Evidence of protective effects of recombinant ADAMTS13 in a humanized model of sickle cell disease

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Supplementary Methods

Mice and efficacy study design. Townes mice, humanized for human sickle hemoglobin ($Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1,HBB*)Tow}$, HbS, SS mice) or human normal hemoglobin ($Hba^{tm1(HBA)Tow}$ $Hbb^{tm3(HBG1,HBB*)Tow}$, HbA, AA mice) were either directly supplied by The Jackson Laboratory (Jackson Laboratories, US/ Charles River Laboratories, Sulzfeld, Germany) or bred in Verona University (CIRSAL). Mice were housed in isolated ventilated cages containing wood fiber bedding, under controlled conditions for room temperature 20–24 °C, and 40-70% relative humidity, and a 12h:12h light-dark cycle. Chow and municipal water were available ad libitum for all animals. At study start mice were randomly assigned to study groups by body weight.
Animals with either skin lesions or Hb levels < 6 g/dL were excluded. Animals were anesthetized with isofluorane, and randomly assigned to experimental groups. Whole blood was collected from each mouse via retro-orbital venipuncture by heparinized microcapillaries. In anesthetized animals, organs were immediately removed and divided into two and either immediately frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin for histology.

Plasma assays. Human ADAMTS13 antigen was determined by an ELISA method using in-house developed anti-ADAMTS13 antibodies. Microtiter plates were coated with polyclonal guinea pig anti-human ADAMTS13 IgG followed by blocking of the non-specific binding sites with a solution containing human serum albumin. Test samples were then incubated in a total volume of 100 µL per well. After several washing steps, specific binding was detected by the addition of polyclonal rabbit anti-human ADAMTS13 antibody followed by HRP conjugated donkey anti-rabbit IgG and addition of Ultra TMB substrate. The reaction was stopped by 1.9 M H_2SO_4 and the OD was read at 450 nm and 620 nm (background correction). Samples were measured against a preparation of rADAMTS13 used as reference standard.
Human ADAMTS13 activity was determined by a fluorescence resonance energy transfer (FRET) assay using “FRETS-VWF73” substrate (Peptanova), a synthetic fluorogenic peptide of 73 amino acids derived from the VWF A2 domain covering the cleavage site of ADAMTS13 (38). Samples were diluted (in 100 µL total volume), transferred to a microtiter plate and the reaction was started by addition of the

substrate (100 µL FRETS-VWF73; 2 µM final concentration). Fluorescence was measured every two minutes for 60 minutes at λ_{ex} = 340 nm and λ_{em} = 450 nm at 30°C, against a reference standard of pooled human plasma from George King Bio-Medical with 1 U/mL ADAMTS13 concentration.
Mouse VWF antigen was determined by a commercially available ELISA kit (Asserachrom VWF:Ag, Diagnostica Stago, Asnières sur Seine, France).
Mouse VWF activity was assessed by a VWF collagen binding ELISA method (Zymutest VWF:CBA, Hyphen BioMed by CoaChrom Diagnostica GmbH, Austria).
Mouse VWF multimer analysis was performed using low resolution agarose gel electrophoresis in combination with immunostaining by an anti-human VWF antibody (Hydragel system, Sebia, Lisses, France).

Determination of plasma VWF and ADAMTS13 antigen and activity under normoxia and hypoxia. Blood samples were collected from both SS and AA male mice under normoxic conditions, and VWF and ADAMTS13 antigen and activity in plasma were analyzed. The effect of hypoxic conditions on these parameters was also investigated. Hypoxic conditions (5 hours at 7% oxygen) followed by normoxic conditions (1 hour at 21% oxygen) were used to trigger VOC in SS mice.

Western blot analysis. Frozen lung, kidney or aorta from AA and SS mice were homogenized and lysed with ice cold lysis buffer (150 mM NaCl, 25 mM bicine, 0.1% SDS, 2% Triton X-100, 1 mM EDTA, protease inhibitor cocktail tablets, 1 mM Na3VO4 final concentration) followed by centrifugation for 30 minutes at 4°C at 12,000g. Proteins were quantified then separated by mono-dimensional SDS-PAGE. Proteins were transferred to nitrocellulose membranes for western blot analysis with specific antibodies: Phospho- Ser536 NF-kB p65 (pNF-kB p65, # 3031, Cell Signaling Technology), NF-kB p65 (clone C22B4, Cell Signaling Technology), VCAM-1 (R and D Systems, Minneapolis, MN, USA), ICAM-1 (clone YN1.17) was kindly gifted by dr. Gabriella Constantin (University of Verona), Endothelin-1 (ET-1, AbCam, Cambridge, UK), Thromboxane Synthase (TXAS, Cayman Chemical, Michigan, USA), Heme oxygenase 1 (HO-1, clone A-3, Santa Cruz Biotechnology, Inc), E-Selectin (clone H-300, Santa Cruz Biotechnology, Inc), eNOS (NOS3, clone C-20, Santa Cruz Biotechnology, Inc), iNOS (NOS2, clone C-11, Santa Cruz Biotechnology, Inc). Anti-GAPDH (clone D6, Santa Cruz Biotechnology) was used as

loading control. Secondary donkey anti-rabbit IgG and anti-mouse IgG HRP conjugates were from GE Healthcare Life Sciences, secondary donkey anti-goat HRP conjugate was from Santa Cruz Biotechnology, Inc. Blots were developed using the Luminata Forte Chemiluminescent HRP Substrate from Merck KGaA, and images were acquired using the Alliance Q9 Advanced imaging system (Uvitec, UK). Densitometric analyses were performed with the Nine Alliance software (Uvitec, UK). Oxidized proteins were monitored by using the Oxyblot Protein Oxidation Detection Kit (EMD Millipore) following the manufacturer instructions.

