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Plasma Peptidylarginine Deiminase IV Promotes VWF-Platelet String Formation and Accelerates Thrombosis after Vessel Injury

Nicoletta Sorvillo[1,2], Daniella de Moraes Mizurini[1,2], Carmen Coxon[3], Kimberly Martinod[1,2], Ronak Tilvawala[4], Deya Cherpokova[1,2], Ari J. Salinger[4], Robert J. Seward[5], Caleb Staudinger[1], Eranthie Weerapana[6], Nate I. Shapiro[7], Catherine E. Costello[5], Paul Thompson[4], Denisa D. Wagner[1,2]

background

- activated ENDothelial cells shed Von Willebrand Factor (VWF)
- VWF remains on END cell surface → attracts leukocytes & platelets
- VWF-platelet strings contribute to formation of stable occlusive thrombi
- VWF linked to: thrombosis, microvascular occlusion,…

background

- ADAMTS13 = disintegrin & metalloproteinase w/ thrombospondin type1 motif-13
  - normally lears VWF-platelet strings → conversion into smaller less thrombogenic fragments
- lack/reduced ADAMTS13 activity linked to:
  - Thrombotic thrombocytopenic purpura (TTP)
  - Disseminated intravascular coagulation (DIC)
  - Stroke
  - Deep vein thrombosis (DVT)

background

- Platelets, fibrin and Neutrophil Extracellular Traps (NETs) observed in thrombi
- NETs promote coagulation, thrombosis, inflammatory responses, …
- PAD4 = peptidylarginine deiminase type IV
  - driving force in NETosis
  - enzyme that citrullinates arginine residues on target protein upon Ca^{2+} activation

Elevated PAD4 plasma levels	
Elevated cit-Protein levels	
Rheumatoid Arthritis (RA) Multiple Sclerosis (MS) Alzheimer‘s Disease	Sepsis Patients bearing malignant tumors

hypothesis

Extracellular PAD4, released during inflammatory responses, citrullinates plasma proteins
→ thereby affecting thrombus formation

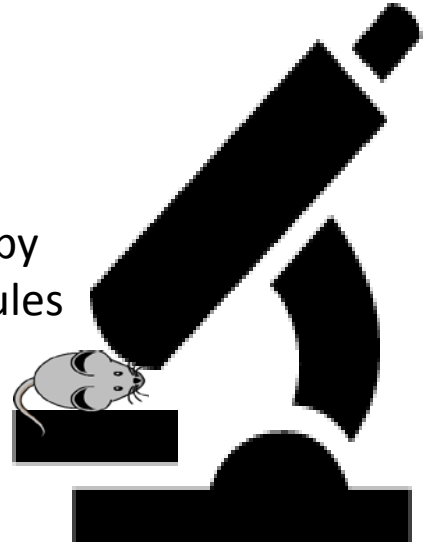
What is the effect of PAD4 in circulation in the context of VWF-platelet string clearance by ADAMTS13?

