Dietary ω-3 fatty acids protect against vasculopathy in a transgenic mouse model of sickle cell disease

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SUPPLEMENTARY METHODS

Hematological parameters and red cell indices.

Hemoglobin levels were determined by spectroscopic measurement of the cyanmet derivative. The hematocrit was determined in a microhematocrit centrifuge (19-21). Erythrocyte cellular indices were determined by Technicon and analyzed using mouse specific software program (Siemens Medicl solutions Diagnostic Tarrytown, NY; USA) (19-21). Reticulocyte counts were determined using thiazole orange labelling as previously reported (19-21).

Red cell fatty acid composition. To extract red cells membrane total lipids, chloroform and methanol (Fisher, Fair Lawn, NJ) were added at a ratio of 2:1 followed by a potassium chloride (Aldrich, Milwaukee, WI) salt wash to isolate the total lipid fraction. Total lipids were extracted from 40 to 150 μl of plasma. Tricosanoic free fatty acid (Sigma, St. Louis, MO) was added to each sample as an internal standard. The lipids were saponified with 0.5 N methanolic sodium hydroxide (Sigma, St. Louis, MO) and the fatty acids were converted to methyl esters with 14% BF_3/methanol (Sigma-Aldrich, St. Louis, MO) at 100°C for 30 minutes (51). Butylatedhydroxytoluene (Sigma-Aldrich, St. Louis, MO) was added before saponification and all samples were purged with N_2 throughout the process to minimize oxidation. Fatty acid methyl esters were analyzed by gas liquid chromatography using a Hewlett Packard 6890 equipped with a flame ionization detector. Peaks were identified by comparison of retention times with external fatty acid methyl ester standard mixtures from NuCheck Prep (Elysian, MN) (22).

Echocardiographic measurements. Transthoracic echocardiography was performed with a Vevo 2100 echocardiograph (Visual Sonics, Toronto, Canada) equipped with a 22-55 MHz transducer (MicroScan Transducers, MS500D). Mice were anesthetized by isoflurane inhalation (2%), maintained by mask ventilation (isoflurane 1%) and placed in a shallow left lateral decubitus position, with strict thermoregulation (37+-1°C) to optimize physiological conditions and reduce hemodynamic variability. Echocardiographic parameters were measured at the level of the papillary muscles in the parasternal short-axis view (M mode). LV fractional shortening was calculated as follows: FS = ((LVEDD - LVESD)/ LVEDD) X 100, where LVFS indicates LV fractional shortening; LVEDD, LV end-diastolic diameter;

and LVESD, LV end-systolic diameter. LV ejection fraction was calculated automatically by the echocardiography system. Cardiac output was calculated as the product of stroke volume (SV) and heart rate (HR): LVOT SV X HR/1000 where LVOT SV indicates left ventricular outflow tract stroke volume. The left atrial area was measured in the apical four-chamber view. All measurements were averaged on 3 consecutive cardiac cycles per experiment and cardiac function was assessed when heart rate was 400-450 bpm (16).

Lung and liver histopathology

Lung. Based on previous reports (19, 23), the pathological criteria for lung histopatholgy were as follows: i) Bronchus: Mucus: 0: no mucus; +: mucus filling less than 50% of the area of the bronchus section; ++: mucus filling more than 50% of the area of the bronchus section. (ii) Inflammatory infiltrate density: 0: less than 5 inflammatory cells per field; +: 5-30 inflammatory cells per field; ++ more than 30 inflammatory cells per field. (iii) Thrombi: 0: no thrombus; + presence of a thrombus in one field, at magnification 250.

Liver. Pathological score:0: no hepatocellular damage; 1: mild injury characterized by cytoplasmic vacuolization and focal nuclear pyknosis; 2: moderate injury with dilated sinusoids, cytosolic vacuolization, and blurring of intercellular borders; 3: moderate to severe injury with coagulative necrosis, abundant sinusoidal dilatation, red blood extravasation into hepatic chords, hypereosinophilia and migration of neutrophils; 4: severe necrosis with loss of hepatic architecture, disintegration of hepatic chords, haemorrhage and neutrophils infiltration. We also evaluated the inflammatory cell infiltrate and the presence of thrombi. Morphologic analysis was performed blindly and independently by two pathologists and consisted of the evaluation of the tissue architecture and changes induced by hypoxia and/or treatment regimens. The inter-observer difference measure was <5% (19, 23).

