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Supplemental information

Cardiomyocyte-fibroblast interaction regulates ferroptosis and fibrosis after myocardial injury

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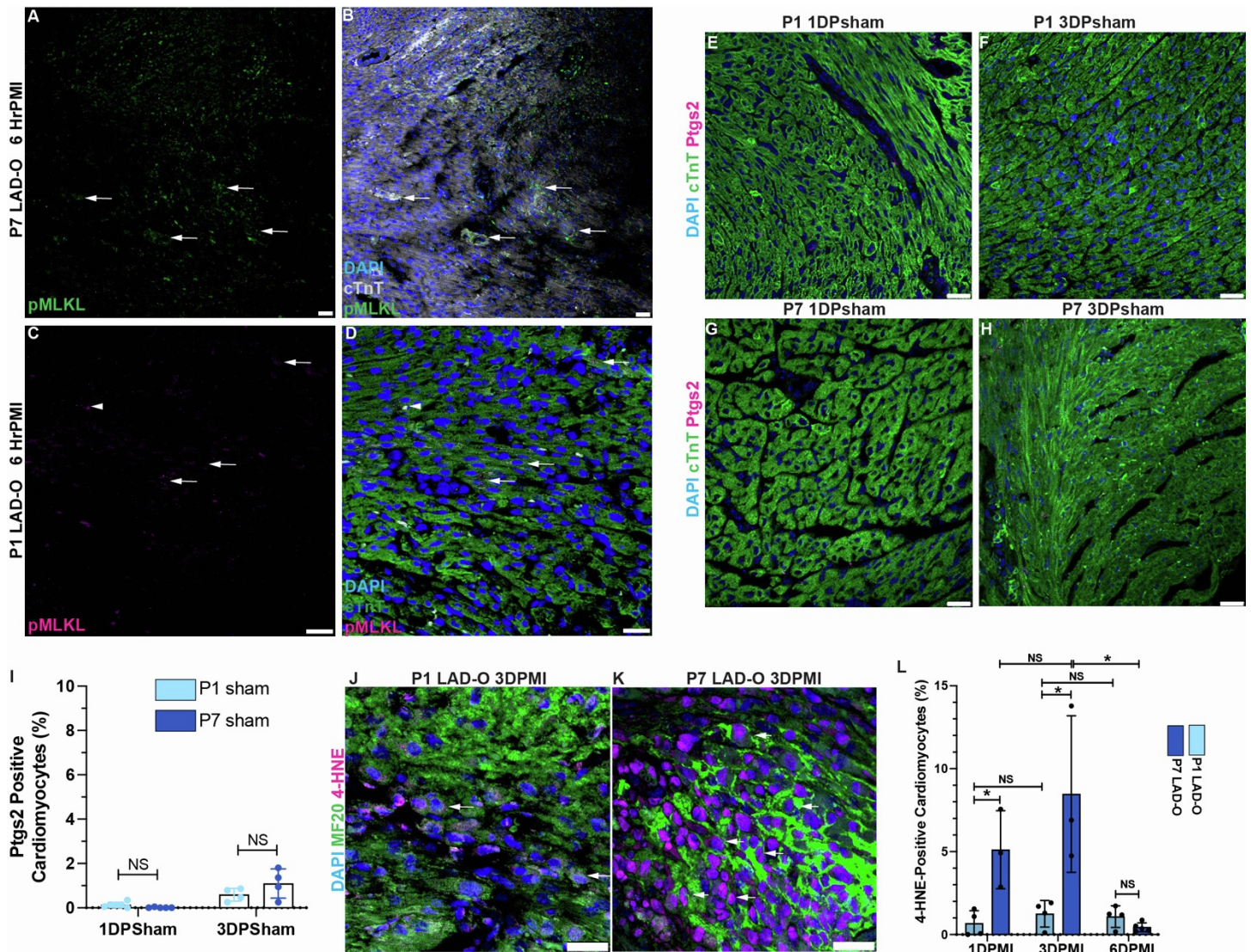


Figure S1. Necroptosis and ferroptosis in cardiomyocytes after MI, related to Figure 1. (A, B) Heart tissue section of wild type mouse stained for pMLKL (Green) and cTnT (grey) at 6 hours after P7 LAD-O. (C, D) Heart tissue section of wild type mouse stained for pMLKL (magenta) and cTnT (green) at 6 hours after P1 LAD-O. (E-I) Sham procedure was performed at either P1 (E, F) or P7 (G, H), heart section were prepared at 1 (E, G) or 3 (F, H) day-post-sham (DPsham) and stained for Ptgs2 (Magenta), cTnT (green) and DAPI (blue), ratio of Ptgs2-positive cardiomyocytes was quantified in (I). (J, K) Mouse heart tissue stained for 4-HNE (magenta) and MF20 (green) at 3 days after P1 (J) or P7 (K) LAD-O. Arrows, cardiomyocytes positive for 4-HNE. (L) Ratio of cardiomyocytes positive for 4-HNE at 1, 3 and 6 days after P1 or P7 LAD-O. DAPI in blue. Arrows, cardiomyocytes positive for pMLKL. All bar graphs represent mean \pm SD. *, $p < 0.05$. NS, not significant. Scale bar, 25 μ m.