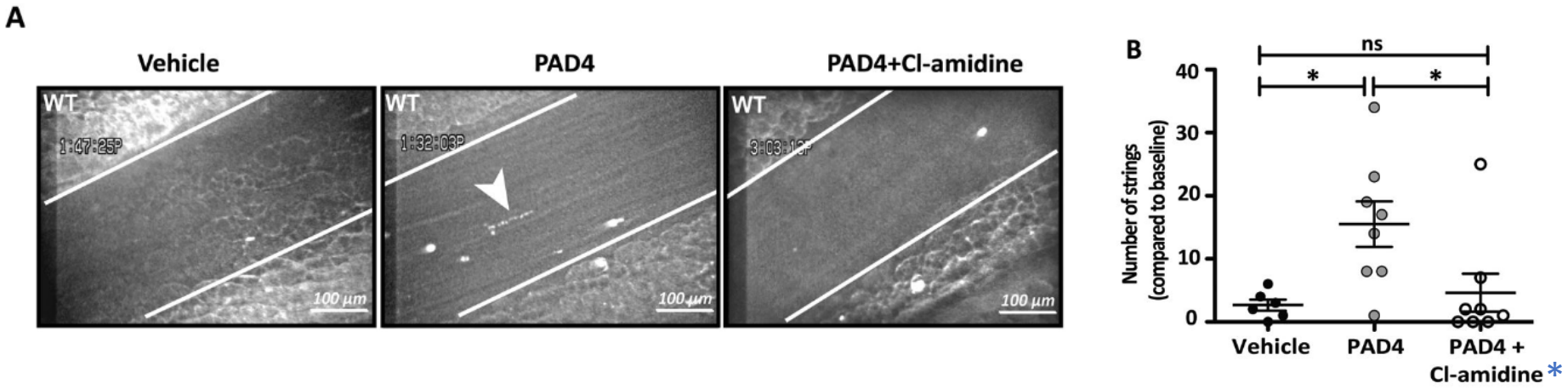
r-huPAD4 / vehicle

+/- PAD4 inhibitor

WT mice

intravital microscopy
of mesenteric venules



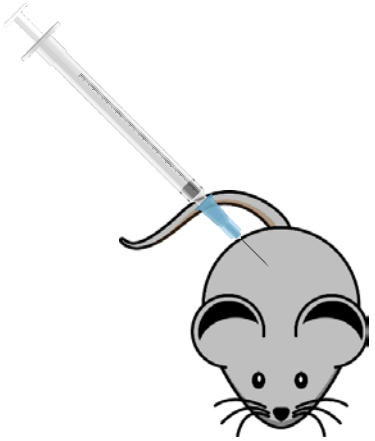


- spontaneous VWF-platelet string formation in PAD4 injected mice
- short lived strings (<30 seconds)
- significantly higher number in PAD4 treated mice compared to vehicle treated mice
- PAD4 inhibitor preserved natural clearance

* Cl-amidine = irreversible small molecule PAD4 inhibitor

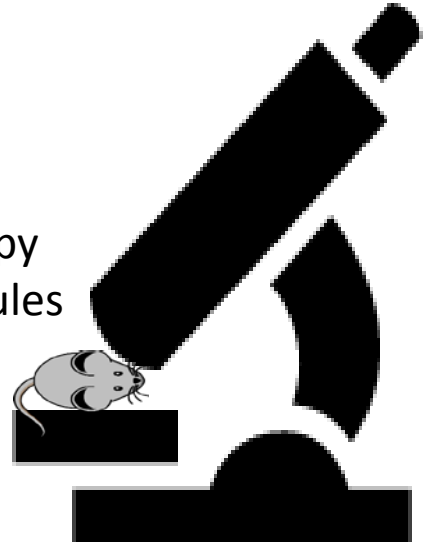
Does PAD4 render VWF-platelet strings uncleavable for ADAMTS13?

r-huPAD4 / r-huADAMTS13

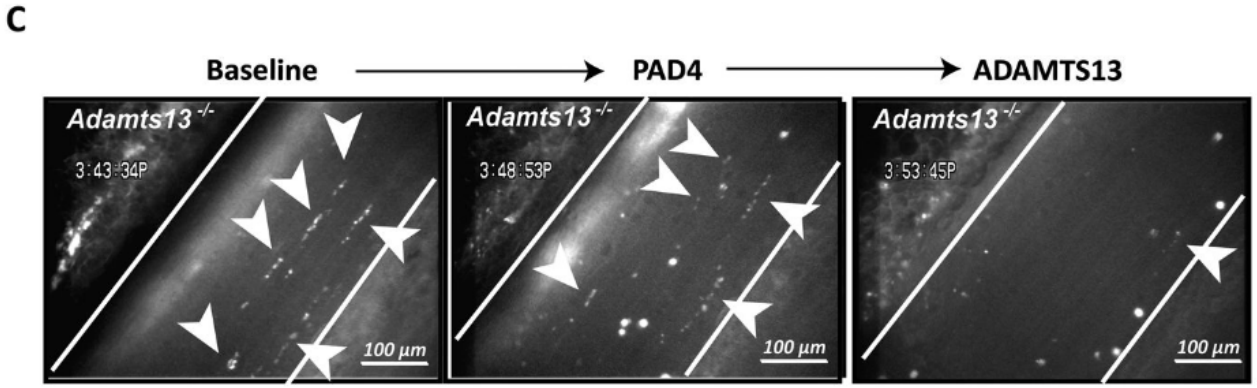


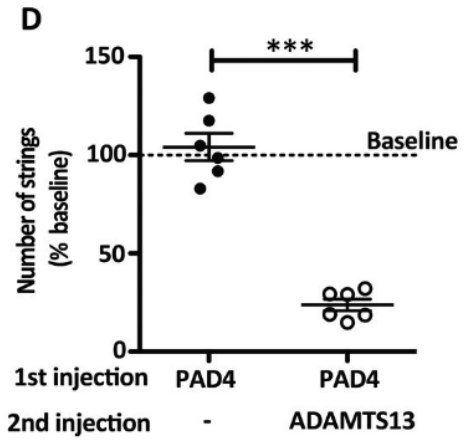
ADAMTS13 $^{-/-}$

intravital microscopy
of mesenteric venules



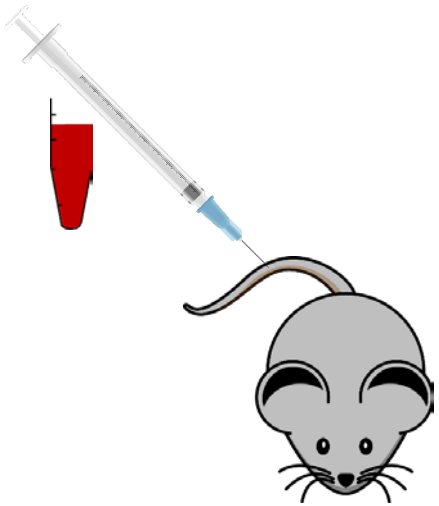
→ ADAMTS13 deficiency linked to pre-activation of END
→ release of VWF & accumulation on mesenteric venules