Lung, liver and aorta Immunoblot analysis. Gels were transferred to nitrocellulose membranes for immuno-blot analysis with specific antibody: anti Endothelin-1 (ET-1), anti Heme Oxygenase-1 (HO-1), anti Superoxide Dismutase 1 (SOD-1), anti GAPDH from SCBT (Santa Cruz, CA, USA); anti VCAM-1 (R and D Systems, Minneapolis, MN, USA); anti Peroxiredoxin 2 (Prx-2, Clone 1E8, AbCam, Cambridge, UK); anti Endothelin receptor B (ET-B receptor, Capralogics, Hardwick, MA, USA); anti Actin (Sigma Aldrich, Saint Louis, MO, USA), used as loading control. Secondary donkey anti-rabbit IgG and anti-mouse IgG HRP conjugated were from GE Healthcare Life

Sciences (Little Chalfont, UK); secondary donkey anti goat IgG HRP conjugated was from SCBT. Blots were developed using the Immobilon Western Chemiluminescent HRP Substrate from Millipore (Billerica, MA, USA) and images were acquired using Image Quant Las Mini 4000 Digital Imaging System (GE Healthcare Life Sciences). Densitometric analyses were performed using the ImageQuant TL software (GE Healthcare Life Sciences) (25).

Fig. 1S

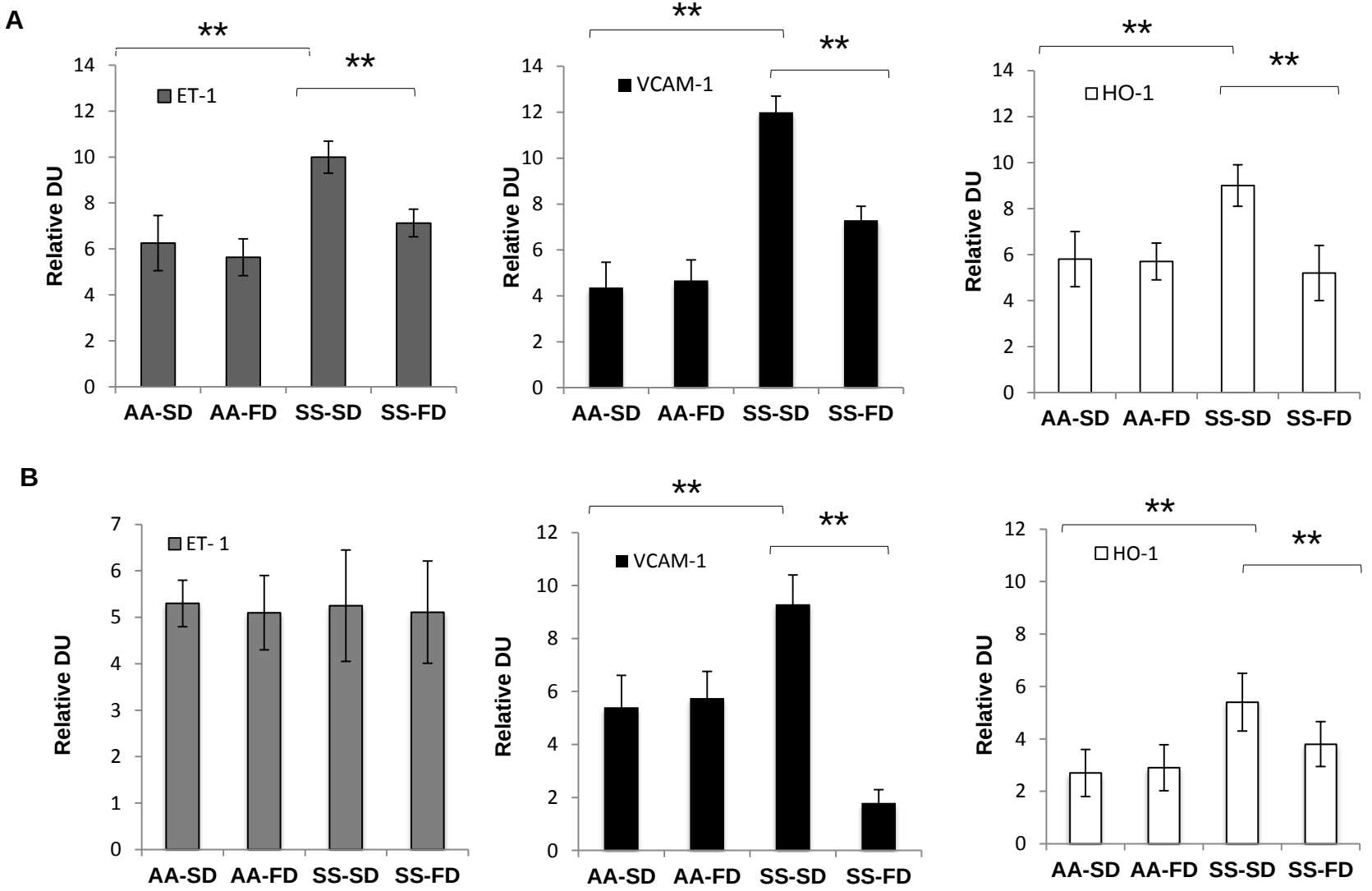

Fig. 1S. (A) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in aorta from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) (see Fig. 1B for relative immunoblots**)**. Data are presented as means ±SD (*n*=6). ***P*<0.005 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD. **(B) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in heart** from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) (see Fig. 1C for relative immunoblots**)**. Data are presented as means ±SD (*n*=6). ***P*<0.005 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD

Fig. 2S

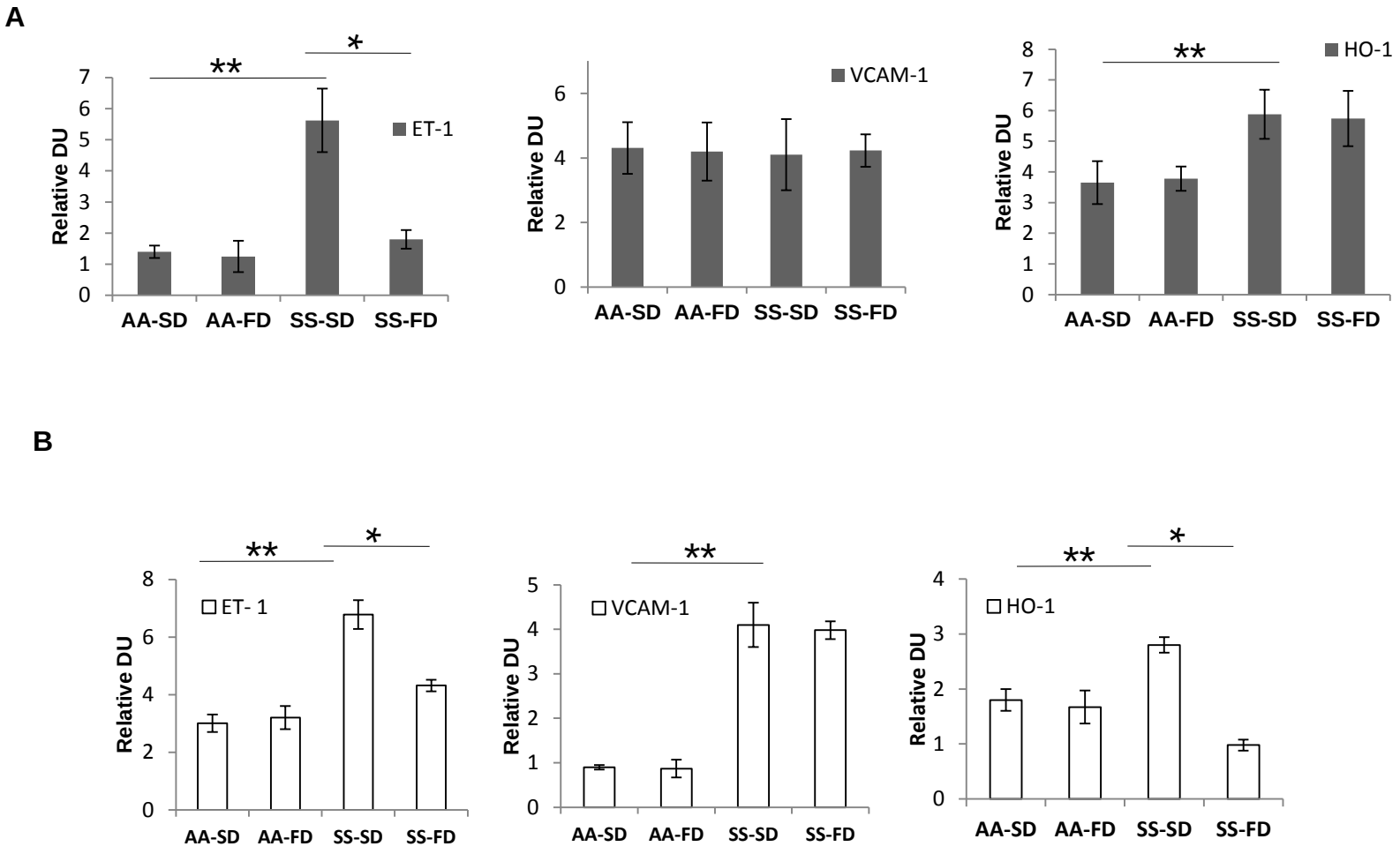

Fig. 2S. (A) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in lung from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) (see Fig. 2B for relative immunoblots**)**. Data are presented as means ±SD (*n*=6).*P<0.005; **P<0.002 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD. **(B) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in liver** from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) (see Fig. 2C for relative immunoblots**)**. Data are presented as means ±SD (*n*=6).*P<0.005; **P<0.002 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD.