Quantitative RT-PCR. mRNA was isolated and reverse transcribed into high-purity cDNA using lMACS One-step cDNA Kit according to the manufacturer's instructions (Miltenyi Biotec). qRT-PCR was performed by SYBR Green PCR Master Mix (Applied Biosystems) by using the Applied Biosystems Model 7900HT Sequence Detection System. Relative gene expression was calculated by using the 2-DCt method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold. The DCt was computed by calculating the difference of the average Ct between the X-gene and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Detailed primer sequences are shown in Supplementary Table 1S.

Supplementary Table 1S. List of primers used in quantitative real-time PCR.

Gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
Il-1b	GCTGAAAGCTCTCCACCTCAA	TTGTCGTTGCTTGGTTCTCCT

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1S. Human and mouse ADAMTS13 show high homology in crucial protein domains. (A) Schematic representation of the ADAMTS13 functional domains. The percentage of identity and similarity for each domain, aligning mouse and human ADAMTS13 by the "Matcher software" (https://www.ebi.ac.uk), are shown. PP: pro-peptide and signal peptide; TSP-1: thrombospondin type-1 motif; Cys: cysteine–rich domain; CUB: Complement components C1r and C1s. (**B)** Aligned sequences of the aminoacidic residues of mouse and human ADAMTS13 performed using the ClustalW2 software (www.ebi.ac.uk). Different domains are identified by color code; the crucial residues for ADAMTS13/VWF interactions are highlighted in yellow. * perfect alignment; ":" strong similarity; "." weak similarity.

Supplementary Figure 2S. Effects of hypoxia on VWF antigen and VWF activity/antigen ratio. (A) VWF multimer gel analysis of SS (n=4) and AA (n=3) mice. **(B)** Experimental study design to characterize ADAMTS13 and VWF in the humanized sickle cell mice under normoxia (21% oxygen) and hypoxia (5 hours at 7% oxygen followed by 1 hour of reoxygenation at 21% oxygen). **(C)** Plasma from AA or SS mice in normoxic (21% oxygen) or SS mice in hypoxic (7% oxygen) conditions were analyzed for VWF activity and antigen. The VWF activity/antigen ratio was calculated. (n=5-6, age-matched male mice). * $p < 0.05$ vs AA 21%. P values were calculated using unpaired one-tailed t-test with Welch's correction. **(D)** Experimental study design to mimic sickle cell related vaso-occlusive crisis in humanized sickle cell mice: 10 hours of hypoxia (8% oxygen) followed by 3 hours of reoxygenation (21% oxygen). Whole blood was collected under normoxia (21% oxygen) 1 hour before and 3 hours after hypoxia. Organs were collected 3 hours after hypoxia. **(E)** Hematocrit in healthy (AA) and SS mice under normoxia and exposed to H/R treated with either vehicle or rADAMTS13; * $p < 0.05$ compared to AA; ^ $p < 0.05$ compared to vehicle. **(F)** Red cell distribution histograms generated for red blood cell volume (RBC Volume) and cell hemoglobin concentration (RBC-HC) of RBCs from humanized AA mice under normoxia and treated with either vehicle or rADAMTS13 and exposed to H/R**.** One experiment representative of six others with similar result is shown. **(G)** Plasma lactate dehydrogenase (LDH) from

AA and SS mice under normoxia and exposed to hypoxia/reoxygenation stress with or without rADAMTS13 treatment; * p <0.05 vs AA; ^ p<0.05 compared to vehicle treated mice. P values were calculated using unpaired one-tailed t-test with Welch's correction.

Supplementary Figure 3S. Effects of recombinant ADAMTS13 on *in vitro* platelets adhesion and systemic inflammatory response. The degree of platelet adhesion is expressed as % of coverage by fluorescently labeled cells over the total surface. **(A)** Time dependent decrease of platelet adhesion (SS mouse blood, n=2) after addition of buffer (black symbols and line) or rADAMTS13 at 200 U/mL (blue symbols and line). **(B)** Representative images of calcein labeled platelets (SS mouse blood, n=2) adhering in the perfusion system at 5 and 150 seconds after addition of buffer (top) or rADAMTS13 (bottom), respectively. **(C)** C-reactive protein (CRP) plasma levels in AA and SS mice exposed to H/R: hypoxia (8% oxygen; 10 hours), followed by reoxygenation (21% oxygen; 3 hours) treated with vehicle or rADAMTS13. Data are mean ± SD (n=3-6, age-matched male and female mice) * p <0.05 compared to AA, ^ p <0.05 compared to vehicle treated animals, one-way ANOVA for repeated measures.

Supplementary Figure 4S. Effects of recombinant ADAMTS13 on local inflammatory response, inflammatory lung vascular activation in SS mice exposed to H/R stress. (A) Densitometric analysis of immunoblots reported in Figure 2C. Data are presented as means ±SEM (n=3 age-matched male female mice in each group); ^ p <0.05 compared to normoxia, * p <0.05 compared to AA; $^{\#}$ p <0.05 compared to vehicle treated animals by two-tailed unpaired Student t-test with Bonferroni correction for multiple comparisons. DU: densitometric units. **(B)** Il-1 mRNA values in lung tissues (normalized to GAPDH) from SS mice in H/R treated with vehicle or rADAMTS13. $^{\#}$ p<0.05 compared to vehicle treated animals. Each sample is a pool from 3 mice. Representative of three independent experiments **(C)** Densitometric analysis of immunoblots reported in Figure 2D. Data are presented as means ± SEM (n=3 in each group); ^ p <0.05 compared to normoxia, * p <0.05 compared to AA; $^{\#}$ p <0.05 compared to vehicle treated animals by two-tailed unpaired Student t-test with Bonferroni correction for multiple comparisons. DU: densitometric units.