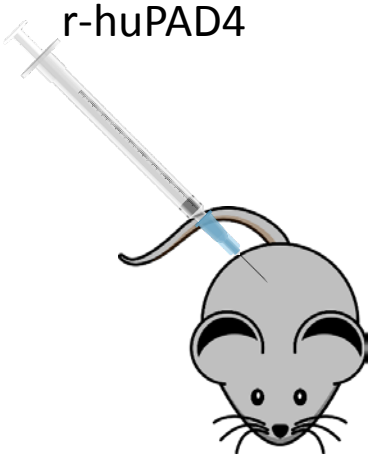




- PAD4 treatment did not affect „baseline“ VWF-platelet string formation
- Subsequent ADAMTS13 treatment could resolve endogenous VWF-platelet strings

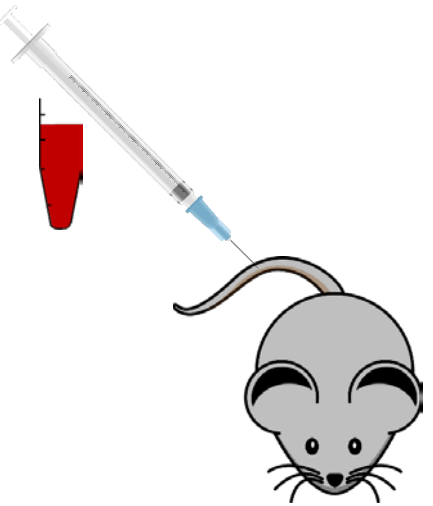
Does PAD4 directly affect ADAMTS13 activity in circulation?



