IL-6 signaling accelerates iron overload by upregulating DMT1 in endothelial cells to promote aortic dissection

Authors: Qiang Xie[1,2], Jianji Wang[1], Runqiao Li[1], Hao Liu[1], Yongliang Zhong[1], Qinfeng Xu[1], Yipeng Ge[1], Chengnan Li[1], Lizhong Sun[1] and Junming Zhu[1,*]

[1] Department of Cardiovascular Surgery, Beijing Aortic Disease Center, Beijing Anzhen Hospital, Capital Medical University, Beijing 100029, China

[2] Department of Thoracic Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.

*** Correspondence:** Professor Junming Zhu, MD. Department of Cardiovascular Surgery, Beijing Aortic Disease Center, Beijing Anzhen Hospital, Capital Medical University, 2 Anzhen Avenue, Beijing 100029, China.

Tel: +86 13801132276

E-mail: anzhenzjm@ccmu.edu.cn; anzhenzjm@163.com

Supplementary Figure count: 6

Supplementary Table count: 5

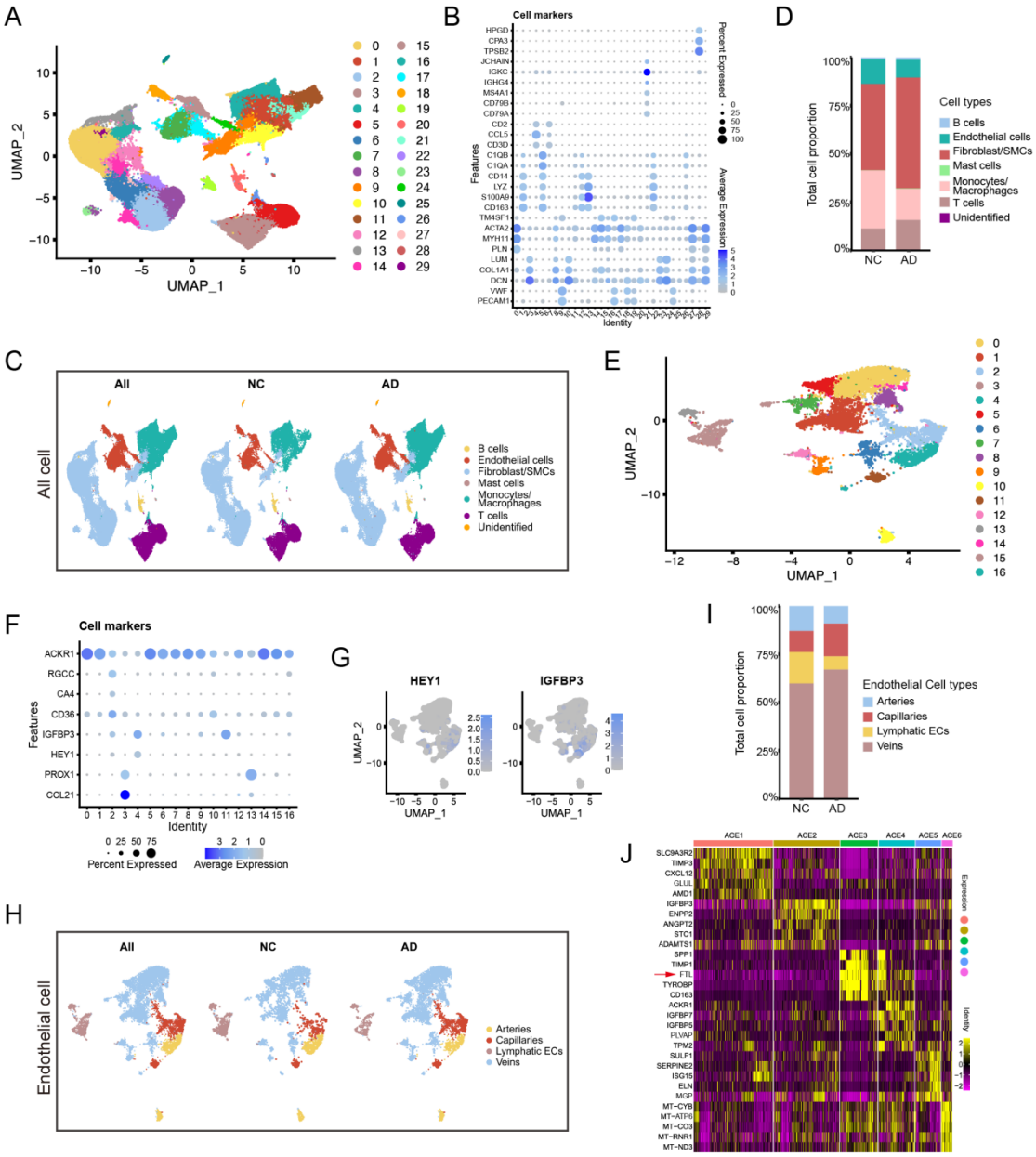

Fig. S1. Dimensionality reduction and clustering of aortic cells in scRNA-seq data. (**A**) UMAP plot of all cells and separate clusters. (**B**) Gene expression profiles of selected markers in separate clusters. (**C**) UMAP plot of seven cell types in the aortas. (**D**) Proportion of different cell types in the aortas. (**E**) Separate clusters of all endothelial cells (ECs). (**F**) Gene expression profiles of selected markers in separate clusters of ECs. (**G**) UMAP plot of the expression of aortic endothelial cell (AEC) marker genes (*HEY1* and *IGFBP3*). (**H**) UMAP of EC types in the aortas. (**I**) Proportion of different ECs. (**J**) Heatmap showing the top five genes in AECs.