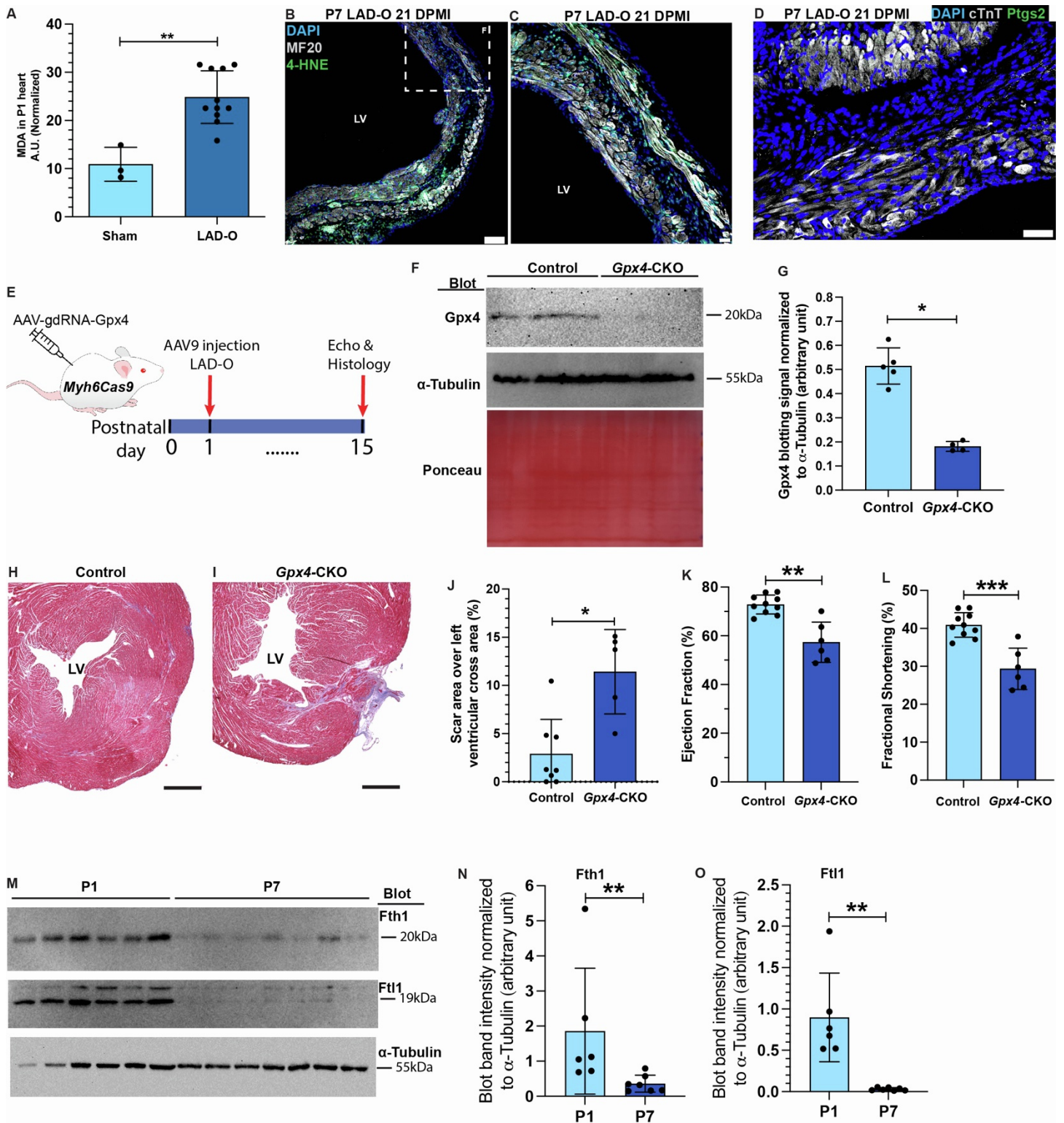


Figure S2. Ferroptosis occurs in cardiomyocytes after MI and affects neonatal heart regeneration, related to Figure 1. (A) MDA assay quantified lipid peroxidation level in ventricular myocardium after P1 LAD-O or sham procedure. (B, C) Mouse heart tissue stained for 4-HNE (green) and MF20 (grey) at 21 days after P7 LAD-O. (D) Heart tissue stained for Ptgs2 (green) and MF20 (grey) at 21 days after P7 LAD-O. (E) Schematic plan for F-L. AAV9-gdRNA-Gpx4 vectors were injected into *Myh6Cas9* mice to generate *Gpx4*-CKO hearts. Wild type littermates with AAV9 injection were used as control. (F, G) Western blot of Gpx4 and α -Tubulin in left ventricular tissue of control and *Gpx4*-CKO hearts at 15 DPMI. Band