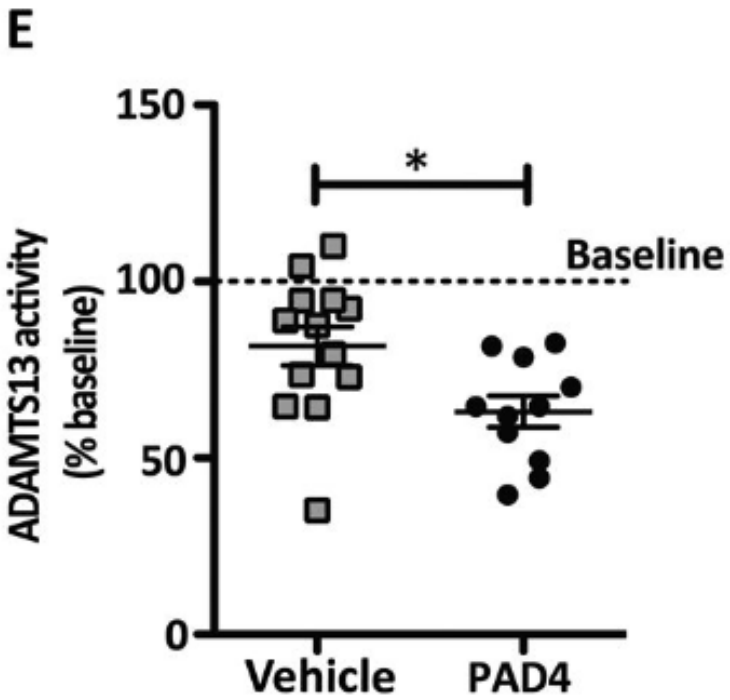


r-huPAD4

WT



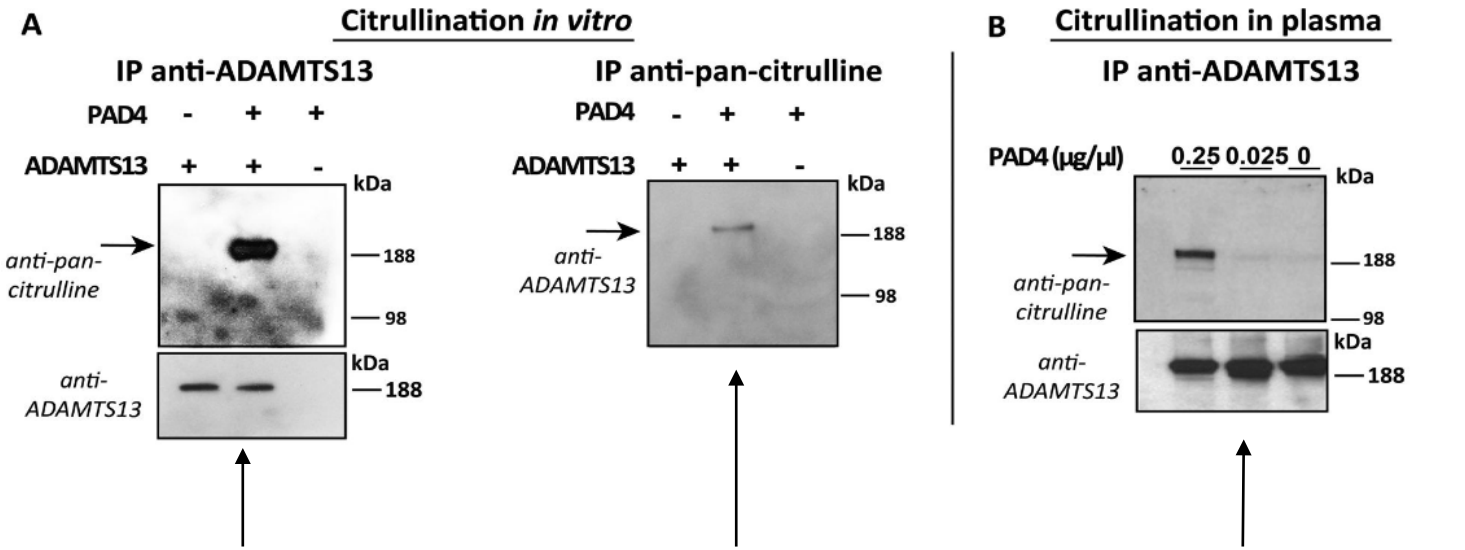
plasma samples → r-huPAD4 injection → plasma samples



- presence of r-huPAD4 reduces endogenous ADAMTS13 activity

Conclusion so far…

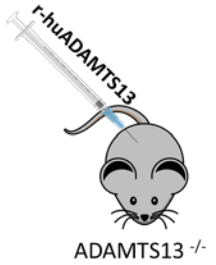
- Injection of PAD4 leads to VWF-platelet string formation and reduces ADAMTS13 activity
- PAD4 inhibition preserved natural clearance
- r-huADAMTS13 treatment cleaved VWF-platelet strings despite prior r-huPAD4 treatment
- PAD4 presence in circulation reduces ADAMTS13 activity

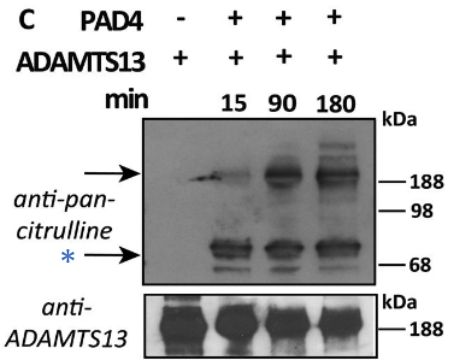


Immunopurification with **α-ADAMTS13** Antibody

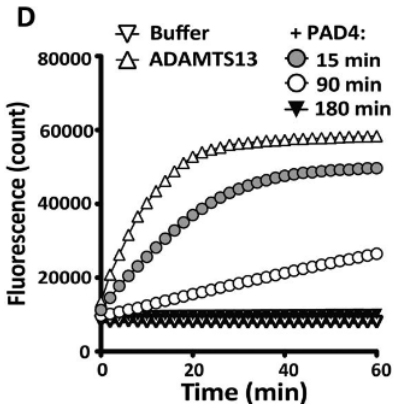
Immunopurification with **α-pan-citrulline** Antibody