Fig. 3S

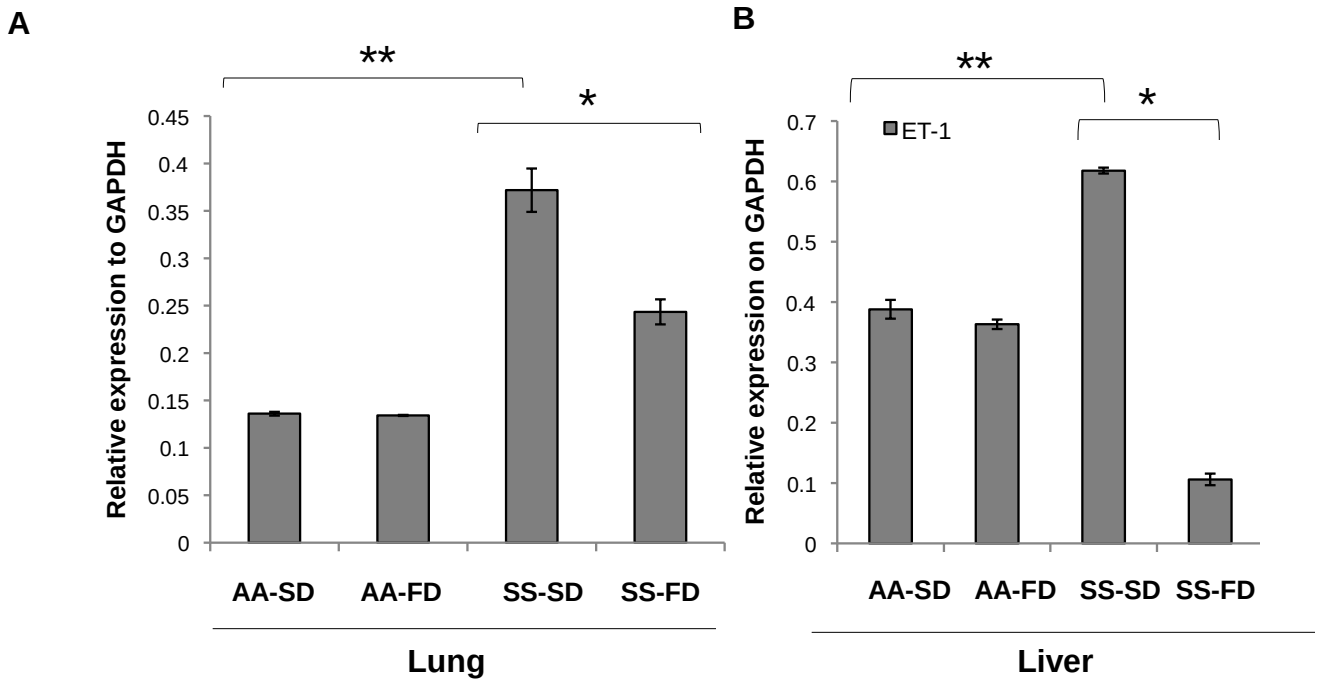

Fig.3S. Effects of FD supplementation on mRNA expression levels of ET-1 in lung and liver of AA and SS mice in normoxia conditions. (A) ET-1 mRNA levels in lung tissues (normalized to GADPH) from AA and SS mice on soy-diet (SD) or supplemented with FD in normoxia conditions. Data are presented as means ±SD (*n*=5 mice from each group); * *P*<0.05 (SS *vs* SS-FD); * **P* <0.001 (AA-SD and AA-FD *vs* SS-SD and SS-FD).; **(B)** ET-1 mRNA levels in liver tissues (normalized to GADPH) from AA (*n*=5 mice from each group) and SS (*n*=5 mice from each group) mice on soy-diet (SD) or supplemented with FD in normoxia conditions. . Data are presented as means ±SD (*n*=5 mice from each group); * *P* <0.005 (SS-SD *vs* SS-FD); ** *P* <0.002 (AA-SD and AA-FD vs SS-SD and SS-FD). AA-SD: healthy mice on soy-diet; AA-FD: healthy mice on fish-oil diet; SS-SD: sickle mice on soy-diet; SS-FD: sickle mice fish-oil diet.

