Iron deficiency-induced thrombocytosis increases thrombotic tendency in rats

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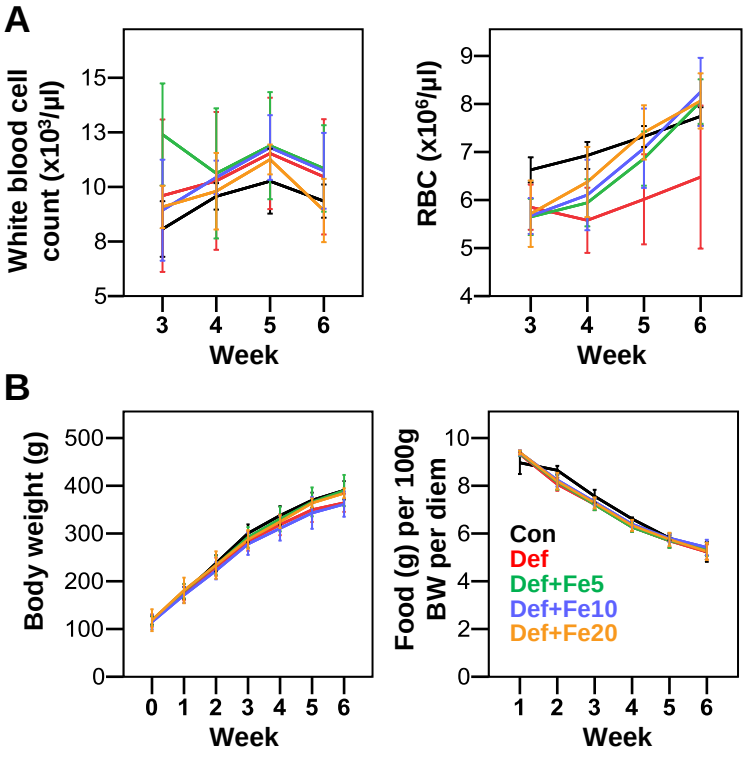
Supplemental Figure 1



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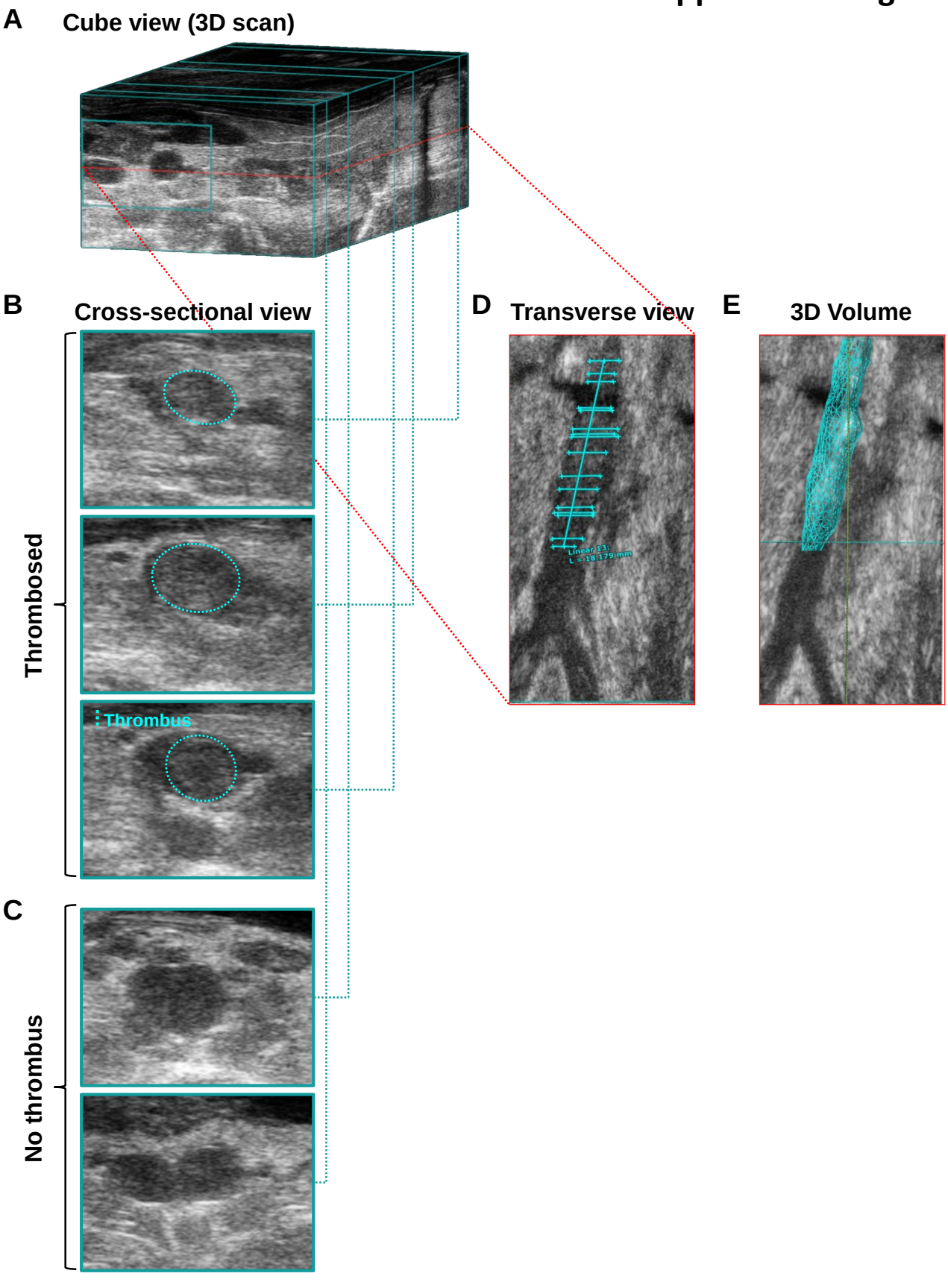