intensity of Gpx4 was quantified in G. (H-J) Trichrome of control and *Gpx4*-CKO hearts at 15 DPMI, with scar area quantified in J. (K, L) Ejection fraction (K) and fractional shortening (L) measured by echocardiography in control and *Gpx4*-CKO hearts at 15 DPMI. (M-O) Western blot of Fth1, Ftl1 and α -Tubulin in left ventricular tissue of P1 and P7 mouse heart. Band intensity of Fth1 and Ftl1 quantified in N and O. LV, left ventricle. DAPI in blue. All bar graphs represent mean \pm SD. *, p < 0.05. **, p < 0.01. NS, not significant. Scale bar, 25 μ m (C, D), 100 μ m (B), 500 μ m (H, I).

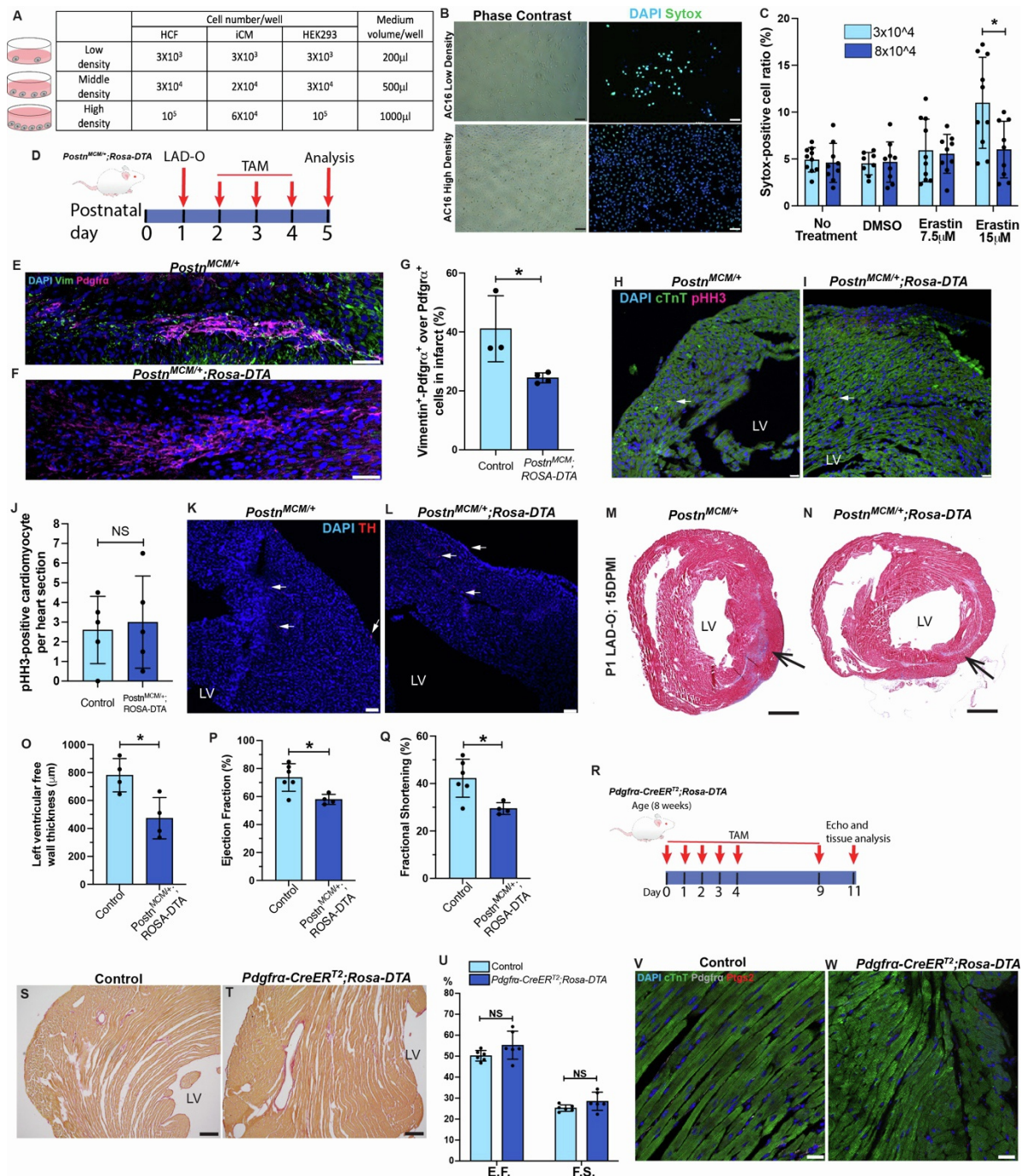


Figure S3. Cell density regulates ferroptosis *in vitro* and *in vivo*, related to Figure 2 and Figure 3.

