ONLINE SUPPLEMENT

CARDIAC METABOLIC ALTERATIONS IN HYPERTENSIVE OBESE PIGS

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METHODS

1. Experimental protocol

The study was approved by the Institutional Animal Care and Use Committee. Littermate Ossabaw pigs (Swine Resource, Indiana University) were randomized to 4 groups (n=7 each): Lean-sham, Obese-sham, Lean-hypertension (HT), and Obese-HT. Lean and obese pigs were fed starting at the age of 3-months 16 weeks of standard chow or an high-fat/high fructose diet (5B4L; Purina Test Diet, Richmond, Indiana)[1], respectively. HT was induced by establishing unilateral renal artery stenosis at 12 weeks of diet.[2] Four weeks later, cardiac structure, function and oxygenation were studied with multi-detector computed-tomography (MDCT) and blood-oxygenation-level-dependent (BOLD)-magnetic resonance imaging (MRI), and blood samples collected. For each in vivo study animals were weighed, induced with Telazol and xylazine (5 mg/kg and 2 mg/kg, respectively, intramuscular injection) and intubated. For MDCT, animals were anesthetized by continuous intravenous infusion of Ketamine and ventilated with room air. During MRI, pigs were maintained anesthetized by inhalation of 2% isoflurane-containing oxygen.

Three days following the completion of in vivo studies, pigs were euthanized by intravenous sodium pentobarbital (100mg/kg, Fatal Plus, Vortech Pharmaceuticals, Fort Washington, PA).[3] Hearts were removed, preserved, and prepared for ex-vivo tissue studies.

2. Induction of renal artery stenosis and blood pressure measurement

Under fluoroscopic guidance, a percutaneous transluminal balloon 7.0 mm in diameter (Cordis, Miami, Fla) wrapped with a local-irritant coil was advanced through the femoral artery into the proximal middle section of the right renal artery. The balloon was inflated (6 atm) for 45 seconds, deflated, and removed, leaving the local-irritant copper coil embedded in the vascular wall.[4] The procedure elicits gradual increase of blood pressure within the following 10 days, accompanying progressive luminal narrowing.[5, 6] The degree of renal artery stenosis was determined by angiography 4 weeks later. A telemetry transducer (TA-D70, Data Sciences International, MN)[5] was implanted in the left femoral artery during renal artery stenosis induction[7, 8] to monitor and record systolic and diastolic blood pressure for the following 4 wks. Mean arterial pressure (MAP) was calculated using the formula: MAP=1/3 systolic pressure+2/3 diastolic pressure. MAP was averaged from the last 3 days prior to an in vivo experiment.

3. Systemic parameters

Blood samples collected during MDCT study were examined for total cholesterol, low-density lipoprotein, triglycerides and basal homeostasis model assessment insulin resistance. Plasma renin activity was assessed, and tumor necrosis factor-α and sE-selectin, and 8-epi-Isoprostane measured for systemic inflammatory and oxidative stress status.[1, 8, 9]

4. LV function and oxygenation

Cardiac function and structure were assessed in vivo using 64-slice MDCT (Somatom Definition-64, Siemens Medical Solution, Forchheim, Germany).[8, 10, 11] Two parallel 6-mm-thick mid-left ventricle (LV) levels were selected for evaluation of myocardial perfusion and LV function. A bolus injection of nonionic, low osmolar contrast medium (Isovue-370, 0.33 ml/Kg over 2 seconds) into the right atrium was followed by a 50-s flow study during respiratory suspension. After a 15-minutes interval, the same process was repeated during a 5-minute intravenous infusion of adenosine (400μg/kg/min).[8, 10, 12] LV myocardial perfusion was measured at both baseline and after adenosine infusion to assess microvascular function. Subsequently, the entire LV was scanned 20 times throughout the cardiac cycle to obtain parameters of cardiac function, including LV end diastolic volume, E/A ratio, stroke volume, and ejection fraction. LV muscle mass (LVMM) was acquired at the end-diastole by tracing the LV endocardial and epicardial borders. The rate pressure product (RPP; systolic blood pressure x heart rate) served as an index of oxygen demand. The images were analyzed with the Analyze™ software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN).[10]

To assess LV myocardial oxygenation, pigs were positioned in the MRI scanner (Signa EXCITE 3T system, GE, Waukeshau, WI) and BOLD images (4-5 axial-oblique) acquired along the cardiac short axis during suspended respiration.[10, 13] Gated Fast Gradient Echo sequence was used with TR/TE/number of echoes/Matrix size/FOV/Slice thickness/Flip angle=6.8 ms/1.6-4.8 ms/8/128x128/35/0.5 cm/30°. In each slice on T2*-weighted images obtained, the BOLD index, R2*, was estimated in each voxel by fitting the MR signal intensity vs. echo times to a single exponential function and calculating the MR intensity decay rate. Images were subsequently analyzed using MATLAB 7.10 (MathWorks, Natick, MA).