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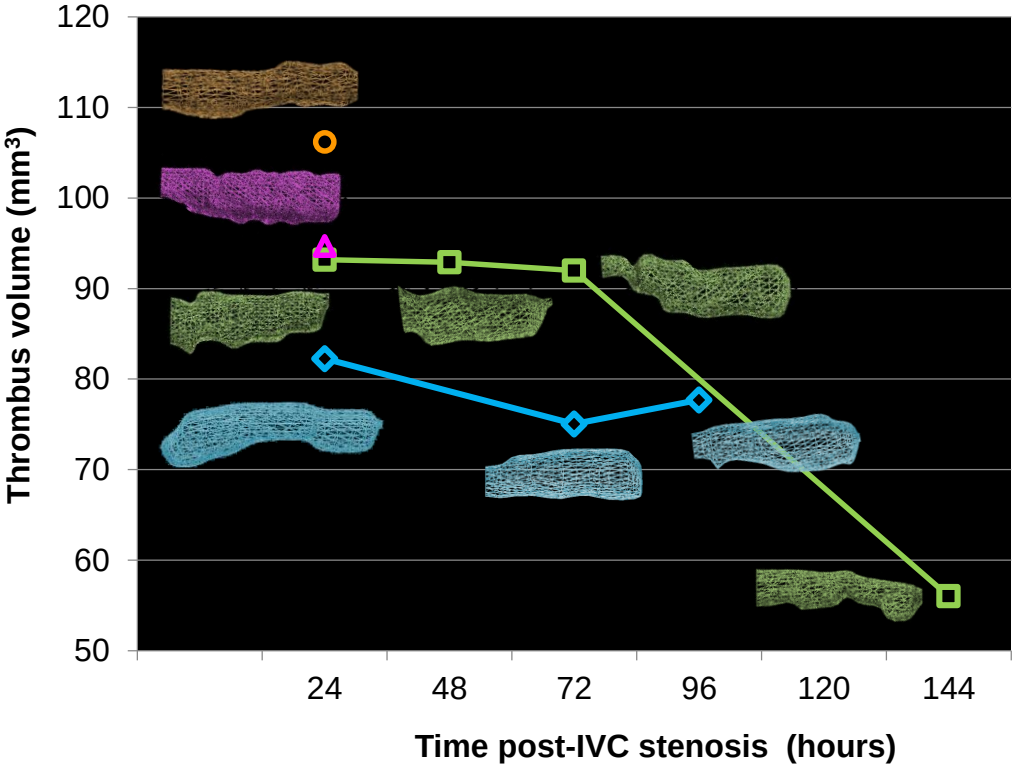

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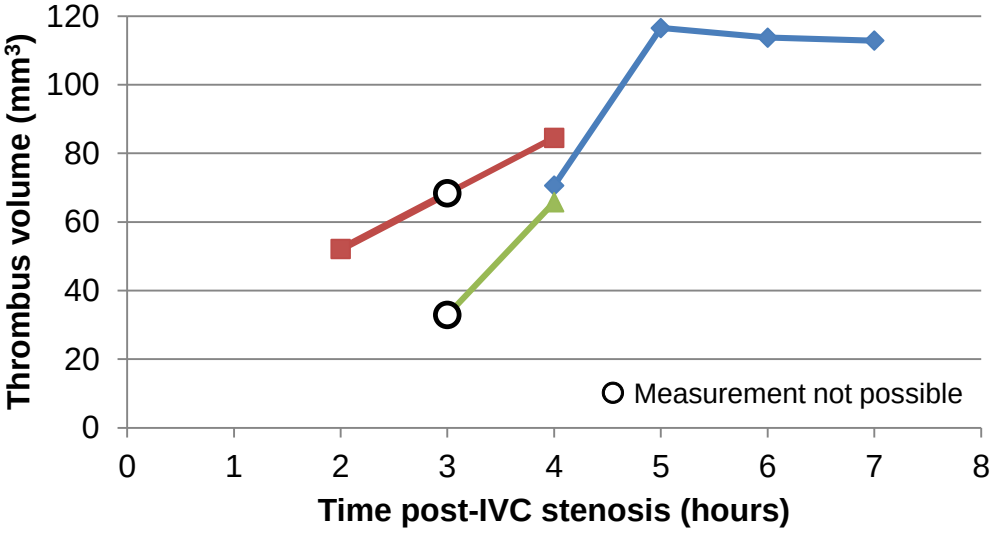