(A) Schematic of cell density assays using 24-well culture plates, see Figure 2 and 3. (B) AC16 cells cultured at low (3×10^4 /well) and high (8×10^4 /well) density and treated with erastin. Dying cells were stained with Sytox (green). (C) Ratio of Sytox-positive AC16 cells. (D) Schematic plan for E-G and Figure 2H-P. (E, F) Heart tissue of controls (*Postn*^{MCM/+}, E) and *Postn*^{MCM/+}; *Rosa-DTA* (F) mice were stained for Vim (green) and Pdgfra (magenta) at 4 DPMI after P1 LAD-O. (G) Ratio Vim⁺-Pdgfra⁺ cells over total Pdgfra⁺ cells. (H-J) Controls (*Postn*^{MCM/+}, H) and *Postn*^{MCM/+}; *Rosa-DTA* (I) mice were stained for cTnT (green) and pHH3 (magenta) at 4 DPMI after P1 LAD-O. Arrows, cardiomyocytes positive for pHH3. Number of pHH3-positive cardiomyocytes was quantified in J. (K, L) Controls (*Postn*^{MCM/+}, K) and *Postn*^{MCM/+}; *Rosa-DTA* (L) mice were stained for tyrosine hydroxylase (TH, red) at 4 DPMI after P1 LAD-O. Arrows, cells expressing TH. (M-Q) Trichrome of controls (*Postn*^{MCM/+}, M) and *Postn*^{MCM/+}; *Rosa-DTA* (N) at 15 DPMI after P1 LAD-O. Arrows, scar zone. Thickness of left ventricular free wall was measured and quantified in O. Ejection fraction and Fractional shortening were quantified in P and Q. (R) Schematic plan for S-W and Figure 2S-X. (S, T) Picosirius red staining of adult control (*Rosa-DTA*, S) and *Pdgfra-CreER*^{T2}; *Rosa-DTA* (T) mice. (U) Ejection fraction (E.F.) and fractional shortening (F.S.) of control and *Pdgfra-CreER*^{T2}; *Rosa-DTA* mice. (V, W) Adult control (*Rosa-DTA*, S) and *Pdgfra-CreER*^{T2}; *Rosa-DTA* (T) heart section stained for cTnT (green), Pdgfra (grey) and Ptgs2 (red). Nuclei stained with DAPI (blue). TAM, tamoxifen. All bar graphs represent mean \pm SD. *, $p < 0.05$. NS, not significant. Scale bar, 25 μ m (E, F, H, I, V, W), 75 μ m (K, L), 100 μ m (B, S, T), 500 μ m (M, N).

