, researchers seek to identify efficacious treatments. Current approaches to COVID-19 therapeutics focus on antiviral agents, convalescent plasma, monoclonal antibodies, immunomodulators and more traditional therapies such as steroids [1-6]. Reversing disturbances in coagulation has also been identified as a priority area for candidate therapies, such as through the Accelerating COVID-19 Therapeutic Interventions and Vaccines 4 adaptive clinical trial (ACTIV-4) which is currently evaluating aspirin, heparins and apixaban [7]. Since there is a clear relationship between mechanisms of coagulation and the immune response, it is possible that reversing disturbances in coagulation may diminish the dysregulated immune response observed in COVID-19. The basis for this hypothesis is described below and is followed by discussion of a proposed candidate therapy - activated protein C. By treating COVID-19 patients using a novel approach, which does not focus on immune-based or antiviral treatments, but instead which addresses both the anti-thrombotic and inflammatory consequences of infection, the hope is that new therapeutic targets can be considered and new candidate therapies, such as activated protein C, may be evaluated.

Radiation Injury

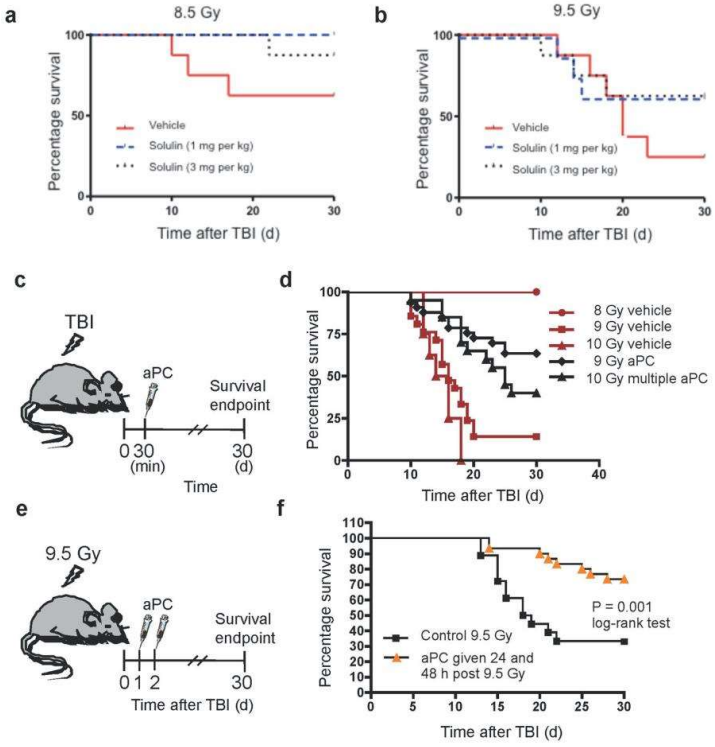


Crit Care Med 2004 Vol. 32, No. 5 (Suppl.)

Nat Med. 2012 July ; 18(7): 1123–1129. doi:10.1038/nm.2813.

Pharmacological targeting of the thrombomodulin–protein C pathway mitigates radiation toxicity

Hartmut Geiger[1,2,*], Snehalata A Pawar[3,*], Edward J. Kerschen[4,*], Kalpana J. Nattamai[1,*], Irene Hernandez[4], Hai Po Liang[4], Jose Á Fernández[5], Jose A. Cancelas[1], Marnie A. Ryan[1], Olga Kustikova[6], Axel Schambach[6], Qiang Fu[3], Junru Wang[3], Louis M. Fink[7], Karl-Uwe



Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Letter to the Editors-in-Chief

Low admission protein C levels are a risk factor for disease worsening and mortality in hospitalized patients with COVID-19

ARTICLE INFO

Keywords
Anticoagulant
COVID-19
Fibrinolysis
Protein C
SARS-CoV-2

At present, the clinical course of coronavirus disease 2019 (COVID-19) is unpredictable and can rapidly develop, causing severe and deadly complications. Therefore, there is an urgent need to identify reliable biomarkers related to COVID-19 disease progression and death for diagnostics as well as to identify pathways that are amenable to existing or new therapeutics. While biomarkers of coagulation (e.g. D-dimer), inflammation (e.g. interleukin-6 [IL-6] and C-reactive protein [CRP]), cell damage (e.g. lactate dehydrogenase [LDH]) and immunity (e.g. lymphocyte count) as well as clinical scoring systems (e.g. International Society on Thrombosis and Hemostasis [ISTH] disseminated intravascular coagulation [DIC] score) [1] can be helpful in predicting clinical course and outcome in patients with COVID-19, there is a need for additional biomarkers.

As COVID-19 progresses, inflammatory responses lead to a coagulopathy associated with a high incidence of thrombotic events, especially in the microvasculature [2]. The pattern of changes in hemostatic variables in COVID-19-associated coagulopathy appears to be different to that in sepsis and DIC, and there are gaps in knowledge as to which hemostatic proteins that may be most informative for the early identification of patients with poor prognosis in COVID-19 [2].

We aimed to characterize admission plasma levels of 12 hemostatic proteins in hospitalized COVID-19 patients in order to identify proteins associated with risk of disease worsening including death within 28 days. The data used here is from a publicly available longitudinal COVID-19 cohort collected at the Massachusetts General Hospital (MGH), Boston, USA (with institutional review board approval; https://www.olink.com/mgh-covid-study/), which has recently been described in detail [3].

This study is based on 231 COVID-19 patients presenting at the emergency department with moderate or severe illness, i.e. requiring oxygen (n = 152) or intensive care (n = 79). The World Health Organization (WHO) COVID-19 outcomes scale was used on day one and again at 28-day follow-up to classify patients as mild (WHO 5–6), moderate (WHO 4), severe (WHO 2–3) or dead (WHO 1). Of the 152 patients presenting with moderate COVID-19, 128 improved, 2 remained unchanged, 5 deteriorated and 17 died; of the 79 patients with severe COVID-19, 23 improved, 34 remained unchanged, 0 deteriorated and 22 died.

Plasma was isolated from blood collected in EDTA tubes on admission. Plasma protein levels were measured using proximity extension assay (PEA) technology with the OLINK Explore 1536 panel (OLINK, Uppsala, Sweden). In total 12 proteins belonging to the Kyoto Encyclopedia of Genes and Genomes (KEGG) coagulation cascade were present on the OLINK Explore panel and analyzed for association with 28-day disease worsening in the present study.

Available clinical and admission laboratory measures previously reported to associate with COVID-19 clinical course and/or outcomes that were included as covariates in the multivariate analyses in the present study were coded as binary variables as indicated in Table 1 (for original definitions, see https://www.olink.com/mgh-covid-study/ and [3]). Given that IL-6 has been robustly associated with poor prognosis in COVID-19 patients, IL-6 levels measured with the OLINK Explore panel were also included as a covariate. Identifying variables such as sex and ethnicity were unavailable. In line with previous studies, patients whose condition deteriorated (including those who died) were more likely to be older, have comorbidities, reduced lymphocyte count, and increased creatinine, CRP, D-dimers, LDH and IL-6 than patients who improved (Table 1).

Univariate ordinal logistic regression analyses for 28-day disease worsening were performed for each of the 12 hemostatic proteins. Patients whose condition deteriorated had elevated admission plasma levels of proteinase-activated receptor 1 (PAR1, $p = 0.004$), tissue factor

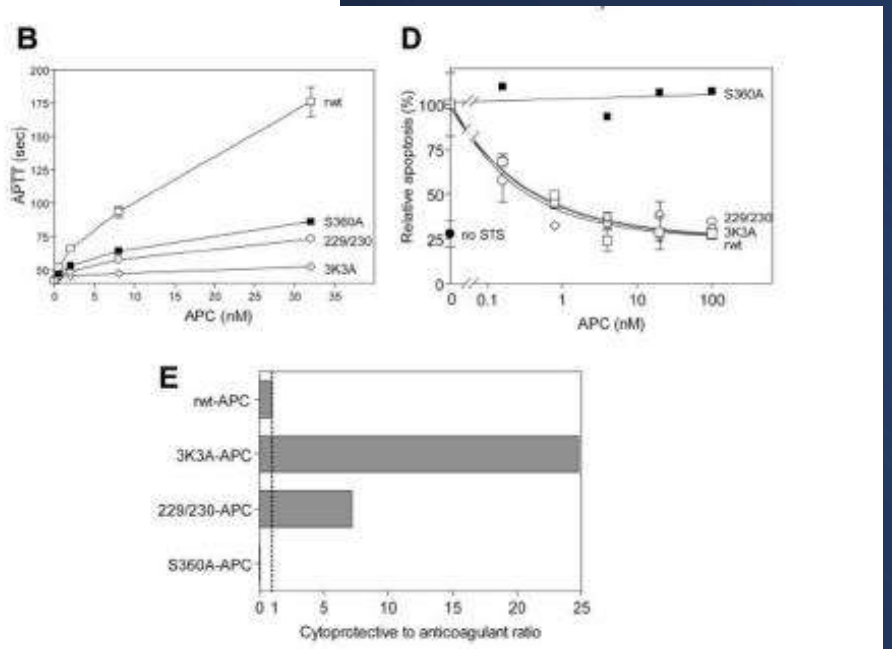
Abbreviations: APC, activated protein C; DIC, disseminated intravascular coagulation; CRP, C-reactive protein; FVII, coagulation factor VII; FIX, coagulation factor IX; IL-6, interleukin-6; LDH, lactate dehydrogenase; PAI-1, plasminogen activator inhibitor type 1; PAR-1, proteinase-activated receptor 1; TF, tissue factor; TFPI, tissue factor pathway inhibitor; t-PA, tissue-type plasminogen activator; TM, thrombomodulin; u-PA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; vWF, von Willebrand factor.

https://doi.org/10.1016/j.thromres.2021.05.016
Received 27 January 2021; Received in revised form 6 May 2021; Accepted 25 May 2021
Available online 29 May 2021
0049-3848/

Protein C Endothelial Permeability and Cytoprotection: Trauma and a Promise for Threat X

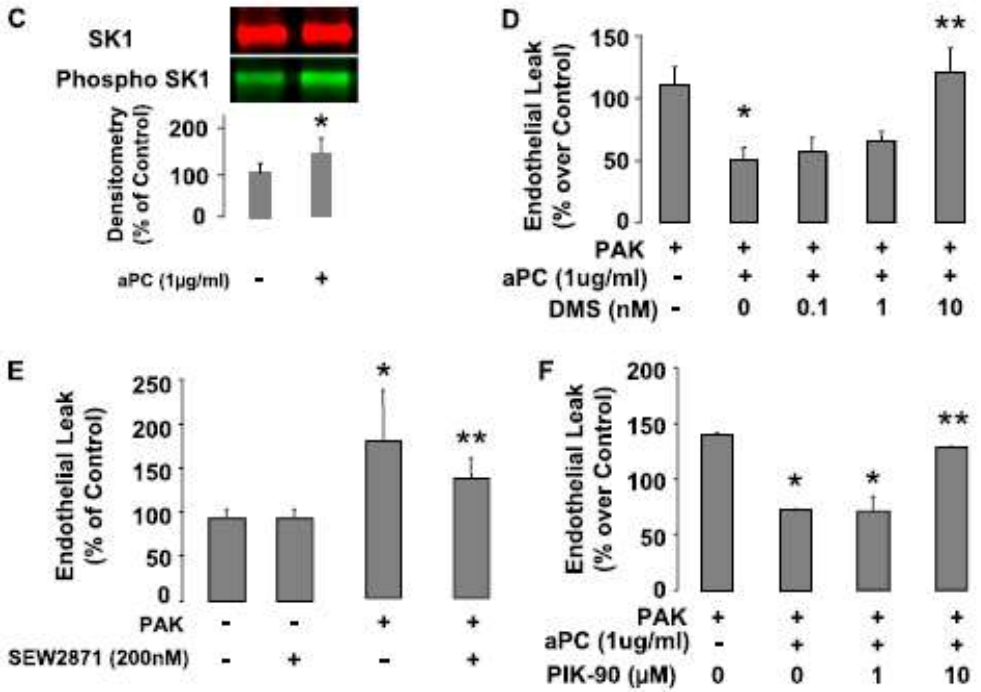
aPC mutants

- 8-20% of anticoagulant activity
- Preserved cytoprotective activity
- 3K3A-APC in phase 2 stroke trials



Mosnier LO, Gale AJ, Yegneswaran S, Griffin JH. Activated protein C variants with normal cytoprotective but reduced anticoagulant activity. Blood. 2004 Sep 15;104(6):1740-4. doi: 10.1182/blood-2004-01-0110. Epub 2004 Jun 3. PMID: 15178575.

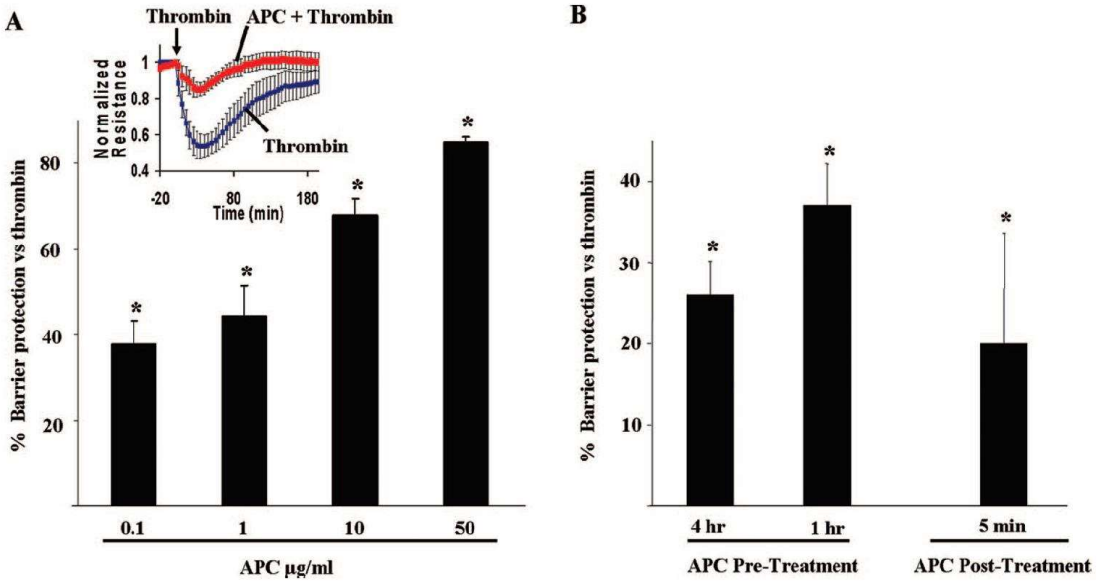
APC increases S1P production

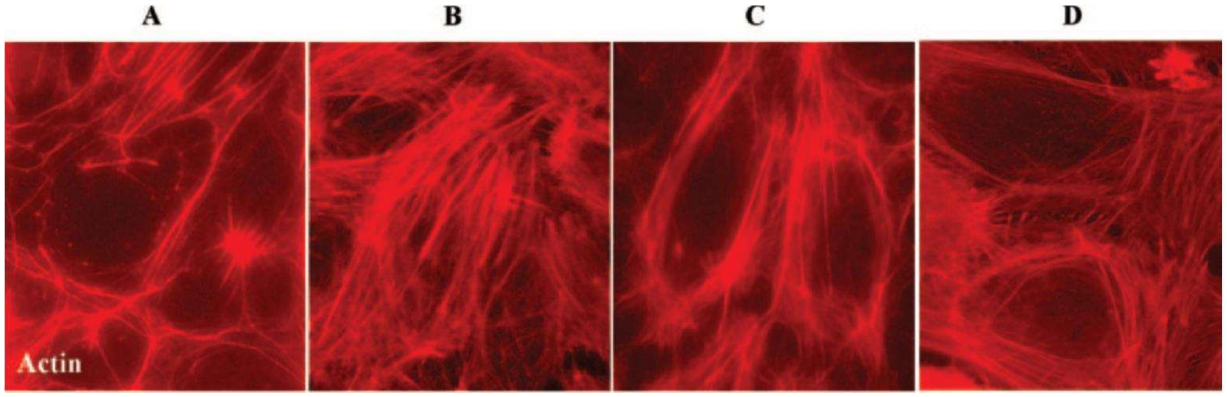


- APC increased SK1 phosphorylation
- Protection against endothelial perm is lost with SK1 inh
- $S1PR_1$ Agonist decreases lung leak
- Protection is lost with PI3K inhibition

Bir N, Lafargue M, Howard M, Goolaerts A, Roux J, Carles M, Cohen MJ, Iles KE, Fernández JA, Griffin JH, Pittet JF. Cytoprotective-selective activated protein C attenuates Pseudomonas aeruginosa-induced lung injury in mice. Am J Respir Cell Mol Biol. 2011 Sep;45(3):632-41. doi: 10.1165/rcmb.2010-0397OC. Epub 2011 Jan 21.