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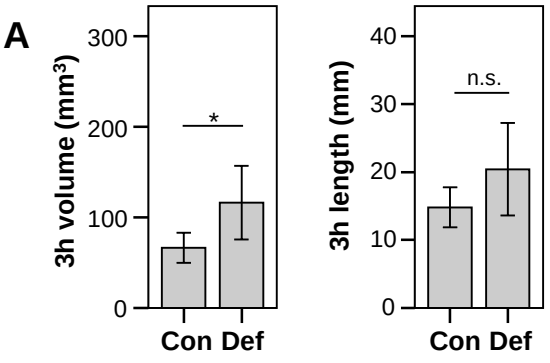

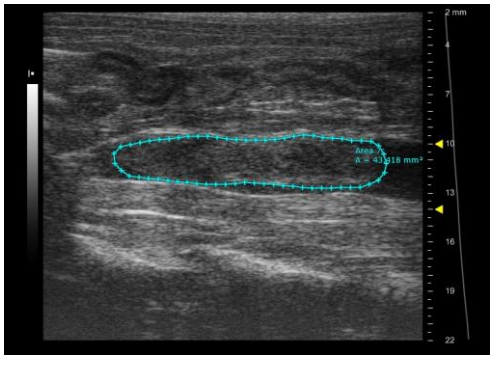

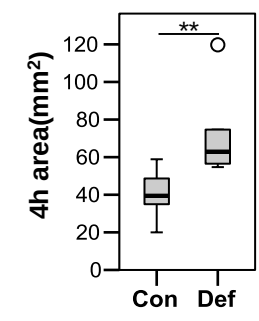

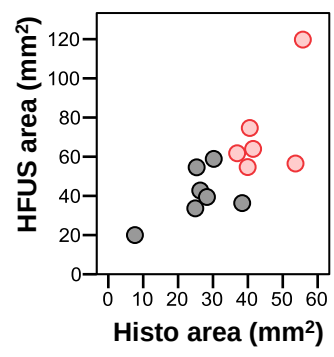

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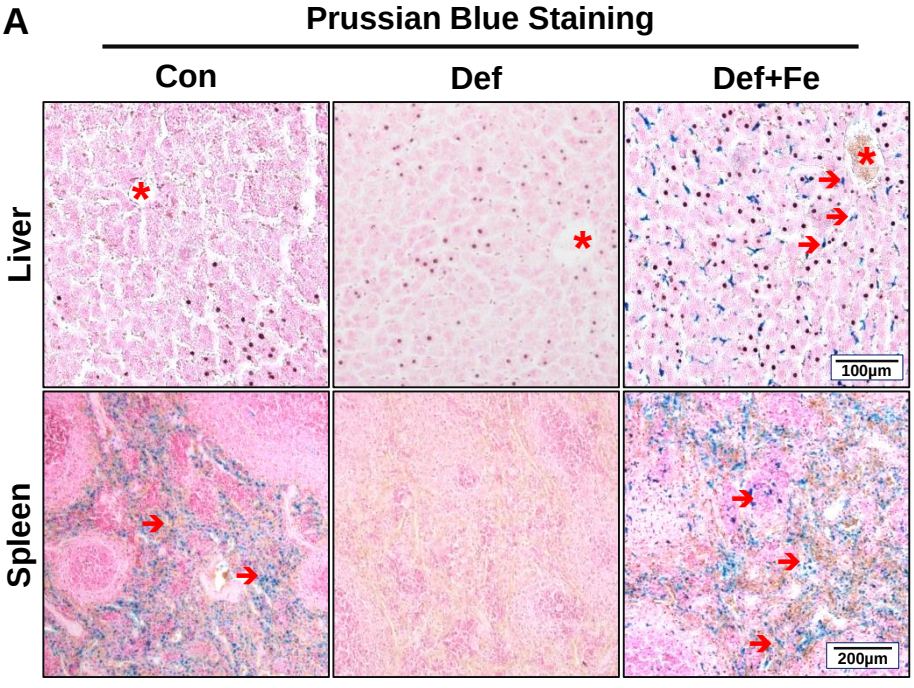