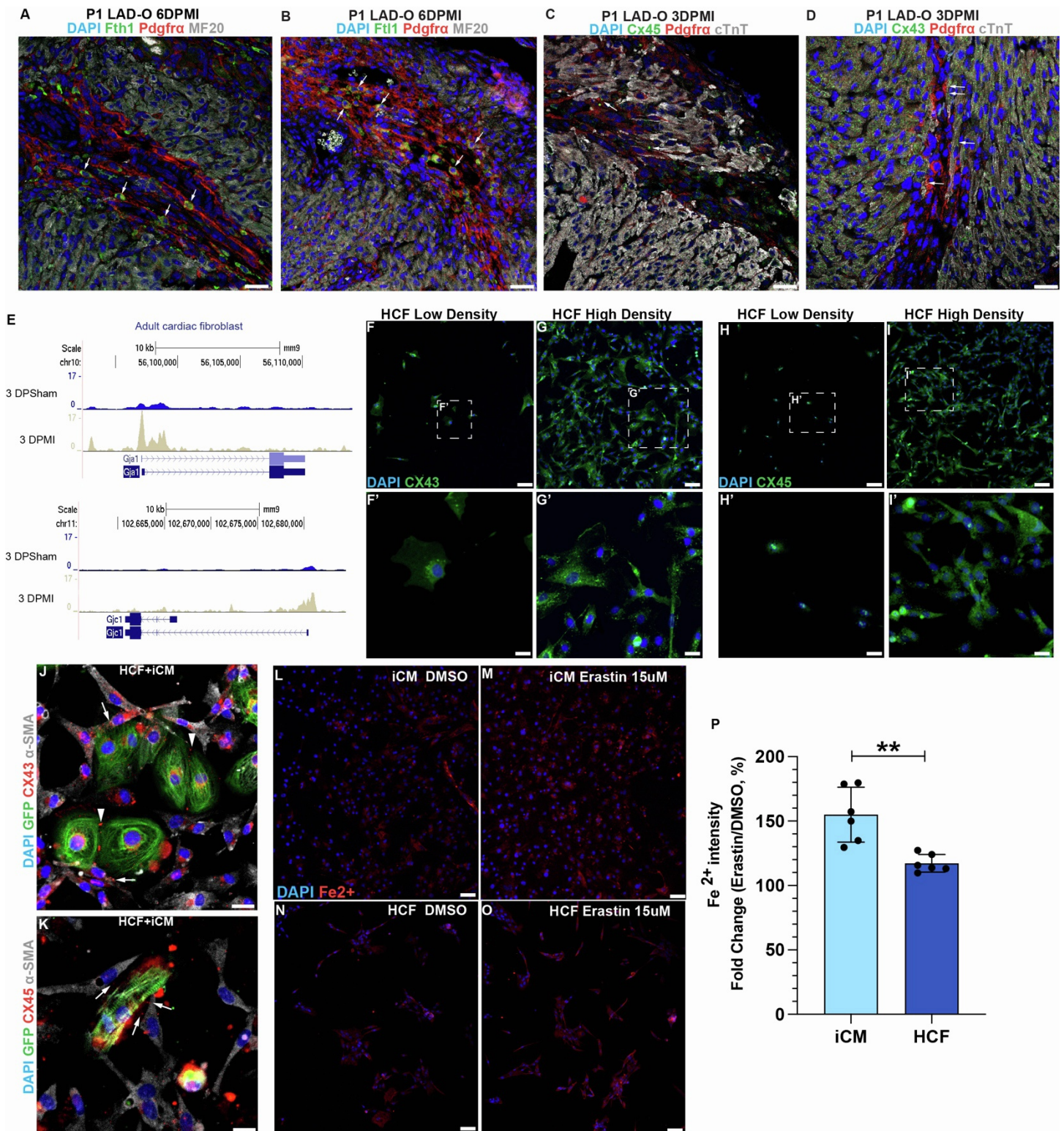


Figure S4. Cardiac fibroblasts interact with cardiomyocytes to share free iron, related to Figure 5. (A, B) Mouse heart sections stained for Fth1 (green, A) or Ftl (green, B), with Pdgfra (red) and MF20 (grey) at 6 days after P1 LAD-O. Arrows, Pdgfra-labeled cells positive for Fth1 (A) or Ftl (B). (C, D) Mouse heart section stained for Cx45 (green, C) or Cx43 (green, D) with Pdgfra (red) and cTnT (grey) after P1 LAD-O. Arrows, potential locations of gap junctions between cardiomyocytes and fibroblasts. (E) ATAC-Seq shows open chromatin region at loci of *Gja1* (*Cx43*) and *Gjc1* (*Cx45*) in adult cardiac fibroblasts at 3 days after LAD-O or sham procedure. (F-I') HCF cultured in low or high density and stained for CX43 (green, F-G') and CX45 (green, H-I'). (J-K) Co-cultured iCMs (marked by TITIN-GFP, green) and HCFs stained for CX43 (red, J) or CX45 (red, K), with αSMA (grey). Arrowheads in J, gap junctions between iCMs. Arrows in J and K, gap junctions between iCM and HCF. (L-P) iCMs (L, M) and HCF (N, O) stained for Fe²⁺ (red) after DMSO (L, N) or erastin (M, O) treatment. Fold change of Fe²⁺ fluorescent intensity after erastin treatment in iCM and HCF quantified in P. Nuclei stained with DAPI (blue). All bar graphs represent mean ± SD. **, p<0.01. Scale bar, 25 μm (A-D, F', G', H', I', J, K), 100 μm (F, G, H, I, L-O).