APC protects against thrombin- induced permeability

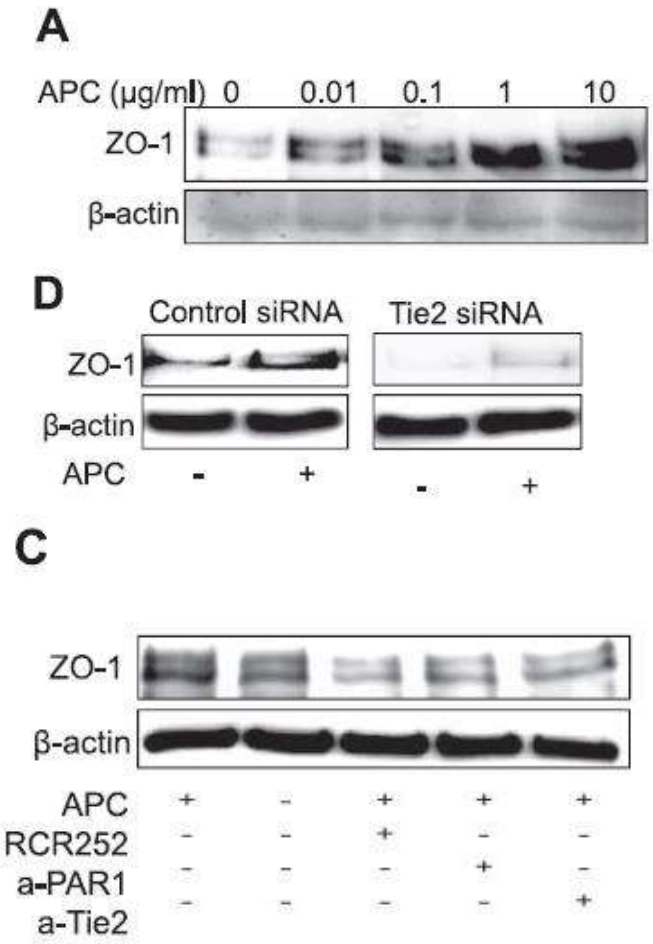




- APC increases Rac1 expression
- Induces MLC phosphorylation
- Attenuates thrombin-induced stress fiber formation

Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. J Biol Chem. 2005 Apr 29;280(17):17286-93. doi: 10.1074/jbc.M412427200. Epub 2005 Feb 14. PMID: 15710622.

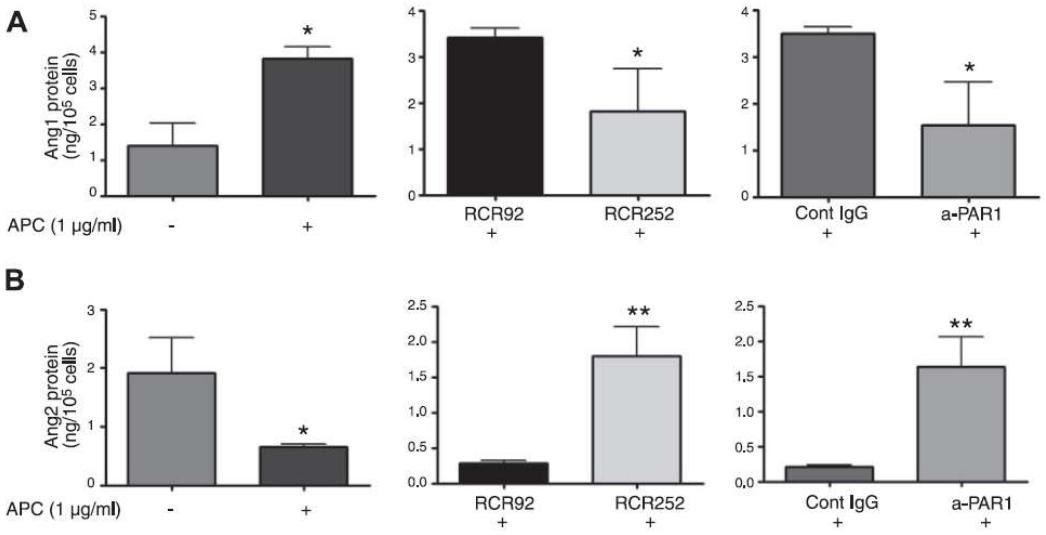
aPC signaling through Tie2 increases ZO-1



- Tie2 is an angiopoietin (Ang) receptor
- Signals through PI3K/Akt to stabilize endothelium and reduce inflammation
- APC increases Tie2 expression and Ang1 expression and translation in endothelial cells
- APC increases ZO-1 expression in a Tie-2 dependent manner

Minhas N, Xue M, Fukudome K, Jackson CJ. Activated protein C utilizes the angiopoietin/Tie2 axis to promote endothelial barrier function. FASEB J. 2010 Mar;24(3):873-81. doi: 10.1096/fj.09-134445. Epub 2009 Oct 26. PMID: 19858095.

APC decreases Ang2

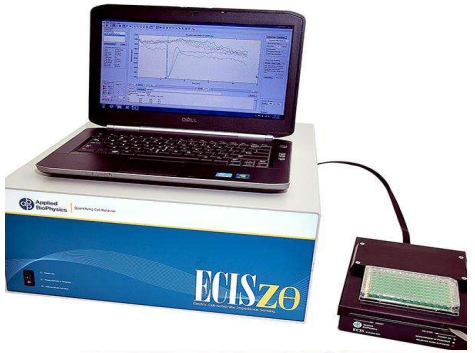


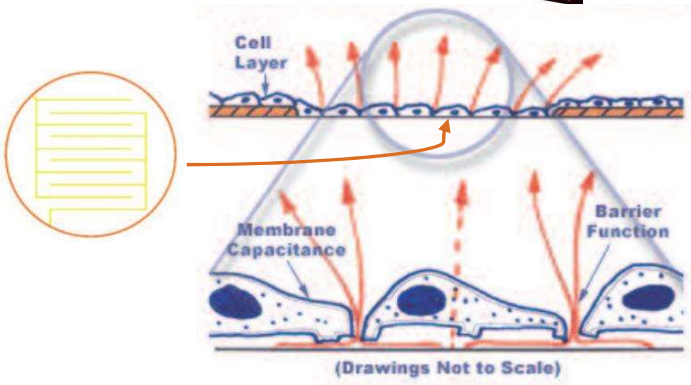
- Ang2 is antagonistic to Ang1 signaling.
- Ang2 is released early after trauma and associated with coagulopathy, endothelial activation, and worse clinical outcomes
- APC increases Ang1 and decreases Ang2, which may increase its benefit shortly after trauma

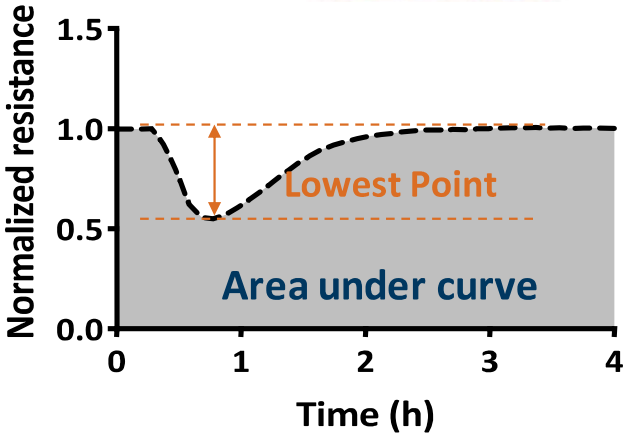
Ganter MT, Cohen MJ, Brohi K, Chesebro BB, Staudenmayer KL, Rahn P, Christiaans SC, Bir ND, Pittet JF. Angiopoietin-2, marker and mediator of endothelial activation with prognostic significance early after trauma? Ann Surg. 2008 Feb;247(2):320-6. doi: 10.1097/SLA.0b013e318162d616. P

ECIS

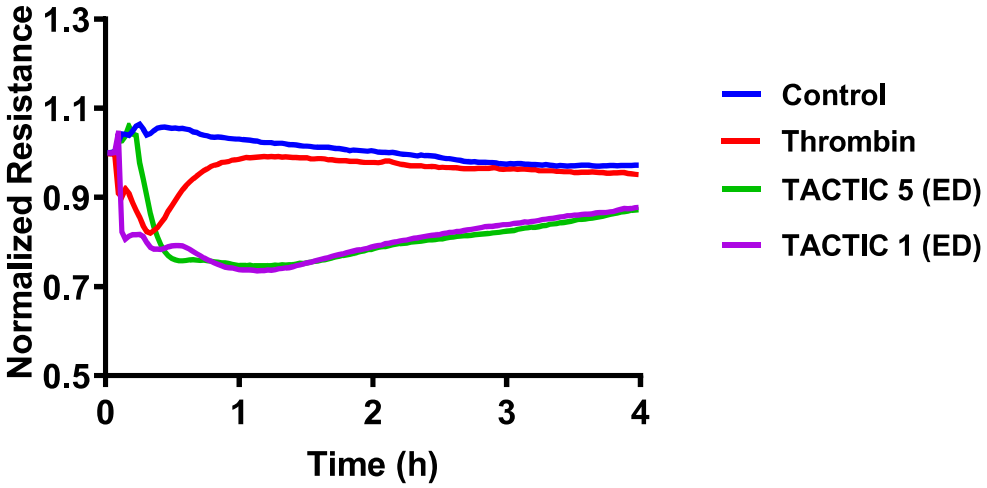
- ECIS uses impedance to measure permeability of cells
- Decreased resistance -> increased permeability
- Normalized resistance: Lowest Point and area under the curve (AUC)