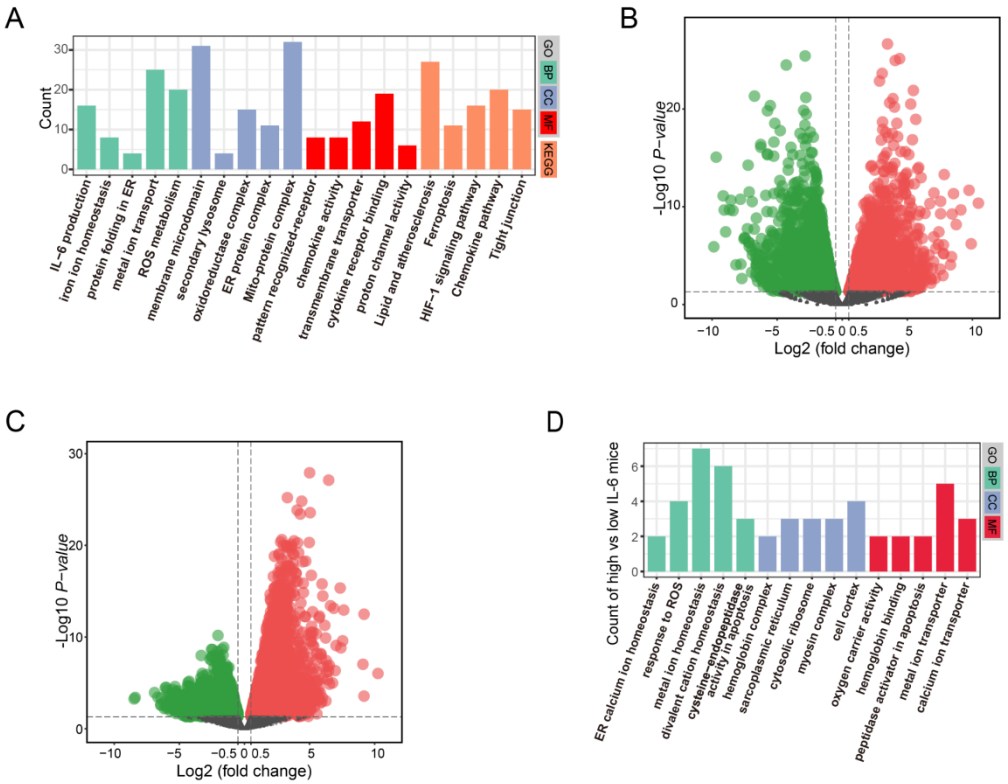

Fig. S2. Volcano plot and enrichment analysis of differentially expressed genes (DEGs) in scRNA-seq and bulk RNA-seq. (**A**) GO and KEGG analyses of DEGs in aortic endothelial cell 3 of scRNA-seq. (**B**) Volcano plot of bulk RNA-seq showing DEGs in aortas of dissection patients and transplant donors. (**C**) Volcano plot showing DEGs in the aortas of six control and six acute AD mice. (**D**) GO and KEGG analyses of DEGs between acute control and AD mouse aortas.

