SUPPLEMENTAL MATERIAL
Data S1. Supplemental Methods

Spectrophotometric Assays for ETC Enzymes

Ground tissues from the left ventricle (LV) were homogenized in ice-cold homogenization buffer (20mM Tris, 40mM KCl, 2mM EGTA, pH=7.4, with 50mM sucrose added day of homogenization). Samples were centrifuged at 600g for 10 minutes at 4°C to remove cellular debris. Supernatant was collected and used to assess the electron transport chain (ETC) enzyme activity of NADH:ubiquinone oxidoreductase (COX I), succinate dehydrogenase (SDH, COX II), decylubiquinol cytochrome c oxidoreductase (COX III), NADH cytochrome c oxidoreductase (COX I + III), succinate cytochrome c reductase (COX II + III), cytochrome c oxidase (COX IV) and citrate synthase (CS). Enzyme activity (nmol·min⁻¹·mg⁻¹) was normalized to volume and protein concentration, following protein determination with Bradford assay. Specifically, it is calculated based on the following equation: enzyme activity (nmol·min⁻¹·mg⁻¹) = \( \frac{\Delta \text{Absorbance/min} \times 1000}{(\text{extinction coefficient} \times \text{volume of sample loaded in ml}) \times (\text{protein concentration of sample in mg/ml})} \). Furthermore, the reaction specificity was assured by subtracting the inhibitor-resistant activity from the total enzymatic activity, which were conducted in parallel. The inhibitor for COX I (1mM rotenone), COX II (1M malonate), COX III (1mg/ml antimycin A), COX I + III (1mM rotenone), COX II + III (1M malonate), and COX IV (10mM KCN) were added to each corresponding reaction mixture prepared separately. Measurements were performed in triplicate.
Spectrophotometric Assays for Antioxidant Enzymes

As described previously, flash-frozen LV tissues were homogenized by using TissueLyser II (r=25rpm, 3min; Qiagen) and total protein were extracted in ice-cold CelLytic™ M (C2978, Sigma, MO, USA) supplemented with protease (Complete Protease Inhibitor) and phosphatase inhibitor (PhosSTOP EasyPack) (Roche, Mannheim, Germany) cocktails. The homogenate was centrifuged at 14,800rpm (21,100g) for 12mins (4°C), and the enzymatic activities were examined in the aliquoted supernatants after quantitation of protein concentration using Bradford assay. All measurements were repeated in duplicate, and the average value was used.

**CAT Enzyme Assay.** Catalase (CAT) activity was measured according to the method described previously with minor modification. Briefly, 20-40μl tissue homogenate (100-300μg protein) was added to 600μl assay buffer, which contained 50mM KH₂PO₄ and 50mM Na₂HPO₄ (pH 7.0), and baseline absorbance was recorded at 240nm for 3 minutes at room temperature (RT) using a quartz cuvette. Reactions were started upon addition of 300μl H₂O₂ (30mM) and the changes to absorbance was followed for 5 minutes. Specific activity (units/mg) was defined as the rate of H₂O₂ consumption per minute per milligram of protein sample.

**SOD Enzyme Assay.** Superoxide dismutase (SOD) activity was assayed based on the competition for O₂⁻ between (ferri-)cytochrome c and SOD following its spontaneous dismutation. One unit of activity was defined as the amount of SOD required to inhibit the initial reduction rate of ferri-cytochrome c by 50%. A reaction cocktail containing 50mM KH₂PO₄/0.1mM EDTA (pH 7.8), 50μM xanthine (X0626, Sigma, MO, USA) and 10μM cytochrome c (C2867, Sigma, MO, USA) was prepared at RT. Xanthine oxidase (6nM, X4376, Sigma, MO, USA) was added to the mixture to obtain a stable baseline reading (0.015-0.025
Abs/min) at 418 nm for 3 minutes. Reactions were started by the addition of whole cell lysate (3-15 μg protein) to a quartz cuvette and absorbance was continuously monitored for 5 minutes to calculate the total SOD activities (SOD1-3). Mitochondrial SOD (SOD2, Mn/Fe-SOD) activity was determined by adding 100mM KCN to a matched reaction mixture prepared from the same sample. The overall Cu/Zn-SOD activities from cytosol (SOD1) and extracellular matrix (SOD3) are completely inhibited by the KCN (100mM) added. The purity of cytochrome c (potential SOD contamination) was checked by adding 1mM KCN to reaction mixtures prior to the addition of any SOD-containing samples; no significant increase in cytochrome c reduction rate were noted after addition of KCN.