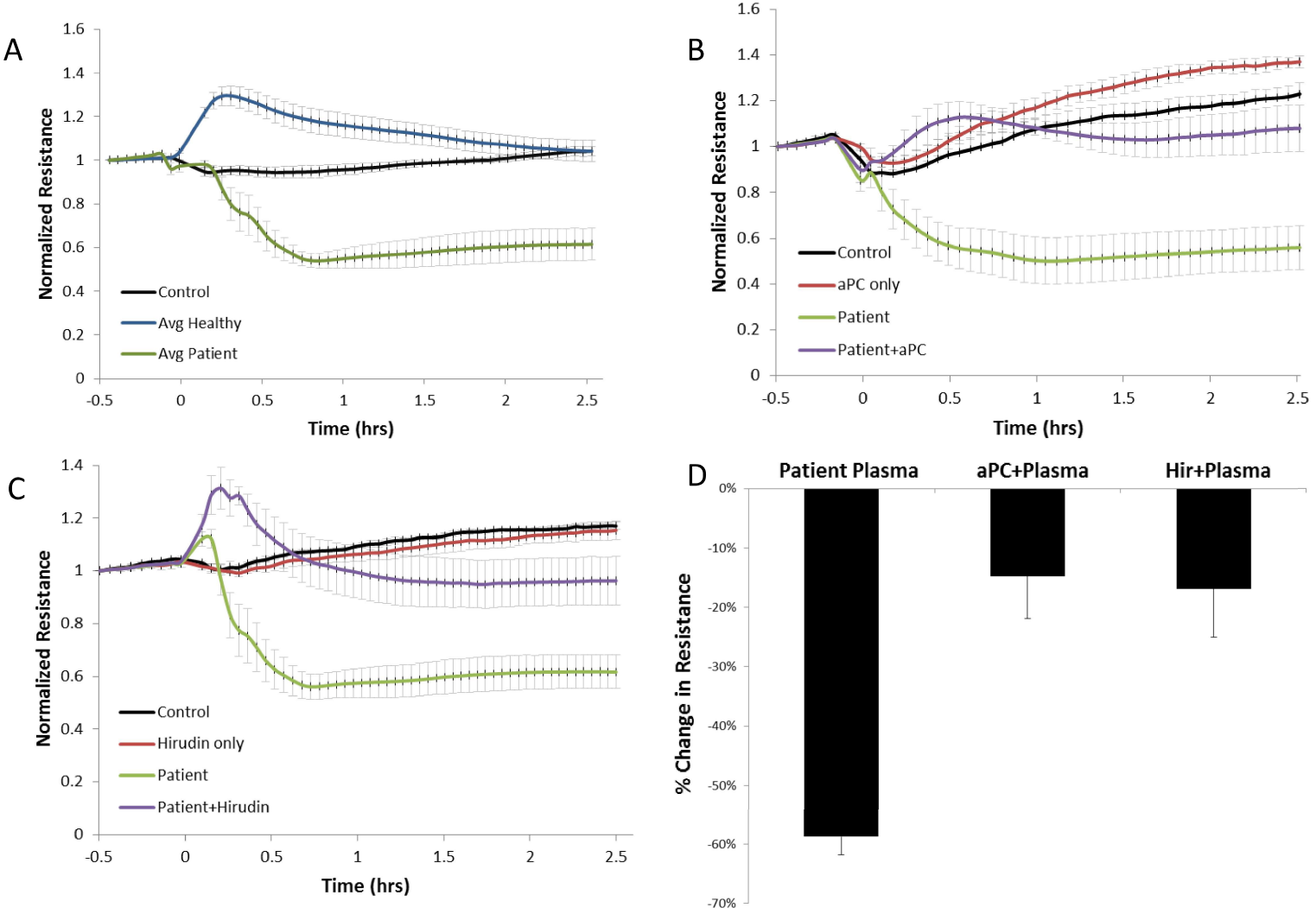


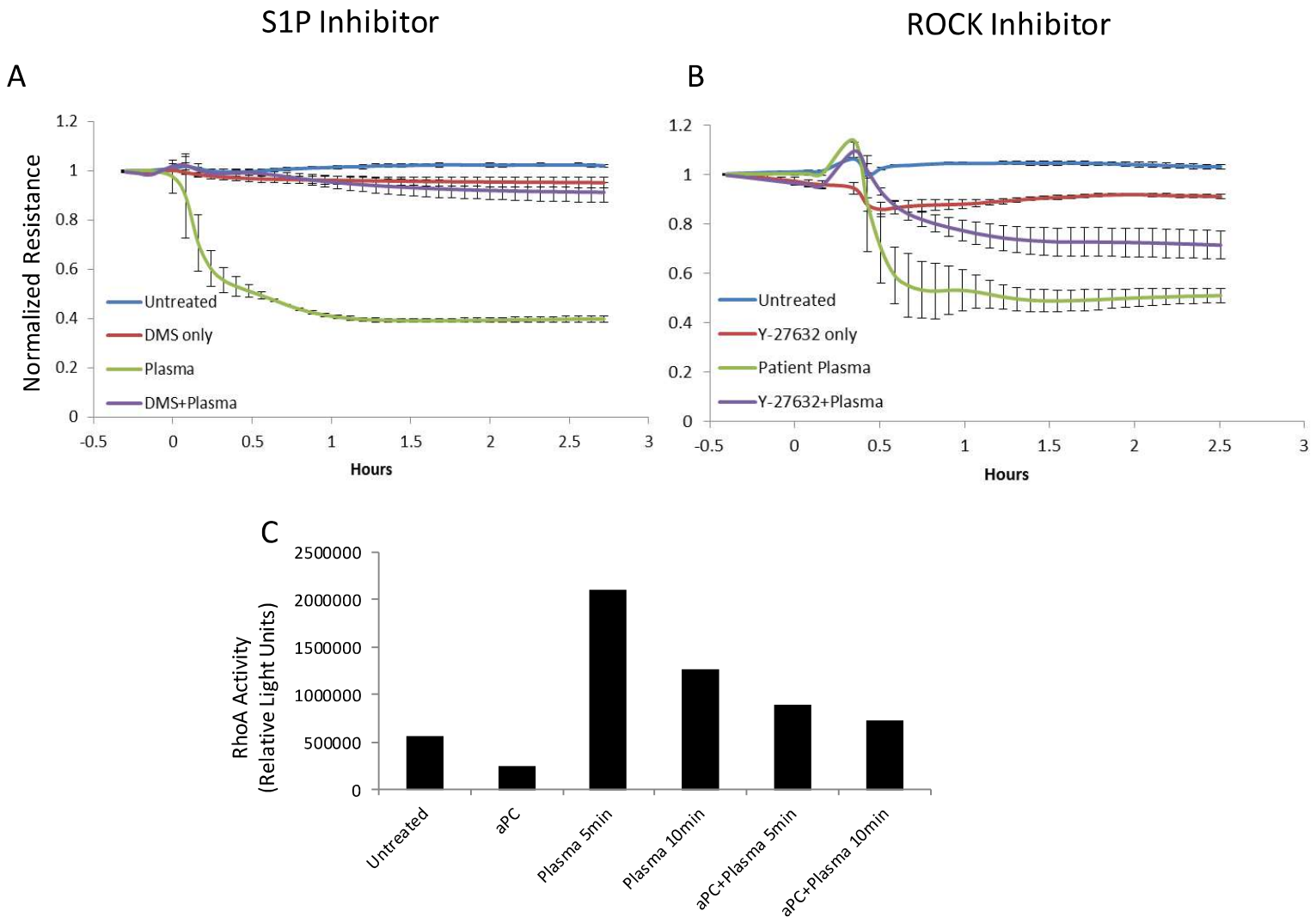
Ex Vivo Trauma Plasma Induces Endothelial Permeability

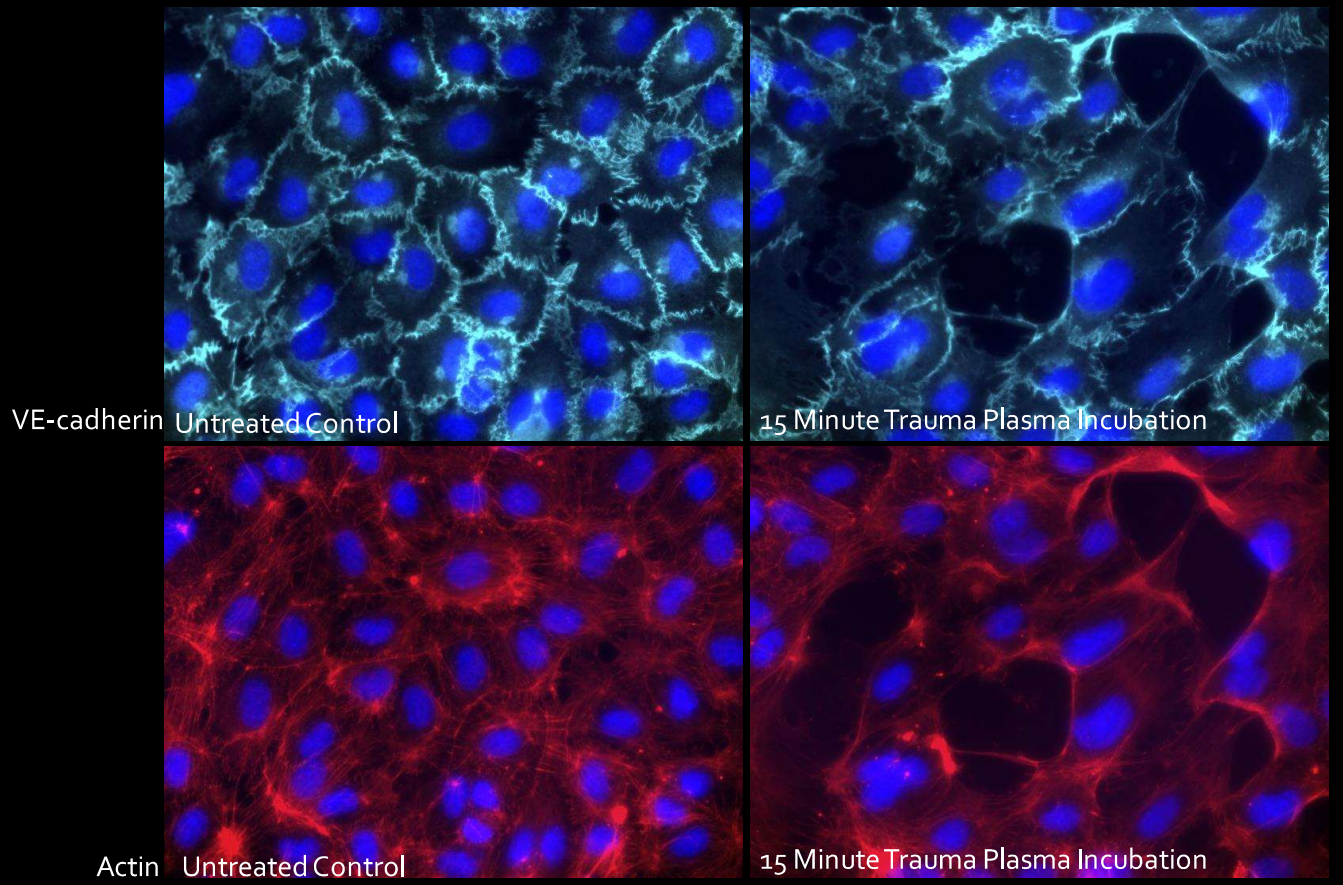


- Cells are bathed in trauma plasma from severely injured patients and resistance is measured continuously to obtain permeability tracings using ECIS
- Ex vivo trauma plasma from severely injured patients (ISS>15, BE<-6) induces endothelial permeability

Fig. 1 Patient Plasma Induces Barrier Dysfunction.

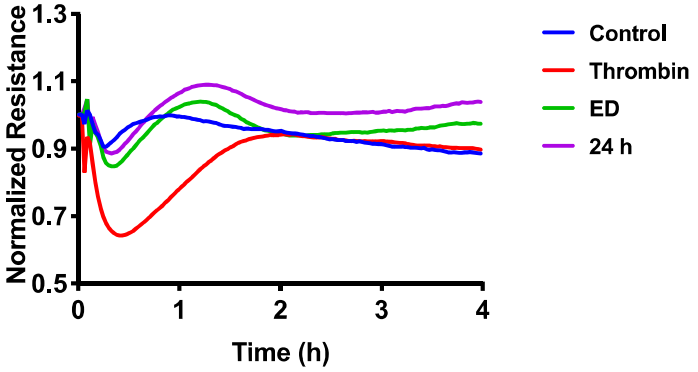



S1P Inhibitor
ROCK Inhibitor
A
B
C
Normalized Resistance
Hours
Untreated
DMS only
Plasma
DMS+Plasma
Y-27632 only
Patient Plasma
Y-27632+Plasma
RhoA Activity
(Relative Light Units)
Untreated
aPC
Plasma 5min
Plasma 10min
aPC+Plasma 5min
aPC+Plasma 10min

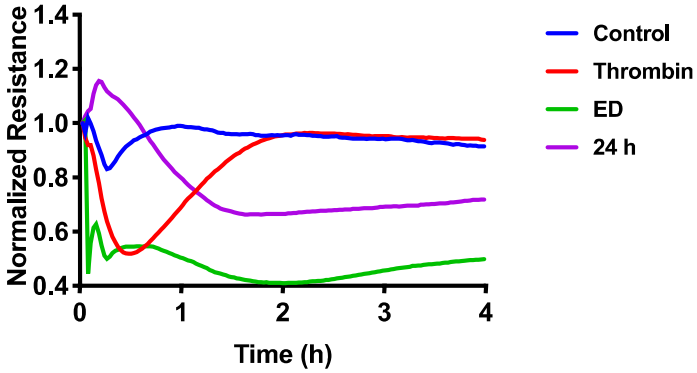

VE-cadherin
Untreated Control
15 Minute Trauma Plasma Incubation
Actin
Untreated Control
15 Minute Trauma Plasma Incubation