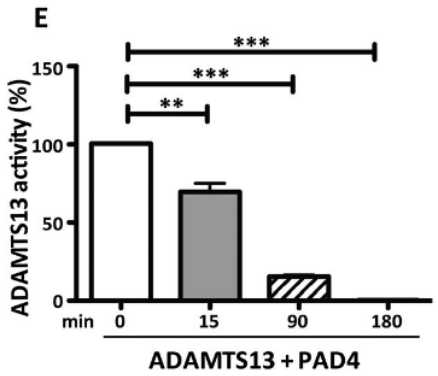
ex vivo PAD4-treated plasma



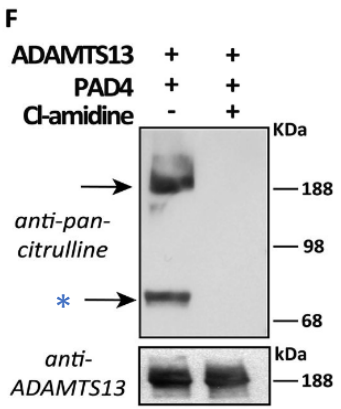


incubation of r-huPAD4 with r-huADAMTS13 over time

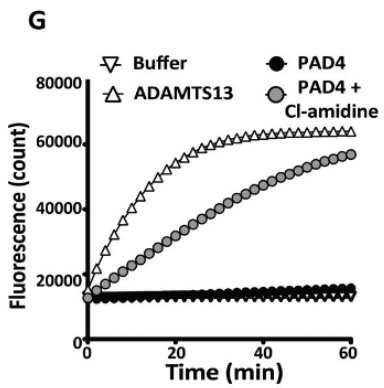


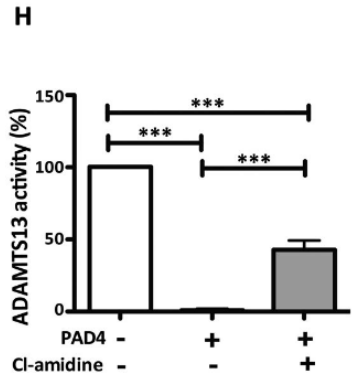


FRETS-VWF73 activity assay



incubation of r-huPAD4 with r-huADAMTS13 and PAD4-inhibitor





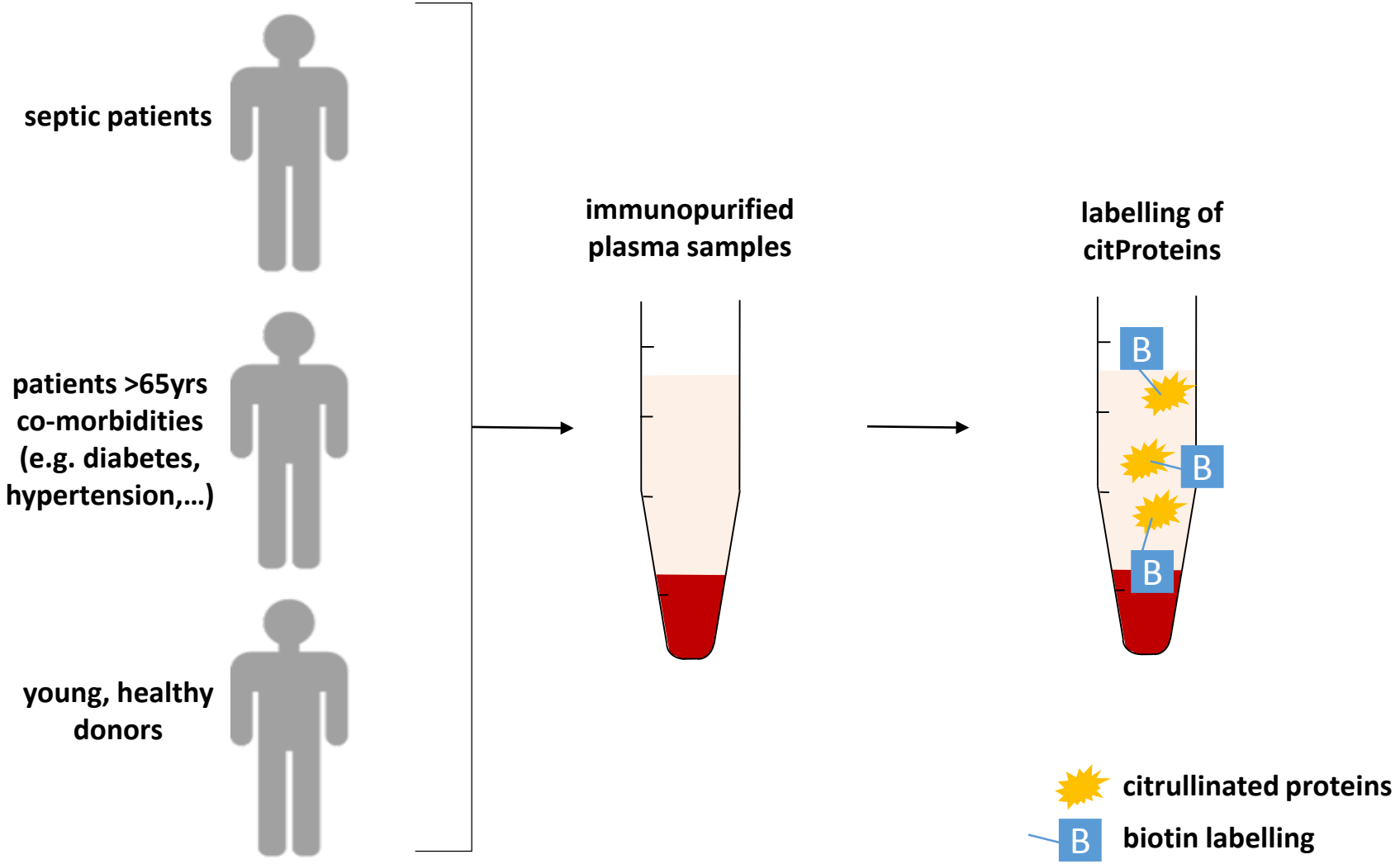
FRETS-VWF73 activity assay

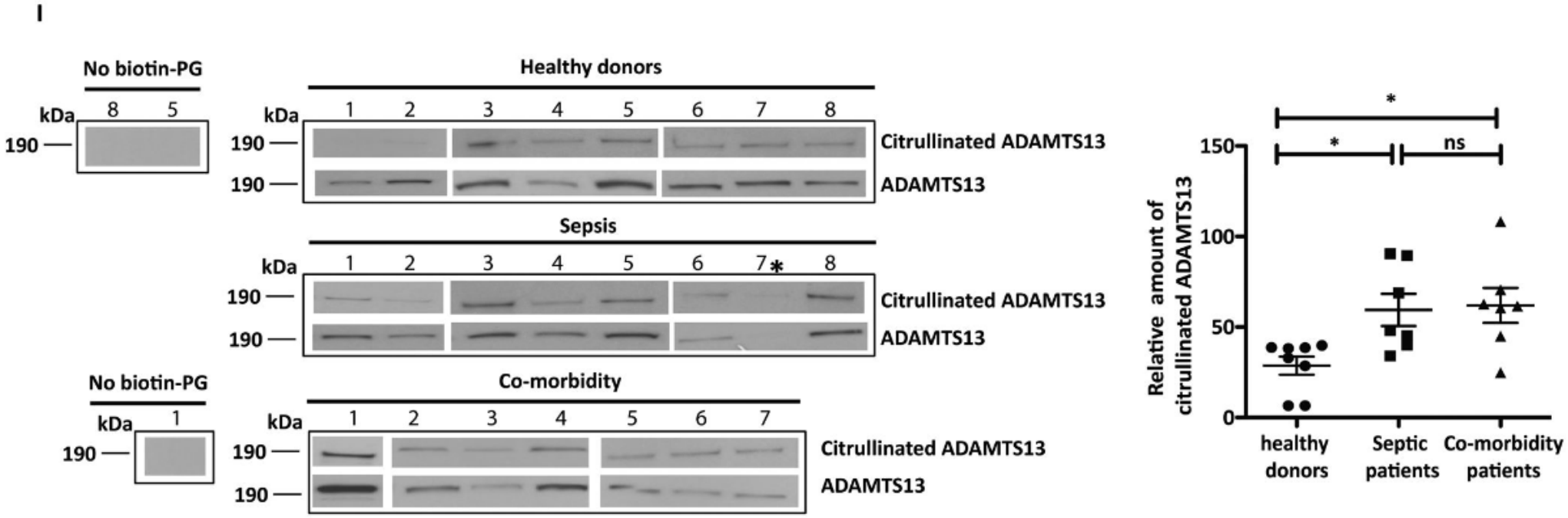