**GPX Enzyme Assay.** Glutathione peroxidase (GPX) activity was measured based on the oxidation of reduced glutathione (GSH) by GPX coupled to the disappearance of NADPH catalyzed by glutathione reductase (GR). The rate of NADPH oxidation was monitored spectrophotometrically at 340nm. Briefly, two assays (A & B) were prepared each containing 0.1M K₂HPO₄/1mM EDTA (pH 7.0), 10mM L-glutathione reduced (G4251, Sigma, MO, USA), 2.4unit/ml glutathione reductase (G3664, Sigma, MO, USA). Both assays were firstly pre-incubated at 37°C for 10 minutes in the presence (assay A) and absence (assay B) of the whole cell lysate (50-150 μg protein). The H₂O₂-independent NADPH oxidation in both assay reactions were monitored for 3 minutes immediately after the addition of 1.5mM NADPH (10107824001, Sigma, MO, USA). Next, pre-warmed 1mM sodium azide (catalase inhibitor; S2002, Sigma, MO, USA) and 1.5 mM H₂O₂ were added simultaneously to both assays and the reduced NADPH optical density was read every 30 seconds for 5 minutes. The non-enzymatic and H₂O₂-independent NADPH depletion were subtracted from the total GPX activity, by comparing the
absorbance changes after addition of H₂O₂ in two assays. Activities were normalized to the added lysate volume and protein concentration.

**Measurement of Myocardial Lipid Peroxidation**

Malondialdehyde (MDA), as an established indicator of lipid peroxidation, was measured colorimetrically using a commercial kit (Abcam, ab233471) according to the manufacturer’s instructions. In detail, flash-frozen myocardium samples (~150 mg) from LV were chopped into smaller pieces and then fully homogenized using Dounce homogenizer in low pH lysis buffer (500μl/each, 20mM NaH₂PO₄ & 0.5% TritonX-100, pH 3.0-3.2) with the addition of protease (Complete Protease Inhibitor) and phosphatase (PhosSTOP EasyPack) (Roche, Mannheim, Germany) inhibitor cocktails. Following a 10-min incubation on ice, the tissue lysates were centrifuged at top speed for 6 min (13,000 rpm, 4°C) and the clear supernatants were collected or stored at -80°C for further studies. Protein concentrations were quantitated using the Bio-Rad BCA assay as aforementioned, and all the prepared reagents and materials were gently agitated after equilibrated to RT. Next, 50 μl of each sample lysate and serially diluted MDA standards (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μM) were pipetted into a 96-well clear bottom microplate, immediately followed by adding 10 μl of MDA Color Reagent solution to each well and incubating at RT for 15 min in the dark. Finally, 40 μl of Reaction Solution was added to each mixture with another 45-min incubation at RT. The absorbance increases were monitored by a microplate reader (SpectraMax iD5, Molecular Devices, San Jose, CA) with path-check correction at 695 nm. The absorbance readings of blank controls (with dilution buffer or lysis buffer only) were used as the negative controls, and were subtracted from the detected values
from both the standards and experimental samples. The total concentration of free MDA (\(\mu M/mg\)) was determined by reference to the MDA standard curve correcting for the sample lysate dilution as well as total amount of protein loaded.\textsuperscript{26,35} The assay conditions (e.g., low pH) served to minimize potential interferences from other lipid peroxidation natural by-products, such as 4-hydroxyalkenals (4-HNE), and our protocol specifically probed the free MDA level within the myocardium. Each sample was assayed in duplicate, with the average value accepted.

**Tissue Glutathione Level (GSH/GSSG) Measurement**

Reduced (GSH), oxidized (GSSG) myocardial glutathione and their redox ratio (GSH:GSSG) were quantitated by enzymatic recycling method as described previously.\textsuperscript{27,30,36} Each sample was analyzed in triplicate, and the average value was used.

**Subcellular Fractionation and Western Blot**

Subcellular fractionations were performed as previously described with modifications.\textsuperscript{40} Tissues from LV were lysed and homogenized (20 rpm/minute, 2 minutes, 4\(^\circ\)C) in 500 \(\mu l\) radioimmunoprecipitation assay (RIPA, 50 mM Tris-HCl, 150mM NaCl and 1mM EDTA, pH=7.4) buffer with the addition of 1X protease inhibitor cocktail (Roche), followed by centrifugation (2900 g, 20 minutes, 4\(^\circ\)C) to precipitate the crude nuclear from the cytosolic and membrane proteins (first supernatant). The pellet was gently washed and homogenized again using the above methods, followed by a second homogenization (25 rpm/minute, 3 minutes, 4\(^\circ\)C) in 200 \(\mu l\) commercial RIPA buffer (ThermoFisher, 25mM Tris-HCl, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with 1X protease inhibitor cocktail
(Roche), producing pure nuclear fraction. The first supernatant was further ultra-centrifuged (29000 g, 45 minutes, 4°C) to pellet the membrane and simultaneously harvest cytosolic components from the second supernatant. The purity of each fraction was further validated by using anti-rabbit TLR-4 (Santa Cruz, sc-10741; membrane marker), anti-rabbit Caspase-3 (Cell Signaling, 9662S; cytosolic marker) and anti-rabbit Histone H3 (Cell Signaling, 4499s; nuclear marker).\textsuperscript{41}

For mitochondrial fractionation, frozen LV tissues were ground and homogenized in fractionation buffer containing 250mM sucrose, 10mM Tris-HCL, 1mM EDTA, 1mM sodium orthovanadate, 1mM sodium fluoride, 10μg/L aproptinin, 2μg/L leupeptin, and 100μg/L pepstatin.\textsuperscript{33} Homogenate was first centrifuged for 10 minutes at 700g (4°C) to remove the debris. The supernatant was decanted and centrifuged for 20 minutes at 10,000g (4°C) to obtain the “crude” mitochondrial fractions as pellet. Subsequently, the mitochondrial pellet was resuspended in fractionation buffer and protein concentrations was calorimetrically determined using the Bio-Rad BCA protein assay kit (with bovine serum albumin as standards).