Ex vivo Trauma Permeability Phenotypes

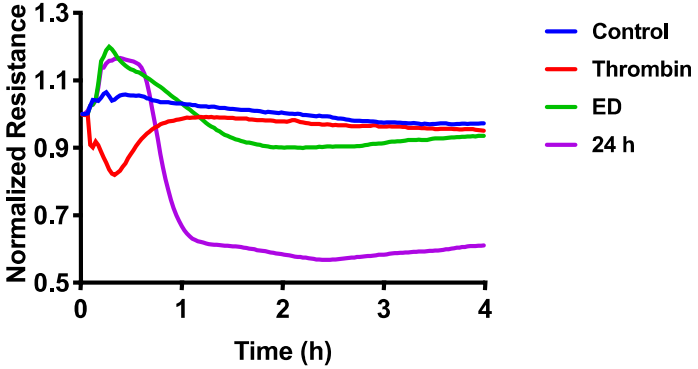
No Injury, No Shock

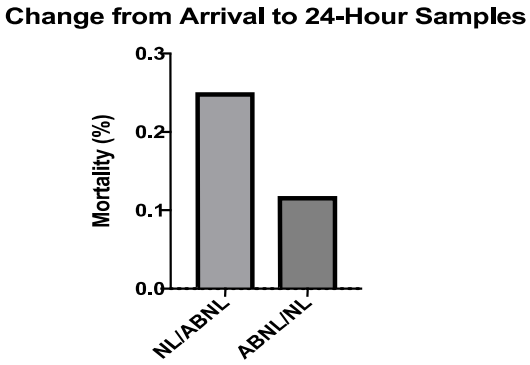


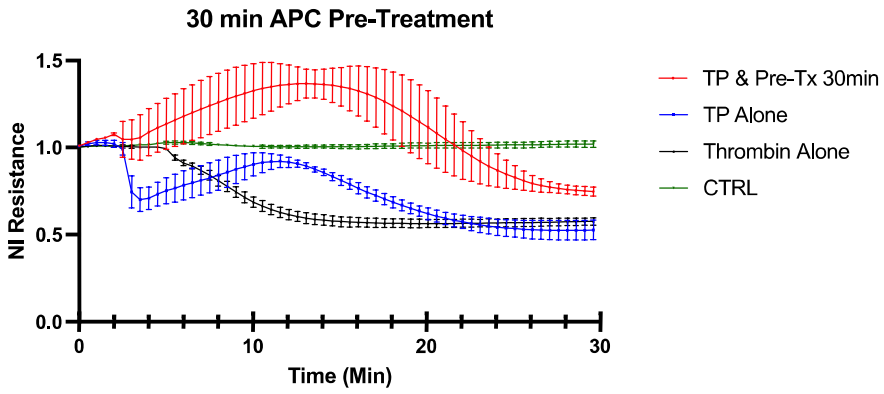
ISS 22, Shock, Resuscitated

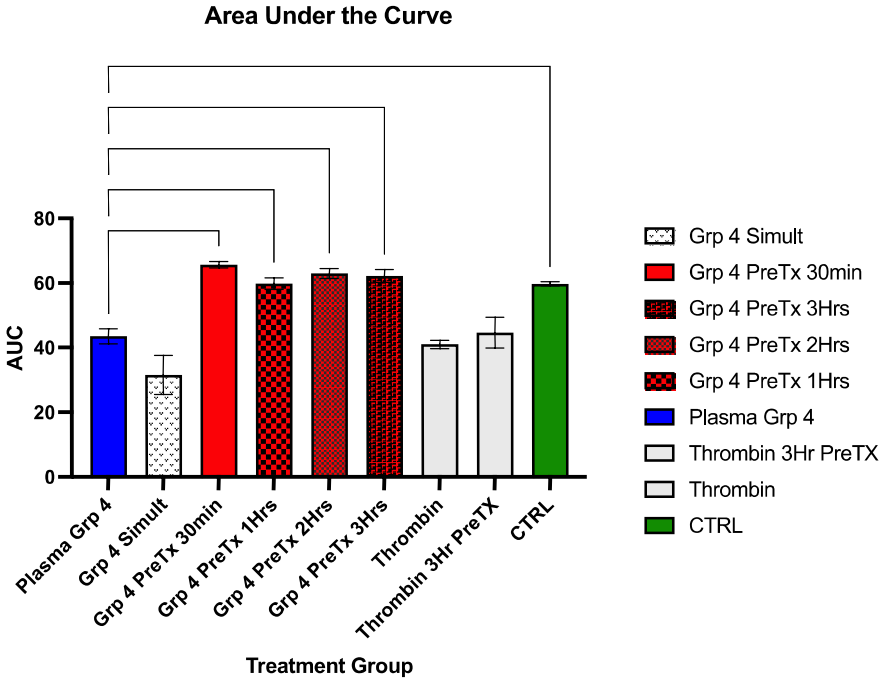


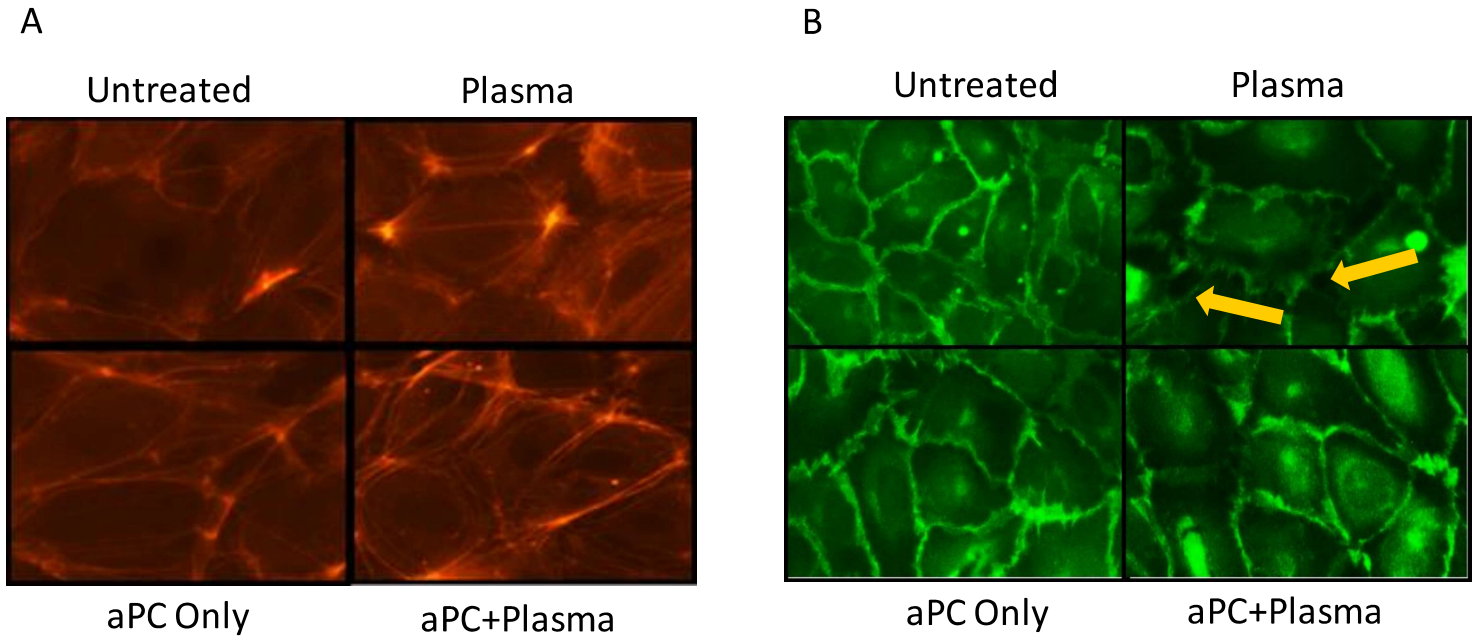
ISS 35, Shock, MOF

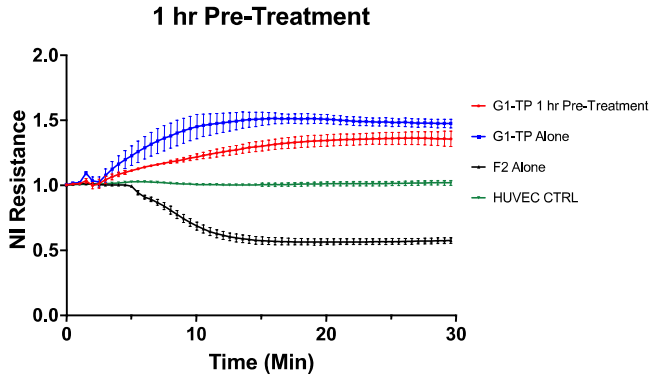


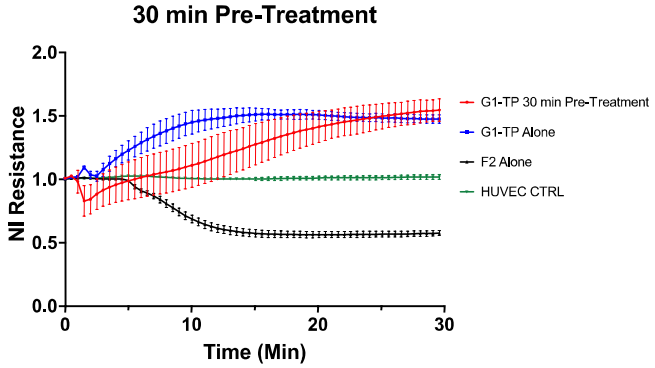


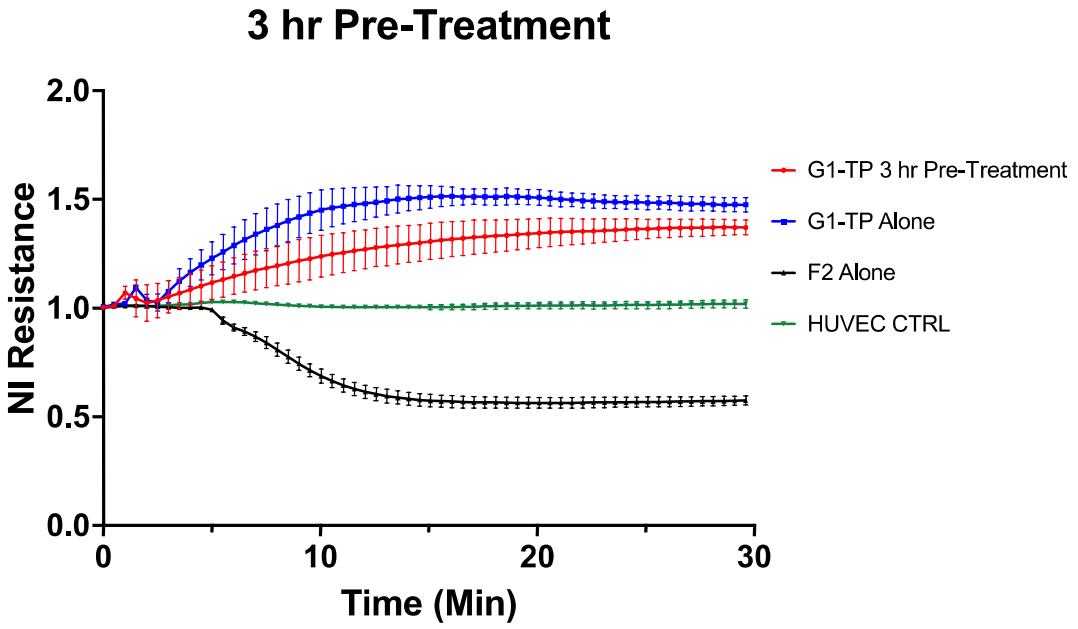

30 min APC Pre-Treatment
NI Resistance
1.5
1.0
0.5
0.0
0
10
20
30
Time (Min)
TP & Pre-Tx 30min
TP Alone
Thrombin Alone
CTRL


Area Under the Curve
AUC
80
60
40
20
0
Plasma Grp 4
Grp 4 Simult
Grp 4 PreTx 30min
Grp 4 PreTx 1Hrs
Grp 4 PreTx 2Hrs
Grp 4 PreTx 3Hrs
Thrombin
Thrombin 3Hr PreTX
CTRL
Treatment Group
Grp 4 Simult
Grp 4 PreTx 30min
Grp 4 PreTx 3Hrs
Grp 4 PreTx 2Hrs
Grp 4 PreTx 1Hrs
Plasma Grp 4
Thrombin 3Hr PreTX
Thrombin
CTRL


A
Untreated
Plasma
aPC Only
aPC+Plasma
B
Untreated
Plasma
aPC Only
aPC+Plasma


1 hr Pre-Treatment
NI Resistance
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Time (Min)
G1-TP 1 hr Pre-Treatment
G1-TP Alone
F2 Alone
HUVEC CTRL


30 min Pre-Treatment
NI Resistance
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Time (Min)
G1-TP 30 min Pre-Treatment
G1-TP Alone
F2 Alone
HUVEC CTRL


3 hr Pre-Treatment
NI Resistance
2.0
1.5
1.0
0.5
0.0
0
10
20
30
Time (Min)
G1-TP 3 hr Pre-Treatment
G1-TP Alone
F2 Alone
HUVEC CTRL

Calcium Signaling in Endothelial Permeability

Am J Physiol Lung Cell Mol Physiol
279: L815–L824, 2000.

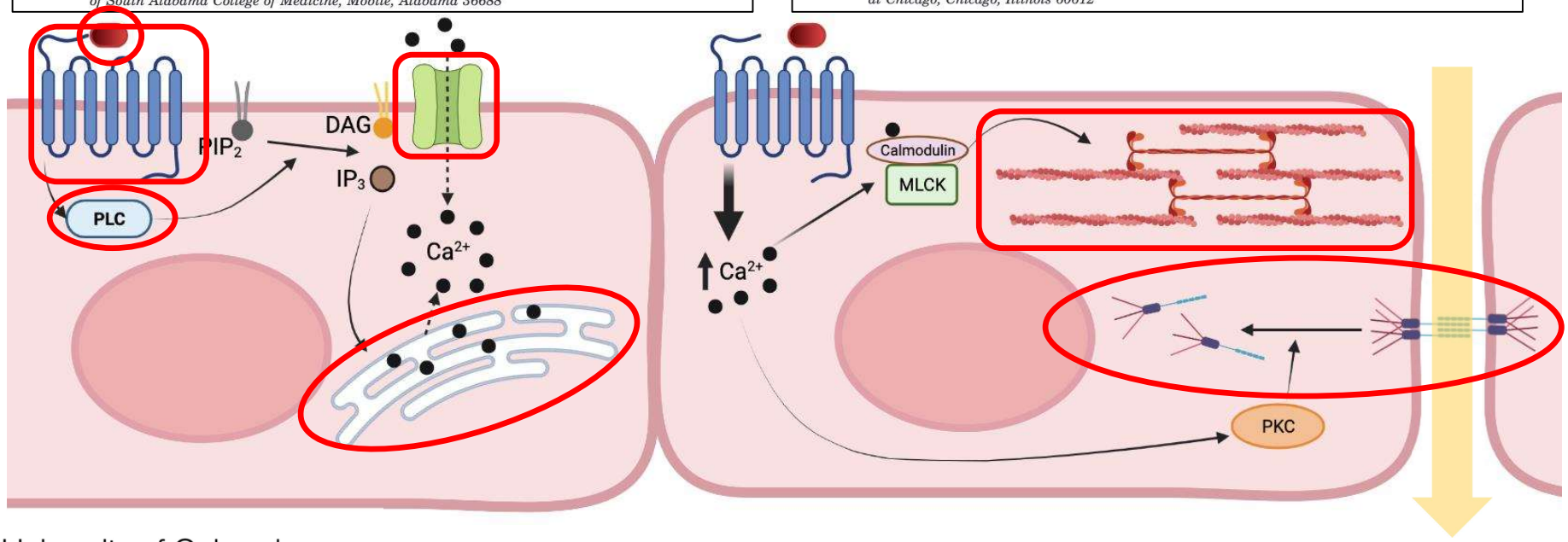
Store-operated calcium entry and increased endothelial cell permeability

NATALIE NORWOOD,[1] TIMOTHY M. MOORE,[1] DAVID A. DEAN,[2] RAKESH BHATTACHARJEE,[1] MING LI,[1] AND TROY STEVENS[1]
Departments of [1]Pharmacology and [2]Microbiology, University of South Alabama College of Medicine, Mobile, Alabama 36688

Am J Physiol Lung Cell Mol Physiol
280: L239–L247, 2001.

Requirement for Ca^{2+} signaling in the mechanism of thrombin-induced increase in endothelial permeability

RAUDEL SANDOVAL, ASRAR B. MALIK, TABASSUM NAQVI, DOLLY MEHTA, AND CHINNASWAMY TIRUPPATHI
Department of Pharmacology, College of Medicine, The University of Illinois at Chicago, Chicago, Illinois 60612



- RhoA Activation
  - Mediates endothelial permeability
  - Dependent on intracellular Ca^{2+} flux
- C4 Activation after injury
  - Associated with adverse outcomes
  - C4a→ ↑intracellular Ca^{2+}→permeability
- Post-injury hypocalcemia
  - Influx from extracellular space?

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 11, pp. 7833–7843, March 16, 2007

$G\alpha_q$-TRPC6-mediated Ca^{2+} Entry Induces RhoA Activation and Resultant Endothelial Cell Shape Change in Response to Thrombin*

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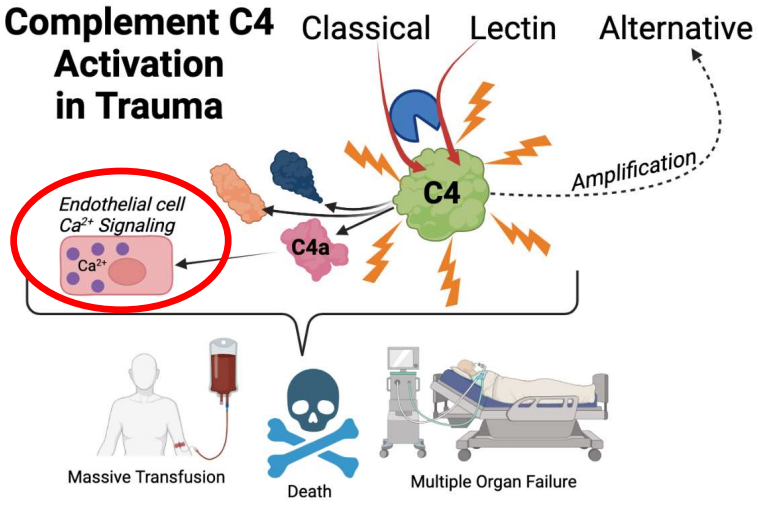
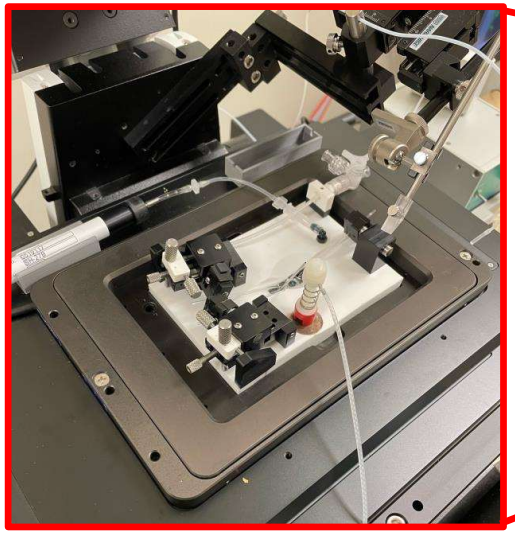
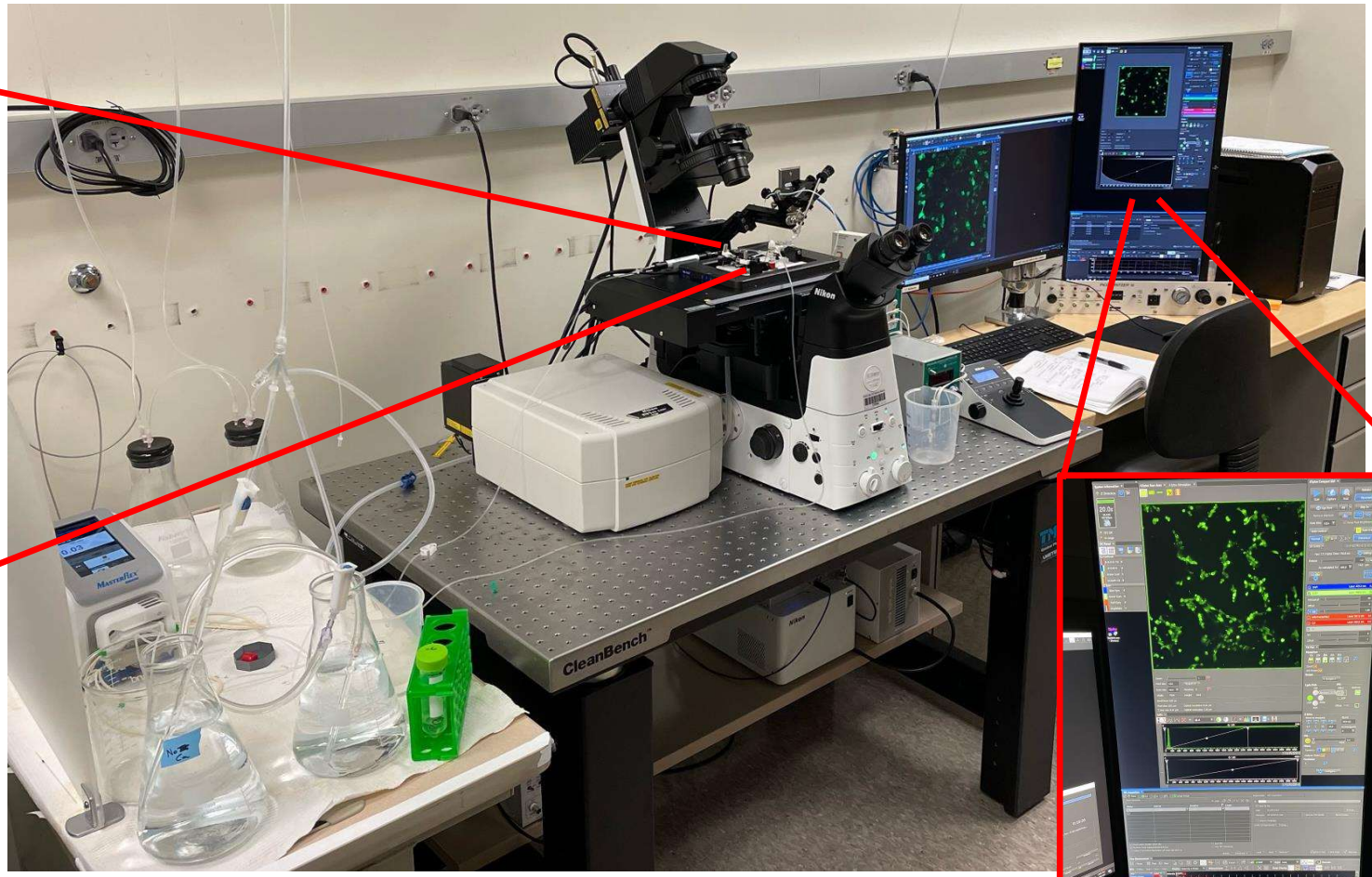
Itender Singh, Nebojsa Knezevic, Gias U. Ahmmed, Vidisha Kini, Asrar B. Malik, and Dolly Mehta[1]