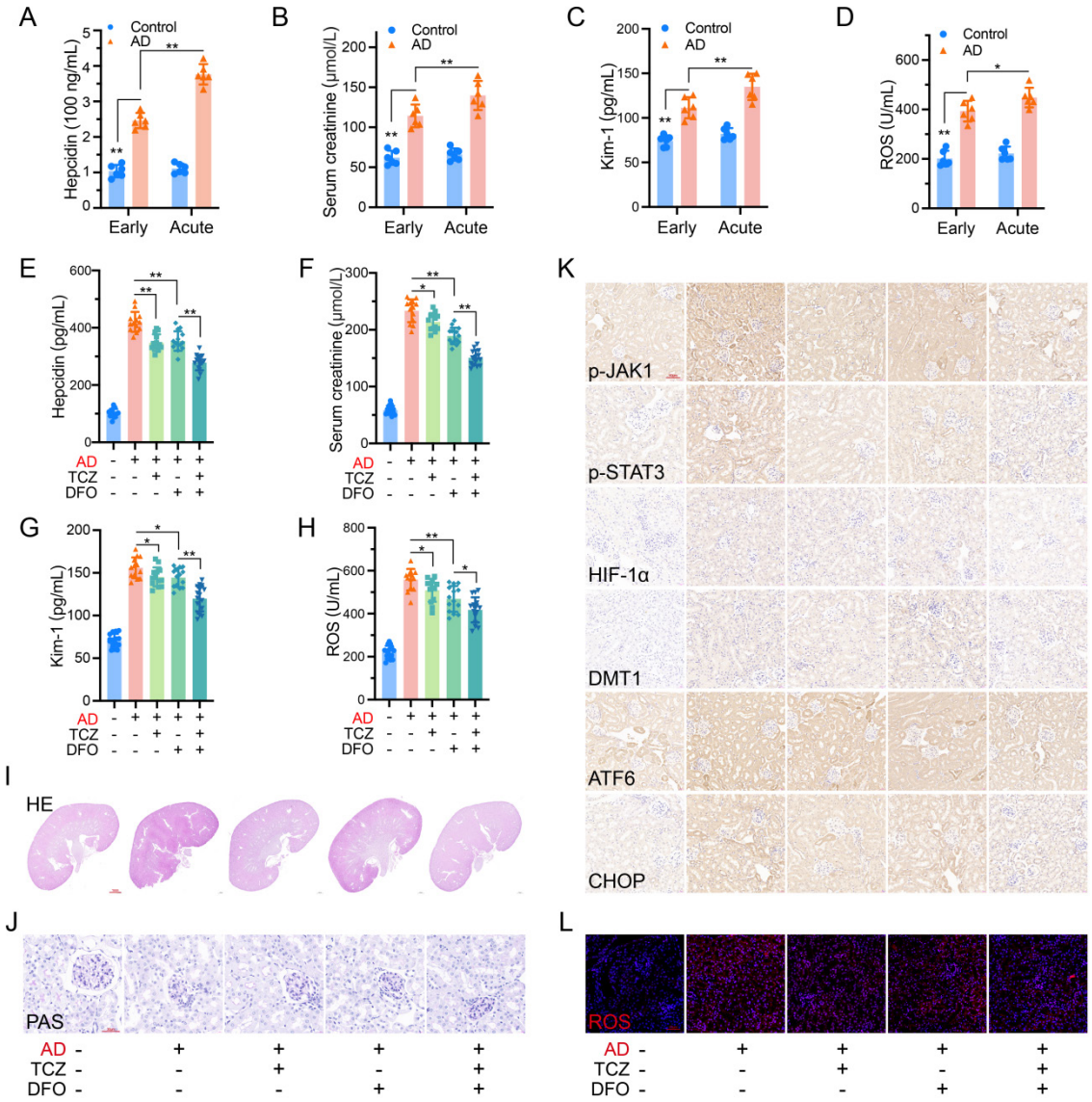

Fig. S3. Serum biomarker levels and renal section staining in mice with aortic dissection. Contents of serum hepcidin (**A**), creatinine (**B**), Kim-1 (**C**), and ROS (**D**) in the first animal experiment. TCZ and DFO inhibited serum levels of hepcidin (**E**), creatinine (**F**), Kim-1 (**G**), and ROS (**H**). (**I**) HE staining of coronal sections of mouse kidneys. (**J**) PAS staining of renal sections. (**K**) IHC staining of renal sections. (**L**) ROS staining of renal tissues. Data are presented as representative images or means ± SDs. **P* < 0.05, ***P* < 0.01, ns, not significant.