Western blotting was performed on flash snap-frozen human myocardium tissues.\textsuperscript{40, 41} In total, 500 μg protein was extracted; and aliquots of protein (45 - 60 μg) were separated on 6% - 20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.2μm PVDF membranes. They were subject to immunoblotting with the following primary antibodies: anti-rabbit TFR-1 (Cell Signaling, 13208s); anti-rabbit FPN (Novus, NBP1-21502); anti-rabbit FTN (Abcam, ab75973); anti-mouse DMT-1 (Abcam, ab55735), and subsequently incubated with HRP-conjugated secondary antibodies at 1/5000 dilution (Cell Signaling). The protein loadings were visualized by MemCode\textsuperscript{TM} reversible stain
(24585, Thermo Scientific™) of the PVDF membranes as a loading control, and all blots were scanned by ImageQuant LAS 4000 (28955810, GE Health Care, Biosciences, Uppsala, Sweden). To avoid exhausting valuable samples, some blots were reprobed with a second targeted protein after incubation in Restore™ Western Blot Stripping Buffer (ThermoFisher 21059) for 15-30 minutes (RT), followed by vigorous washing using combination of 1X TBST (3rpm, 5min x3) and 1X TBS (3rpm, 5min x3), and complete blocking by 5% non-fat milk for 1 hour (3rpm, RT). ImageJ software (NIH, USA) was used for band intensity quantitation.

**Histological Analysis**

The excised transmural biopsies were immediately fixed in 10% buffered formalin (containing 4% formaldehyde) followed by embedding in paraffin. Thin sections (5μm) of the tissue were stained with picro-sirius red (PSR) and Masson’s trichrome stain for morphometric analysis. The tissue sections were first deparaffinized in xylene and alcohol grades, then rehydrated in water and subjected to respective staining protocols as described previously.26, 27 The fibrotic pattern was assessed by visualization under a bright field microscope (DM 4000 B, Leica), together with fibrillar content quantification under Olympus IX81 fluorescence microscope. Image analysis was performed on MetaMorph software (Basic version 7.7.0.0, Molecular Devices, Inc). From each heart, n=2 sections were stained with n=20-25 random images analyzed from each section in a blinded manner.

**Dihydroethidium Staining and Densitometry**
Dihydroethidium (DHE), a cell-permeable oxidative fluorescent probe, was applied to directly reflect the total superoxide levels from the LV specimens as previously demonstrated.\textsuperscript{26, 35, 37-39}

Briefly, the 5-10 μm OCT-embedded cryosections were washed using Hank’s Balanced Salt Solution (HBSS, #14025092, Gibco\textsuperscript{TM}) with calcium chloride and magnesium chloride at RT for 5 min. A sufficient amount of (100-200 μl) TrueBlack Lipofuscin Quencher (1:20 in 70% ethanol, #23007, Biotium) was quickly applied to both experimental and control sections at RT for 5-10 min, followed by washing with HBSS (5 min/each) for another 3 times. Then, the experiment sections were incubated with DHE (1:100 in HBSS, D1168, Invitrogen) at RT for 20 min in the dark, while the negative control sections were incubated with 1X HBSS simultaneously. The sections were finally mounted using prolong gold DAPI antifade (#P36931, Invitrogen). In situ generation of superoxide was then detected qualitatively using an Olympus IX81 fluorescence microscope with multi channels (i.e., TxRed:DHE and DAPI:nuclei) selected, and the overall oxidative stress was represented as the red fluorescence intensity of the product upon oxidation – (oxy)ethidium – within the nuclei, which was readily quantifiable by MetaMorph software (Basic version, 7.7.0.0, Molecular Devices, Inc.). Specifically, the RGB pictures were converted into 8-bit gray scale (intensity profile: 0 to 255), and regions (n=5-10) congruent to the cell nuclei boundaries from both experimental and negative control sections were randomly drawn to calculate the average pixel intensities as the background noise. Given that all the image acquisition settings (i.e., exposure time, brightness/contrast, etc.) remained unvaried between groups, the actual oxidized DHE fluorescence was obtained by subtracting the background signal from the average pixel intensity of the nuclei using Fiji ImageJ (National
Institute of Health, Bethesda, MD, USA) software.\textsuperscript{35} n=20 images/sample were blindly taken as the technical replicate with n=20-25 nuclei analyzed from each tissue section.