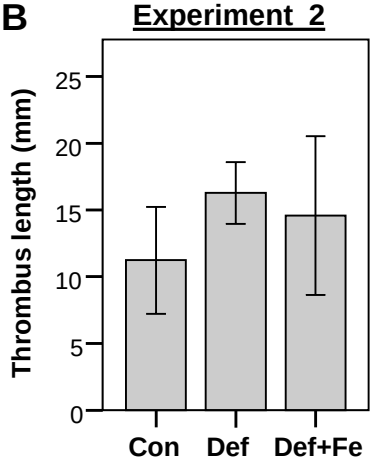

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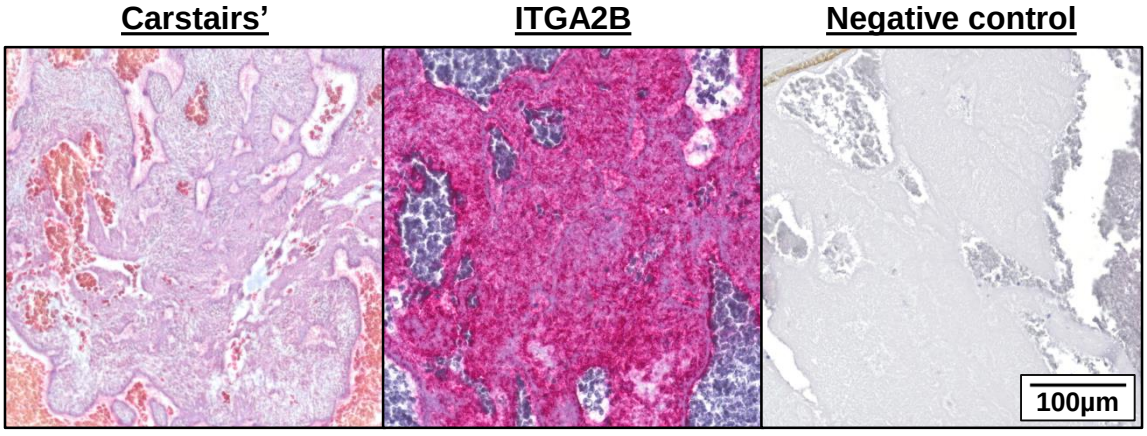

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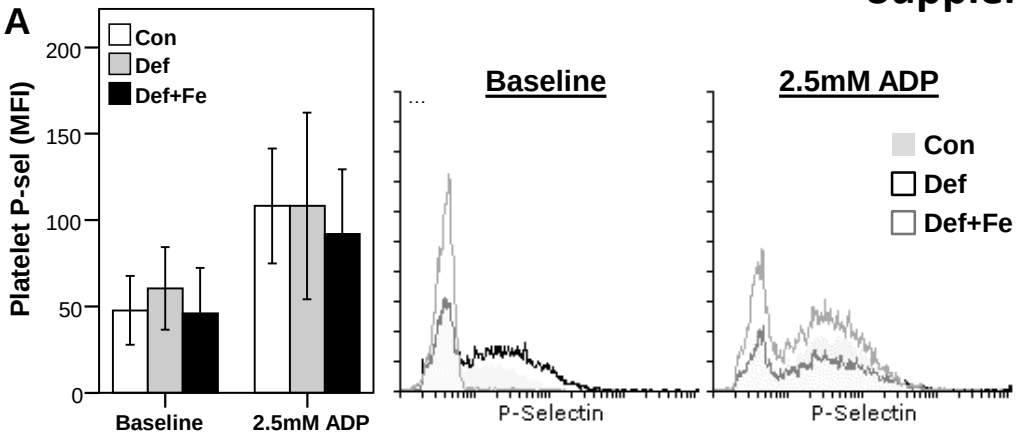