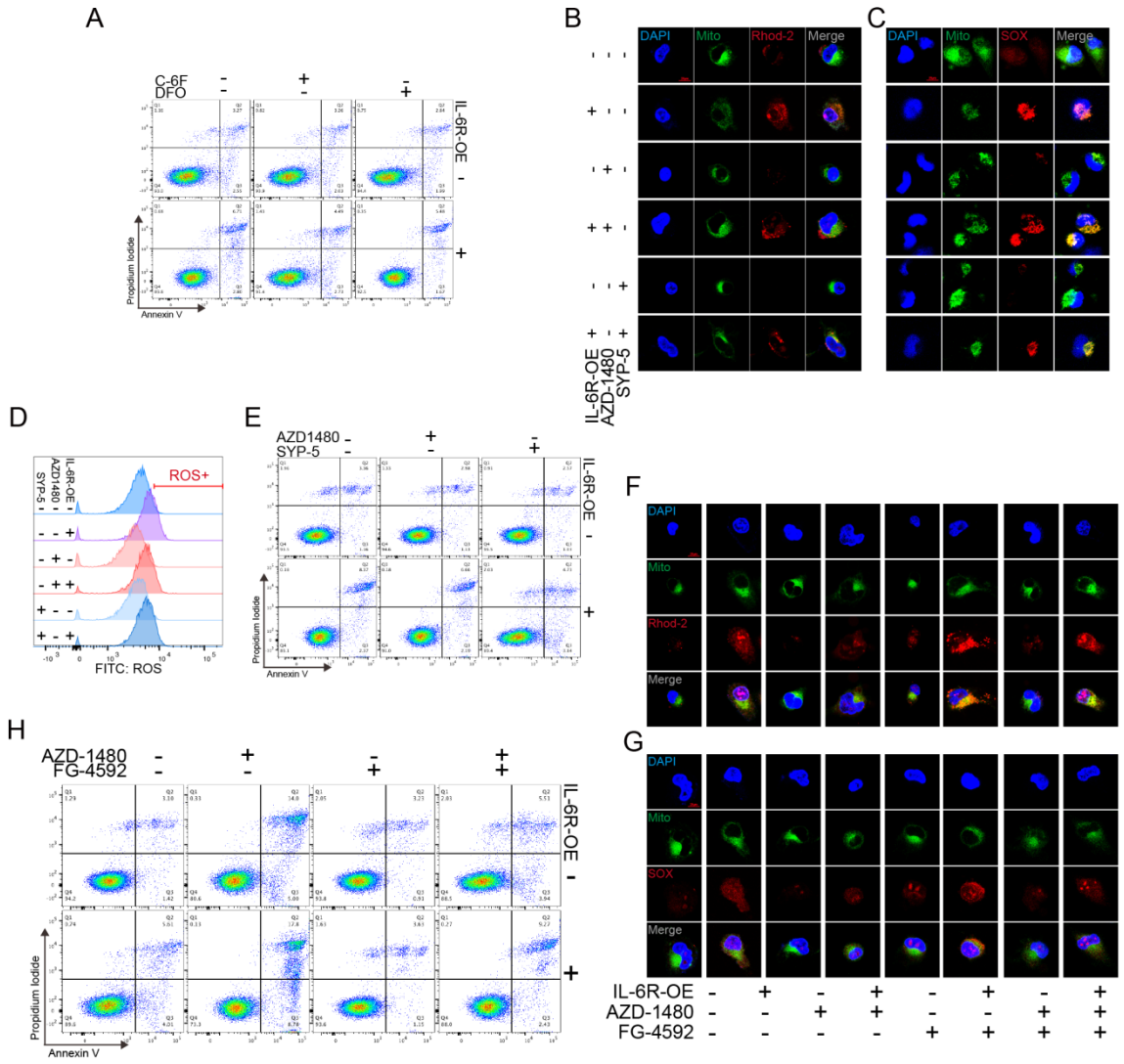

Fig. S4. IL-6R overexpression induces ERS and apoptosis in HAECs. (**A**) C-6F and DFO reduced apoptosis in HAECs. (**B**) Confocal images of mito-Ca^{2+} fluorescence staining. (**C**) Confocal images of mitoSOX fluorescence staining. (**D**) AZD1480 and SYP-5 reduced ROS in HAECs. (**E**) AZD1480 and SYP-5 reduced apoptosis in HAECs. (**F**) Fluorescence images of mito-Ca^{2+}. (**G**) MitoSOX fluorescence staining. (**H**) Apoptosis of HAECs was detected by flow cytometry. Data are presented as representative images.