Supplementary Figure 5S. Effects of recombinant ADAMTS13 on lung E-selectin expression in SS mice exposed to H/R stress

Left panel. Immunoblot analysis using specific antibodies against E-Selectin in lung from AA and SS mice in normoxia or exposed to hypoxia (8% oxygen; 10 hours), followed by reoxygenation (21% oxygen; 3 hours) treated with vehicle or rADAMTS13 (2940 U/kg). GAPDH serves as protein loading control. One representative gel from 3 with similar results is shown. **Right panel.** Densitometric analysis of the immunoblot. Data are presented as means ±SEM (n=3 age matched male and female mice in each group); ^ $p < 0.05$ compared to normoxia, * $p < 0.05$ compared to AA; # $p < 0.05$ compared to vehicle treated animals by one-way ANOVA for repeated measures. DU: densitometric units.

Supplementary Figure 6S. In SCD mice, rADAMTS13 reduces H/R induced lung oxidation and modulates e/iNOS expression. (A) Left panel. Lung soluble fraction from AA and SS mice mice under normoxia or exposed to hypoxia (8% oxygen; 10 hours), followed by reoxygenation (21% oxygen; 3 hours) treated with either vehicle or rADAMTS13 (2940 U/kg). Samples were analyzed on 11% SDS-PAGE and subjected to OxyBlot. The carbonylated proteins (1 mg) were detected by treating with 2,4-dinitrophenylhydrazine and blotted with anti-DNP antibody. **Right panel.** Quantification of band area was performed by densitometry and expressed as percentage to AA mice in normoxia. Data are presented as means ±SEM (n=3 age-matched male and female mice in each group); ^ $p < 0.05$ compared to normoxia, * $p < 0.05$ compared to AA; # $p < 0.05$ compared to vehicle treated animals by two-tailed unpaired Student t-test with Bonferroni correction for multiple comparisons. **(B) Left panel.** Immunoblot analysis, using specific antibodies against eNOS and iNOS of lung from AA and SS mice under normoxia or exposed to hypoxia (8% oxygen; 10 hours), followed by reoxygenation (21% oxygen; 3 hours) treated with either vehicle or rADAMTS13 (2940 U/kg). GAPDH was used as protein loading control. One representative gel from 3 with similar results is shown. **Right panel.** Densitometric data are presented as means ±SEM (n=3 age matched male and female mice in each group); ^ $p < 0.05$ compared to normoxia, * $p < 0.05$ compared to AA; # $p < 0.05$ compared to vehicle treated animals by two-tailed unpaired Student t-test with Bonferroni correction for multiple comparisons. DU: densitometric units.

Supplementary Figure 7S. In SCD mice, recombinant ADAMTS13 reduces H/R induced kidney damage and vascular inflammatory activation. (A) Densitometric analysis of immunoblots reported in Figure 3C. Data are presented as means ±SEM (n=3 in each group); ^ p <0.05 compared to normoxia, * p <0.05 compared to AA; # p <0.05 compared to vehicle treated animals. DU: densitometric units. **(B)** Densitometric analysis of immunoblots reported in Figure 3D. Data are presented as means ±SEM (n=3 age matched male female mice in each group); ^ p <0.05 compared to normoxia, * p <0.05 compared to AA; # p <0.05 compared to vehicle treated animals by two-tailed unpaired Student t-test with Bonferroni correction for multiple comparisons. DU: densitometric units.

Figure 1S

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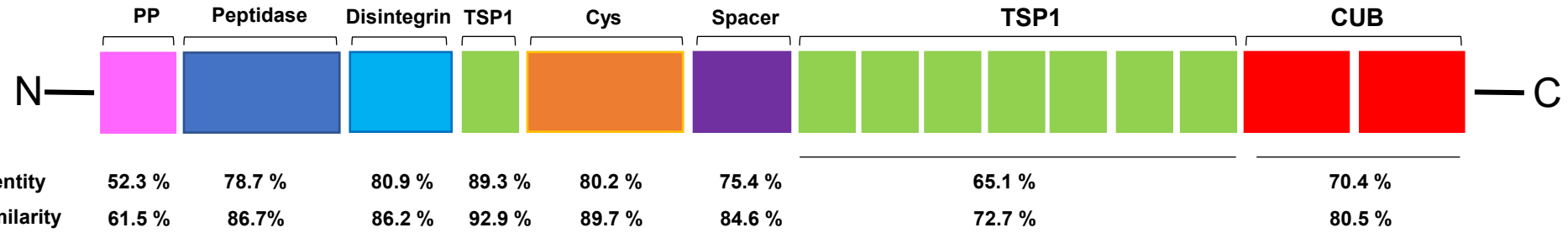