From the Department of Pharmacology and Center for Lung and Vascular Biology, College of Medicine, University of Illinois, Chicago, Illinois 60612



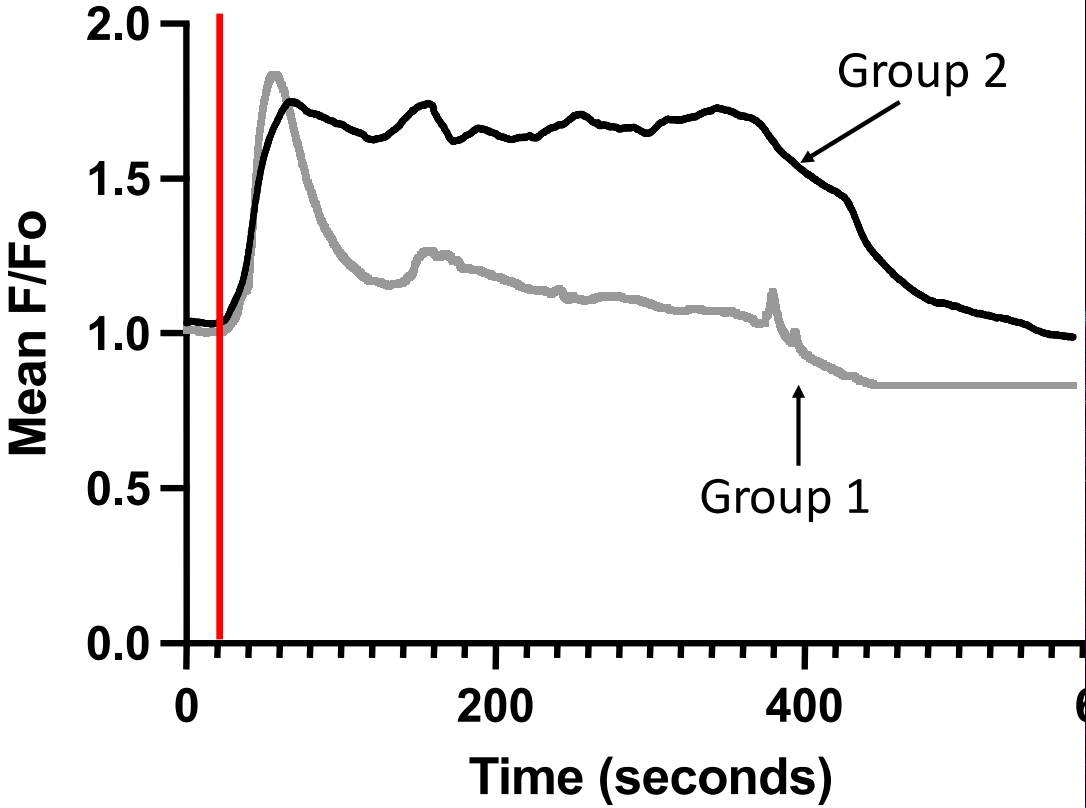
University of Colorado
Anschutz Medical Campus

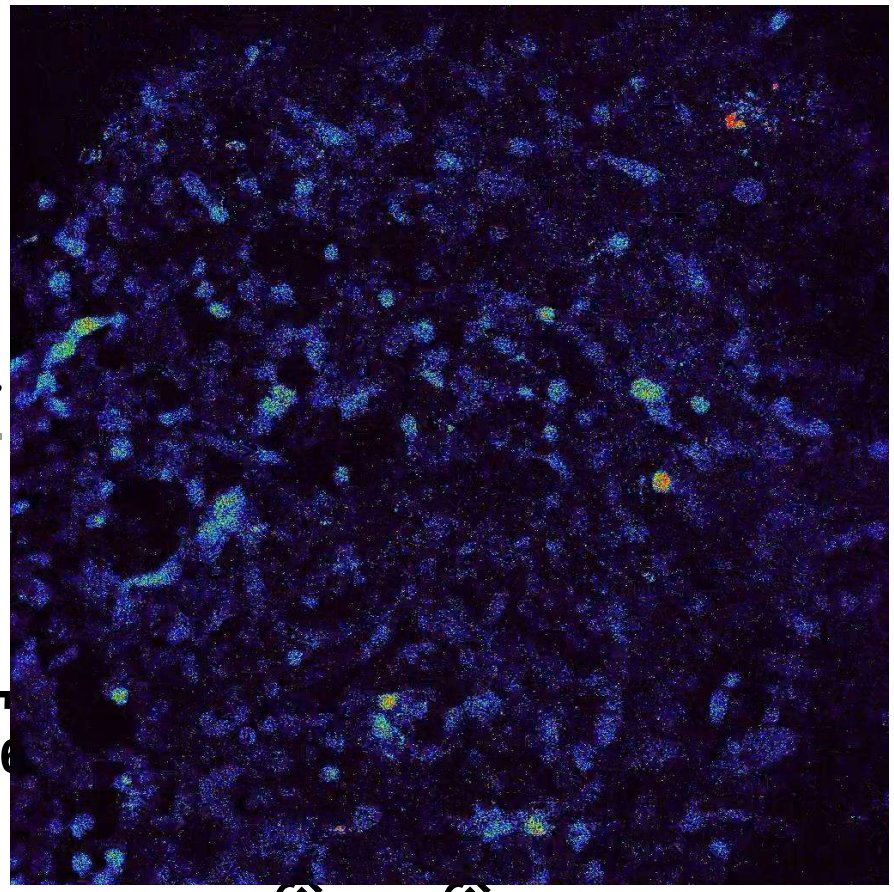




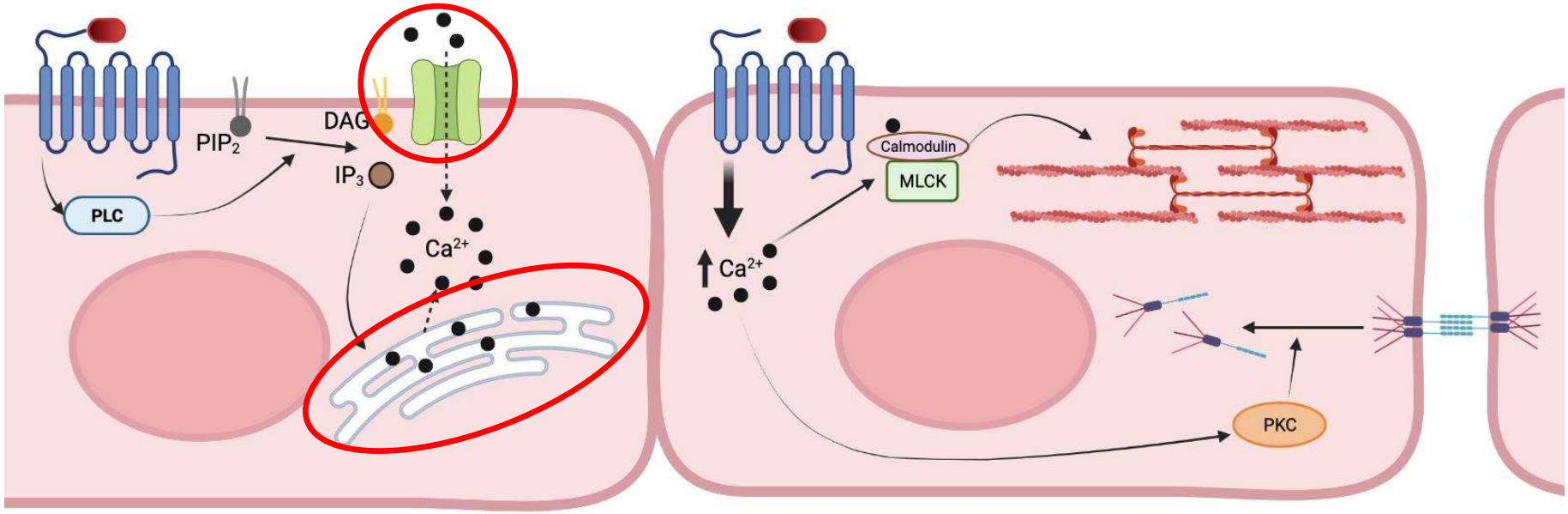
University of Colorado
Anschutz Medical Campus

More intracellular calcium flux in ex vivo severe injury.



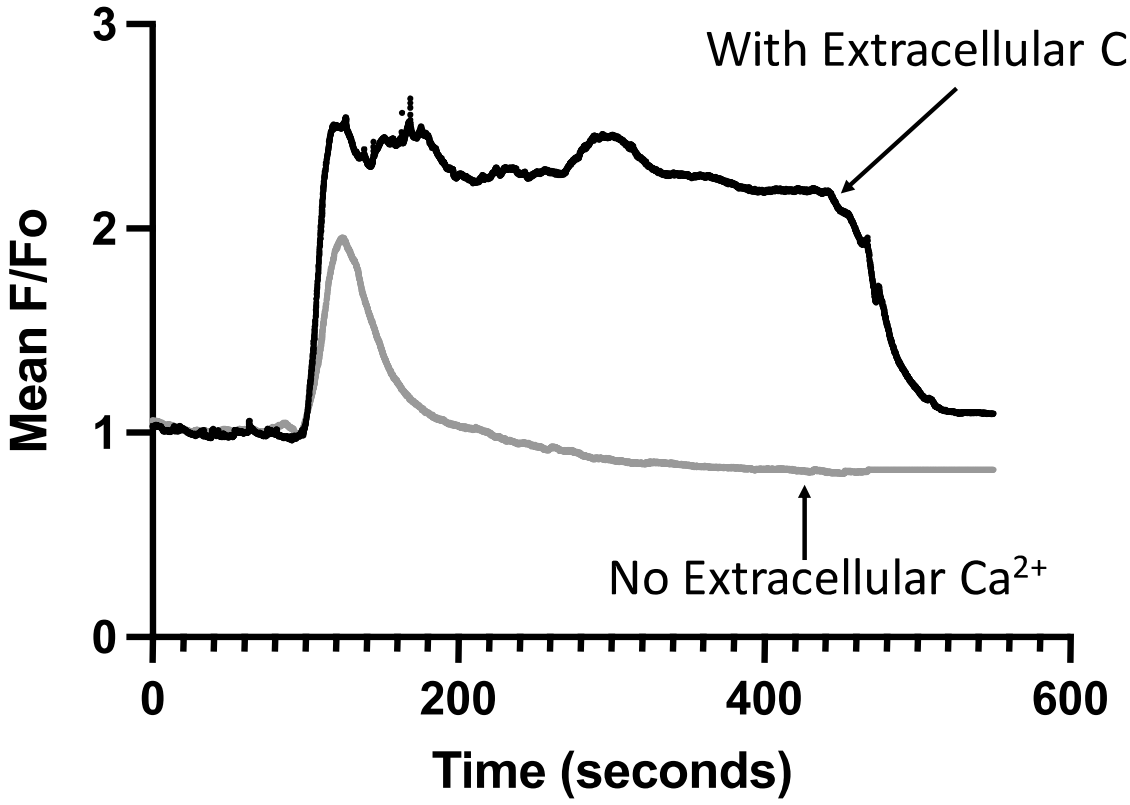


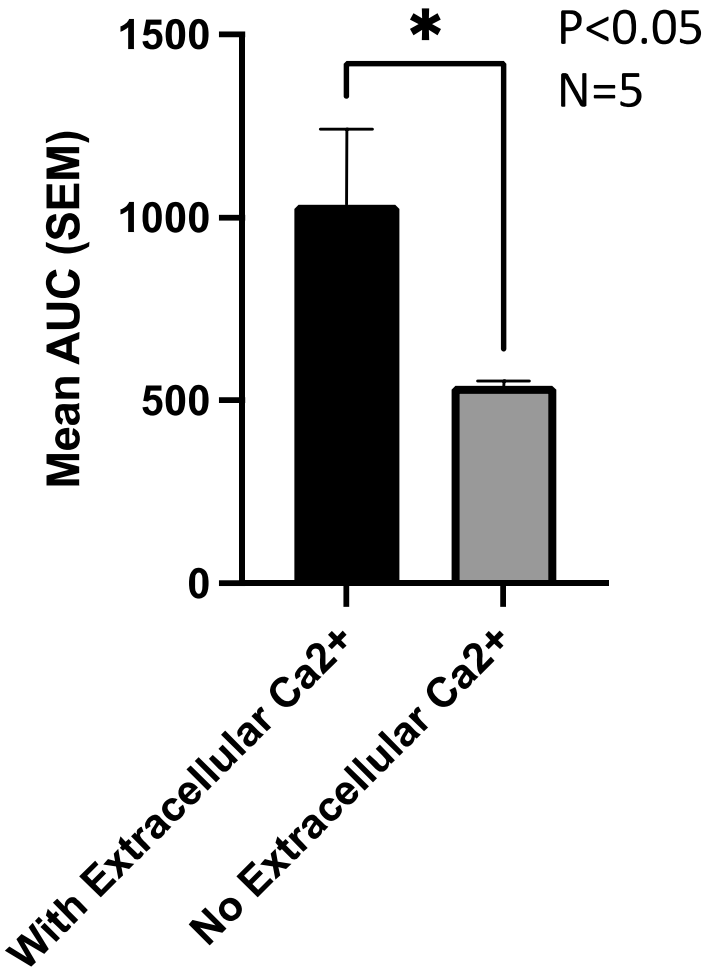
Where does the Ca^{2+} come from?