**Immunofluorescence (IF) and Fluorescence Microscopy**

Cardiomyocyte morphology was evaluated fluorescently by applying wheat germ agglutinin (WGA) staining on the optimal cutting temperature (OCT) compound-mount (TFM, General Data Company) tissue blocks, which was snap frozen in liquid nitrogen as previously published.\textsuperscript{21, 26, 40} Similarly, the 5-10 μm cryo-sectioned slices were fixed with 4% paraformaldehyde for 20 mins and then rehydrated in 1X PBS for 30 mins at RT. Sections were permeabilized using 100% precool methanol (-20°C) for 10 mins, followed by blocking with 4% BSA for 1 hr at RT. After thorough washings, the sections were incubated with WGA (1:200, W11261, Invitrogen) for 30 mins at RT and then applied with 20μl/section DAPI gold anti-fade mountant (#P36931, Invitrogen). The plasma membrane was fluorescently visualized under Olympus IX81 fluorescence microscope and analyzed using MetaMorph software (Basic version 7.7.0.0, Molecular Devices, Inc). From each heart, n=2 sections (including one technical control) were examined, with n=20-25 random images captured from each section in a blinded manner. Within each image, n=25 cardiomyocytes were unbiasedly sampled from whole regions (four corners & center) into our analyses.

**Autofluorescence Quench and Confocal Microscopy**

Non-specific autofluorescences (mainly lipofuscin) from the human OCT-embedded blocks were significantly eliminated by applying TrueBlack® Lipofuscin Quencher (#23007, Biotium)
to the cryosections for 5 mins at RT, followed by standardized tissue fixation, deparaffinization, antigen retrieval and permeabilization as described above. The sections were gently washed with 1XPBS for 3 times, blocked with 5% serum for 1 hr at RT, and incubated with primary antibody as per manufacturer instructions, namely anti-rabbit TFR-1 (Cell Signaling, 13208s), anti-rabbit FPN (Novus, NB1-21502), and anti-mouse DMT-1 (Abcam, ab55735) overnight in a humidified hood at 4°C. Next the sections were incubated with Alexa Fluor 594-conjugated secondary antibodies (Invitrogen, USA) against the host species of individual primary antibody for 2 hrs at 37°C. Lastly the sections were stained with Alexa Fluor 488-conjugated WGA (W11261, Invitrogen) and mounted with DAPI antifade (#P36931, Invitrogen) to outline plasma membrane and nuclei, respectively. Intracellular protein colocalizations were acquired under laser scanning confocal microscopy (Leica TCS SP5, Leica Microsystems), and quantitative analyses were performed using Fiji ImageJ (National Institute of Health, Bethesda, MD, USA) software.  

 Transmission Electron Microscopy (TEM)  

Human explanted myocardial specimens were collected transmurally as described above. The tissues (<1mm³) were promptly fixed in 2% glutaraldehyde with a physiological pH and 360 mOsm osmolarity at 37°C. After stored in 4°C fridges overnight, the specimens were post-fixed in 1.5% K₄Fe(CN)₆ and 2% osmium tetroxide (OsO₄) followed by complete washing with 0.1M sodium cacodylate (pH 7.2) and 0.1M sodium acetate (pH 5.2) buffers. Next, the post-fixative samples were immersed in solution of 2% uranyl acetate (UA) and 0.1M sodium acetate (pH 5.2) for high-contrast en bloc staining. Samples were dehydrated with graded ethanol and acetone
solutions, immediately followed by infiltration with Spurr’s Resin (Leica Electron Microscopy Sciences, Hatfield, PA, USA). After 24 hours, two resin blocks per sample were sectioned with an ultramicrotome diamond knife along the longitudinal axis of myofilaments to produce four non-consecutive ultrathin sections (70 μm), which were further post-stained with 4% UA and 4% lead citrate.

Four 100 μm² regions were randomly selected to obtain n=1 image at 2000X resolution, n=4 images at 4000X resolution, and n=6 images at 10000X resolution per sections for a total of 44 images per sample (H7650, Hitachi, Tokyo, Japan). Two investigators independently evaluated cardiomyocytes for the presence and severity of intramitochondrial inclusions, mitochondrial cristae quality as well as sarcomeric ultrastructural integrity (ImageJ software, National Institute of Health, Bethesda, MD, USA). Each mitochondrial long axis, short axis and cross-sectional surface area were measured. The cross-sectional area was defined as the region enveloped by the mitochondria outer membrane, and mitochondrial long axis was defined as the longest distance between two points on the outer membrane, while short axis was defined as the shortest distance perpendicular to the long axis. We established a scoring system in which a higher score signified a greater severity of dysfunction and based on the collective score of individual mitochondria, each heart specimen was ranked as healthy or varying degree (mild, moderate, or severe) of abnormalities (Table S1). For consistency, sarcomere or mitochondria whose outer membrane was cut off by the image field of view were excluded from analysis. Blinded assessment of all images was randomly carried out in triplicate by two examiners, and a third adjudicator was involved should any discrepancies arise between the individual assessments.
Cardiac Magnetic Resonance Imaging (CMR)