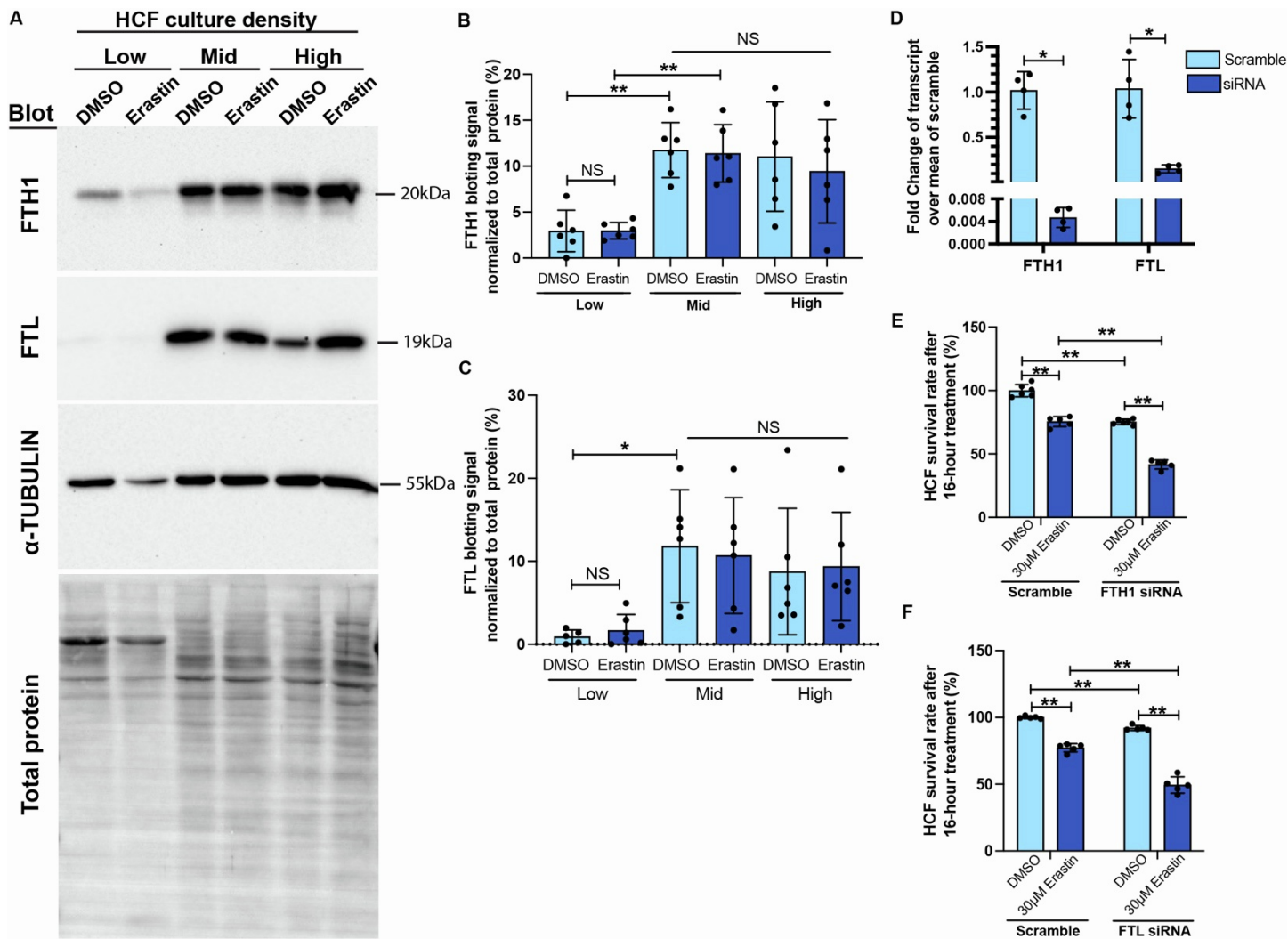


Figure S5. Cardiac fibroblasts increase Ferritin expression to resist ferroptosis, related to Figure 5. (A-C) Western blot of FTH1, FTL and α -TUBULIN in HCFs cultured at low, mid and high density, with DMSO or erastin treatment. Target band signal intensity quantified in B (FTH1) and C (FTL). (D) qPCR shows the knockdown of *FTH1* and *FTL* with siRNA in HCFs. (E, F) Survival rate of HCFs after erastin (30 μ M) treatment, with *FTH1* (E) or *FTL* (F) knockdown compared to scramble siRNA. All bar graphs represent mean \pm SD. *, $p < 0.05$. **, $p < 0.01$. NS, not significant.