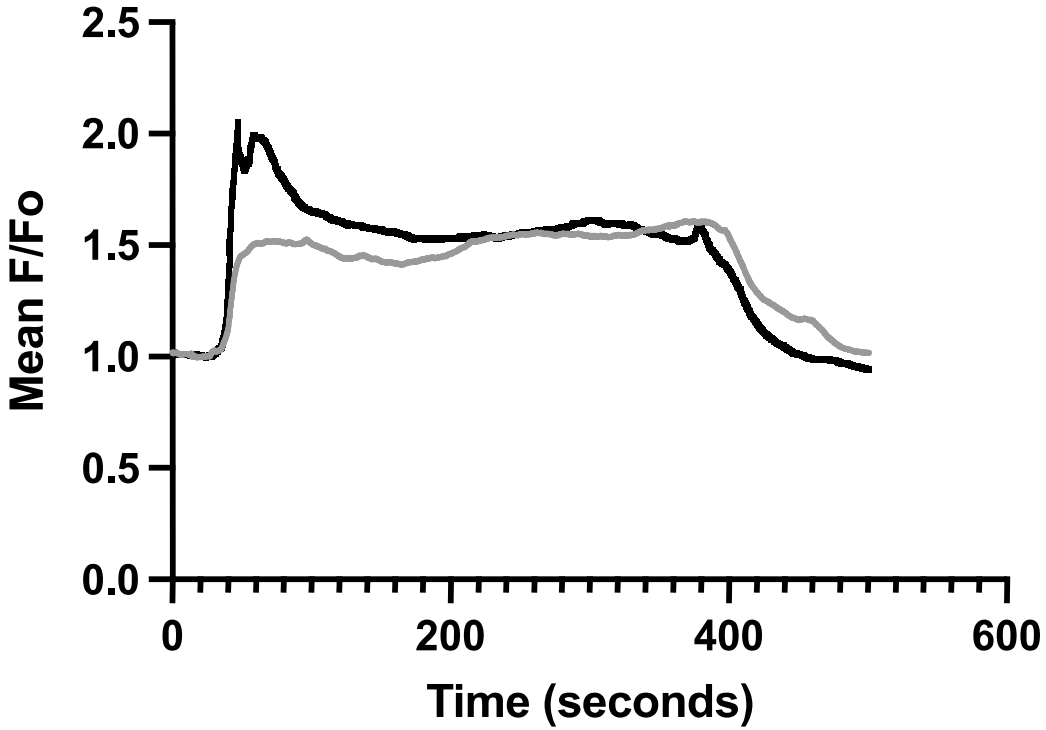
- Buffer without $CaCl_2$ + EGTA → No Extracellular Ca^{2+}
- Group 2 patient samples (n=5)

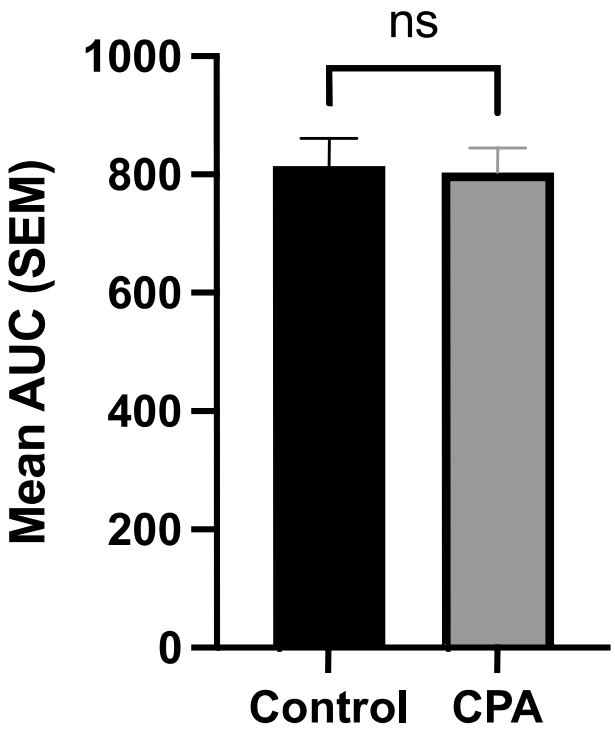
Less intracellular Ca^{2+} flux without extracellular Ca^{2+}

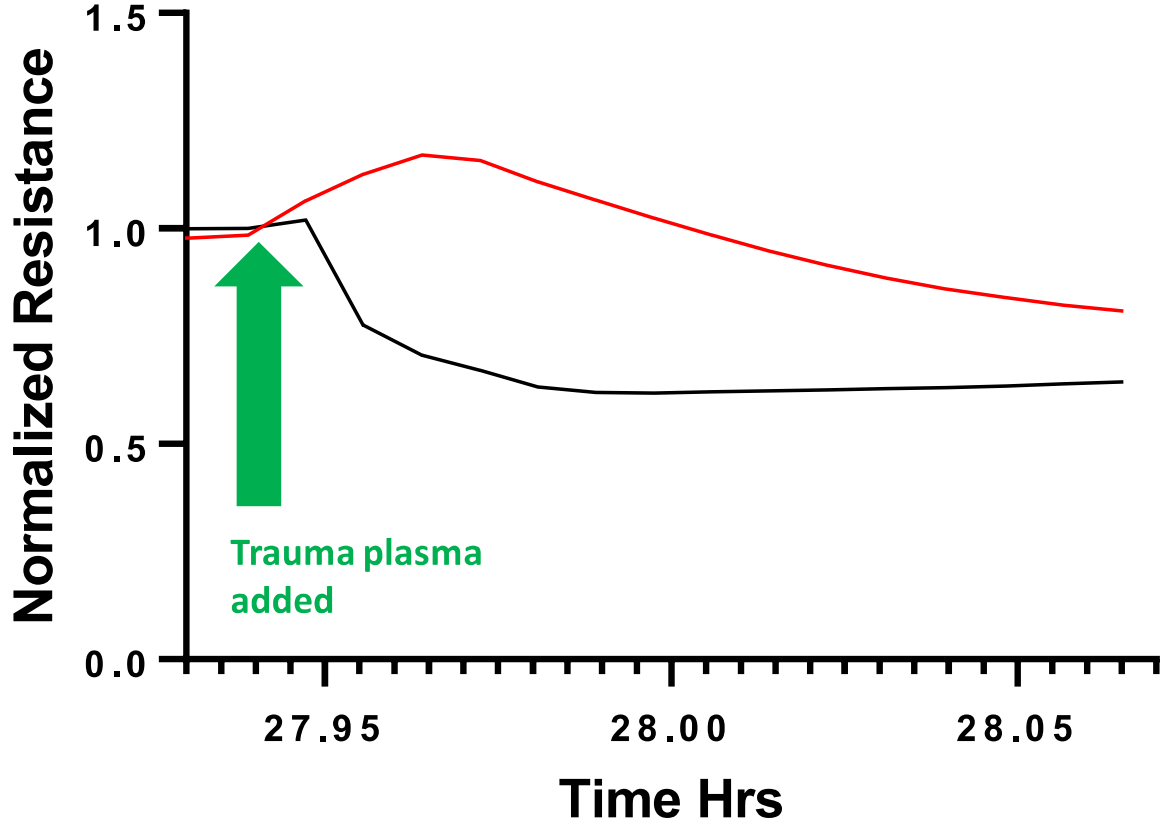




There is no difference in Ca^{2+} flux when intracellular stores are depleted with CPA







TP aCSF (Ca^{2+}Depleted)

TP aCSF (w/Ca^{2+})

COAGULATION

Targeted clinical control of trauma patient coagulation through a thrombin dynamics model

Amor A. Menezes,[1,2] Ryan F. Vilardi,[3] Adam P. Arkin,[1,2,4]* Mitchell J. Cohen[5,6]*

We present a methodology for personalizing the clinical treatment of severely injured patients with acute traumatic coagulopathy (ATC), an endogenous biological response of impaired coagulation that occurs early after trauma and shock and that is associated with increased bleeding, morbidity, and mortality. Despite biological characterization of ATC, it is not easily or rapidly diagnosed, not always captured by slow laboratory testing, and not accurately represented by coagulation models. This lack of knowledge, combined with the inherent time pressures of trauma treatment, forces surgeons to treat ATC patients according to empirical resuscitation protocols. These entail transfusing large volumes of poorly characterized, nontargeted blood products that are not tailored to an individual, the injury, or coagulation dynamics. Massive transfusion mortality remains at 40 to 70% in the best of trauma centers. As an alternative to blunt treatments, time-consuming tests, and mechanistic models, we used dynamical systems theory to create a simple, biologically meaningful, and highly accurate model that (i) quickly forecasts a driver of downstream coagulation, thrombin concentration after tissue factor stimulation, using rapidly measurable concentrations of blood protein factors and (ii) determines the amounts of additional coagulation factors needed to rectify the predicted thrombin dynamics and potentially remedy ATC. We successfully demonstrate in vitro thrombin control consistent with the model. Compared to another model, we decreased the mean errors in two key trauma patient parameters: peak thrombin concentration after tissue factor stimulation and the time until this peak occurs. Our methodology helps to advance individualized resuscitation of trauma-induced coagulation deficits.

INTRODUCTION

Trauma is the leading cause of death and disability between the ages of 1 and 44 (*1*), with bleeding contributing to the vast majority of these deaths (*2*). Such hemorrhage is a clinical problem that is complicated by an endogenous biological response called acute traumatic coagulopathy (ATC) (*3*). ATC results in impaired coagulation, increased bleeding, greater transfusion needs, and a fourfold increase in mortality (*3*). After the initial phase of hypocoagulobility, ATC patients often dynamically transition to a hypercoagulable thrombotic state manifested by excessive clotting (*3*). The resulting deep vein thrombosis, myocardial infarction, stroke, and organ failure (*4*) all contribute to an extremely poor outcome in patients who survive their initial injuries.

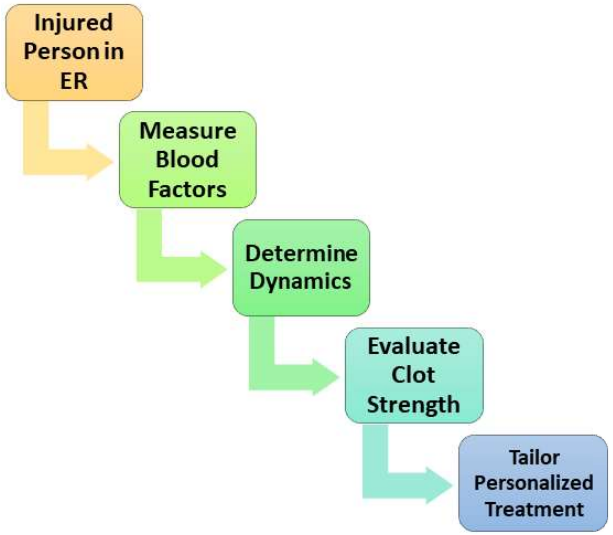
Despite considerable research (*4*) on the molecular mechanisms of ATC, there remains a mechanistic and predictive knowledge gap that stems from an inadequate understanding of coagulation mechanisms after an injury and a lack of adequate prediction and real-time decision support for clinicians who care for the severely injured. These failings impede improvements to urgent resuscitation. Thus, there is a need to characterize coagulation mechanisms in trauma patients and to use this characterization to improve the precision of individual treatments.

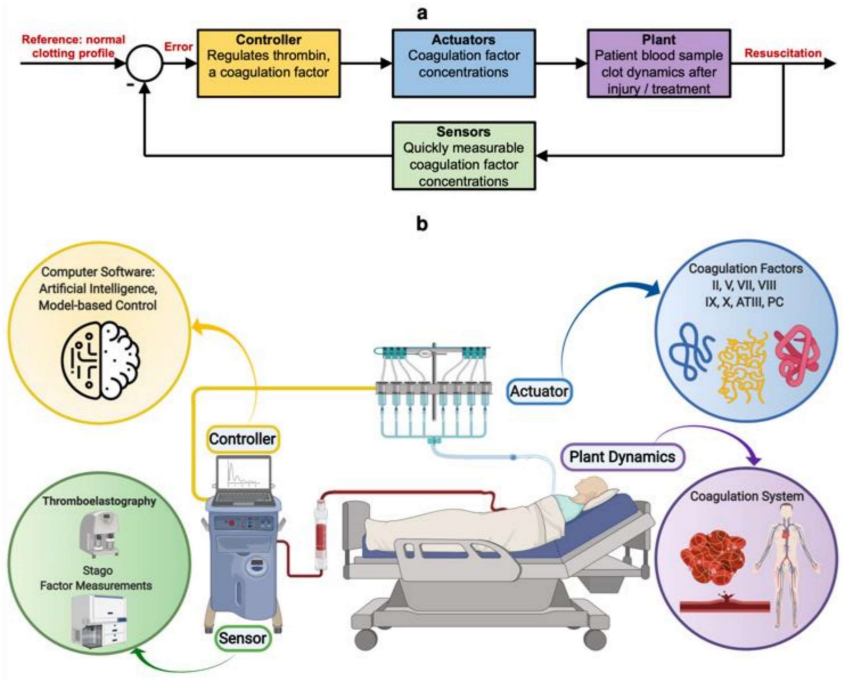
In the absence of dynamic diagnostics and decision support, current trauma resuscitation practices (*4*) center on the nontargeted repair of the coagulation cascade (*5*) (Fig. 1A) and the production of its principal protein thrombin through the transfusion of large volumes of poorly characterized fresh-frozen plasma containing multiple clotting proteins and inhibitors in concentrations that vary from unit to unit. These urgent-care therapies indiscriminately actuate many interacting elements of the coagulation process, resulting in variable untargeted treatment for every patient and with every administration, which is further exacerbated by a lack of clarity about treatment effects on the patient's physiological and biological trajectories resulting from the missing diagnostics and decision support. Such blunt treatment is often either not enough (ATC and bleeding continue) or too much (thrombosis occurs). Both of these extremes contribute to dysregulated inflammation and poor outcomes (*4*). The mortality from massive transfusion remains at 40 to 70% in the best of trauma centers (*6*). Retrospective (*7*) and prospective (*8*) studies connect the blunt addition of fresh-frozen plasma to poor outcomes, even when the plasma is augmented with empiric ratios of platelets and red blood cells. Transfusion of fresh-frozen plasma is independently associated with a higher risk of multiple organ failure and poor outcomes in patients with hemorrhagic shock (*9*). Meanwhile, individual interventions consisting of personalized blood protein factor concentrations that are tailored to specific clotting perturbations have been shown to be beneficial (*4*), although no consensus yet exists on the amount and type of coagulation factors to administer. There is, however, a clinical desire for specific blood products to treat trauma coagulopathy (*10*). In sum, in an era of increasing personalized medicine, there is an urgent need for targeted, patient-specific trauma coagulation therapies.