* r-huPAD4; undergoes auto-citrullination

Conclusion so far…

- PAD4 citrullinates ADAMTS13 *in vitro* and *ex vivo*
- citrullination of ADAMTS13 significantly reduces its enzymatic activity
- enzymatic activity of ADAMTS13 was partly recovered by specific PAD4 inhibitor

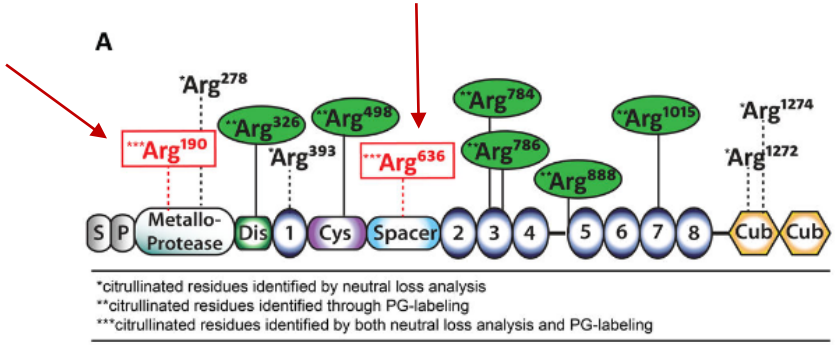
Does citrullination of ADAMTS13 occur during inflammatory events?





- significantly higher levels of citADAMTS13 in septic & older patients with co-morbidities