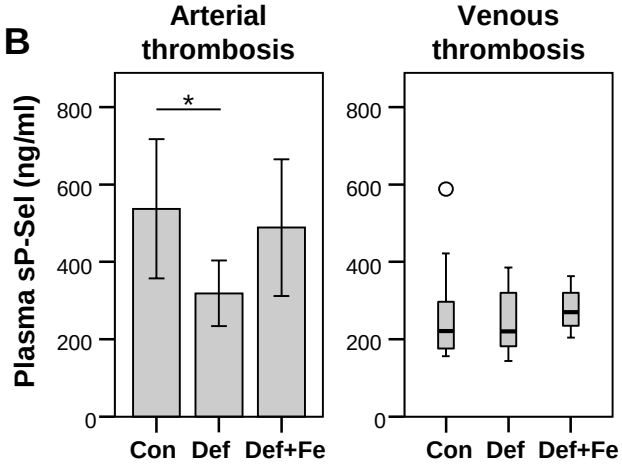

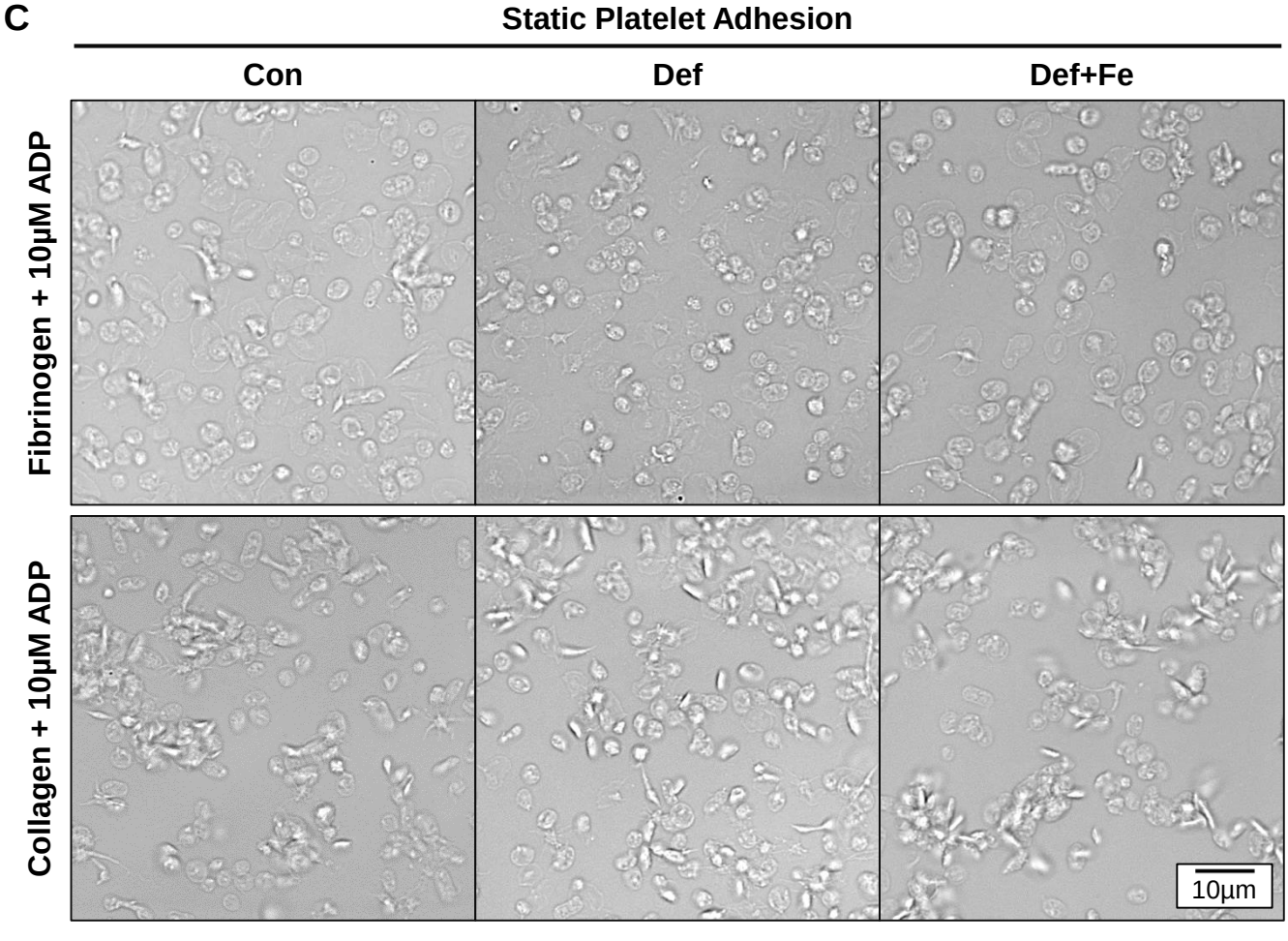

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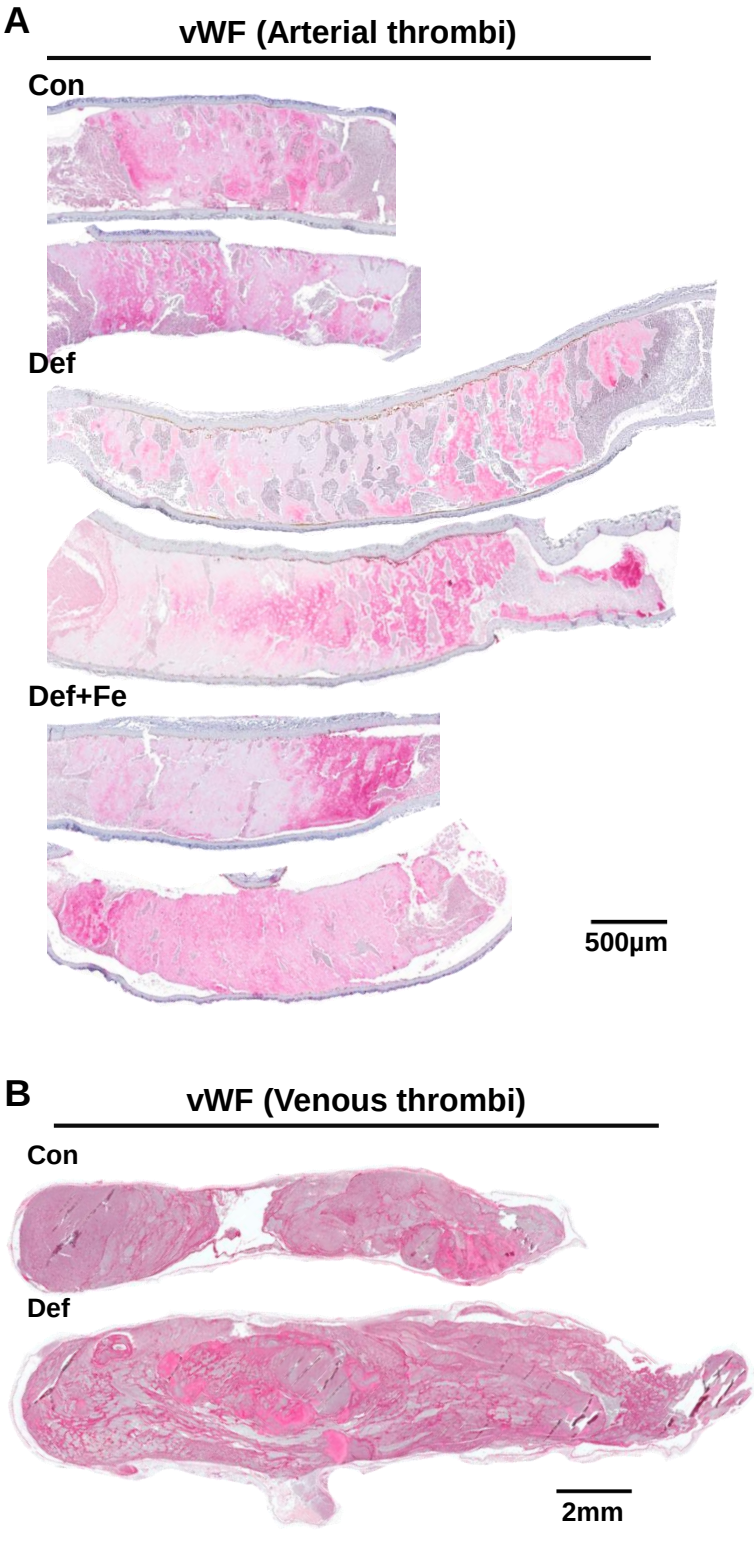