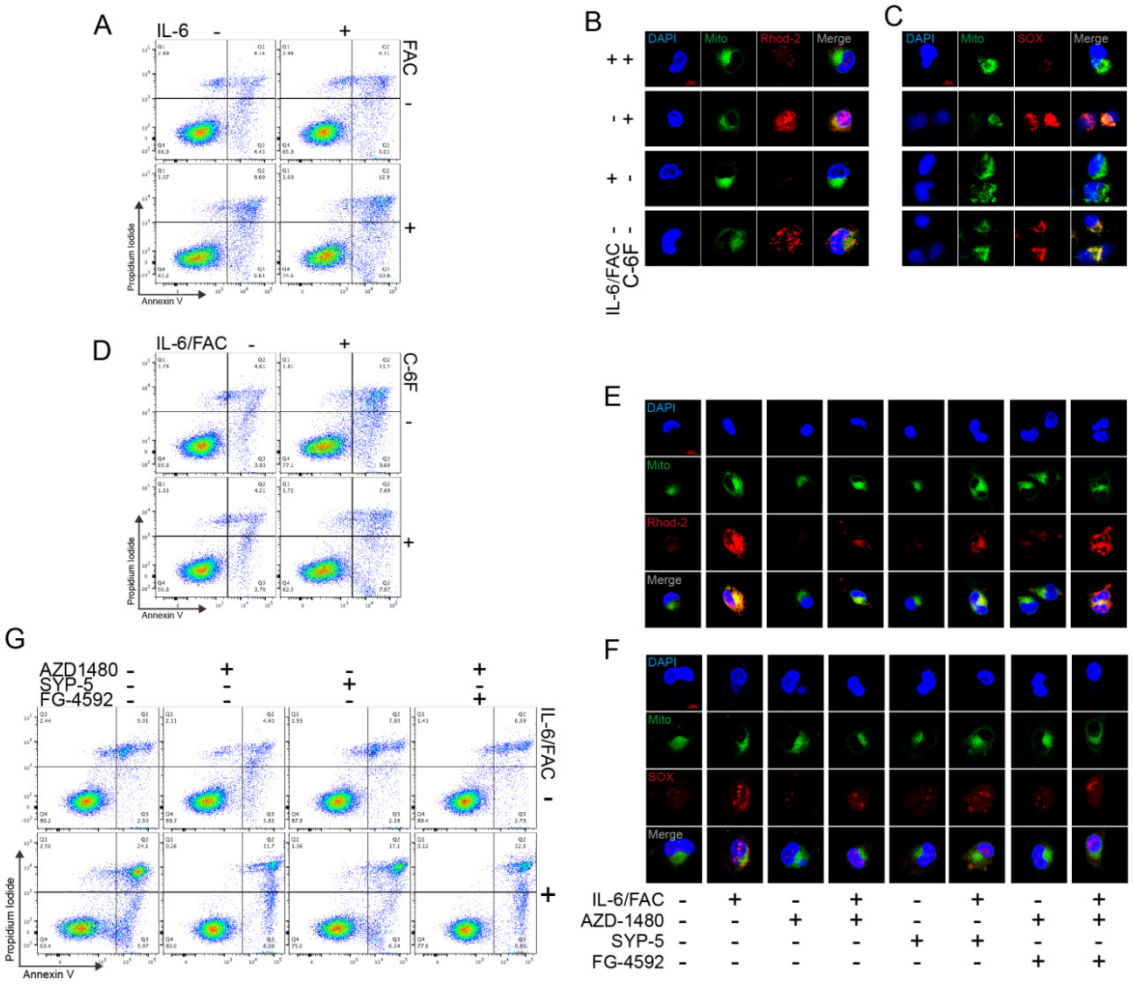

Fig. S5. Excessive IL-6 sensitized FAC-induced ERS and apoptosis in HAECs. (**A**) IL-6 sensitized FAC-induced apoptosis in HAECs. (**B**) Confocal images of mito-Ca^{2+} fluorescence staining. (**C**) Confocal images of mitoSOX fluorescence staining. (**D**) C-6F reduced apoptosis induced by IL-6 and FAC in HAECs. (**E**) Fluorescence images of mito-Ca^{2+}. (**F**) MitoSOX fluorescence staining. (**G**) Apoptosis of HAECs was detected by flow cytometry. Data are presented as representative images or means ± SDs.