In addition, myocardial microvascular remodeling and function were evaluated by media/lumen area fraction by α-smooth muscle actin (SMA, abcam) staining and endothelial nitric oxide synthase (eNOS, abcam 1:1000) by western blotting, respectively.

5. Myocardial mitochondrial metabolic dynamics and activity

Protein expression of the mitochondrial biogenesis regulators peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α (abcam 1:1000) and its coactivator nuclear respiratory factor (NRF)-1 (abcam 1:500) were examined by western blotting. The mitochondrial inner membrane phospholipid cardiolipin was quantified for mitochondrial content by immunofluorescence staining. Mitochondrial DNA regulator mitochondrial transcription factor A (TFAM, abcam 1:1000) and mitochondrial DNA-encoded-NADPH dehydrogenase (ND)2 gene copy number (Life Technology) were examined by Western blotting and real time quantitative polymerase chain reaction (PCR), respectively. S18 ribosome DNA was used as reference. In addition, circulating plasma levels of ND1, ND2, and respiratory chain complex IV (COX) subunit 3 (all Life Technology) were measured to indicate mitochondrial component spillover secondary to cellular mitochondrial injury.[14] Their primers' sequences are as follow:
ND2 (CCWR2L5):

ND2_CCWR2L5_F ATATTATCAATTTTAATCGGAGGGTGAGGAG18
ND2_CCWR2L5_R TGTGCGATTGATGAGTATGCTATGA18

18S (CCT959P):
18S_CCT959P_F GCCCGAAGCGTTTACTTTGAA18
18S_CCT959P_R CATTATTCCTAGCTGCGGTATCCA18

ND1 (CCOW57):
ND1_CCOW57_F CAAGCCTAGCAGTCTACTCTATCCT
ND1_CCOW57_R GATTGTTTGGGCTACTGCTCGTA

COX3 (CCNIFF2):
COX3_ CCNIFF2_F GGAGCCCTATCAGCCCTTTTAATA
COX3_ CCNIFF2_R TTGTCAAAGTATTGGTTAATAGTCCTAGAGATAGT

Activities of mitochondrial respiratory chain complex IV subunits COX1 and 4 were determined by ELISA (both Abcam). Mitochondrial autophagy was assessed by protein expression of dynamin related protein-1 (Cell Signaling 1:1000) by western blotting, and co-localization of mitochondrial outer membrane marker Tom20 and Parkin (both Santa Cruz, 1:50, immunoflurosecent staining), the latter translocating from the cell plasma to the mitochondrial outer membrane, mediating mitophagy.[15, 16]

6. Myocardial oxidative stress and fibrosis

Dihydroethidium staining was performed to assess tissue oxidative stress. Fibrosis was examined by trichrome staining.

7. Adipose tissue assessment

Pericardial and abdominal fat volumes were quantified on MDCT images, as described previously.[10]

8. Statistical analysis

Results are expressed as mean±SEM. JMP software package version 9.0 (SAS Institute, Cary, NC) was used to perform two-way ANOVA to analyze the individual and interactive effects of factors diet and HT, followed by Tukey's test as appropriate. Significant factors are indicated following ♠; significance from post-hoc tests is indicated as *, $, and †. Paired Student's t-test was performed for comparisons within groups (myocardial perfusion response to adenosine). The link of mitochondrial biogenesis and mitophagy to myocardial fibrosis was detected by Pearson correlation analysis. Results were considered significant for $p<0.05$.