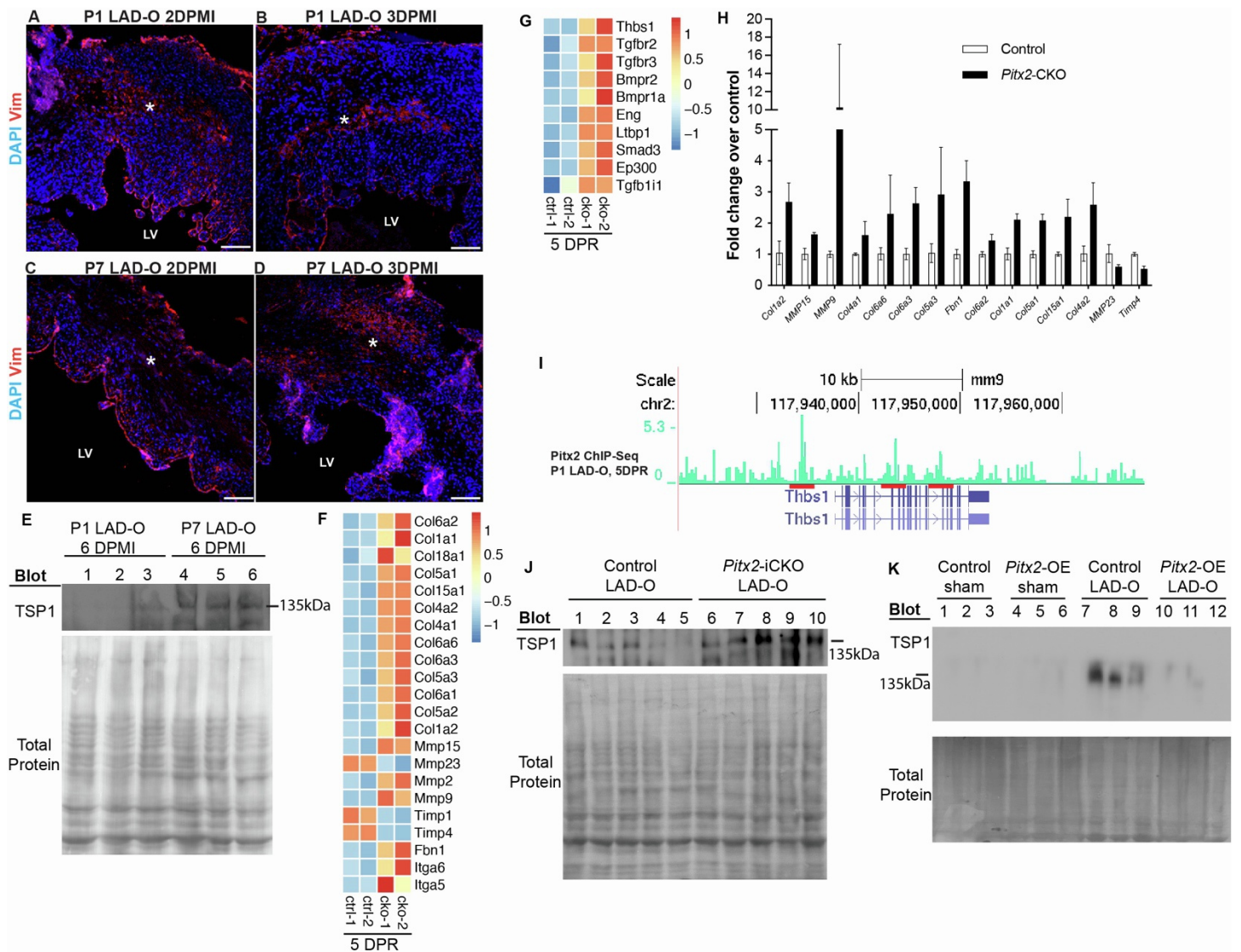


Table S1. Quantification of common paracrine cytokines and chemokines in the HCF-conditioned medium using cytokine array, related to Figure 4.

Analyte	Ctrl-1	Ctrl-2	Ctrl-3		H2O2-1	H2O2-2	H2O2-3	t test, p value	p<0.05
Adiponectin	0.85	0	0		0	1.375	0	0.8125	
Apolipoprotein A-I	0.15	0	0		0	0	0	0.4226	
Angiogenin	10.95	27.15	2.775		2.525	24.175	1.65	0.1970	
Angiopoietin-1	0	0	0		0	0	0	N/A	
Angiopoietin-2	3.35	0	0		0	3.925	0	0.9357	
BAFF	0	0	0		0	0	0	N/A	
BDNF	1.65	0	0		0	1.175	0	0.8646	
Complement Component C5/C5a	0	0	0		0	0	0	N/A	
CD14	1.1	0	0		0	1.625	0	0.8455	
CD30	3.65	0	0		0	4.925	0.55	0.8288	
CD40 ligand	0	0	0		0	0.925	0	0.4226	
Chitinase 3-like 1	0	0	0		0	0	0	N/A	
Complement Factor D	0	0	0		0	0	0	N/A	
C-Reactive Protein	0	0	0		0	0	0	N/A	
Cripto-1	0	0	0		0	0	0	N/A	
Cystatin C	8.65	21.8	8.975		0	3.675	0	0.0617	
Dkk-1	19.5	25.45	11.475		24.875	46.625	28.45	0.0917	
DPPIV	0	0	0		0	0	0	N/A	
EGF	4.48	4.62	3.74		7.343	10.949	3.91	0.2221	
EMMPRIN	11.3	17.35	1.925		12.375	30.775	8.8	0.1839	
ENA-78	0	0	0		0	0	0	N/A	
Endoglin	0	1.1	0		0	1.775	0	0.4226	
Fas Ligand	0	0	0		0	0	0	N/A	
FGF basic	0	0	0		0	0	0	N/A	
FGF-7	0	0	0		0	0	0	N/A	
FGF-19	4.15	8.85	0		4.425	13.125	1	0.2716	
Flt-3 Ligand	0	0	0		0	0	0	N/A	
G-CSF	0	0	0		0	0	0	N/A	
GDF-15	0	1.05	0		1.975	10.375	0	0.3156	
GM-CSF	0	0	0		0	2.125	0	0.4226	
GRO α	0	1.4	0		0	0	0	0.4226	
Growth Hormone	0	0	0		0	0	0	N/A	
HGF	0	0.7	0		0	0	0	0.4226	
ICAM-1	0	0	0		0	0	0	N/A	
IFN- γ	0	0	0		0	0	0	N/A	
IGFBP-2	16.1	30.55	9.075		0	1.725	0	0.0895	
IGFBP-3	20.95	34.35	22.775		3.325	6.275	0	0.0170	*
IL-1 α	2	2.5	0		0	2.775	0	0.5067	
IL-1 β	0	0	0		0	0	0	N/A	
IL-1ra	0	0	0		0	0	0	N/A	