B

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Mouse   LYITRFDLELPDGNQQVRGVTQLGGACSLSWSCLITEDTGFDLGVTIAHEIGHSFGLDHDGAPGSGSTCKASGHVMAADGATPTGGTLEWSACSQRQLQHLLSTGQMHCFQDPPGLQSGLTRHQLMAQPGLYYSADDQCRVAFGSGAVACTFSREGLDVCQALSCHTDPLDQSSCSRLLV 357
Human   LYITRFDLELPDGNRQVRGVTQLGGACSPTWSCLITEDTGFDLGVTIAHEIGHSFGLEHDGAPGSG--CGPSGHVMASDGAAPRAG-LAWSPCSRRQLLSLLSAGRARCVWDPPRPQPGSAGHPPDAQPGLYYSANEQCRVAFGPKAVACTFAREHLDMCQALSCHTDPLDQSSCSRLLV 352
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Mouse   PLLDGTECGVEKWCSKARCRSLAELAPVAAVHGHWSSWGPHSPCSRSCGGGVITRRRWCNNPRPAFGGRACVGEDLQAKMCNTQACEKTQLEFMSEQCAQTDRQPLQLSQGTASFYHWDAAVQYSQGDTLCRHMCWAVGESFIVSRGDRFLDGTRCVPSGPQDDGTLSLCLLGSCRTFGC 537
Human   PLLDGTECGVEKWCSKGRCRSLVELTPIAAVHGRWSSWGPRSPCSRSCGGGVVTRRRQCNNPRPAFGGRACVGADLQAEMCNTQACEKTQLEFMSQQCARTDGQPLRSSPGGASFYHWGAAVPHSQGDALCRHMCRAIGESFIMKRGDSFLDGTRCMPSGPREDGTLSLCVSGSCRTFGC 532
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Mouse   DGRMDSQKVWDACQVCGGDNSTCSSRNGSFTAGRAREYVTFLIVTPNMTNAHIVNRRPLFTHLAVRIQGHYIVAGKTSISPNTTYPSLLEDYRVEYRVTLTEDQLPHLEEIHIRGPVRDDIEIQVYRRYGGEYGDLTHPDITFSYFQLKQQAAWVWTAKRGPCSVSCGAGLRWVTYSCQD 717
Human   DGRMDSQQVWDRCQVCGGDNSTCSPRKGSFTAGRAREYVTFLTVTPNLTSVYIANHRPLFTHLAVRIGGRYVVAGKMSISPNTTYPSLLEDGRVEYRVALTEDRLPRLEEIRIWGPLQEDADIQVYRRYGEEYGNLTRPDITFTYFQPKPRQAWVWAAVRGPCSVSCGAGLRWVNYSCLD 712
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Mouse   QAQDKWVKNAQCQGSPQPPAWQEPCVSAPCSPYWVAGDFSPCSVSCGGGLRERSLRCVETQDGFLKTLPPARCRAVAQQPAAEVENCNSQPCPTRWEVSDPGPCMSSACEAGLDSRNVTCVSRAGDPEKPETAGPCRTDEMSAMLEPCSRSLCSPGLGQVDNTMSLGEEAPSPVGSDKPG 897
Human   QARKELVETVQCQGSQQPPAWPEACVLEPCPPYWAVGDFGPCSASCGGGLRERPVRCVEAQGSLLKTLPPARCRAGAQQPAVALETCNPQPCPARWEVSEPSSCTSA-GGAGLALENETCVPGADGLEAPVTEGPGSVDEKLPAPEPCVGMSCPPGWGHLDAT-SAGEKAPSPWGSIRTG 890
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Human   IADCTYRWHVGTWMECSVSCGDGIQRRRDTCLGPQAQAPVPADFCQHLPKPVTVRGCWAGPCVGQGTPSLVPHEEAAAPGRTTATPAGASLEWSQARGLLFSPAPQPRRLLPGPQENSVQSSACGRQHLEPTGTIDMRGPGQADCAVAIGRPLGEVVTLRVLESSLNCSAGDMLLLWGRL 1248
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Human   TWRKMCRKLLDMTFSSKTNTLVVRQRCGRPGGGVLLRYGSQLAPETFYRECDMQLFGPWGEIVSPSLSPATSNAGGCRLFINVAPHARIAIHALATNMGAGTEGANASYILIRDTHSLRTTAFHGQQVLYWESESSQAEMEFSEGFLKAQASLRGQYWTLQSWVPEMQDPQSWKGKEGT 1427
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Figure 2S