Frozen myocardium from the middle of interventricular septum were adopted to evaluate the tissue iron content by CMR mappings.\textsuperscript{42} Based on LV iron level, n=10 and n=4 samples were retrospectively included in the NFC group and each HF subgroup, respectively. However, the subsequent sample preparation, image acquisition, and analytical processing were conducted in a double-blinded manner. After rapid thawing (15 seconds) on ice, each frozen specimen was cut into cuboids with an approximate dimension of 10mm x 10mm x 5mm (length x width x thickness) with smooth edges. They were then immersed in sufficient 0.9\% saline solution to gently equilibrate the tissues to RT (21°C) avoiding possible interferences from temperature,\textsuperscript{42} and the fresh saline solution was replaced every 10 minutes for a total of three times. To eliminate artifacts from air bubbles, the final saline buffer (50ml) was simultaneously prepared by heating the 50ml conical tube in a water bath for 30 minutes, followed by thorough sonication (FS30H, Fisher Scientific, MA, USA) for additional 30 minutes at RT. Similarly, the tapping water filling the ultrasonic bath was replaced every 10 minutes in order to equilibrate the heated buffer solution back to RT without time delay. A 10ml Pyrex\textsuperscript{®} glass beaker (CLS100010, Aldrich, MO, USA) was assembled into the conical tube, where the prepared tissue was surrounded by homogenous deaired buffer solution and well situated at the bottom center with muscle fiber orientation in parallel to the magnetic field. All measurements were completed in duplicate.

CMR experiments were performed on a 3T MRI scanner (MAGNETOM Prisma; Siemens Healthcare; Erlangen, Germany) with body coil excitation and a 2.5 cm surface coil for signal reception. Longitudinal relaxation time (T\textsubscript{1}) images were acquired with a saturation-
recovery gradient-echo pulse sequence with the following parameters: 10 slices (no gap), 1 mm slice thickness, 30 mm by 60 mm field of view, 128 phase-encoding and 256 readout points for 0.23 mm in-plane spatial resolution. Saturation-recovery images with a recovery time of TS = 1000 ms and full recovery were used to calculate $T_1$ in each pixel. Transverse relaxation time ($T_2$) images were acquired with a spin-echo sequence with identical spatial coverage and resolution as the $T_1$ acquisition, with echo-times of TE=11 ms in steps of 11 ms to 88 ms. $T_2^*$ images were acquired with a multi-echo gradient-echo sequence with identical spatial coverage and resolution as the $T_1$ and $T_2$ acquisitions, with echo-times of TE=3.26 ms in steps 6.28 ms to 47.22 ms. Mono-exponential recovery was assumed for calculation of $T_1$, $T_2$ and $T_2^*$ (signal (TS)=1-exp(-TS/$T_1$) for saturation recovery imaging and signal(TE)=exp(-TE/$T_2$) and signal (TE)=exp(-TE/$T_2^*$) for $T_2$ and $T_2^*$ multi-echo imaging) with pixel-by-pixel relaxation maps generated for all samples. Averaged relaxation values (measured in msec) from all pixels within each tissue sample were automatically selected for analyses; all measurements were completed in duplicate.
Table S1. Qualitative Scoring Criteria for Mitochondrial Ultrastructural Morphology and Architecture for Intra-mitochondrial Inclusion (A) and Cristae (B)

| A.          | Percentage of Individual Mitochondrial Inclusion Scores |
|-------------|--------------------------------------------------------|
|             | % Mitochondria with score = 0 | % Mitochondria with score = 1-2 | % Mitochondria with score = 3 |
| Overall Patient Score | | | |
| Healthy     | >75% | <5% | <5% |
| Mild        | <75% | <5% | <5% |
| Moderate    | <75% | >5% | <5% |
| Severe      | X    | X   | >5% |

| B.          | Percentage of Individual Mitochondrial Cristae Scores |
|-------------|-------------------------------------------------------|
|             | % Mitochondria with score = 0 | % Mitochondria with score = 1-2 | % Mitochondria with score = 3 |
| Overall Patient Score | | | |
| Healthy     | >80% | <10% | <5% |
| Mild        | <80% | <10% | <5% |
| Moderate    | <80% | >10% | <5% |
| Severe      | X    | X   | >5% |

Intramitochondrial inclusions score of 0 indicates no inclusions, and scores 1-3 represent the presence of mitochondrial inclusions with increasing severity. Patients with >5% mitochondria having a score of 3 are considered severe regardless of other factors. Mitochondria cristae quality score of 0 indicates healthy cristae, and scores 1-3 represent decreasing cristae quality. Patients with >5% mitochondria having a score of 3 are considered severe regardless of other factors.
A multiple linear regression was performed, where the myocardial iron level is the outcome variable and the three multiparametric cardiac magnetic resonance mapping (CMR, including T1, T2, and T2*) and one etiological category (1: non-failing control; 2: heart failure) are the predictor variable. The “Enter” method (direct entry) was adopted for the variable selection in the linear regression model. Results show that the overall model could significantly predicted the myocardial iron content by CMR sequences and HF etiology, with $F(4,29)=3.705$ ($p<0.05$, $r=0.581$; Fig. 6D). However, only T2* ($\beta=-0.607$, $p=0.089$) and etiology ($\beta =-0.378$, $p=0.088$) are marginally significant predicting variables, whereas T1/T2 does not significantly contribute to the prediction model. More specifically, the myocardial iron levels are predicted to be larger with lower T1 and/or T2* mappings and among healthy individuals. IBM SPSS Statistics for Windows, version 21 (IBM Corp., N.Y., USA) was used for data analysis and narrative interpretation.
Table S3. Basic Clinical Profile of Patients with End-stage Heart Failure Secondary to Dilated Cardiomyopathy (DCM) and Coronary Artery Disease (CAD)