REFRENCES FOR ONLINE SUPPLEMENT

1. Li Z, Woollard JR, Wang S, Korsmo MJ, Ebrahimi B, Grande JP, Textor SC, Lerman A, Lerman LO. Increased glomerular filtration rate in early metabolic syndrome is associated with renal. adiposity and microvascular proliferation. *Am J Physiol Renal Physiol*. 2011;301:F1078-1087
2. Caceres VHU, Lin J, Zhu XY, Favreau FD, Gibson ME, Crane JA, Lerman A, Lerman LO. Early experimental hypertension preserves the myocardial microvasculature but aggravates cardiac injury distal to chronic coronary artery obstruction. *Am. J. Physiol.-Heart Circul. Physiol.* 2011;300:H693-H701.
3. Juillard L, Lerman LO, Kruger DG, Haas JA, Rucker BC, Polzin JA, Riederer SJ, Romero JC. Blood oxygen level-dependent measurement of acute intra-renal ischemia. *Kidney Int*. 2004;65:944-950.
4. Chade AR, Rodriguez-Porcel M, Grande JP, Krier JD, Lerman A, Romero JC, Napoli C, Lerman LO. Distinct renal injury in early atherosclerosis and renovascular disease. *Circulation*. 2002;106:1165-1171.
5. Lerman LO, Schwartz RS, Grande JP, Sheedy PF, Romero JC. Noninvasive evaluation of a novel swine model of renal artery stenosis. *J Am Soc Nephrol*. 1999;10:1455-1465.
6. Zhang X, Eirin A, Li Z-L, Crane JA, Krier JD, Ebrahimi B, Pawar AS, Zhu X-Y, Tang H, Jordan KL, Lerman A, Textor SC, Lerman LO. Angiotensin receptor blockade has protective effects on the poststenotic porcine kidney. *Kidney Int*. 2013;84:767-775.
7. Zhu XY, Rodriguez-Porcel M, Bentley MD, Chade AR, Sica V, Napoli C, Caplice N, Ritman EL, Lerman A, Lerman LO. Antioxidant intervention attenuates myocardial neovascularization in hypercholesterolemia. *Circulation*. 2004;109:2109-2115.
8. Zhu XY, Daghini E, Chade AR, Napoli C, Ritman EL, Lerman A, Lerman LO. Simvastatin prevents coronary microvascular remodeling in renovascular hypertensive pigs. *J Am Soc Nephrol*. 2007;18:1209-1217.
9. Galili O, Versari D, Sattler KJ, Olson ML, Mannheim D, McConnell JP, Chade AR, Lerman LO, Lerman A. Early experimental obesity is associated with coronary endothelial dysfunction and oxidative stress. *Am J Physiol Heart Circ Physiol*. 2007;292:H904-911.
10. Li ZL, Woollard JR, Ebrahimi B, Crane JA, Jordan KL, Lerman A, Wang SM, Lerman LO. Transition from obesity to metabolic syndrome is associated with altered myocardial autophagy and apoptosis. *Arterioscler Thromb Vasc Biol*. 2012;32:1132-1141.
11. Rodriguez-Porcel M, Herrman J, Chade AR, Krier JD, Breen JF, Lerman A, Lerman LO. Long-term antioxidant intervention improves myocardial microvascular function in experimental hypertension. *Hypertension*. 2004;43:493-498.
12. Urbieta Caceres VH, Lin J, Zhu XY, Favreau FD, Gibson ME, Crane JA, Lerman A, Lerman LO. Early experimental hypertension preserves the myocardial microvasculature but aggravates cardiac injury distal to chronic coronary artery obstruction. *Am J Physiol Heart Circ Physiol*. 2011;300:H693-701.

13. Warner L, Glockner JF, Woollard J, Textor SC, Romero JC, Lerman LO. Determinations of renal cortical and medullary oxygenation using blood oxygen level-dependent magnetic resonance imaging and selective diuretics. *Invest Radiol*. 2011;46:41-47.
14. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. Circulating mitochondrial damps cause inflammatory responses to injury. *Nature*. 2010;464:104-107.
15. Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RL, Kim J, May J, Tocilescu MA, Liu W, Ko HS, Magrane J, Moore DJ, Dawson VL, Grailhe R, Dawson TM, Li C, Tieu K, Przedborski S. Pink1-dependent recruitment of parkin to mitochondria in mitophagy. *Proc Natl Acad Sci U S A*. 2010;107:378-383.
16. Arena G, Gelmetti V, Torosantucci L, Vignone D, Lamorte G, De Rosa P, Cilia E, Jonas EA, Valente EM. Pink1 protects against cell death induced by mitochondrial depolarization, by phosphorylating bcl-xl and impairing its pro-apoptotic cleavage. *Cell Death Differ*. 2013;20:920-930.