Supplemental Figure Legends

Figure S 1.

A, White blood cell (WBC) and erythrocyte (RBC) counts measured weekly from 3 to 6 weeks after start of experiment (n=4 per group). Error bars: Mean ± SD. **B**, Body weight and amount of food consumed measured weekly from the beginning to end of experiment (n=4 per group). Error bars: Mean ± SD. **C**, Area under the curve (AUC) measured from Multiplate whole blood impedance aggregometry after stimulation with ADP or collagen (n=2-4 per group)

Figure S 2.

A, Cube view of a 3D abdominal scan beginning from the iliac bifurcation until the level of the IVC ligation. **B**, Cross-sectional views of the IVC tracing the thrombus diameter at several points along the length of the thrombus. **C**, Cross-sectional views showing an empty IVC nearing the iliac bifurcation. **D**, Transverse-view of the measured thrombus, each horizontal line depicting a plane in which the thrombus diameter was measured. **E**, 3D volume reconstruction of the thrombus based on the sequential thrombus diameter measurements.

Figure S 3.

A, Thrombus volumes from 24 hours after stenosis of the IVC (n=4). Wireframes depict the 3D reconstruction of the thrombus within the IVC, based upon high-frequency ultrasound scans. **B**, Thrombus volume (n=3) measured in the hours following stenosis of the IVC according to high-frequency ultrasound scans. Empty circles depict timepoints where a clear view of the thrombus could not be acquired.

Figure S 4.

A. Thrombus volume and length at 3 hours post-IVC stenosis on HFUS (n=5 per group). **B**, B-mode image from a longitudinal view of the thrombus inside the IVC. **C**, Thrombus area at 4 hours post-IVC stenosis on HFUS (n=6-7 per group). **D,** Thrombus area measured via HFUS as compared to thrombus area measured histologically.

Figure S 5.

A, Representative images of liver and spleen upon Prussian blue staining.. Red arrows mark positive staining cells. Red asterisks (*) mark central veins. **B**, Venous thrombus length measured by Fiji. Experiment 1: n=9 per group, Experiment 2: n=4-5 per group. *P <0.05.

Figure S 6.

A, Area under the flow curve (AUC) of the percent reduction in flow over time. n=9-11 per group. **B**, Carstairs´ staining correlated with ITGA2B immunohistochemical staining for arterial thrombi

Figure S 7.

A, P-selectin mean fluorescence intensity (P-sel MFI) of platelets before and after stimulation with 2.5mM ADP. Error bars are mean ± SD (n=4-5 per group). Representative histograms overlay treatment groups with and without stimulation with ADP. **B**, Plasma P-selectin concentration by ELISA after arterial thrombosis (n=7-10 per group, mean ± SD) or venous thrombosis (n=4-9 per group). **C**, Representative images of washed platelets adhering to a fibrinogen or collagen coated surface after stimulation with 2µM ADP.

Figure S 8.

Representative images of immunohistochemical staining for von Willebrand factor (vWF) in **A**, Venous and **B**, arterial thrombi.

Supplemental Video 1.

Sample video generated from high frequency ultrasound scan along the length of the inferior vena cava. Video begins from the caudal end at the level of the bifurcation, ending at the ligation near the left renal branch.