Identification of citrullinated arginine residues by tandem mass spectrometry

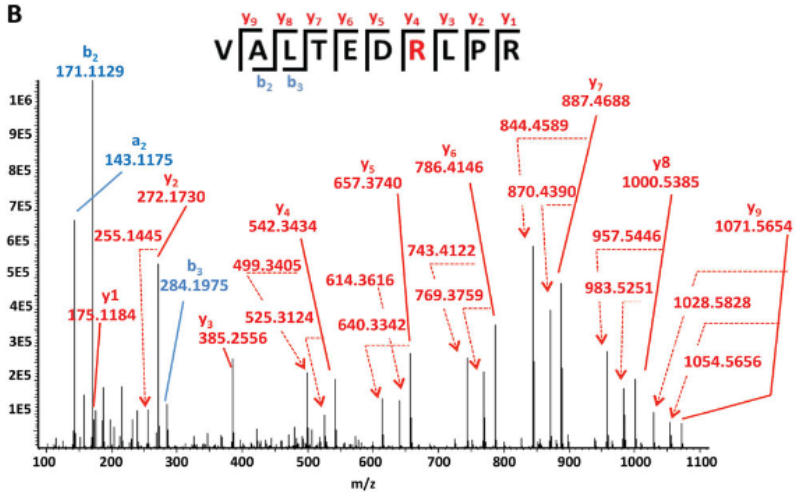


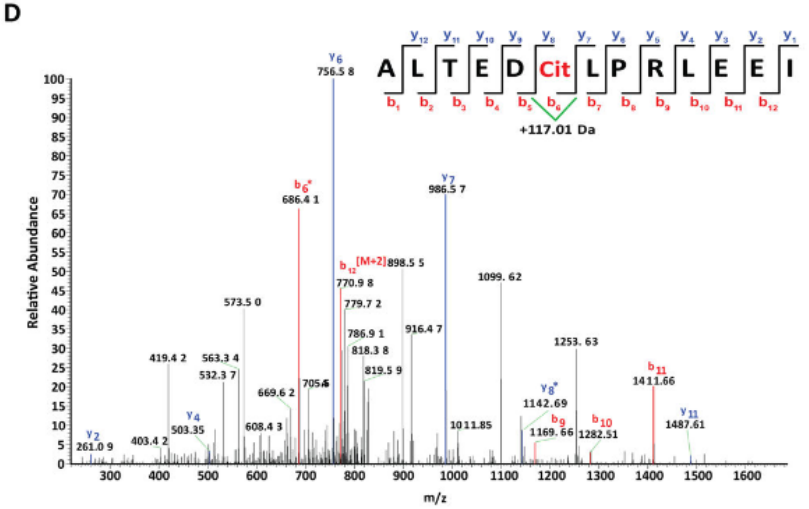
C

PG-Labelling

Residue	Peptide	Charge	Calculated Mass	Experimental Mass
190	RFDLELPDGN(Cit)*QV	2	838.407	838.407
326	TFA(Cit)*EHL	2	495.738	495.737
498	MK(Cit)*GDSFLDGT	2	620.783	620.782
498	(Cit)*GDSFLDGTR	2	672.301	672.298
636	ALTED(Cit)*LPRLEEI	2	971.026	970.995
784-786	LPPA(Cit)*C(Cit)*A	2	587.771	587.758
888	SAGEKAPSWGSI(Cit)*TGAQAA	2	1029.998	1029.994
1015	CSLEPCPP(Cit)*WKV	2	823.379	823.378

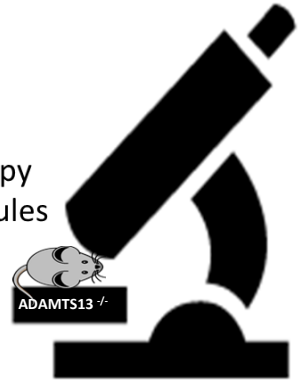
*Indicates the additional mass of PG (+117.010 Da)



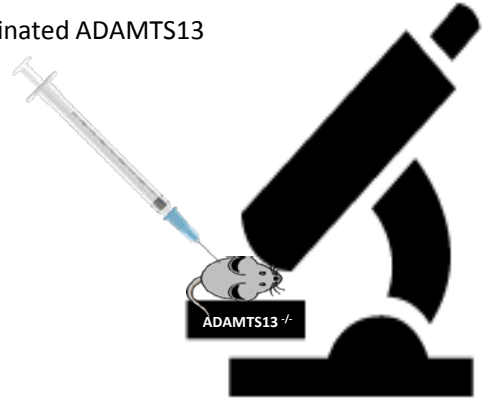


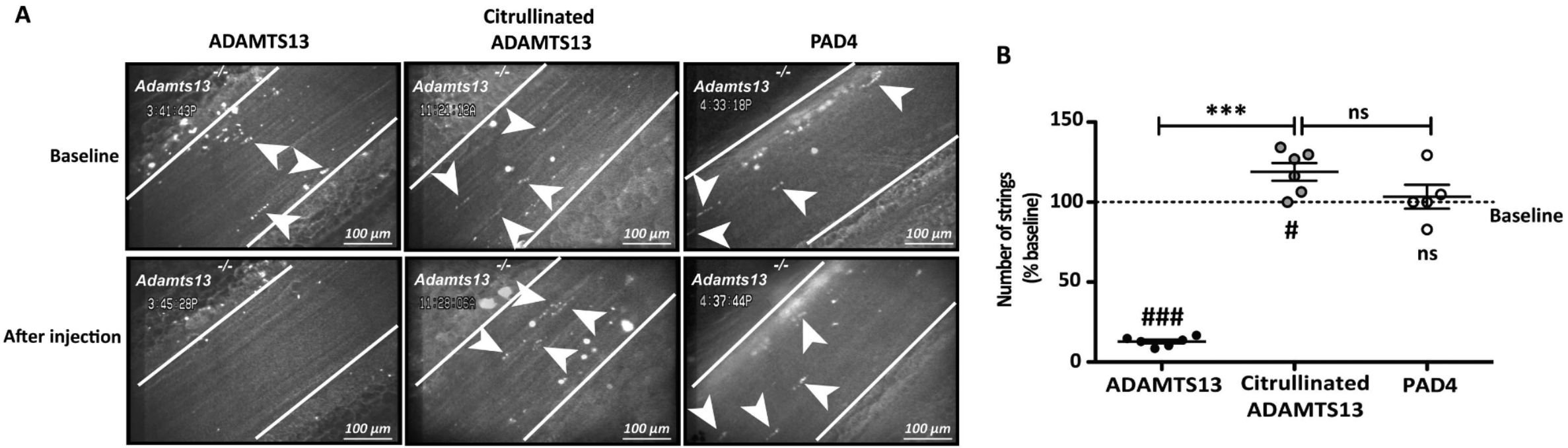
Does citrullination of ADAMTS13 interfere with VWF-platelet string clearance *in vivo*?