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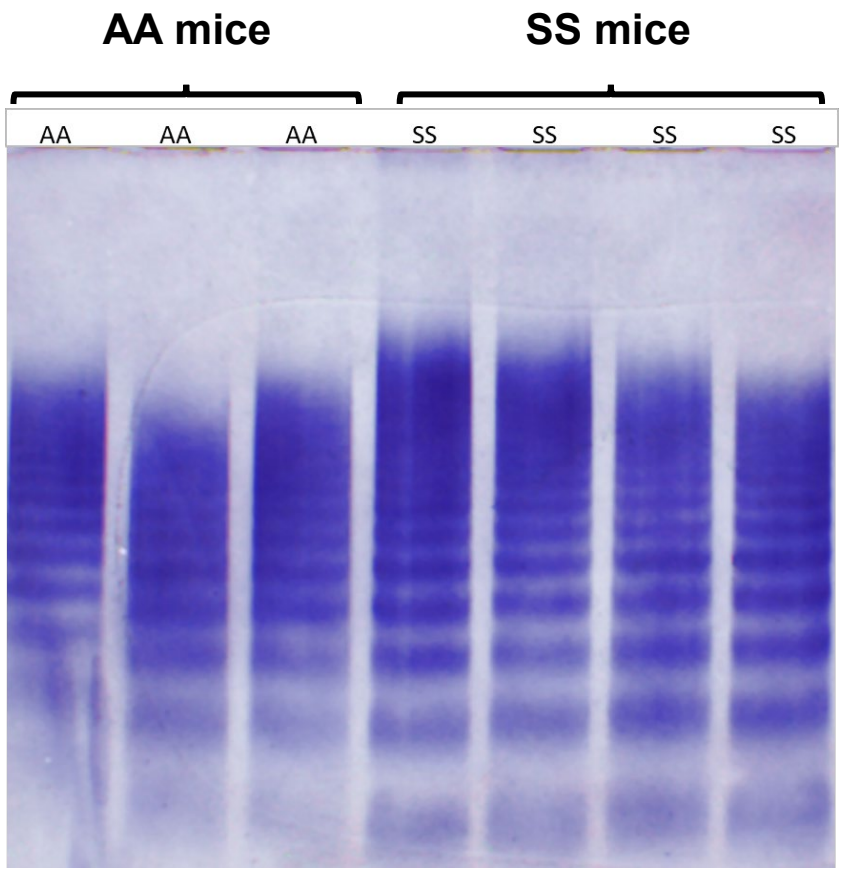
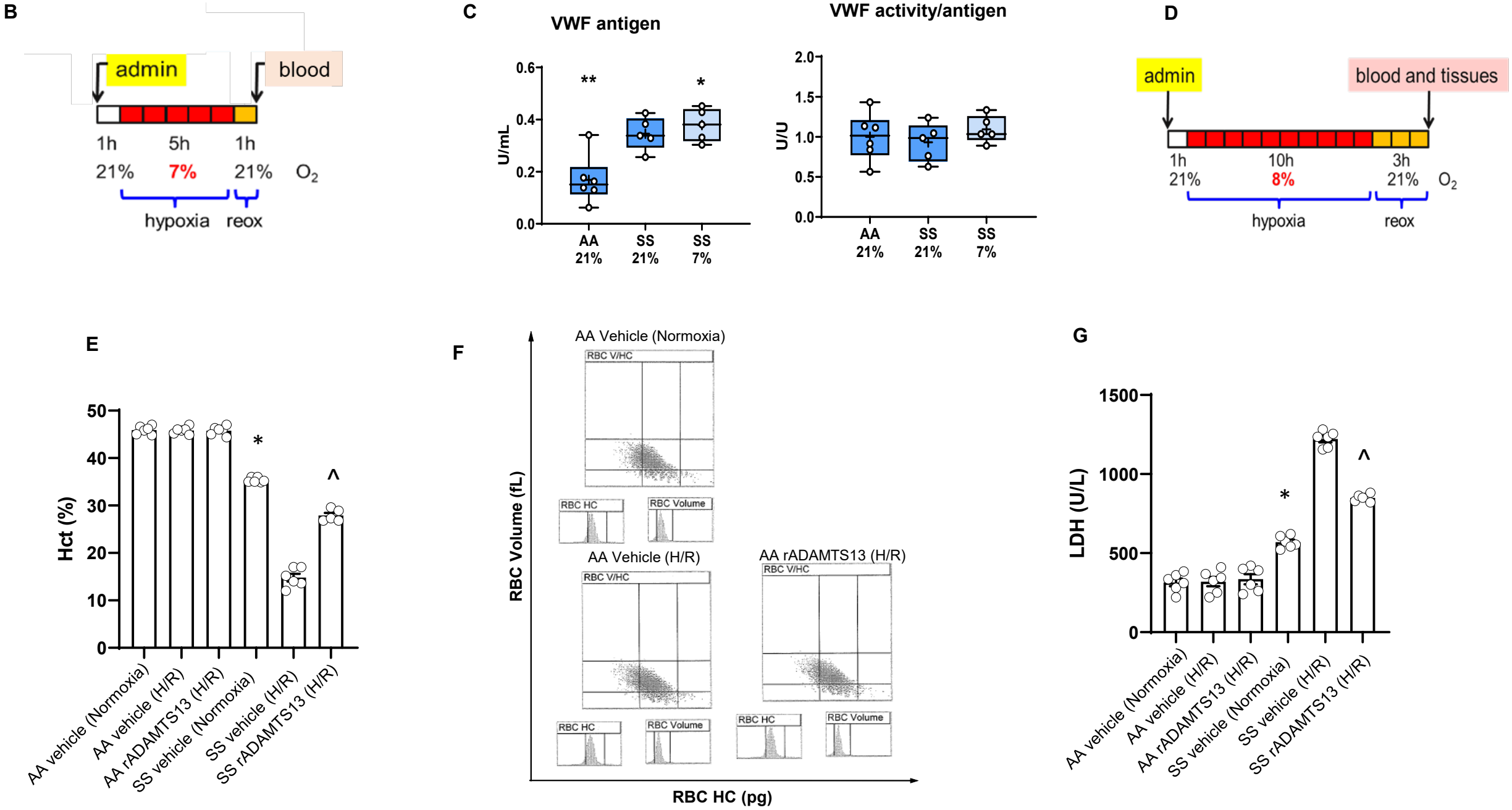


Figure 2S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VWF activity/antigen
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U/U
AA 21%
SS 21%
SS 7%
D
admin
blood and tissues
1h
10h
3h
21%
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O2
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reox
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SS vehicle (H/R)
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F
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AA Vehicle (H/R)
AA rADAMTS13 (H/R)
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Figure 3S

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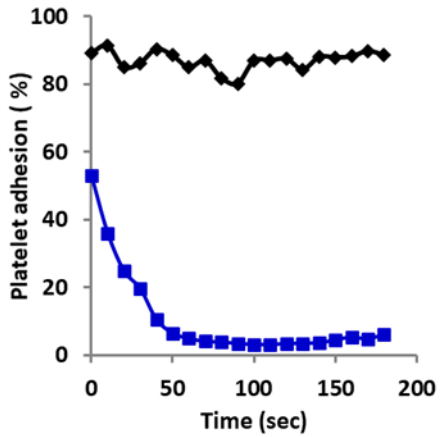

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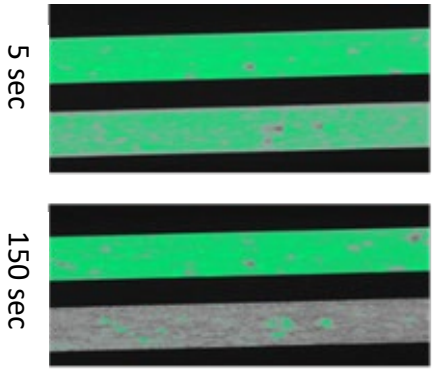

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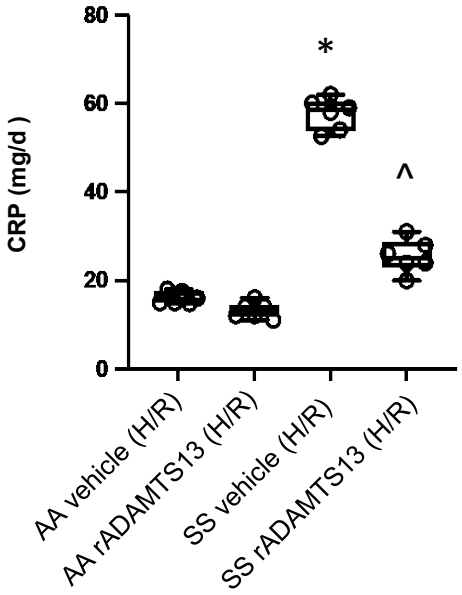