Supplemental Tables

Table S 1. Hematological parameters: Dose finding study

		Hb (g/dl)	MCV (fl)	MCH (pg)	RBC ($x10^6$/µl)	HCT%	PLT ($x10^3$/µl)
Week 3	**Con (n=4)**	13.5±0.2	62 (61.8-62.2)	20.4±0.5	6.6±0.3	47.8±1.9	776.3±171.6
	Def (n=16)	9.4±0.8*	53.1 (52.7-54)*	16.5±0.6*	5.7±0.5*	40.6±7.1*	1288.8±403.2*
Week 6	**Con (n=4)**	14.6 (14.5-14.7)	61.8±2.7	18.8 (18.6-19.1)	7.7 (7.6-7.9)	47.2 (46.5-49.1)	779 (736-796)
	Def (n=4)	9.6 (8.3-9.6)	48±0.3*	13.6 (13.2-15.1)	7.1 (5.6-7.3)	34 (27.1-35)	1714 (1555-2916)
	Def+Fe5 (n=4)	11.5 (10.9-11.8)	50.4±1.2*	14 (13.5-14.6)	8 (7.7-8.4)	40.3 (38.9-42.2)	1417 (1159-1735)
	Def+Fe10 (n=4)	12.4 (11.3-13.4)	54.3±3.7*	15.9 (14-16.1)	8.3 (7.8-8.7)	45.4 (42.6-46.8)	953 (883-1121)
	Def+Fe20 (n=4)	13.2 (13-13.59)	57.6±3.5*,†	16.6 (15.8-17.2)	7.8 (7.7-8.4)	46.5 (45.1-47.5)	998 (888-1108)

Median (IQR) or Mean ± SD; * p ≤ 0.05 vs Con; # p ≤ 0.05 vs Def; Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, RBC: Erythrocyte count, HCT: Hematocrit, PLT: Platelet

Table S 2.Hematological parameters: Thrombosis Models

			Hb (g/dl)	MCV (fl)	MCH (pg)	RBC ($x10^6$/µl)	HCT%	PLT ($x10^3$/µl)
Venous Thrombosis Model	Experiment 1	Con (n=7)	13.6±1.5	58.2±2	20.1±0.8	6.8±0.7	39.5±4	888±158
		Def (n=7)	7.8±1.7*	38.6±2.4*	10.6±1.6*	7.4±1	28.4±4.5*	1236±237*
	Experiment 2	Con (n=4)	14.6±0.3	57.9±1.7	20.0±0.3	7.3±0.1	42.2±1.3	916±103
		Def (n=4)	9.2±1.1*	40.0±4*	12.4±0.9*	7.4±0.3	29.7±3.8*	1210±116*
		Def+Fe (n=4)	13.0±0.6*,†	49.2±4.4*,†	16.1±1.4*,†	8.12±0.6*	39.9±3.1*,†	945±136*,†
Arterial Thrombosis Model		Con (n=9)	16.8 (16.3-17)	57.6 (56.9-59.1)	19.7 (19.1-19.8)	8.5 (8.4-8.7)	49.4 (48.5-50)	909 (835-1053)
		Def (n=11)	8.3 (6.9-9.8)*	35.6 (29-37.4)*	9 (8.2-10.3)*	8.9 (8.5-9.1)	32.4 (24.5-35.1)*	1380 (1261-1605)*
		Def+Fe (n=9)	15.1 (15-15.3)*,†	53.6 (51-54)*,†	16.3 (15.9-16.8)*,†	9.1 (9-9.4)*	48.3 (47-49.6)†	973 (937-1054)†

Median (IQR) or Mean ± SD; * p ≤ 0.05 vs Con; # p ≤ 0.05 vs Def; Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, RBC: Erythrocyte count, HCT: Hematocrit, PLT: Platelet

Table S 3. Hematological parameters: Platelet function tests

	Hb (g/dl)	MCV (fl)	MCH (pg)	RBC ($x10^6$/µl)	HCT%	PLT ($x10^3$/µl)
Con (n=5)	15±0.3	58.2±2	19.3±0.7	7.8±0.4	45.4±0.9	931±97
Def (n=5)	8.5±0.7*	37.9±1.1*	9.7±0.6*	8.8±0.4*	33.3±2.1*	1322±66*
Def+Fe (n=5)	14.2±0.8†	52.4±2.2*,†	15.9±0.6*,†	8.9±0.4*	46.7±2.7†	1041±127†

Median (IQR) or Mean ± SD; * p ≤ 0.05 vs Con; # p ≤ 0.05 vs Def; Hb: Hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, RBC: Erythrocyte count, HCT: Hematocrit, PLT: Platelet

Supplemental methods

Animal anesthesia

Animal experiments were carried out at the Department for Biomedical Research of the Medical University of Vienna. Blood counts (Advia 2120i, Siemens, Germany). were taken prior to surgery to confirm the phenotype, and thrombosis induction was performed on the 7th week after the start of experiment. Anesthesia was initiated with 5% isoflurane followed by subcutaneous piritramide (3mg/kg) and intraperitoneal 100mg/kg ketamine and 5mg/kg xylazine in the arterial thrombosis model. Animals were euthanized using an overdose of pentobarbital, after which artery and containing thrombi were collected for histology. For venous thrombosis, ketamine and xylazine were omitted as they increased animal mortality. Surgery was then performed under 1-2% inhalational isoflurane. High frequency ultrasound measurements were likewise performed under 2% inhalational isoflurane, with monitoring of cardiac and respiratory rate, after which the animal was immediately euthanized. For the venous thrombosis model, animals that did not undergo high frequency ultrasound were given drinking water with piritramide for pain relief during the recovery period. Four hours post-ligation, animals were euthanized and IVC with thrombi were collected.