|                      | End-stage HF (N=138) | DCM (N=71) | CAD (N=67) | p-value  |
|----------------------|----------------------|------------|------------|----------|
| **Demographic**      |                      |            |            |          |
| Age (years)          | 54.5 (47.0-61.0)     | 50.0 (40.0-58.5) | 57.0 (48.5-63.0) | <0.001** |
| Sex, Male            | 116 (84)             | 59 (83)    | 57 (85)    | 0.751    |
| Heart Weight (gram)  | 465.0 (374.5-563.0)  | 476.0 (374.5-546.0) | 443.0 (375.5-564.8) | 0.928    |
| **Comorbidities**    |                      |            |            |          |
| CVD                  | 21 (15)              | 12 (17)    | 9 (13)     | 0.571    |
| PVD                  | 12 (9)               | 4 (6)      | 8 (12)     | 0.189    |
| COPD/Asthma          | 46 (33)              | 22 (31)    | 24 (36)    | 0.547    |
| DM2                  | 36 (26)              | 11 (15)    | 25 (37)    | 0.004**  |
| Dyslipidemia         | 41 (30)              | 12 (17)    | 29 (43)    | <0.001** |
| Thyroid Disease      | 22 (16)              | 14 (20)    | 8 (12)     | 0.212    |
| Kidney Disease       | 74 (54)              | 39 (55)    | 35 (52)    | 0.751    |
| Liver Disease        | 24 (17)              | 13 (18)    | 11 (16)    | 0.769    |
| Obesity              | 88 (64)              | 40 (56)    | 48 (72)    | 0.061    |
| PAH                  | 37 (27)              | 22 (31)    | 15 (22)    | 0.254    |
| Hypertension         | 33 (24)              | 12 (17)    | 21 (31)    | 0.047*   |
| **Electrocardiography** |                      |            |            |          |
| PR Interval (ms)     | 171.0 (146.0-202.0)  | 172.0 (148.5-199.0) | 168.0 (144.0-205.0) | 0.555    |
| QRS Duration (ms)    | 124.0 (93.0-154.0)   | 134.0 (102.0-162.0) | 112.0 (88.5-148.0) | 0.049*   |
| AF                   | 26 (19)              | 16 (23)    | 10 (15)    | 0.253    |
| IVCD                 | 35 (25)              | 22 (31)    | 13 (19)    | 0.118    |
| LBBB                 | 14 (10)              | 10 (14)    | 4 (6)      | 0.115    |
| RBBB                 | 23 (17)              | 8 (11)     | 15 (22)    | 0.080    |
| **Blood Parameters** |                      |            |            |          |
| Ferritin (μg/L)      | 133.0 (56.0-276.0)   | 135.0 (62.5-276.0) | 110.4 (53.0-261.8) | 0.484    |
| Serum Iron (μmol/L)  | 10.0 (7.0-13.5)      | 10.0 (6.0-14.0) | 11.0 (8.6-12.0) | 0.764    |
| TIBC (μmol/L)        | 54.0 (48.0-67.0)     | 57.0 (50.0-65.0) | 54.0 (47.3-71.5) | 0.795    |
| sTF (%)              | 19.0 (11.3-26.5)     | 16.0 (11.0-25.0) | 20.0 (15.0-27.0) | 0.401    |
| Hemoglobin (g/L) | 127.0 (109.5-139.0) | 133.0 (114.0-142.5) | 119.0 (102.0-130.5) | 0.001** |
|-----------------|----------------------|----------------------|----------------------|---------|
| MCV (fL)        | 90.0 (86.0-94.0)     | 90.0 (86.0-95.0)     | 90.0 (86.0-93.0)     | 0.624   |
| MCHC (g/L)      | 336.0 (328.3-342.0)  | 336.0 (327.5-341.5)  | 335.0 (330.0-342.5)  | 0.465   |
| BNP (pg/ml)     | 989.0 (485.0-1749.0) | 942.0 (494.0-1731.0) | 994.5 (476.5-3084.5) | 0.624   |
| NT-proBNP (pg/ml) | 3671.0 (2677.0-7777.0) | 3785.0 (2889.5-8087.0) | 2800.0 (1037.8-5100.8) | 0.208   |
| C-reactive Protein (mg/L) | 6.5 (3.0-31.8) | 7.2 (3.6-42.3) | 6.1 (2.6-19.7) | 0.472   |
| Creatinine (μmol/L) | 121.0 (95.3-150.0) | 120.0 (96.5-147.0) | 121.0 (93.5-152.5) | 0.920   |
| eGFR (ml/min/1.73m^2) | 55.0 (41.2-73.5) | 55.0 (44.0-75.0) | 56.0 (40.0-72.0) | 0.529   |