Fig. 4S

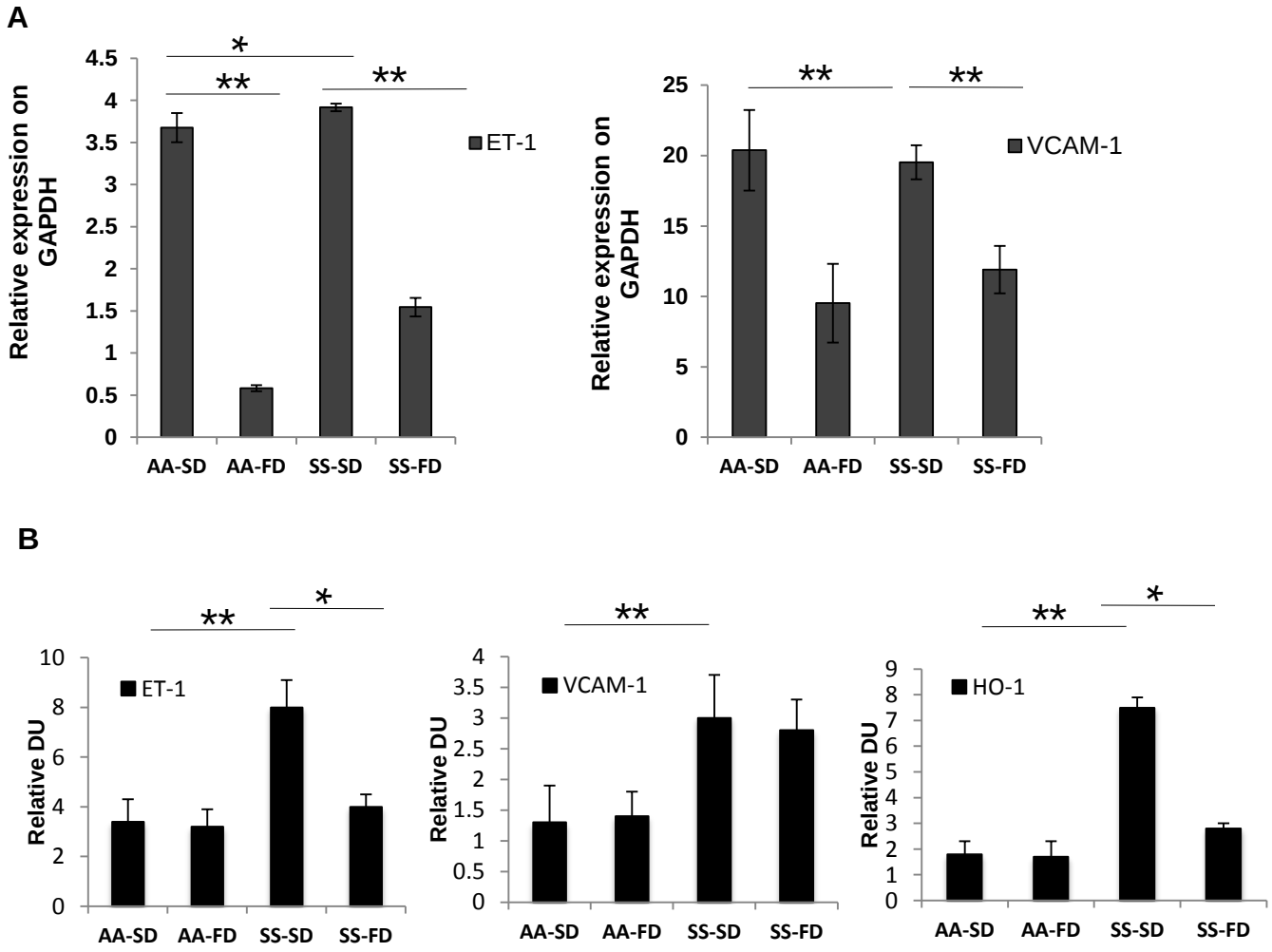

Fig. 4S. (A)) Endothelin-1 (ET-1) and VCAM-1 mRNA levels in lung tissue (normalized to GADPH) from SS mice on soy-diet (SD) or supplemented with fish-oil diet (FD) exposed to H/R stress. **P* <0.05 (SS vs SS-FD). Data are presented as means ±SD (*n*=5 mice from each group); **P*<0.005; ***P*<0.002 compared to to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD. **(B) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in lung** from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) exposed to hypoxia/reoxygenation (see Fig. 4F for relative immunoblots**)**. Data are presented as means ±SD (*n*=6).**P* < 0.005; ***P*<0.002 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD.