intravital microscopy
of mesenteric venules

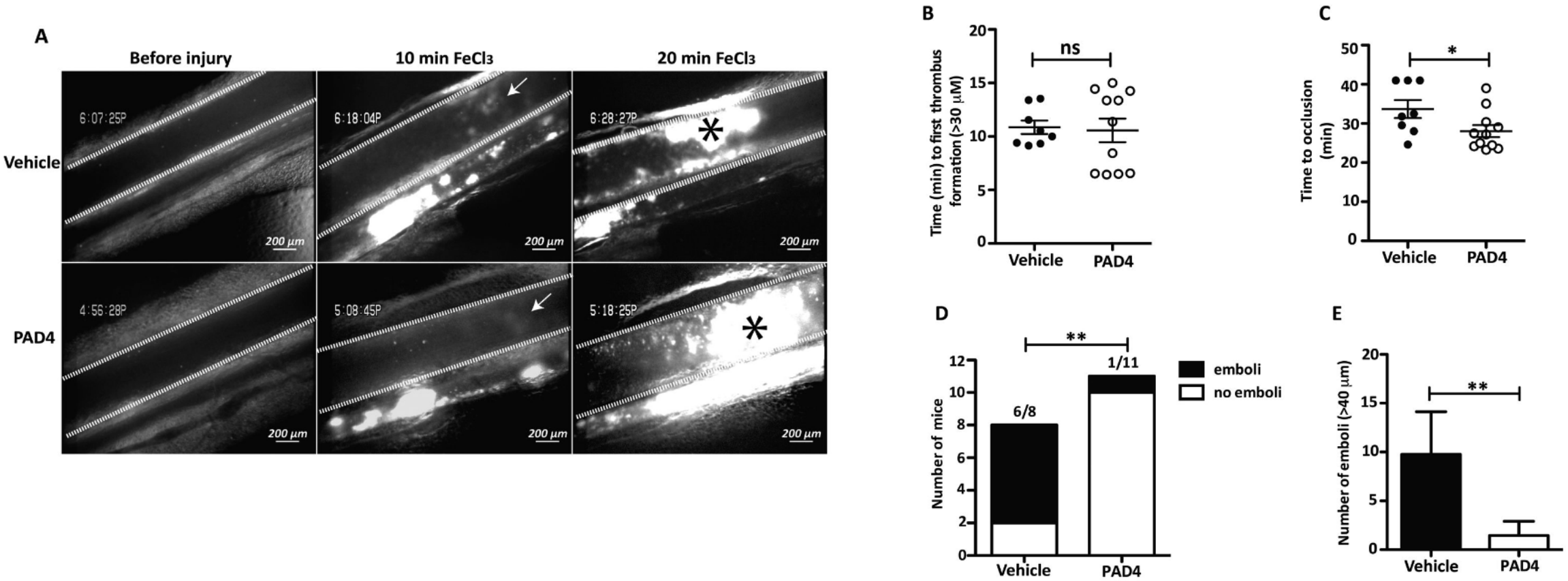


citrullinated ADAMTS13





- spontaneous VWF-platelet string formation in ADAMTS13-/- mice was abolished by r-huADAMTS13 injection
- citrullinated ADAMTS13 could not clear VWF-platelet strings
- PAD4 injection did not alter VWF-platelet string formation



- no difference in length of time to form thrombus
- r-huPAD4 treated mice developed occlusive thrombi (accelerted thrombi development by approx. 20%)
- vehicle injected mice more frequently formed large emboli (6/8, within 40min. observation)
- r-huPAD4 injected mice rarely formed large emboli (1/11)

conclusion

- PAD4 in plasma reduces ADAMTS13 activity => VWF-platelet string accumulation on vessel walls
  → pathological thrombus formation
  → inflammatory cell recruitment
- PAD4 citrullinates ADAMTS13 => reduced enzymatic activity
- citrullinated ADAMTS13 fails to clear VWF-platelet strings *in vitro, ex vivo* and *in vivo*