Current diagnostics and decision support suffer from a dearth of patient-specific coagulation measurements. Although clinical practice uses several global markers [international normalized ratio (INR), partial thromboplastin time (PTT), prothrombin time (PT), platelet count, fibrinogen concentration, etc.] to diagnose the presence of ATC, these conventional coagulation tests are not enough to tailor a specific intervention and support only the decision to administer plasma or not. Cell-based viscoelastic tests are insufficiently predictive, and their use in resuscitation algorithms also results in nontargeted treatment. Moreover,

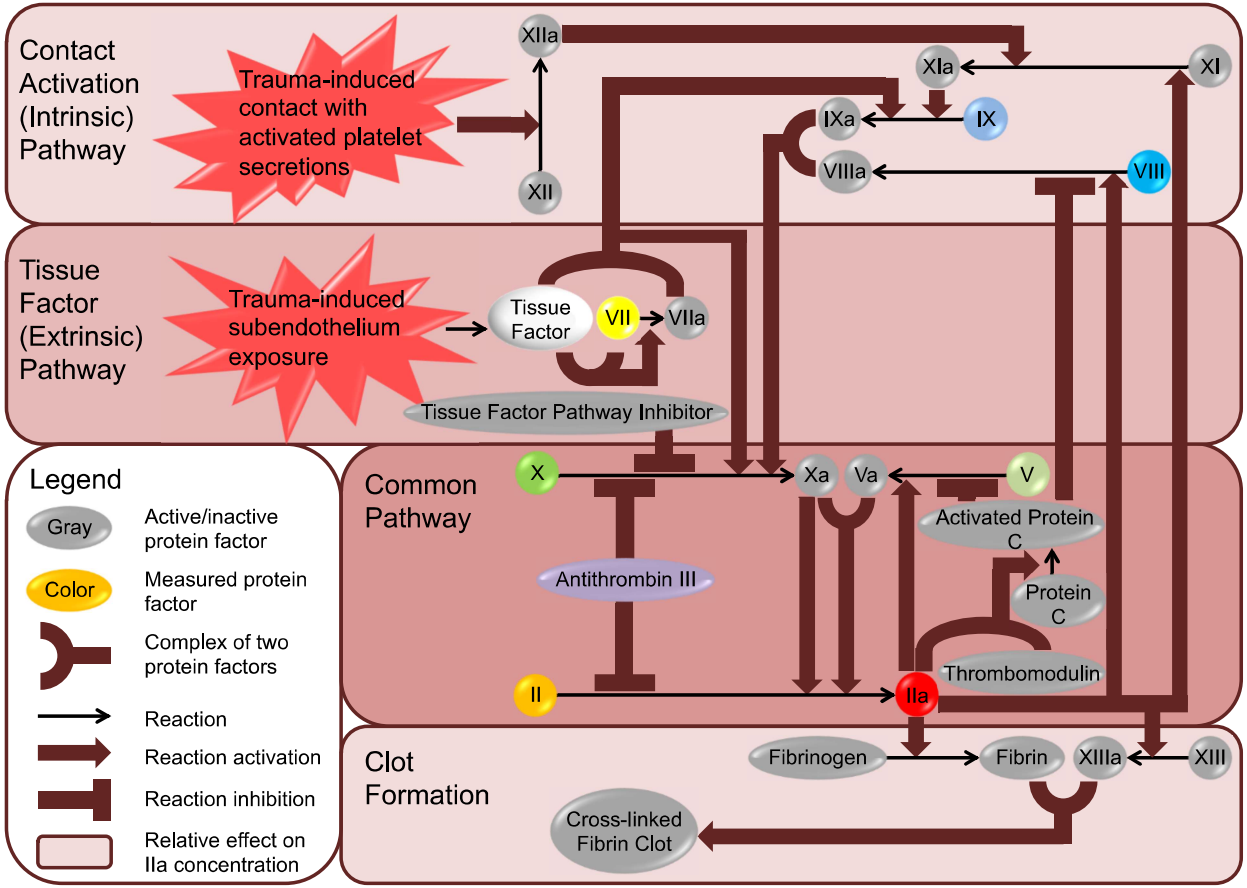
[1]California Institute for Quantitative Biosciences at University of California, Berkeley, 2151 Berkeley Way, Berkeley, CA 94704–5230, USA. [2]Environmental Genomics and Systems Biology Division at E. O. Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Mailstop 955-512L, Berkeley, CA 94720, USA. [3]Department of Laboratory Medicine, University of California, San Francisco, 505 Parnassus Avenue, San Francisco, CA 94143, USA. [4]Department of Bioengineering, University of California, Berkeley, 2151 Berkeley Way, Berkeley, CA 94704–5230, USA. [5]Department of Surgery, Denver Health Medical Center, 777 Bannock Street, Denver, CO 80204–6206, USA. [6]Department of Surgery, University of Colorado, 12631 East 17th Avenue, C-305, Aurora, CO 80045, USA. *Corresponding author. Email: aparkin@lbl.gov (A.P.A.); mitchell.cohen@dhha.org (M.J.C.)

Solution: Personalized dynamic approach

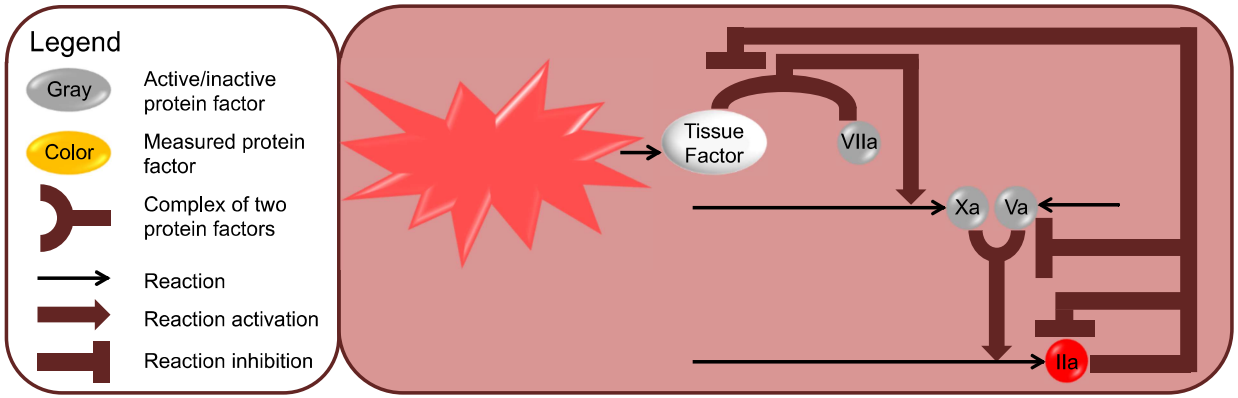




Current Understanding: Coagulation Cascade



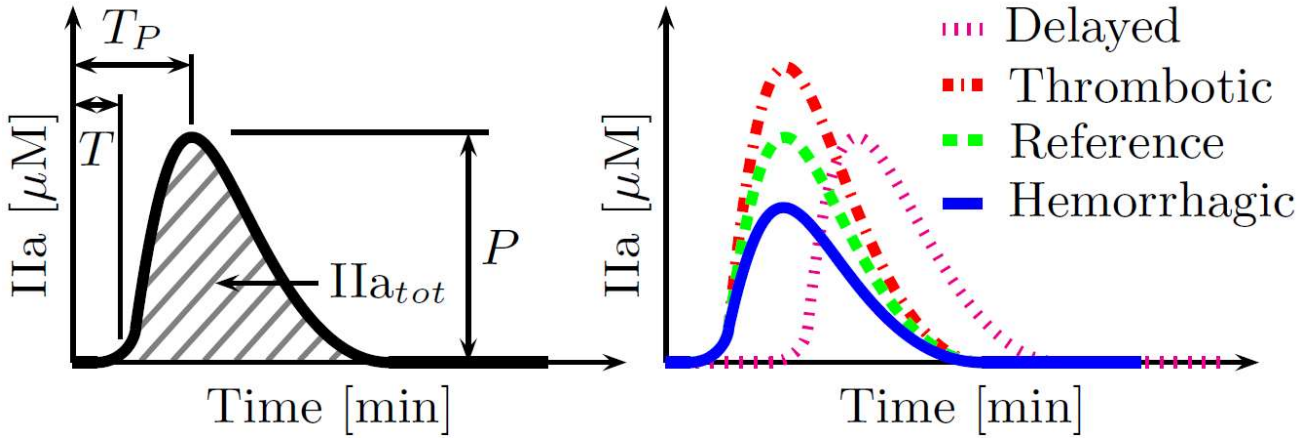
Claim: Possible to Simplify



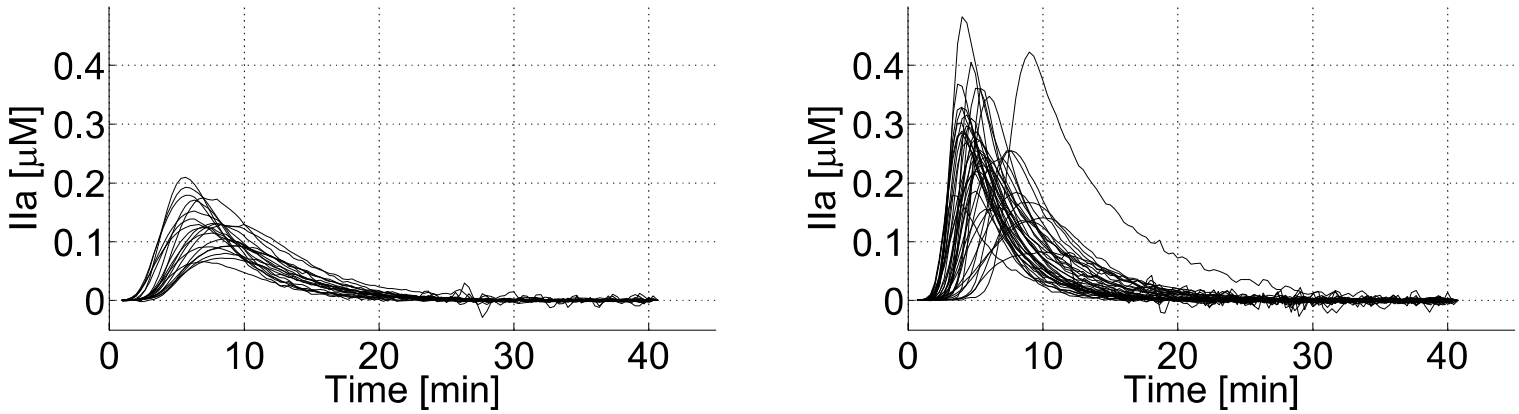
- Dynamical System Input: Tissue Factor
- Dynamical System Output: Thrombin
- Need an input-to-output measurement.

Thrombin Measurement

- The Calibrated Automated Thrombogram (CAT) is a fluorogenic assay that measures the time-history of thrombin generation in a blood sample upon the addition of (typically 5pM of) tissue factor.

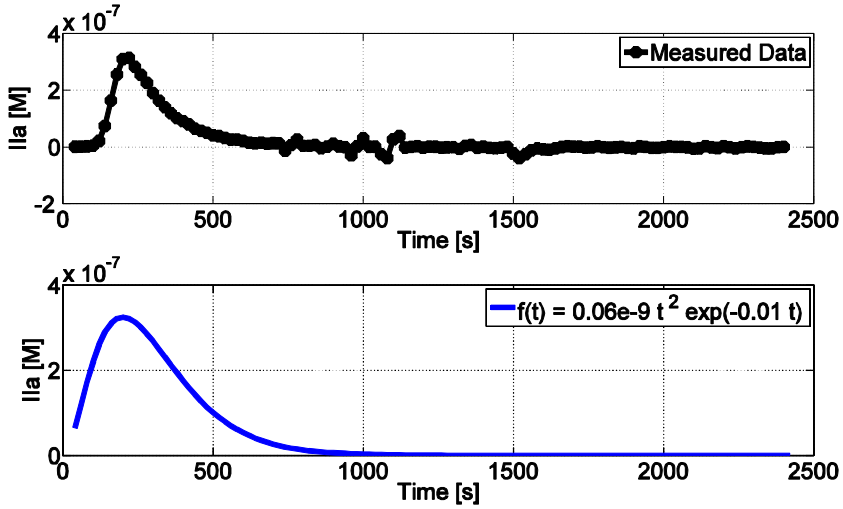


Normal vs. Trauma CATs



- Can we emulate trajectories with a single-input single-output thrombin dynamical system model with a separable delay for treatment guidance? What kind of model?

Building a Black-Box Model



- Can approximate a CAT peak.
- Suppose we choose the following non-delayed function as first approximation:

$$y(t) = \beta t^2 e^{-\alpha t}$$

- t^2 → three states.

- Look at output in frequency domain as the result of some dynamical system:

$$Y(s) = \frac{2\beta}{(s+\alpha)^3} = \frac{2\beta}{s^3 + 3\alpha s^2 + 3\alpha^2 s + \alpha^3}$$

Building a Black-Box Model: 3 states, 5 pars.