Figure S1

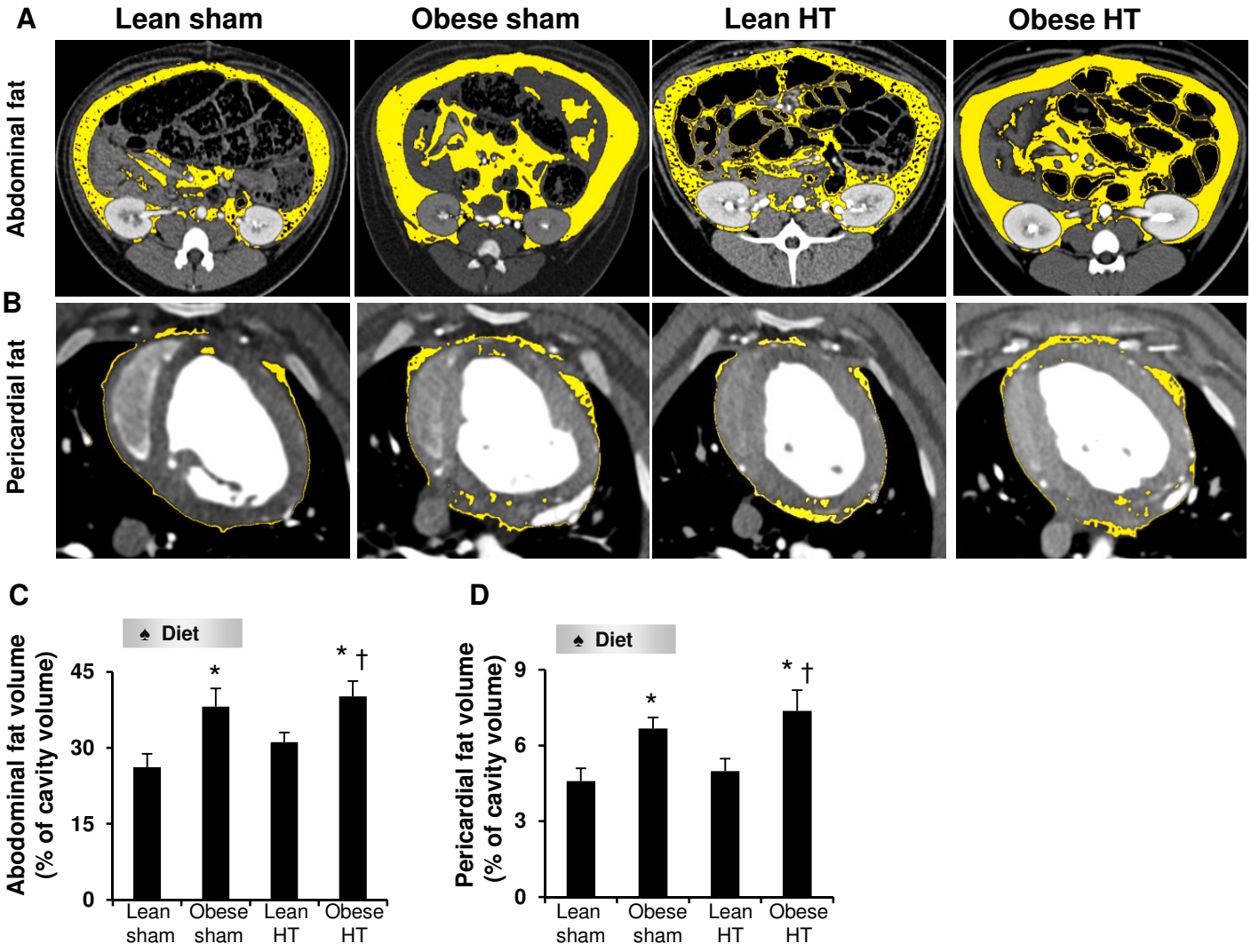