Fig. 5S

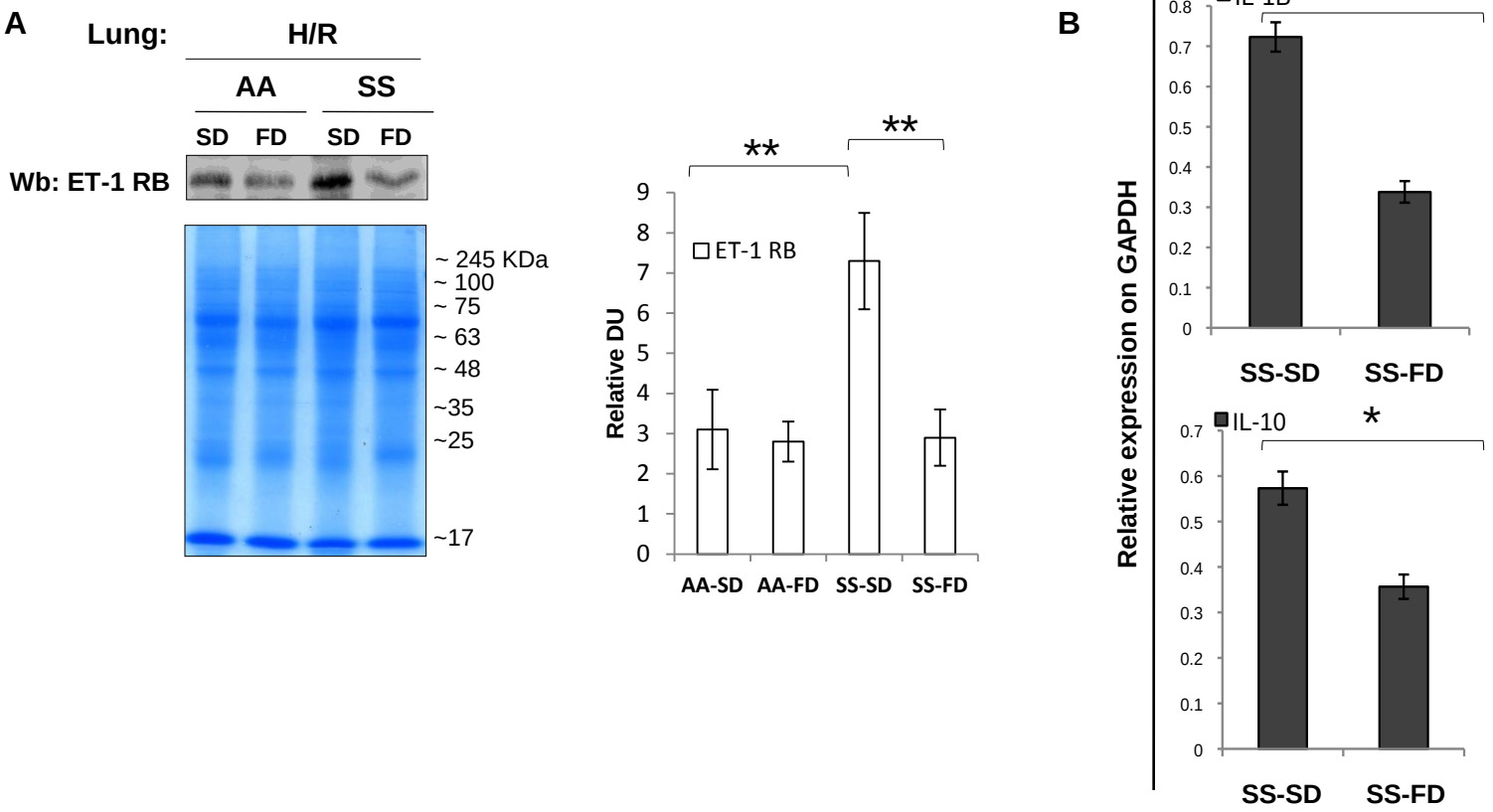

Fig. 5S. (A) Endothelin-1 receptor B expression in lung from healthy and sickle cell mice exposed to hypoxia/ reoxygenation (H/R) Immunoblot analysis with specific endothelin-1 receptor B (ET-1- RB) in lung from healthy (AA) and sickle cell mice (SS mice) on soy diet (SD) or fish-oil diet (FD) exposed to H/R: hypoxia (8% oxygen; 10 hours) followed by reoxygenation (21% oxygen; 3 hours). One representative gel from other 6 with similar results is presented. **Right panel.** Densytometric analysis of ET-1 RB immunoblots; data are presented as means ±SD (*n*=6; ***P*<0.005, ***P*<0.002). **Lower panel.** Colloidal Coomassie stained gel, run in parallel with gel transferred to membrane for immunoblot analysis and showed in the upper. The colloidal Coomassie stained gel was used as proteins loading control. One representative gel from other 6 with similar results is presented. **(B)** IL-1B and IL-10 mRNA levels in lung tissues (normalized to GADPH) from SS mice treated with soy-diet (SD) or fish-oil diet (FD) in H/R stress. * *P* <0.05 (SS-SD *vs* SS-FD). Data are presented as means ±SD (*n*=5 mice from each group); * *P* <0.005 (SS-SD *vs* SS-FD); SS: sickle mice on standard diet; SS-FD: sickle mice fish oil diet.