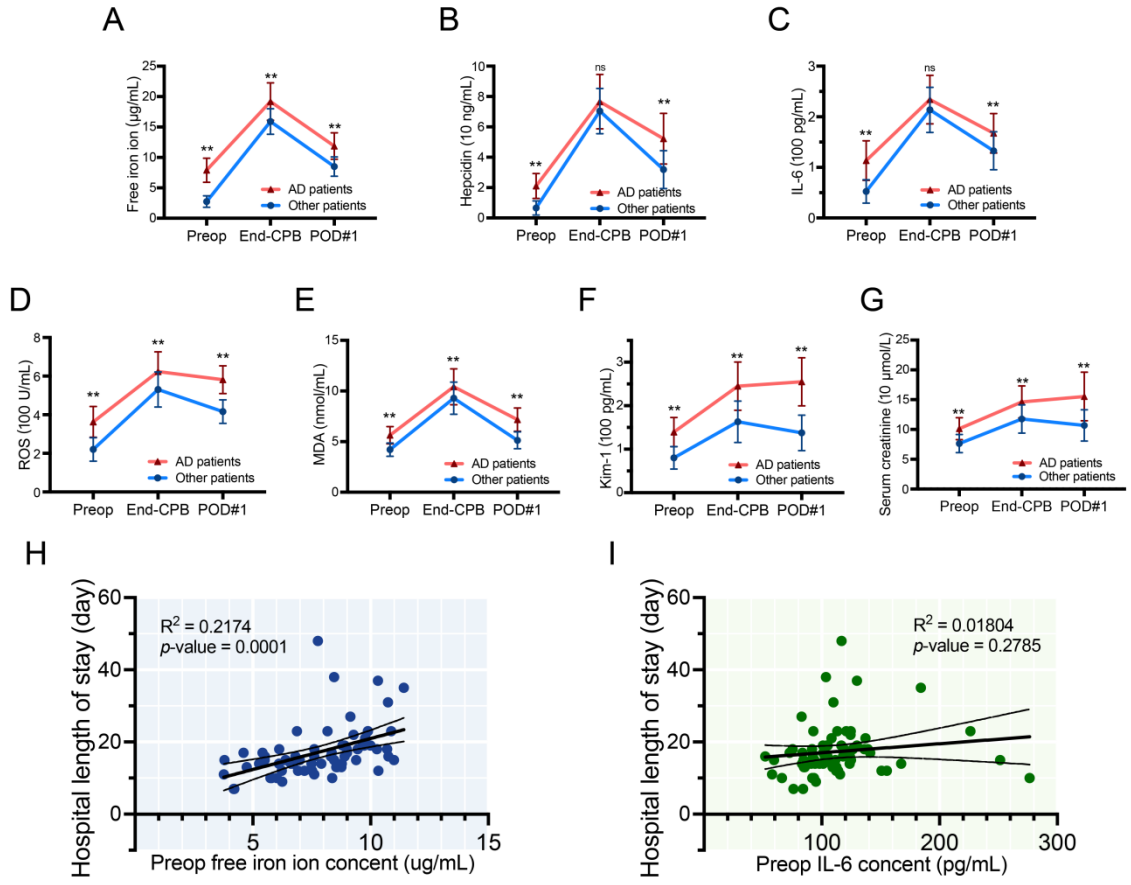

Fig. S6. Changes in perioperative serum biomarkers and the correlation between biomarkers and prognosis. Levels of serum iron ion (**A**), hepcidin (**B**), IL-6 (**C**), ROS (**D**), MDA (**E**), Kim-1 (**F**), and creatinine (**G**) in aortic dissection (AD) patients and other patients requiring aortic surgery at pre-operation (Preop), end of cardiopulmonary bypass (End-CPB), and postoperation day one (POD#1). (**H**) Correlation between preoperative free iron ion and hospital stay in AD patients. (**I**) Relationship between preoperative IL-6 and hospital stay in dissection patients. Data are presented as representative images or means ± SDs. *$P < 0.05$, **$P < 0.01$, ns, not significant.

Table S1. The primers sequence information.

Categories	**Forward (5'--3')**	**Reverse (5'--3')**
RT-PCR assay		
Human		
FTH1	-CCAGAACTACCACCAGGACTC-	-GAAGATTCGGCCACCTCGTT-
FTL	-CAGCCTGGTCAATTTGTACCT-	-GCCAATTCGCGGAAGAAGTG-
HIF-1α	-GAACGTCGAAAAGAAAAGTCTCG-	-CCTTATCAAGATGCGAACTCACA-
SLC39A8	-ATGCTACCCAAATAACCAGCTC-	-ACAGGAATCCATATCCCCAAACT-
SOD2	-GGAAGCCATCAAACGTGACTT-	-CCCGTTCCTTATTGAAACCAAGC-
HMOX1	-AAGACTGCGTTCCTGCTCAAC-	-AAAGCCCTACAGCAACTGTCG-
DMT1	-CGGGTGCTGGAGCTATACAG-	-CGACAGTGTTGACATCAATGGC-
PTPRC	-ACCACAAGTTTACTAACGCAAGT-	-TTTGAGGGGGATTCCAGGTAAT-
IL6R	-CCCCTCAGCAATGTTGTTTGT-	CTCCGGGACTGCTAACTGG-
LYN	-TGCAGAGGGAATGGCATACAT-	-TGACTCGGAGACCAGAACATTAG-
CSF1R	-CTGCCCAGATCGTGTGCTC-	-AGGTTGAGGGTCAGGACTTTTT-
GAPDH	-CGGGAAGCTTGTCATCAATGG-	-GGCAGAGATGATGACCCTTT-
Mouse		
Ptprc	-GTTTTCGCTACATGACTGCACA-	-AGGTTGTCCAACTGACATCTTTC-
Il6r	-GCCACCGTTACCCTGATTTG-	-TCCTGTGGTAGTCCATTCTCTG-

Categories	Forward (5'--3')	Reverse (5'--3')
Lyn	-ATTGTGGTGGCCTTATACCCT-	-ACCATTCCCCATGCTCTTCTA-
Csf1r	-TGTCATCGAGCCTAGTGGC-	-GGTCCAAGGTCCAGTAGGG-
Gapdh	-AGGTCGGTGTGAACGGATTTG-	-GGGGTCGTTGATGGCAACA-
ChIP assay		
DMT1-Site1	-AGGGACCCAGATGAAAGGTCTCTTT-	-GGTTCACGCCTGTAATCCCAGCA-
DMT1-Site2	-TGTTTAAGGTGTACAAGATGGAGG-	-AATCCTTGTAGTAGGCTGGCAC-
DMT1-Site3	-TCCCCTCCTCCCAGATTGAG-	-AGACATCAGAGCCACGCAAA-
Pulldown assay (biotin-labeling)		
DMT1-Site1-probe	-AGGGACCCAGATGAAAGGTCT-	-ACACCTTAAACAATTCCCTTCCATT-
DMT1-Site2-probe	-TGTTTAAGGTGTACAAGATGGAGG-	-AATCCTTGTAGTAGGCTGGCAC-
DMT1-Site3-probe	-AAACTTCTAGAAAACTGCCTGC-	-TAACCCCAAGCCTGACAGTG-
Luciferase plasmids construction		
DMT1-WT -pGL3-Basic	-AGGTACCGAGCTCTTACGCGTAATTCTCCTGCCTGAGCCTCCCA-	-GCTTACTTAGATCGCAGATCTGGTGCAGAACTAGTTTGACTTTCCCAATGC-
DMT1-Mut-1	-ACCACTGTGCCTGGCCGCAA-	-TTGCGGCCAGGCACAGTGGTTCTGTAATCCCAGCACTTTG-
DMT1-Mut-2	-GCTGGGATTGCAGAATTTTTGTGCCAGCCTACTAC-	-AAAAATTCTGCAATCCCAGCACTTTGG-
DMT1-Mut-3	-CCAAGTCCGGAGTTTGCTCTGATGTCTGCTCGTGG-	-AGAGCAAACTCCGGACTTGGTGC-

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction assay; ChIP, chromatin immunoprecipitation; WT, wild type plasmid; Mut, mutant plasmid.