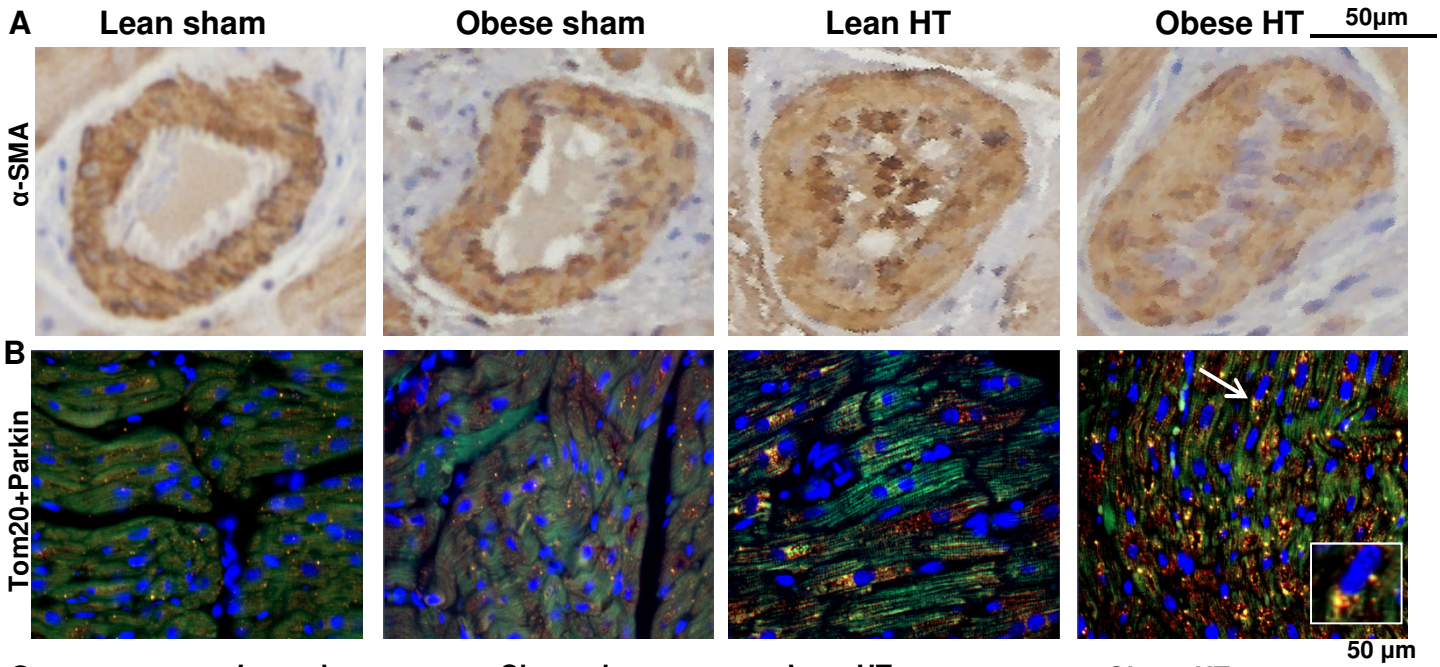
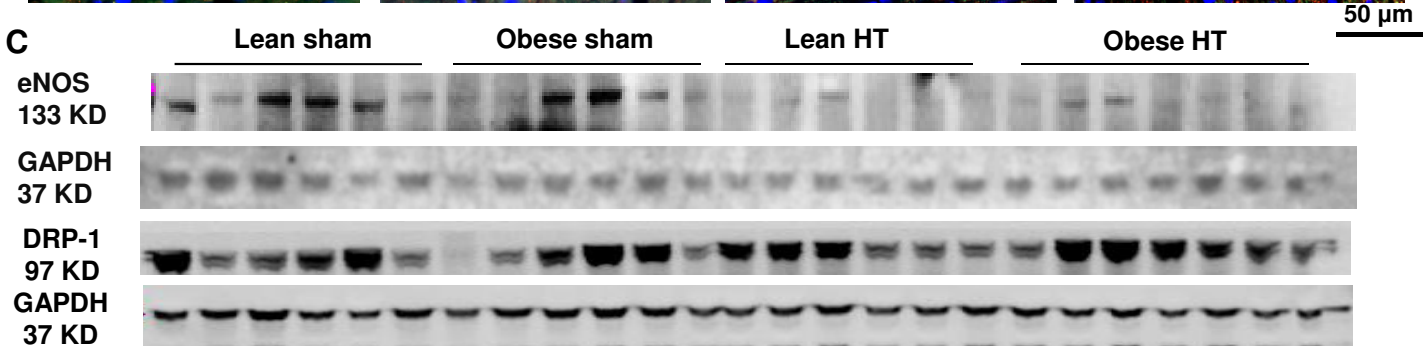