**Medications**

| Medication | Count (%) |
|------------|-----------|
| ACEi/ARB   | 109 (79)  |
| Beta Blocker | 112 (81)  |
| Diuretics  | 110 (80)  |
| MRA        | 80 (58)   |
| Digoxin    | 31 (22)   |
| Antiplatelet | 81 (59)   |
| Anticoagulation | 97 (70) |
| Statin     | 76 (55)   |
| Antiarrhythmic | 61 (44)  |

BMI=body mass index; BSA=body surface area; HR=heart rate; SBP=systolic blood pressure; DBP=diasstolic blood pressure; NYHA=New York Heart Association Functional Classification; CVD=cerebrovascular diseases; PVD=peripheral vascular diseases; COPD=chronic obstructive pulmonary diseases; DM2=type 2 diabetes mellitus; PAH=pulmonary artery hypertension; AF=atrial fibrillation; IVCD=intraventricular conduction delay; LBBB=left bundle branch block; RBBB=right bundle branch block; TIBC=total iron binding capacity; sTF=saturation of transferrin; MCV=mean corpuscular volume; MCHC=mean corpuscular hemoglobin concentration; BNP=brain natriuretic peptide; NT-proBNP=N-terminal pro b-type natriuretic peptide; eGFR=estimated glomerular filtration rate based on MDRD equation; ACEi=angiotensin converting enzyme inhibitors; ARB=angiotensin receptor blockers; MRA=mineralocorticoid receptor antagonists. Data are presented as means ± standard deviations, medians with lower and upper quartiles, or numbers (with percentages), where appropriate. Chi-square test, one-way ANOVA (followed by Tukey post hoc analysis) or Mann-Whitney U test were used as appropriate to compare the variables between groups. A two-tailed p value < 0.05 was considered statistically significant, as indicated by an asterisk. *p<0.05; **p<0.01. Categorical variables reported by count with percentage in parenthesis: sex, comorbidities, diagnosis of AF, IVCD, LBBB and RBBB, and medications. Continuous variables reported by median with interquartile range in parenthesis: age, heart weight, PR interval and QRS duration, and blood parameters.
Figure S1

A

MID-Hb
NID-Hb

B

MD-Ferritin
ND-Ferritin

C

MD-Systemic Iron
ND-Systemic Iron

MID-Ferritin
NID-Ferritin

MID-Hb
NID-Hb
**Figure S3**

**A**

|   | NID | MID |
|---|-----|-----|
| DCM | ![Image of DCM NID and MID](image) | ![Image of DCM NID and MID](image) |
| Peri-infarct | ![Image of Peri-infarct NID and MID](image) | ![Image of Peri-infarct NID and MID](image) |
| CAD | ![Image of CAD NID and MID](image) | ![Image of CAD NID and MID](image) |
| Non-infarct | ![Image of Non-infarct NID and MID](image) | ![Image of Non-infarct NID and MID](image) |

**B**

**Cristae Morphology**

- NFC
- DCM
- DCM

**Inclusion Bodies**

- NFC
- DCM
- DCM

**Circularity**

- NFC
- DCM
- DCM
Whole Cell Lysis

Figure S5
Figure S6

A. Membrane Fraction vs. Cytosolic Fraction

- TLR4: 95KDa
- CASP3: 32KDa

B. Cytosolic Fraction vs. Nuclear Fraction

- CASP3: 32KDa
- Histone H3: 17KDa

C. Comparison of TFR-1 (A.U.) between HF-NID and HF-MID

D. Comparison of DMT-1 (A.U.) between HF-NID and HF-MID

E. Comparison of FPN (A.U.) between HF-NID and HF-MID

MemCode
Figure S7

Transferrin Receptor 1

Cytosol

Membrane
Cytosol

Divalent Metal Transporter 1

Membrane

Figure S8
Figure S9
Figure S10

A

| NFC | DCM | Peri-Infarct | CAD | Non-Infarct |
|-----|-----|--------------|-----|-------------|
| NID | NID | MID          | NID | MID         |

**DAPI**

**WGA**

**TFR-1**

B

**DAPI**

**WGA**

**DMT-1**

C

**DAPI**

**WGA**

**FPN**

Figure S10
Supplemental Figure Legends

**Figure S1.** Lack of correlation between myocardial iron levels and systemic hemoglobin (A), ferritin (B), and serum iron (C) levels in HF cohorts, based on linear regression analysis. MID: myocardial iron deficiency, n=32; NID: no myocardial iron deficiency, n=106.

**Figure S2.** Representative wheat germ agglutinin (A) and picrosirius red staining (B) of normal myocardium (NFC); scale bar = 100 µm (WGA) or 200 µm (PSR). Masson’s trichrome staining (C) confirmed exacerbated interstitial fibrosis in the HF subgroups with MID; scale bar = 200 µm. **D-E** Transmission electron microscopy (TEM) images captured from NFC at different magnifications illustrating pristine and well-aligned sarcomeric and mitochondrial ultrastructure; scale bar = 1 µm (4000X) or 500 nm (10000X). S: sarcomere; M: mitochondrion; G: cytoplasmic granules; DCM: dilated cardiomyopathy; CAD: coronary artery disease; LVFW: left ventricle free wall.