Fig. 6S

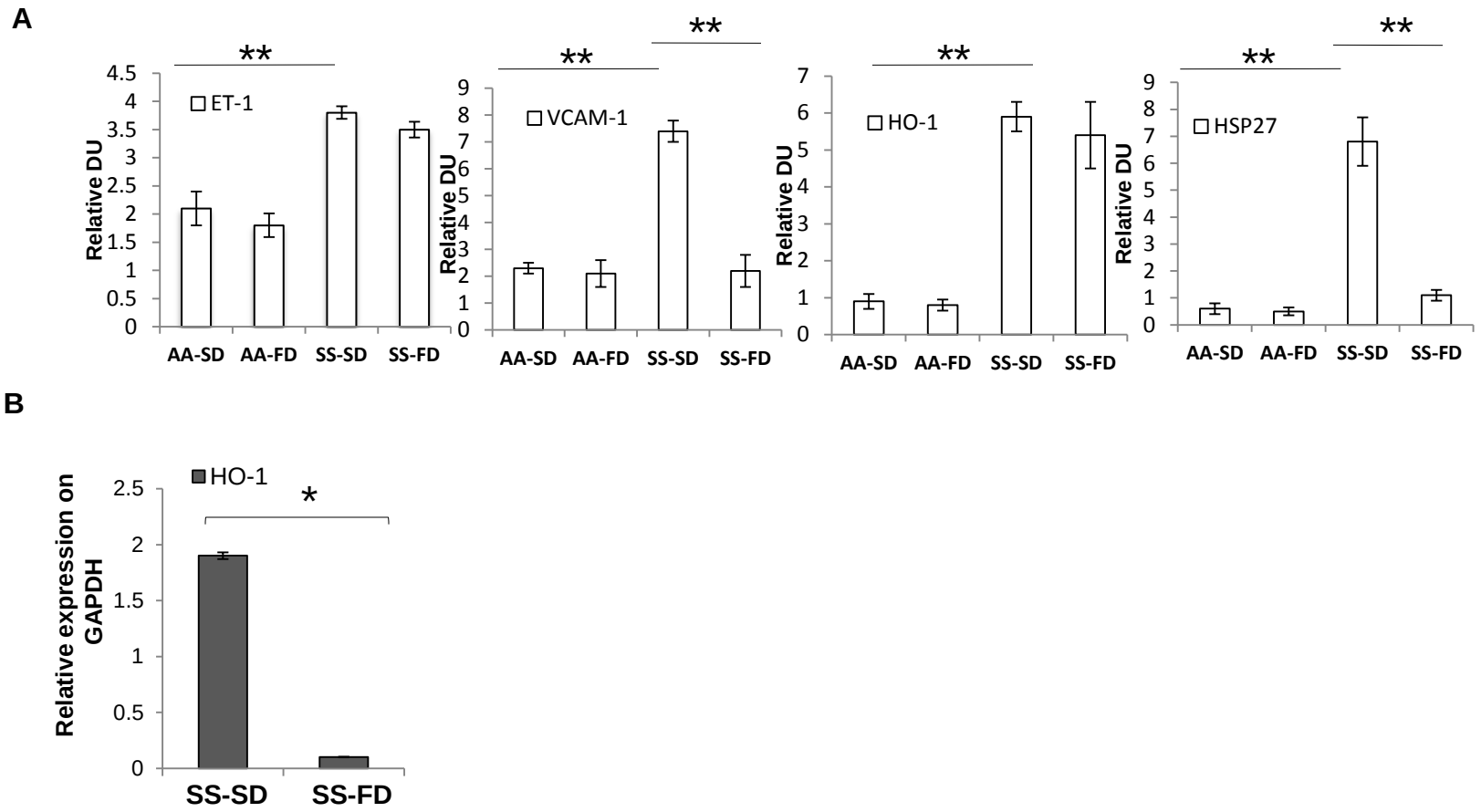

Fig.6S. (A) Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1) in liver from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) exposed to hypoxia/reoxygenation (see Fig. 5E for relative immunoblots**)**. Data are presented as means ±SD (*n*=6).*P < 0.005; **P<0.002 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD. **(B)** HO-1 mRNA levels in liver tissue (normalized to GADPH) from SS mice on soy-diet (SD) or supplemented with fish-oil diet (FD) exposed to H/R stress. *P <0.005 (SS vs SS-FD). Data are presented as means ±SD (*n*=5 mice from each group); *P<0.02 compared to SS-SD mice.

Fig. 7S

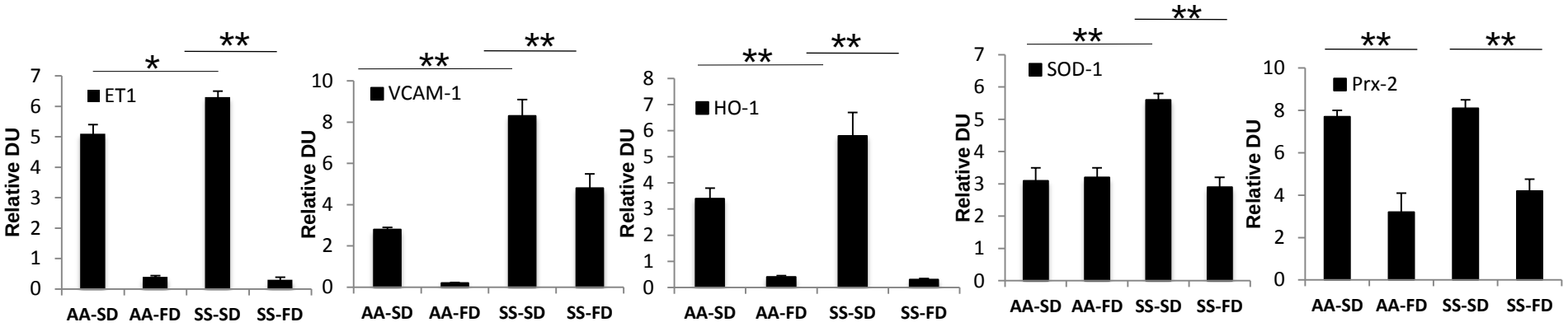

Fig. 7S. Relative quantification of immunoreactivity of endothelin- 1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1) and heme-oxygemase-1 (HO-1), superoxide-dismutase 1 (SOD1) and Peroxiredoxin-2 (Prx2) in aorta from healthy (AA) and sickle cell mice (SS mice) under soy diet (SD) or supplemented with fish-oil diet (FD) exposed to hypoxia/reoxygenation (see Fig. 6A for relative immunoblots**)**. Data are presented as means ±SD (*n*=6).**P* < 0.005; ***P*<0.002 compared to SS-SD *vs* AA-SD or SS-SD *vs* SS-FD.