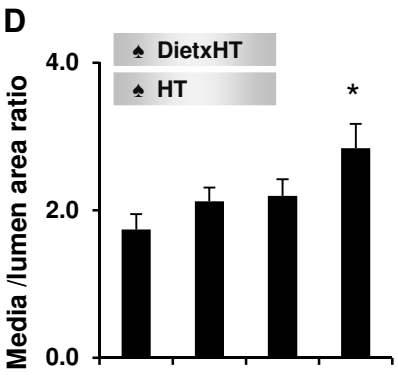
Figure S1. Representative cross-sectional multi-detector computed tomography images for abdominal (A) and pericardial (B) fat (both yellow) from Lean and Obese Ossabaw pigs with or without hypertension (HT), and their quantification (C-D). ♠ Diet: significant effect of diet (Two-way ANOVA). *p<0.05 vs. Lean-sham, †p<0.05 vs. Lean-HT.

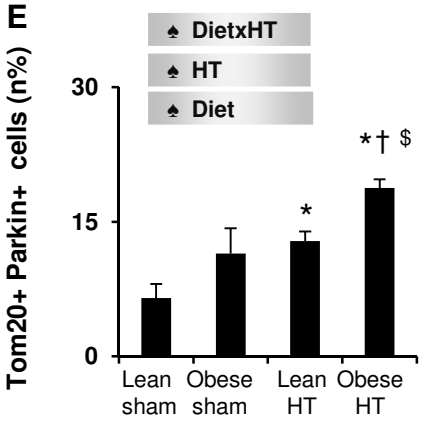
Figure S2. A, D: Representative images of microvascular α-smooth muscle actin (SMA) and media/lumen ratio. B, E: Representative images for co-localization of Tom20 with parkin and its quantification (Tom20 red, parkin green, co-localization orange, blue nuclei).
C, F-G: Expression of endothelial nitric oxide synthase (eNOS) and dynamin-related protein (DRP)-1. ♠ Diet: significant effect of diet. ♠ HT: significant effect of HT. ♠ DietxHT: significant interaction (Two-way ANOVA). *p<0.05 vs. Lean-sham, $p<0.05 vs. Obese-sham, †p<0.05 vs. Lean-HT.

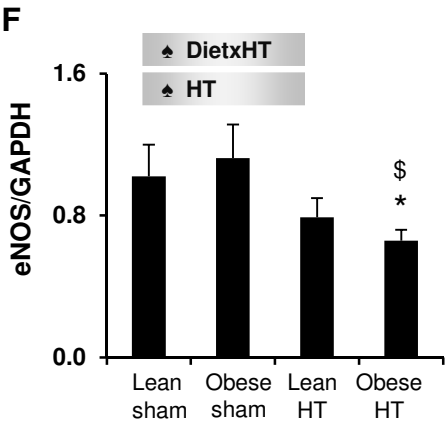
Figure S2


A
Lean sham
Obese sham
Lean HT
Obese HT
50µm
α-SMA
B
Tom20+Parkin
50 µm


C
Lean sham
Obese sham
Lean HT
Obese HT
eNOS
133 KD
GAPDH
37 KD
DRP-1
97 KD
GAPDH
37 KD


D
♠ DietxHT
♠ HT
Media /lumen area ratio
4.0
2.0
0.0
*


E
♠ DietxHT
♠ HT
♠ Diet
Tom20+ Parkin+ cells (n%)
30
15
0
*† $
*
Lean sham
Obese sham
Lean HT
Obese HT


F
♠ DietxHT
♠ HT
eNOS/GAPDH
1.6
0.8
0.0
$
*
Lean sham
Obese sham
Lean HT
Obese HT