Table S2. The antibodies used information.

Categories	Usage and dosage	Product codes	Sources
Primary antibodies			
FTH	WB 1:200 IHC 1:100 IF 1:50	sc-376594	Santa Cruz, California, USA
FTL	WB 1:200 IHC 1:100 IF 1:50	sc-74513	Santa Cruz, California, USA
IL-6R	WB 1:200 IHC 1:100 IF 1:50	sc-373708	Santa Cruz, California, USA
HIF-1α	WB 1:1000 IHC 1:500 IF 1:100 ChIP 2 ug	#36169	CST, Massachusetts, USA
DMT1	WB 1:1000 IHC 1:200 IF 1:100	20507-1-AP	Proteintech, Illinois, USA
CD31	IF 1:100	11265-1-AP	Proteintech, Illinois, USA
p-JAK1	WB 1:1000 IHC 1:200	SAB4300123	SIGMA, Missouri, USA
p-STAT3	WB 1:1000 IHC 1:200	#9145	CST, Massachusetts, USA
ATF6	WB 1:1000 IHC 1:200	24169-1-AP	Proteintech, Illinois, USA
GRP78	WB 1:1000 IHC 1:200	11587-1-AP	Proteintech, Illinois, USA
CHOP	WB 1:1000 IHC 1:200	15204-1-AP	Proteintech, Illinois, USA
Caspase 12	WB 1:500	#58208	CST, Massachusetts, USA
Cleaved-caspase 3	WB 1:500	#9661	CST, Massachusetts, USA
GAPDH	WB 1:10000	10494-1-AP	Proteintech, Illinois, USA
Histone H3	WB 1:2000	17168-1-AP	Proteintech, Illinois, USA
Control Rabbit IgG	ChIP 2 ug	#2729	CST, Massachusetts, USA

Categories	Usage and dosage	Product codes	Sources
Secondary antibodies			
IRDye® 800CW Goat anti-Mouse IgG (H+L)	WB 1:20000	926-32210	LI-COR, Nebraska, USA
IRDye® 800CW Goat anti-Rabbit IgG (H+L)	WB 1:20000	926-32211	LI-COR, Nebraska, USA
Donkey anti-Mouse IgG (H+L) Alexa Fluor™ 488	IF 1:200	AB_2556542	Thermo Fisher Scientific, Massachusetts, USA
Goat anti-Mouse IgG (H+L) Alexa Fluor™ 594	IF 1:200	AB_2556547	Thermo Fisher Scientific, Massachusetts, USA
Donkey anti-RabbitIgG (H+L) Alexa Fluor™ 594	IF 1:200	AB_2556549	Thermo Fisher Scientific, Massachusetts, USA
Goat anti-Rabbit IgG (H+L) Alexa Fluor™ 488	IF 1:200	AB_2556544	Thermo Fisher Scientific, Massachusetts, USA

Abbreviations: WB, western blot; IHC, immunohistochemistry; IF, immunofluorescence; ChIP, chromatin immunoprecipitation.

Table S3. The enzyme-linked immunosorbent assay kits used information.

Categories	**Product codes**	**Sources**
Human		
Free iron ion (Non-transferrin binds iron)	MM-60946H1	Mmbiology, Jiangsu, China
Interleukin-6	MM-0049H1	Mmbiology, Jiangsu, China
Malonaldehyde	MM-2037H1	Mmbiology, Jiangsu, China
Reactive oxygen species	MM-1893H1	Mmbiology, Jiangsu, China
Kidney injury molecule-1	MM-0647H1	Mmbiology, Jiangsu, China
Creatinine	MM-13833H1	Mmbiology, Jiangsu, China
Mouse		
Free iron ion (Non-transferrin binds iron)	MM-46281M1	Mmbiology, Jiangsu, China
Interleukin-6	MM-0163M1	Mmbiology, Jiangsu, China
Malonaldehyde	MM-0897M1	Mmbiology, Jiangsu, China
Reactive oxygen species	MM-43700M1	Mmbiology, Jiangsu, China
Kidney injury molecule-1	MM-0318M1	Mmbiology, Jiangsu, China
Creatinine	MM-0693M1	Mmbiology, Jiangsu, China

Table S4. Clinical details of aortic dissection and other patients.