**Figure S3.** Mitochondrial morphological alterations worsened by iron deficiency in failing explanted hearts. Representative TEM images illustrating worsened intra-mitochondrial ultrastructural derangements in iron-deficient failing hearts captured from samples with both DCM and CAD (A) with qualitative quantification shown from the DCM subgroups (B) demonstrating predominant changes in cristae structures and increased presence of inclusion bodies in the MID subgroup with occasional mitochondrial lysis. n=10 for NFC;
n=5 each for DCM-NID and DCM-MID groups. Scale bar = 500 nm. Mito: mitochondria; 
DCM: dilated cardiomyopathy; CAD-PI: peri-infarcted from coronary artery disease; 
CAD-NI: non-infarcted from coronary artery disease; NID: no myocardial iron deficiency; 
MID: myocardial iron deficiency; TEM: transmission electron microscopy. Arrows 
indicate inclusion bodies. *p<0.05, **p<0.01 compared with NFC; #p<0.05, ##p<0.01 
compared with HF-NID.

Figure S4. Enzymatic activities of antioxidant enzymes including catalase (A), glutathione 
peroxidase (GPX-1, B) in HF subgroups in comparison with non-failing controls (NFC). 
n=20 for NFC; n=12 for each HF subgroup. C. Enzymatic activity of electron transport 
chain complex III (COX III) in HF subgroups compared NFC. DCM: dilated 
cardiomyopathy; CAD-PI: peri-infarcted from coronary artery disease; CAD-NI: non- 
infarcted from coronary artery disease; NID: no myocardial iron deficiency; MID: 
myocardial iron deficiency. n=10 for NFC; n=6 for each HF subgroup.

Figure S5. Original immunoblots of iron transporters using whole cell lysates that 
correspond to the protein quantitation in Figure 5B. TFR-1: transferrin receptor 1; DMT- 
1: divalent metal transporter 1; FPN: ferroportin. Arrowheads indicated the probed proteins 
with targeted molecular weight (TFR-1: 100 KDa, DMT-1: 70-100 KDa, FPN: 62.5 KDa), 
whereas red rectangular boxes showed the part of MemCode that were analyzed for protein 
abundance normalization. The blots selected as representative in Figure 5B were outlined 
with a green border.
**Figure S6.** Western blot analysis demonstrating the validity of our tissue fractionation methods including membrane versus cytosolic (A), cytosolic versus nuclear (B) fractionations by established compartment-specific markers. C-E: Subcellular expression comparisons of TFR-1 (C), DMT-1 (D), and FPN (E) between HF-MID and HF-NID groups, respectively. TLR4: toll-like receptor 4; CASP3: caspase 3. MemCode represents the total protein loading on PVDF membrane. NID: no myocardial iron deficiency; MID: myocardial iron deficiency. n=6 for each HF subgroup.

**Figure S7.** Original immunoblots of cytosolic and membrane TFR-1 that correspond to the protein quantitation in Figure 5D. TFR-1: transferrin receptor 1. Arrowheads indicated the TFR-1 molecular weight across cytosolic (55 KDa) and membrane (100 KDa) fractions, respectively. Rectangular boxes showed the part of MemCode that were analyzed for abundance normalization, whereas red asterisks indicated those underwent additional membrane stripping and downstream protein redetection (i.e., DMT-1). The blots selected as representative in Figure 5C were outlined with a green border.

**Figure S8.** Original immunoblots of cytosolic and membrane DMT-1 that correspond to the protein quantitation in Figure 5G. DMT-1: divalent metal transporter 1. Arrowheads indicated DMT-1 molecular weight across cytosolic (55 KDa) and membrane (70-100 KDa) fractions. Rectangular boxes showed the part of MemCode that were analyzed for abundance normalization, whereas colored asterisks indicated those underwent additional
membrane stripping and downstream protein redetection: red asterisks represented the shared MemCode as total protein loading for both TFR-1 and DMT-1 quantification, while green asterisks for DMT-1 and FPN quantification. The blots selected as representative in Figure 5F were outlined with a green border.

**Figure S9.** Original immunoblots of cytosolic and membrane FPN that correspond to the protein quantitation in Figure 5J. FPN: ferroportin. Arrowheads demonstrated the FPN’s unaltered molecular weight in both cytosolic and membrane (62.5 KDa) fractions. Rectangular boxes showed the part of MemCode that were analyzed for abundance normalization, whereas green asterisks indicated those underwent additional membrane stripping and downstream protein redetection (i.e., DMT-1). The blots selected as representative in Figure 5I were outlined with a green border.

**Figure S10.** Original immunofluorescent images of separated channels that constitute the representative composites of individual iron transporter, including TFR-1 (A, red), DMT-1 (B, red), and FPN (C, green). TFR-1: transferrin receptor 1; DMT-1: divalent metal transporter 1; FPN: ferroportin.