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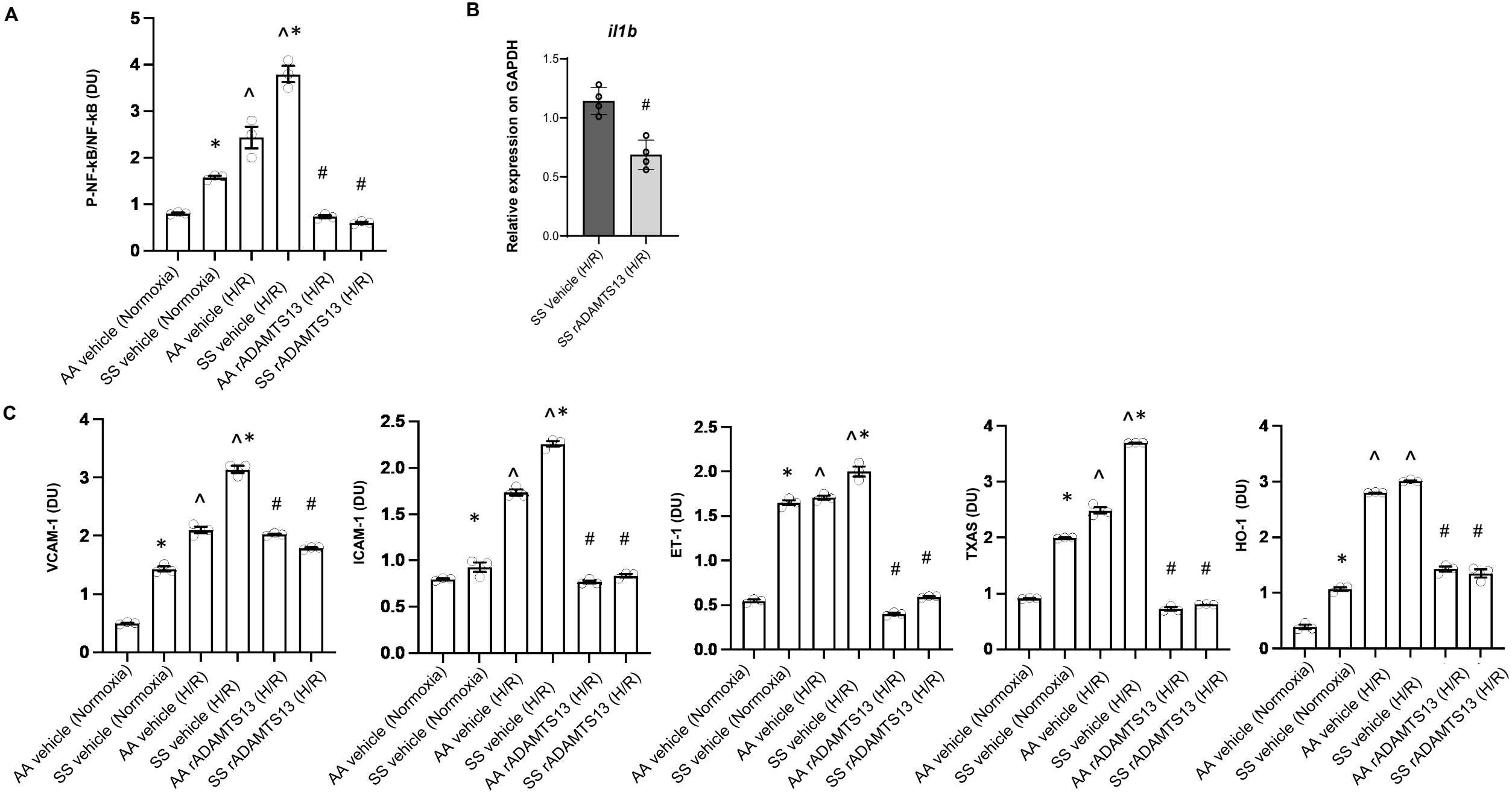
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AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)
B
il1b
Relative expression on GAPDH
SS Vehicle (H/R)
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ET-1 (DU)
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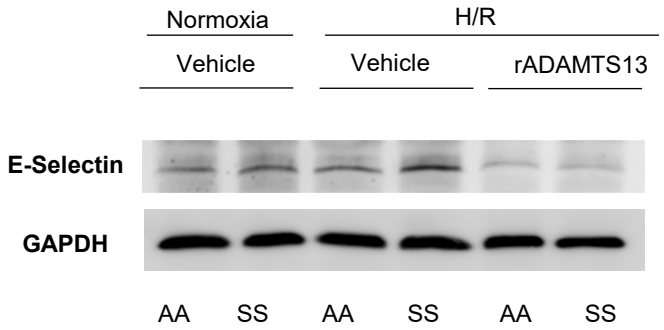
HO-1 (DU)

Figure 5S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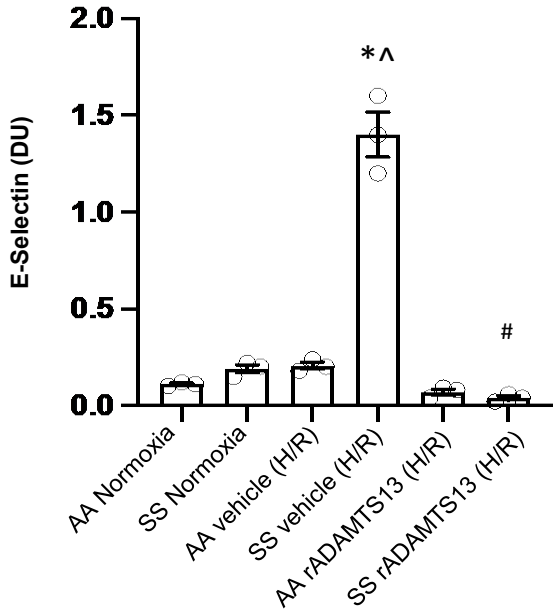
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Figure 6S