Variables	AD (n = 67)	Others (n = 70)	*p*-value
Preoperative variables			
Age (years)	50.0 ± 11.2	50.3 ± 13.0	0.813
Male	46 (68.7%)	57 (81.4%)	0.084
Height (cm)	169.6 ± 7.7	170.4 ± 7.6	0.230
Weight (kg)	74.0 ± 12.2	71.6 ± 11.6	0.244
Body mass index (kg/m^2)	25.7 ± 3.7	24.6 ± 3.4	0.060
Smoking	21 (31.3%)	33 (47.1%)	0.059
Hypertension	44 (65.7%)	35 (50.0%)	0.063
Left ventricular ejection fraction (%)	60.5 ± 4.7	58.1 ± 7.7	0.116
Free iron ion (ug/mL)	7.9 ± 2.0	2.7 ± 0.9	**< 0.001**
Interleukin-6 (pg/mL)	113.3 ± 39.1	52.4 ± 23.1	**< 0.001**
Serum creatinine (ug/mL)	101.2 ± 18.4	76.3 ± 15.2	**< 0.001**
Hepcidin (ng/mL)	21.1 ± 8.2	6.6 ± 4.6	**< 0.001**
Kidney injury molecule-1 (pg/mL)	139.5 ± 33.5	80.1 ± 25.8	**< 0.001**
Malondialdehyde (nmol/mL)	5.6 ± 0.8	4.2 ± 0.6	**< 0.001**
Reactive oxygen species (U/mL)	363.0 ± 79.4	220.2 ± 60.8	**< 0.001**
Operation-related variables			
Length of operation (hour)	7.5 ± 1.7	5.8 ± 1.1	**< 0.001**
Cardiopulmonary bypass time (min)	187.2 ± 46.6	136.9 ± 41.2	**< 0.001**
Cross-clamp time (min)	105.3 ± 28.5	89.8 ± 27.5	**0.003**
Assisted circulation time (min)	73.9 ± 28.4	39.0 ± 21.7	**< 0.001**
Postoperative variables			
Packed red cells on first 24h (units)	1.9 ± 2.2	0.7 ± 1.9	**< 0.001**
Frozen plasma on first 24h (100 mL)	0.9 ± 1.9	0.4 ± 1.3	0.067
Platelet concentrate on first 24h (units)	0.1 ± 0.3	0	**0.006**
Packed red cells (units)	4.3 ± 5.7	1.2 ± 3.6	**< 0.001**

Variables	AD (n = 67)	Others (n = 70)	*p*-value
Postoperative variables			
Fresh frozen plasma (100 mL)	1.6 ± 3.6	0.7 ± 2.6	**0.049**
Platelet concentrate (units)	0.4 ± 0.8	0.1 ± 0.7	**< 0.001**
Hospital length of stay (day)	17.3 ± 7.3	16.9 ± 6.3	0.799
ICU length of stay (hour)	98.8 ± 83.2	38.2 ± 45.6	**< 0.001**
Prolonged ICU of stay (≥ 3 days)	32 (47.8%)	7 (10.0%)	**< 0.001**
Mechanical ventilation (hour)	38.5 ± 48.4	27.6 ± 49.8	**0.004**
Mechanical ventilation (≥ 24 hours)	35 (52.2%)	8 (11.4%)	**< 0.001**
In-hospital mortality	2 (3.0%)	1 (1.4%)	0.534
Renal replacement therapy	5 (7.5%)	0	0.061
Acute kidney injury	28 (41.8%)	13 (18.6%)	**0.003**
Acute kidney injury			**0.006**
None	39 (58.2%)	57 (70.1%)	
Stage 1	15 (22.4%)	10 (14.3%)	
Stage 2	6 (9.0%)	3 (4.3%)	
Stage 3	7 (10.4%)	0	
Extracorporeal membrane oxygenation	0	2 (2.9%)	0.497
Stroke	7 (10.4%)	1 (1.4%)	0.059
Paraplegia	3 (4.5%)	0	0.228
Re-exploration for bleeding	2 (5.0%)	2 (2.9%)	0.228
Serious adverse events	42 (62.7%)	11 (15.7%)	**< 0.001**

Footnote: Variables are displayed as n (%) and mean ± standard deviation. The *p*-values with statistical significance are bolded.

Abbreviations: ICU, intensive care unit.

Table S5. Univariable logistic regression of serious adverse events.

Variables	OR (95% CI)	*p*-value
Age	1.03 (0.99 - 1.08)	0.151
Male	1.29 (0.44 - 3.80)	0.649
Body mass index	0.99 (0.86 - 1.13)	0.869
Smoking	0.71 (0.25 - 2.04)	0.527
Hypertension	1.49 (0.53 - 4.18)	0.452
Left ventricular ejection fraction	1.01 (0.91 - 1.13)	0.830
Free iron ion	1.69 (1.24 - 2.31)	**0.001**
Interleukin-6	1.02 (1.00 - 1.04)	**0.037**
Serum creatinine	1.04 (1.00 - 1.08)	**0.049**
Hepcidin	1.04 (0.97 - 1.10)	0.266
Kidney injury molecule-1	1.00 (0.98 - 1.01)	0.584
Malondialdehyde	0.90 (0.50 - 1.61)	0.717
Reactive oxygen species	1.00 (0.99 - 1.00)	0.549
Cardiopulmonary bypass time	1.01 (1.00 - 1.02)	0.069
Cross-clamp time	1.01 (0.99 - 1.03)	0.170

Footnote: Preoperative and operation-related variables with *p*-values < 0.20 in univariable logistic regression were considered candidates for multivariable regression. The *p*-values with statistical significance are bolded.

Abbreviations: OR, odds ratio; CI, confidence interval.