IL-2	0	0	0		0	0.325	0	0.4226	
IL-3	0	0	0		0	0.925	0	0.4226	
IL-4	0	1.45	0		0	0	0	0.4226	
IL-5	0	0	0		0	0	0	N/A	
IL-6	0	0	0		0	0	0	N/A	
IL-8	11.70 8	15.957 5	14.675		37.583 5	41.01 5	32.06 4	0.0138	*
IL-10	0	0	0		0	0	0	N/A	
IL-11	0	0	0		0	0	0	N/A	
IL-12 p70	0	0	0		0	0	0	N/A	
IL-13	0	0	0		0	0	0	N/A	
IL-15	0	0	0		0	0	0	N/A	
IL-16	0	0	0		0	0	0	N/A	
IL-17A	2.7	6.35	0		3.975	10.77 5	0.15	0.2670	
IL-18 Bpa	0	0	0		0	0.525	0	0.4226	
IL-19	0	0	0		0	0	0	N/A	
IL-22	0	3.45	0		0	1.475	0	0.4226	
IL-23	0	0	0		0	0	0	N/A	
IL-24	0	0	0		0	0	0	N/A	
IL-27	0	0	0		0	0	0	N/A	
IL-31	0	0	0		0	0	0	N/A	
IL-32	0	0	0		0	0	0	N/A	
IL-33	0	0	0		0	0	0	N/A	
IL-34	0	0	0		0	0	0	N/A	
IP-10	0	0	0		0	0	0	N/A	
I-TAC	0	0	0		0	0	0	N/A	
Kallikrein 3	4	2.8	0.125		3.425	3.775	1.7	0.4121	
Leptin	0	0.45	0		0	0	0	0.4226	
LIF	0	0	0		0	0	0	N/A	
Lipocalin-2	0	0	0		0	0	0	N/A	
MCP-1	0	5.6	0		0	0.125	0	0.4226	
MCP-3	0	0	0		0	0	0	N/A	
M-CSF	0	0	0		0	0	0	N/A	
MIF	0	3.45	0		0.925	5.025	0	0.2098	
MIG	0	0	0		0	0	0	N/A	
MIP-1 α /MIP-1 β	0	0	0		0	0	0	N/A	
MIP-3 α	0	0	0		0	0	0	N/A	
MIP-3 β	0	0	0		0	0	0	N/A	
MMP-9	0	1.4	0		1.425	1.025	0	0.5886	
Myeloperoxidase	0	0.9	0		0	0	0	0.4226	
Osteopontin	0	5.2	0		1.075	1.625	0	0.6134	
PDGF-AA	0	0	0		0	0	0	N/A	
PDGF-AB/BB	0	0	0		0	0	0	N/A	
Pentraxin 3	0	6.75	0		1.625	1.625	0	0.6242	
PF4	0	0	0		0	0	0	N/A	
RAGE	0	0	0		0	0	0	N/A	
RANTES	0	0	0		0	0	0	N/A	
RBP-4	0	0	0		0	0	0	N/A	
Relaxin-2	0	0.15	0		0	0	0	0.4226	

Resistin	0	2.25	0		1.575	0.675	0	1.0000	
SDF-1 α	6.15	15.95	7.375		4.025	3.325	0	0.1354	
Serpin E1	64.15	91.5	67.525		69.575	78.675	27.25	0.3541	
SHBG	0	2.2	0		0	0	0	0.4226	
ST2	0	0	0		0	0	0	N/A	
TARC	0	0	0		0	0	0	N/A	
TFF3	0	0	0		0	0	0	N/A	
TfR	0	0.95	0		0	0	0	0.4226	
TGF- α	0	0	0		0	0	0	N/A	
Thrombospondin-1	0	8.4	1.475		0.175	0.275	0	0.3412	
TNF- α	0	0	0		0	0	0	N/A	
uPAR	0.9	12.1	5.975		6.125	10.525	0	0.8341	
VEGF	0	1	0.325		0.925	0	0	0.8351	
Vitamin D BP	0	3.15	0		0.825	0	0	0.5878	
CD31	0	2.5	0		0	0	0	0.4226	
TIM-3	0	0	0		0	0	0	N/A	
VCAM-1	0	0.25	0		0	0	0	0.4226	