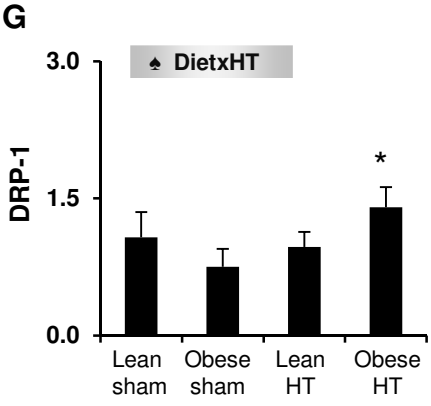

G
♠ DietxHT
DRP-1
3.0
1.5
0.0
*
Lean sham
Obese sham
Lean HT
Obese HT

Figure S3

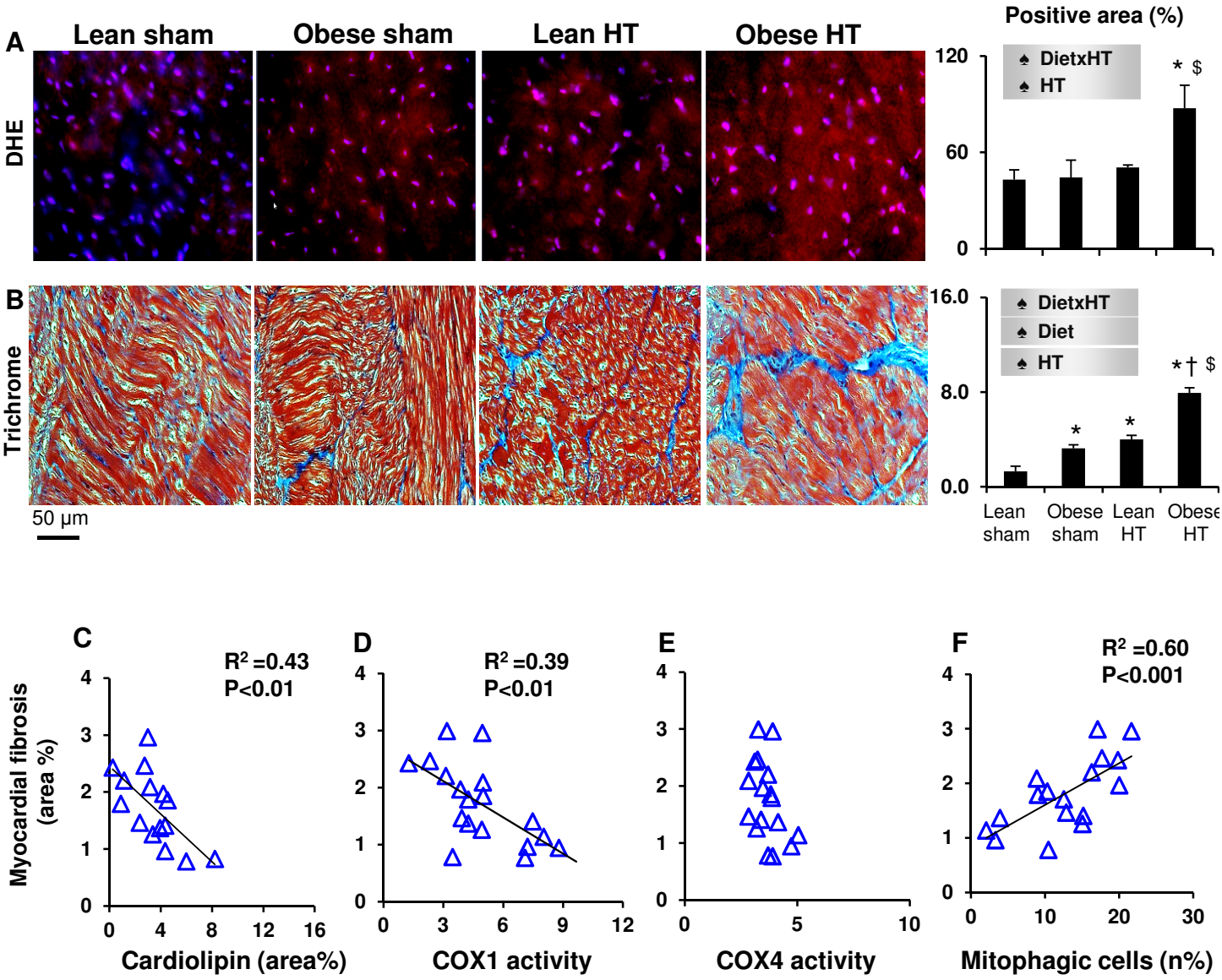


Figure S3. A: Representative images of dihydroethidium (DHE) staining and its quantification. B: Trichrome staining and quantification. C-F: Pearson correlation for association of myocardial fibrosis with mitochondrial content, respiratory chain complex IV enzyme activities, and extent of mitophagy. ♠ Diet: significant effect of diet. ♠ HT: significant effect of HT. ♠ DietxHT: significant interaction (Two-way ANOVA). *p<0.05 vs. Lean-sham, $p<0.05 vs. Obese-sham, †p<0.05 vs. Lean-HT.