Static Adhesion Assay

Wells were covered with 1mg/ml fibrinogen (Sigma-Aldrich) or 1mg/ml collagen (Thermofisher, Massachusets, USA) and left overnight at 37℃. Wells were then washed with PBS, air dried, and stored at 4℃ until usage. Blood was collected via terminal cardiac puncture from anesthetized animals (ketamine and xylazin) into standard citrate tubes and centrifugated at 200g for 15 minutes with no brake. Upper ¾ of supernatant was collected and centrifugated at 2200g for 10 minutes.

Supernatant was removed and platelet pellet resuspended in Tyrode´s buffer (134 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 20 mM HEPES, 5 mM glucose, 1 mM MgCl) supplemented with Prostaglandin E1 (final 1µM), Heparin (final 10U/ml) and Apyrase (final 100U/ml) and incubated for 15 minutes at 37℃. Prostaglandin E1 was added again, and samples centrifugated at 1900g. Platelets were resuspended at 1x 10^8/ml in Tyrode´s buffer supplemented with $CaCl_2$. ADP (final 10µM) was added to separate samples. After incubation for 1 hour at 37℃, samples were gently washed with PBS, imaged, and fixed with 4% paraformaldehyde (Sigma-Aldrich). Wells were washed with D-PBS (Gibco, Thermofisher), and then imaged on an Olympus IX81 inverted microscope.[1]

Flow cytometry

Sublingual blood was collected in heparin tubes (Greiner Bio-One, Kremsmuenster, Austria) for ADP tests, and for thrombin tests in citrate tubes (Greiner Bio-One) with addition of H-Gly-Pro-Arg-Pro-NH2 acetate salt (Bachem, Germany) to prevent clot formation. Platelets were labelled with CD61-FITC (ebioscience, Thermofisher) and platelet activity measured using CD62P-PE (ebioscience, Thermofisher). Samples were diluted in Tyrode's buffer, with $CaCl_2$ was added to citrated samples for recalcification. After 15 minutes, samples were fixed with 0.2% Paraformaldehyde to stop staining and platelet activation. Baseline and post-agonist stimulation (0.016U/ml, 0.25U/ml Thrombin, 2.5mM ADP, Sigma) expression of CD62P were evaluated on a Cell Lab Quanta SC (Beckman Coulter, California, USA)(see Supplementary methods for more detail).[2]

Histology

Liver, spleen, and thrombi in their respective vessels were fixed 24 hours in 10% neutral buffered formalin (Sigma-Aldrich, Missouri, USA), dehydrated and embedded

in paraffin, and cut at 3µm thickness. Immunohistochemistry was performed using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California, USA), with ImmPACT VectorRed as chromogen (Vector Laboratories) and counterstaining with Mayers hematoxylin (Merck, New Jersey, USA). Antigen retrieval was heat-mediated in citrate retrieval buffer (Dako, Agilent, California, USA). Antibodies used were against ITGA2B (1:200, Abcam, Cambridge, UK), and vWF (1:1000, Sigma-Aldrich). Carstairs staining was performed as previously described[21]. Thrombi were imaged using an Olympus BX61US Slide Scanner and analyzed using Fiji/ImageJ[22,23]. Liver and spleen were stained using the standard Perls Prussian blue staining and imaged using an Olympus BX41 microscope.

Analysis of ITGA2B-positive area

For the anaylsis of ITGA2B area, whole thrombus images were subjected to color deconvolution[3] on Fiji[4] to isolate pink areas corresponding to positive staining. This was then converted to a binary image and the corresponding area quantified.

Prussian blue staining

After deparaffinization and rehydration, sample cuts were immersed in staining solution (10% HCl, 5% Potassium ferrocyanide, Sigma) for 20 minutes and then counterstained with nuclear fast red for 3 minutes. After drying and mounting, samples were imaged on an Olympus BX41 microscope.

Tail bleeding assay

Animals were anesthetized with ketamine and xylazine. Tails were transected 4mm from the tip and submerged in a pre-weighed volume of warm 0.9% NaCl. Time until complete cessation of blood flow was measured, and weight of blood calculated from the resultant weight of the NaCl.

ELISA

Citrated blood was collected via cardiac puncture. Platelet poor plasma was generated by 2 centrifugations at 2000g and stored at –80C until use. Plasma concentration of P-selectin (Cusabio, Texas, USA) was evaluated using ELISA.

Real time PCR

RNA was extracted from liver tissue using TRIzol (Thermofisher) and reverse transcribed using the High Capacity cDNA reverse transcription Kit (Applied Biosystems, Thermofisher). Hepcidin (Qiagen, Germany) expression was evaluated using real-time PCR based on SYBR-green (Applied Biosystems, California, USA) on an AB7500 (Applied Biosystems) and normalized to HPRT1 (Qiagen).

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