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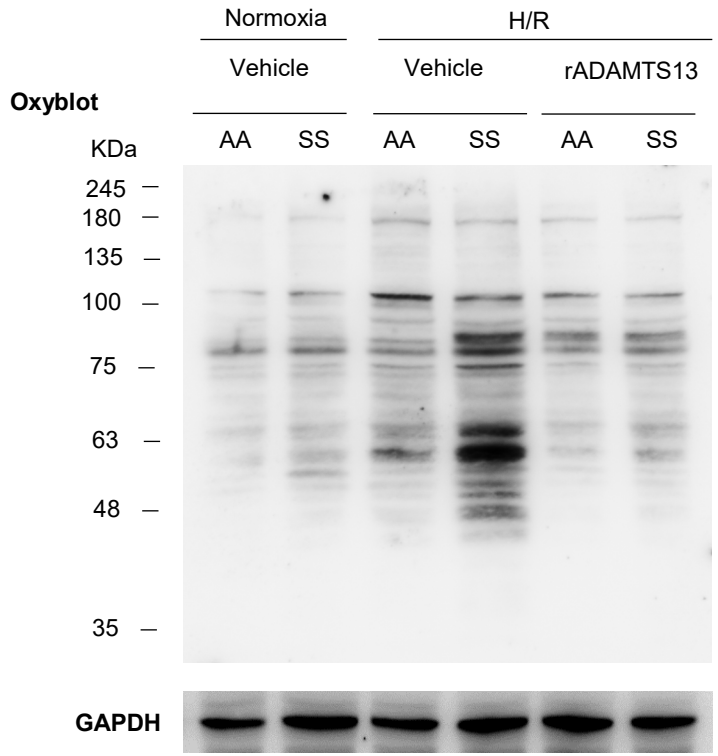
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AA
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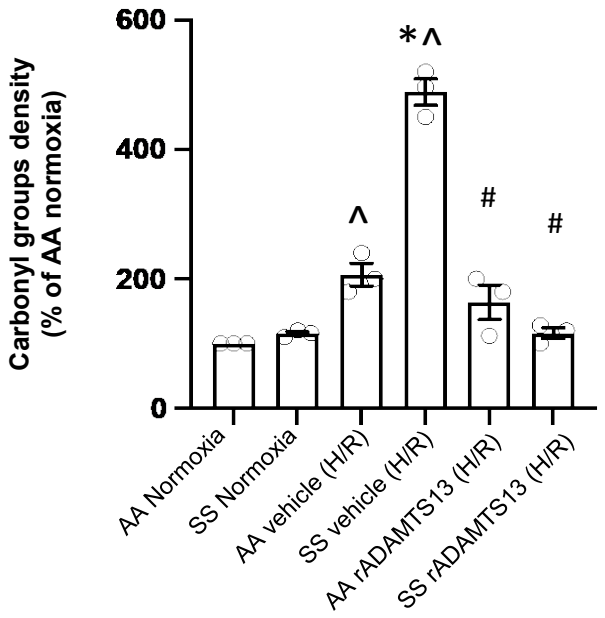
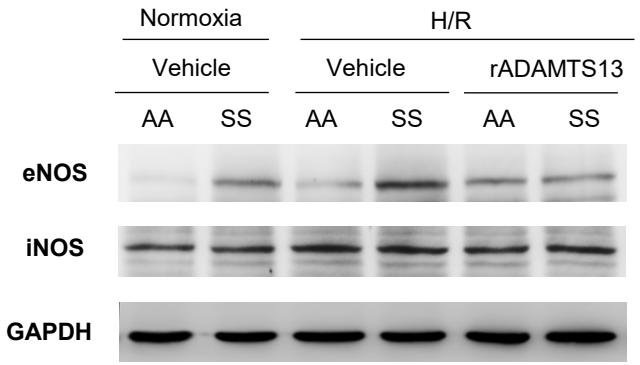
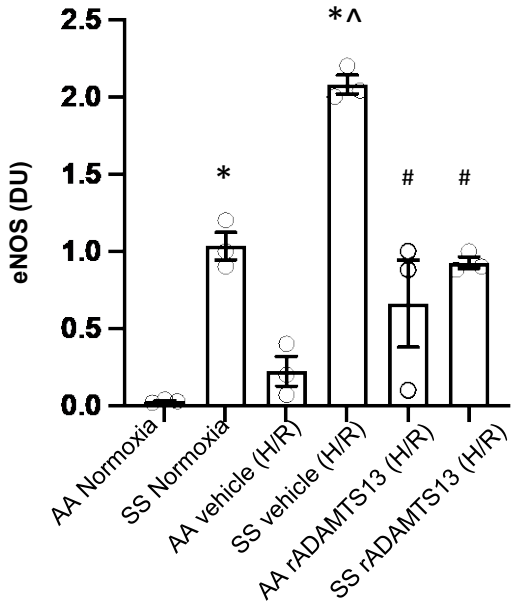
AA
SS
KDa
245
180
135
100
75
63
48
35
GAPDH


Carbonyl groups density
(% of AA normoxia)
600
400
200
0
*^
^
#
#
AA Normoxia
SS Normoxia
AA vehicle (H/R)
SS vehicle (H/R)
AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)