Table 1S. Oligonucleotide primer sequences used for qRT-PCR of analyzed mouse genes

Gene symbol	Forward primer (5'- 3')	Reverse primer (5'- 3')
IL-1b	5′-GCTGAAAGCTCTCCACCTCAA-3′	5′-TTGTCGTTGCTTGGTTCTCCT-3′
ET-1	5′-ACTTCTGCCACCTGGACATC-3′	5′-CTTTCAAGGAACGCTTGGAC-3′
GAPDH	5′-CCACATCGCTCAGACACCAT-3′	5′-AGTTAAAAGCAGCCCTGGTGAC-3′
IL-10	5′-CCTGTGAAAATAAGAGCAAGGCA-3′	5′-TGGCCTTGTAGACACCTTGGTC-3′
VCAM-1	5′- TGTCTGCAAAGGACACTGGAAA-3′	5′- AGTGGCCCACTCATTTTAATTACTG-3′

Table 2S. Red cell membrane fatty acid composition

Fatty Acid	AA-SD (*n*=3)	AA- FD (*n*=3)	SS-SD (*n*=3)	SS – FD (*n*=3)
16:0	42.91 ± 1.78	45.54 ± 2.35	33.68 ± 0.34$^{\#}$	34.33 ± 1.55$^{\gamma}$
16:1	1.07 ± 0.11	1.54 ± 0.38	2.66 ± 0.13$^{\#}$	3.80 ± 0.46$^{\delta\gamma}$
18:0	19.42 ± 0.51	18.25 ± 1.72	9.59 ± 0.23$^{\#}$	10.56 ± 0.56$^{\gamma}$
18:1 n-9	14.72 ± 0.54	15.53 ± 0.92	15.94 ± 0.95	18.55 ± 0.92$^{\delta\gamma}$
18:1 n-7	2.14 ± 0.08	2.21 ± 0.33	3.85 ± 0.27$^{\#}$	4.04 ± 0.32$^{\gamma}$
18:2 n-6	9.15 ± 0.88	2.85 ± 0.23*	7.86 ± 0.51	1.92 ± 0.31$^{\delta}$
18:3 n-6	0.05 ± 0.02	0.02 ± 0.02	0.13 ± 0.02$^{\#}$	0.02 ± 0.01$^{\delta}$
18:3 n-3	**0.05 ± 0.05**	**0.00 ± 0.00**	**0.17 ± 0.03$^{\#}$**	**0.01 ± 0.02$^{\delta}$**
20:3 n-9	0.03 ± 0.03	0.05 ± 0.01	0.21 ± 0.04$^{\#}$	0.18 ± 0.01$^{\gamma}$
20:3 n-6	0.67 ± 0.03	0.21 ± 0.03*	0.62 ± 0.05	0.23 ± 0.04$^{\delta}$
20:4 n-6	5.75 ± 0.76	2.76 ± 0.42*	**13.10 ± 0.38$^{\#}$**	4.23 ± 0.41$^{\delta\gamma}$
20:5 n-3	**0.06 ± 0.01**	**3.16 ± 0.79***	**0.21 ± 0.02**	**5.51 ± 0.49$^{\delta\gamma}$**
22:4 n-6	0.70 ± 0.12	0.10 ± 0.03*	2.67 ± 0.21$^{\#}$	0.20 ± 0.03$^{\delta}$
22:5 n-6	0.23 ± 0.08	0.09 ± 0.02*	**1.33 ± 0.22$^{\#}$**	0.28 ± 0.04$^{\delta}$
22:5 n-3	**0.13 ± 0.03**	**0.73 ± 0.49**	**0.88 ± 0.04**	**3.79 ± 0.75$^{\delta\gamma}$**
22:6 n-3	**1.26 ± 0.37**	**4.75 ± 0.48**	**5.53 ± 0.10**	**7.27 ± 6.31**

AA: healthy mice; SD: soy diet; FD: fish-oil diet; SS: sickle cell mice; ω-6 fatty acid are highlighted in grey ; ω-3 fatty acid are shown in bold; * P<0.05 for AA-FD compared to AA-SD; $^{\#}$ P<0.05 for SS-SD compared to AA-SD; $^{\delta}$ P<0.05 for SS-FD compared to SS-SD; $^{\gamma}$ P<0.05 for SS-FD compared to AA-FD

Table 3S. Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) in wild-type and sickle cell mice exposed to hypoxia/reoxygenation

	AA-SD (*n*=6)	**AA-FD** (*n*=6)	**SS-SD** (*n*=6)	**SS-FD** (*n*=6)
ALT (U/L)	195±23	120± 14°	324±22	250±44*
AST (U/L)	198±31	154±11°	448±31	302±35*

SD: soy-diet; FD: fish-oil diet; AA: wild-type healthy mice; SS: sickle cell mice; °*P*< 0.02 compared to AA-SD; **P*<0.005 compared to SD