- Suppose input is a (unit) impulse, *U(s)* = 1:

$$\frac{Y(s)}{U(s)} = \frac{2\beta}{(s+\alpha)^3} = \frac{2\beta}{s^3 + 3\alpha s^2 + 3\alpha^2 s + \alpha^3}$$

- System transfer function, including delay:

$$\frac{Y(s)}{U(s)} = \frac{b}{s^3 + a_2 s^2 + a_1 s + a_0} e^{-sT}$$

Building a Black-Box Model: Traditional Form

$$\frac{Y(s)}{U(s)} = \left(\frac{Kp}{s+p}\right)\left(\frac{\omega_n^2}{s^2+2\zeta\omega_n s+\omega_n^2}\right)e^{-sT}$$

- Define

$\sigma = \zeta\omega_n$ and $\omega_d = \omega_n\sqrt{1-\zeta^2}$ (i.e., $\omega_n^2 = \sigma^2 + \omega_d^2$), and let

$A = \frac{Kp\omega_n^2}{p^2-2\zeta\omega_n p+\omega_n^2}$; $B = \frac{-Kp\omega_n^2}{p^2-2\zeta\omega_n p+\omega_n^2}$; $C = \frac{Kp\omega_n^2(p-2\zeta\omega_n)}{p^2-2\zeta\omega_n p+\omega_n^2}$;

$D = \left(B\cos\left(\omega_d\left(t-T\right)\right) + \frac{C-\sigma B}{\omega_d}\sin\left(\omega_d\left(t-T\right)\right)\right)$. Then each fitted time-delayed CAT unit impulse response is given by

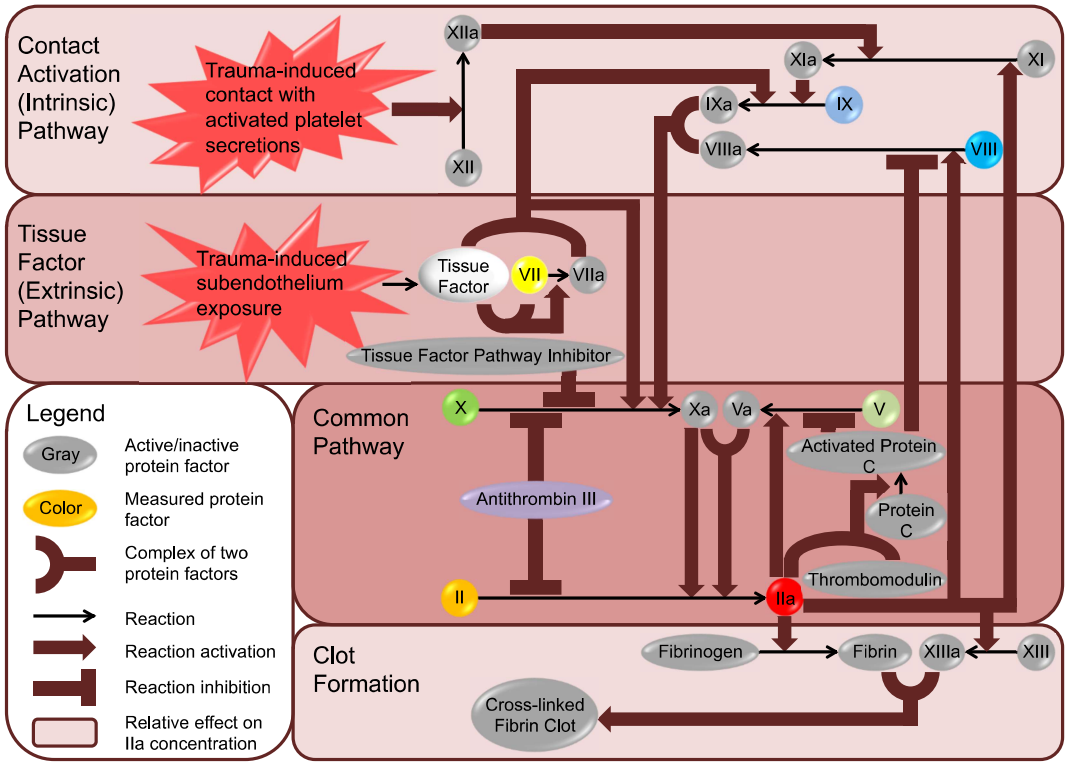
$$y(t) = \begin{cases} 0 & \text{if } t < T; \\ \left(Ae^{-p(t-T)} + De^{-\sigma(t-T)}\right)1(t-T) & \text{if } t \geq T, \end{cases}$$

for some p, ζ, ω_n and T, computed from a_2, a_1, a_0 and T.

Black-Box Model Dynamics Interpretation

$$\dot{x}_1(t) = -a_2 x_1(t) + x_2(t)$$
$$\dot{x}_2(t) = -a_1 x_1(t) + x_3(t)$$
$$\dot{x}_3(t) = -a_0 x_1(t) + bv(t)$$
$$y(t) = x_1(t)$$

- First equation:
  - IIa inhibits own production
  - [IIa] grows proportionately with $x_2(t)$
  - $x_2(t)$ is either [Xa] or [Xa-Va]



Black-Box Model Dynamics Interpretation

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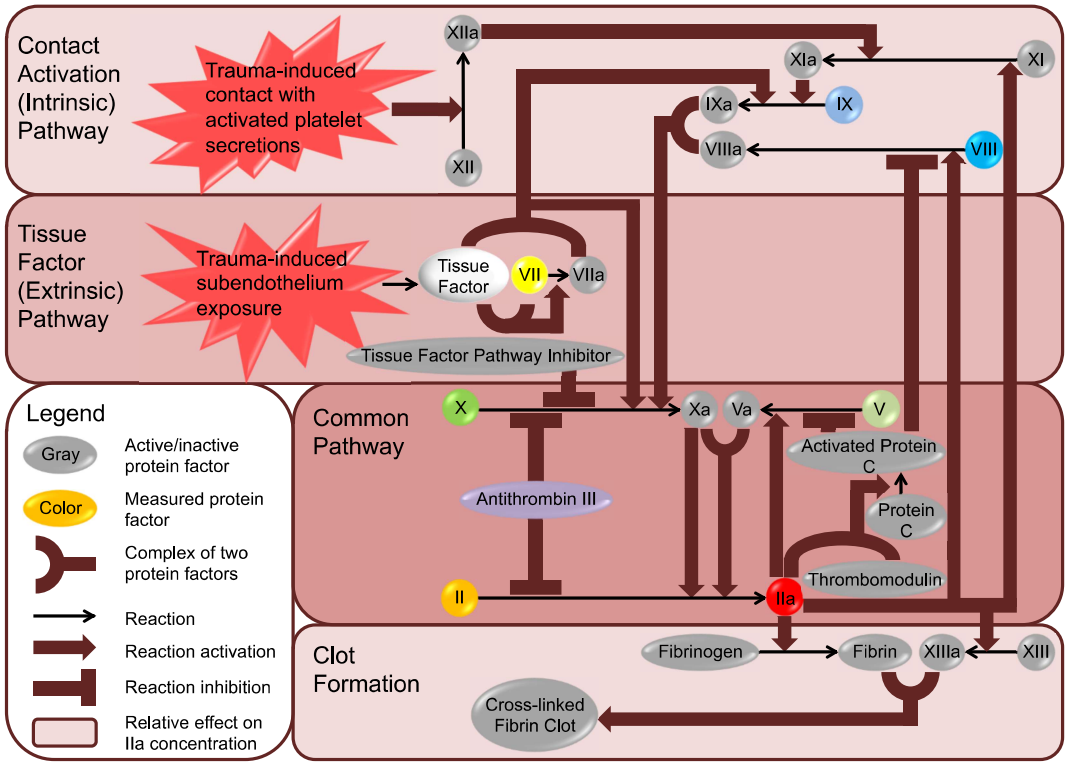
$$\dot{x}_3(t) = -a_0 x_1(t) + bv(t)$$
$$y(t) = x_1(t)$$

- Summary:
  - $x_1(t)$ is [IIa]
  - $x_2(t)$ is [Xa-Va]
  - $x_2(t)$ is [TF-VIIa]



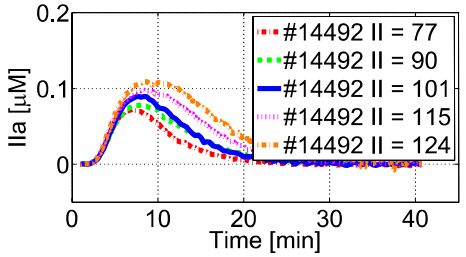
- Model is keeping track of the chief participants of the thrombin generation process.

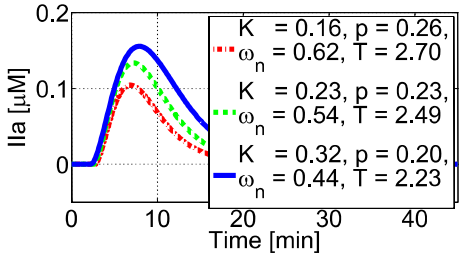
Targeted Clinical Control of Trauma Patient Coagulation Through a Thrombin Dynamics Model

Amor Menezes, Ryan Vilardi, Adam Arkin and Mitchell Cohen (UCB/LBNL, UCSF/SFGH)

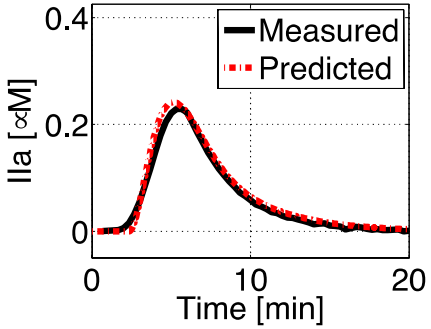
Benefit/Results

Parameters exactly capture behavior caused by protein factor addition (e.g., factor II to normal plasma)

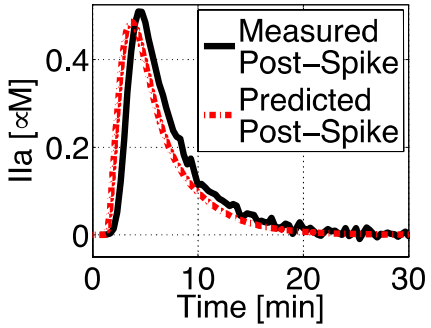




Initial protein factor concentrations can exactly predict a trauma patient CAT (e.g., for a moderate Injury Severity Score)



For this patient's plasma, after adding concentrations of factors II, VIII, X, we can still correctly predict the moved CAT



Have proved the likelihood of authority (at least *in vitro*) to achieve a standard desirable CAT trajectory.

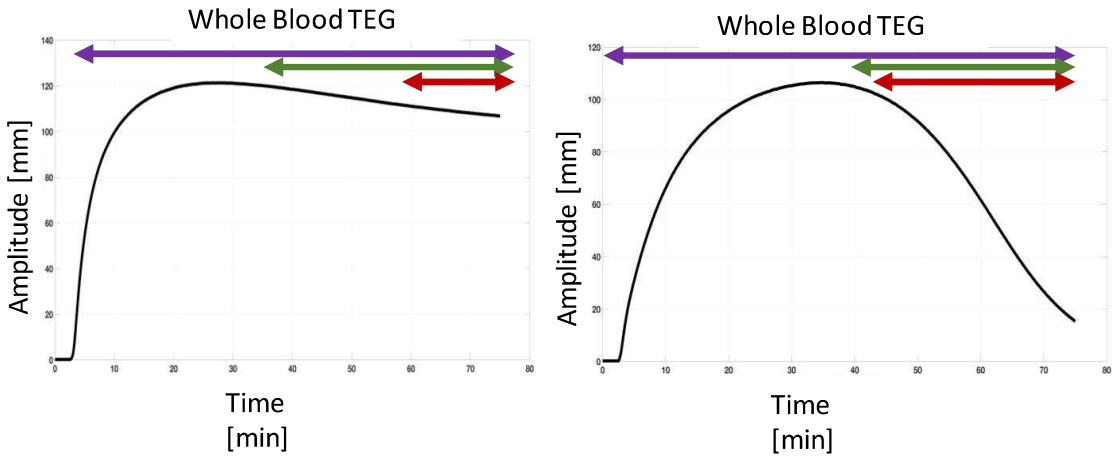
Challenge/Computing Needs

1. Larger study to improve parametric inference and better validate predictions
   i. More data
   ii. Data-model reconciliation
2. Computer/model-aided investigation of controllability, control authority
3. Extensions of model's applicable range

Viscoelasticity of Whole Blood

Factor Concentrations to Whole Blood TEG

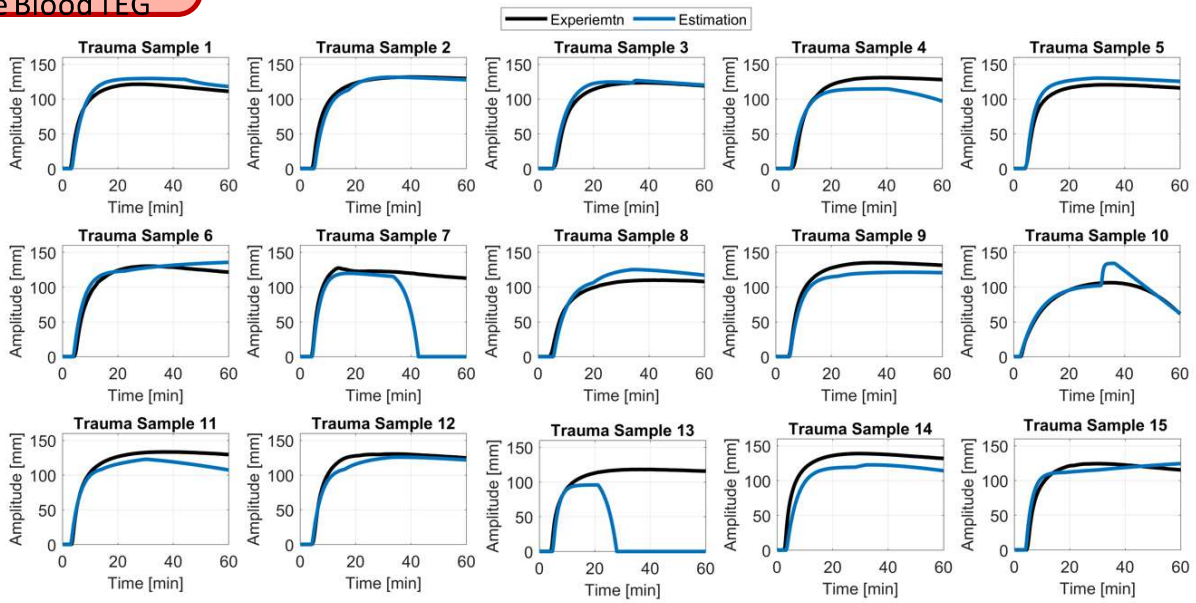
$$\frac{K_{n,1}}{s(K_{p,1}s+1)}e^{-sK_{d,1}} + \frac{-K_{n,2}}{s^2}e^{-sK_{d,2}} + \frac{-K_{n,3}}{s(K_{p,3}s+1)}e^{-sK_{d,3}}$$



Viscoelasticity of Whole Blood

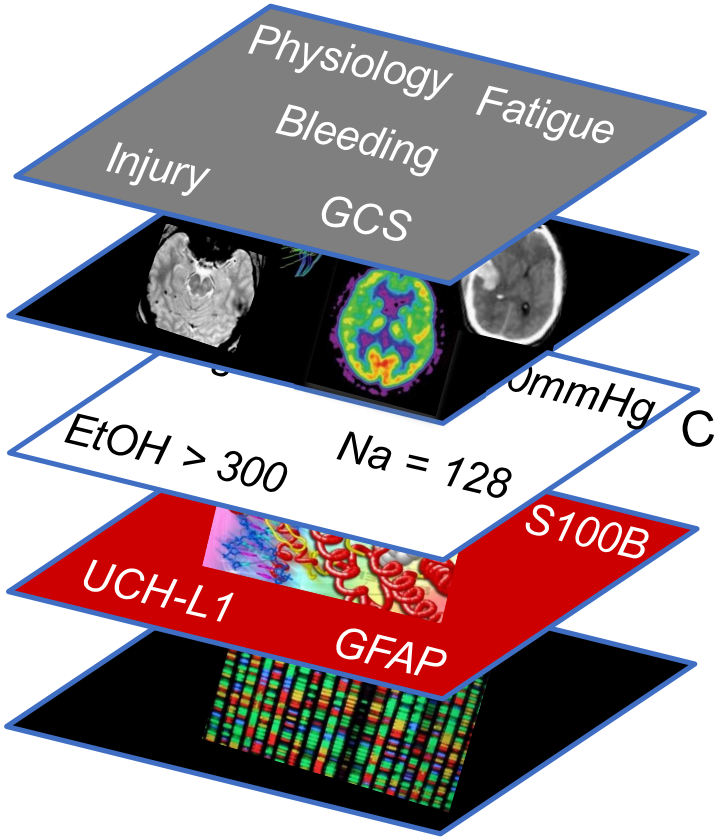
Factor Concentrations to Whole Blood TEG

TEG graph estimation using:
Coagulation factors II, V, VII, VIII, IX, X, ATIII, PC, D-dimer



Precision Medicine

Threat X: a Precision Medicine Approach



Demographic/Injury

Imaging

Clinical Data/Physiology

Proteome

Genome

Mitchell.cohen@cuanschutz.edu