B


Normoxia
H/R
Vehicle
Vehicle
rADAMTS13
AA
SS
AA
SS
AA
SS
eNOS
iNOS
GAPDH


eNOS (DU)
2.5
2.0
1.5
1.0
0.5
0.0
*
*^
#
#
AA Normoxia
SS Normoxia
AA vehicle (H/R)
SS vehicle (H/R)
AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)

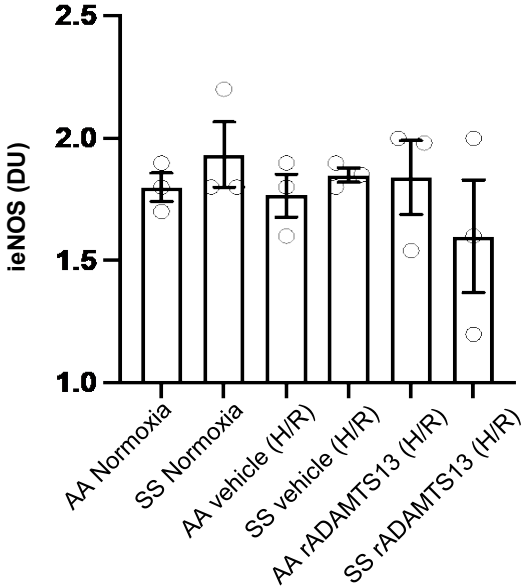
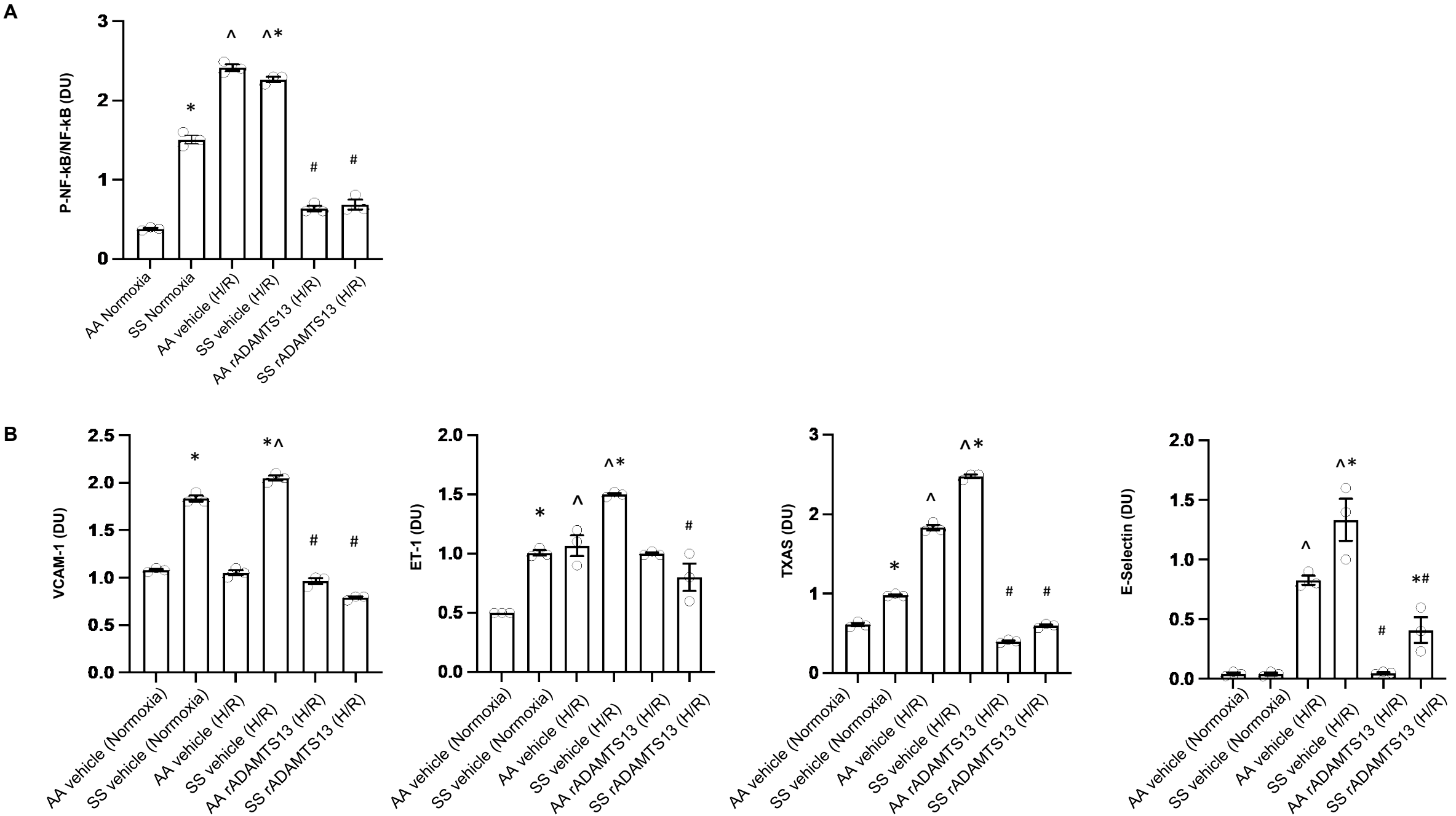

ieNOS (DU)
2.5
2.0
1.5
1.0
AA Normoxia
SS Normoxia
AA vehicle (H/R)
SS vehicle (H/R)
AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)

Figure 7S


A
P-NF-kB/NF-kB (DU)
AA Normoxia
SS Normoxia
AA vehicle (H/R)
SS vehicle (H/R)
AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)
B
VCAM-1 (DU)
ET-1 (DU)
TXAS (DU)
E-Selectin (DU)
AA vehicle (Normoxia)
SS vehicle (Normoxia)
AA vehicle (H/R)
SS vehicle (H/R)
AA rADAMTS13 (H/R)
SS rADAMTS13 (H/R)