Supplementary information for:

Integrated landscape of cardiac metabolism in end-stage human non-ischemic dilated cardiomyopathy

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Sample Procurement

Whole human hearts were explanted from either late-stage heart failure patients undergoing transplant at the Hospital of the University of Pennsylvania (HF) or from brain-dead organ donors from the Gift of Life organ donor program (NF). We received informed consent from transplant patients or next-of-kin of organ donors. Hearts were arrested *in situ* with ice cold, high-potassium cardioplegia (UW formula: 125 mmol/L K^+, 30 mmol/L Na^+, 5 mmol/L Mg^{2+}, 25 mmol/L phosphate, 5 mmol/L SO_4^{2-}, 100 mmol/L lactobionate, 30 mmol/L raffinose, 1 mmol/L allopurinol, 5 mmol/L adenosine, 3 mmol/L glutathione, 5% pentastarch), excised from the body, and stored at 4°C in Krebs-Henseleit Buffer (KHB). Samples were taken from the left ventricular free wall, flash frozen in liquid nitrogen, and stored at -80°C.

Blood samples were collected from donors and transplant patients before administration of Heparin and placed in collection tubes. Tubes were kept at 4°C and spun in a centrifuge at 3000 RPM for 15 minutes to achieve phase separation. The plasma layer was collected, placed into tubes with lithium heparin coating, flash frozen in liquid nitrogen, and stored at -80°C.

Cohort Selection

We performed our studies in two cohorts. The first cohort of n=18 end-stage failing and n=18 non-failing hearts was matched for age, gender, and race. Non-failing status was determined by ejection fraction (EF) $\geq$ 50% with no history of HF. All failing hearts were diagnosed as non-ischemic cardiomyopathy or dilated cardiomyopathy with no coronary artery disease (CAD) or sarcoidosis etiology. Patients with a history of diabetes or left ventricular assist device (LVAD) were excluded from both populations. The second cohort of 30 NF and 21 HF samples was chosen within the same criteria as the first, but without gender and race matching.

RNA-seq

RNA sequencing libraries were prepared using the Illumina TruSeq stranded mRNA kit followed by the Nugen Ovation amplification kit. To avoid confounding by batch effects, libraries were randomly selected into pools of 32, and pools were sequenced on a Hiseq2500 to a depth of ~30 million 100-bp paired-end reads per biological sample. Fastq files were aligned against human reference (hg19/hGRC37) using the STAR aligner. Duplicate reads were removed using MarkDuplicates from Picard tools, and per gene read counts for Ensembl (v75) gene annotations were computed. Expression levels in counts per million (CPM) were normalized and transformed using the VOOM procedure in the LIMMA R package. Surrogate variables to account sources of latent variation such as batch were calculated using the svaseq function from the SVA package. The data are publicly available in NCBI GEO repository with accession GSE14190.

Metabolite extraction

Serum (5 µL) was mixed with 150 µL -20°C 40:40:20 methanol:acetonitrile:water (extraction solvent), vortexed, and immediately centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was collected for LC-MS analysis. To extract metabolites from heart samples, frozen hearts were ground at liquid nitrogen temperature with a Cryomill (Retsch, Newtown, PA). The resulting tissue powder (~20 mg) was weighed and then extracted by adding -20°C extraction solvent (as above), vortexed, and immediately centrifuged at 16,000 x g for 10 min at 4°C. The volume of the extraction solution (µL) was 40x the weight of tissue (mg) to make an extract of 25 mg tissue per mL solvent. The supernatant was collected for LC-MS analysis.

Metabolite measurement by LC-MS

A quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA) operating in negative or positive ion mode was coupled to hydrophilic interaction chromatography via electrospray ionization and used to scan from m/z 70 to 1000 at 1 Hz and 140,000 resolution. LC separation was on a XBridge BEH Amide column (2.1 mm x 150 mm, 2.5 µm particle size, 130 Å pore size; Waters, Milford, MA) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water: acetonitrile, pH 9.45) and solvent B (acetonitrile). The flow rate was 150 µL/min. The LC gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min, 85% B; 30 min, 85% B. Autosampler temperature was 5°C, and injection volume was 3 µL. Data were analyzed using the MAVEN software.

Lipid measurement by LC-MS

Serum (5 µL) and heart tissue powder (~20 mg) were mixed with -20°C isopropanol (extraction solvent), vortexed, and immediately centrifuged at 16,000 x g for 10 min at 4°C. The volume of the extraction solution (µL) was 30x the volume of serum and 40x the weight of tissue (mg), respectively. The supernatant was collected for LC-MS analysis.
A quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA) operating in positive ion mode was coupled to via electrospray ionization and used to scan from m/z 290 to 1200 at 1 Hz and 140,000 resolution. LC separation was on a Atlantis T3 Column (2.1 mm x 150 mm, 3 µm particle size, 100 Å pore size; Waters, Milford, MA) using a gradient of solvent A (1 mM ammonium acetate, 35 mM acetic acid in 90:10 water: methanol) and solvent B (1 mM ammonium acetate, 35 mM acetic acid in 98:2 isopropanol: methanol). Flow rate was 150 µL/min. The LC gradient was: 0 min, 25% B; 2 min, 25% B; 5.5 min, 65% B; 12.5 min, 100% B; 16.5 min, 100% B; 17 min, 25% B; 30 min, 25% B. Autosampler temperature was 4°C, and injection volume was 3 µL. Data were analyzed using the MAVEN software.

Acyl-CoA extraction and analysis

Acyl-CoAs were measured by liquid chromatography- high resolution mass spectrometry as previously described[61]. Briefly, aliquots of pre-weighed tissue (range 5.9-45.2, average 19.7

mg) were spiked with 0.1 mL of $^{13}C_3{}^{15}N_1$-acyl-CoA internal standard prepared as previously described from yeast[62] and 0.9 mL of 10% (w/v) trichloroacetic acid in water. Samples were homogenized by using a probe tip sonicator in 0.5 second pulses 30 times then centrifuged at 17,000 x g for 10 min at 4°C. Calibration curves were prepared from commercially available acyl-CoA standards (Sigma Aldrich). Calibration curve samples were also subjected to sonication and extraction in the same manner as the experimental samples to account for matrix effects, sample losses, and analyte stability. Samples and standards were purified by solid phase extraction (SPE) cartridges (Oasis HLB 10 mg) that were conditioned with 1 mL of methanol and 1 mL of water. Acid-extracted supernatants were loaded onto the cartridges and washed with 1 mL of water. Acyl-CoA metabolites were eluted with 1 mL of 25 mM ammonium acetate in methanol and dried under nitrogen. Samples were resuspended in 50 µL of 5% (w/v) 5-Sulfosalicyilic acid and 10 µL injections were analyzed on an Ultimate 3000 UHPLC using a Waters HSS T3 2.1x100mm 3.5 µm column coupled to a Q Exactive Plus. The analysts were blinded to sample identity during processing and quantification.

Data normalization and analysis

Normalization and analysis were done using R v 3.6.0 and Microsoft Excel v 2103. The two cohorts of metabolomics data were normalized as separate batches. Within each batch, positive and negative mode data was also normalized separately. First, statistical outlier data points were removed from each metabolite. Outliers were determined using the 'boxplot()' function in R. Next, metabolites for which count values were below 10,000 for any sample were removed from analysis. Weak signals (e.g. < 10,000 counts) indicate low-integrity data that could confound analysis. Finally, data was normalized to median value within each sample. Then, each data point for a metabolite was averaged to the average non-failing value of that metabolite.

After separate normalization, positive and negative modes from both cohorts were combined to form one data set. Fold change of each metabolite was calculated as average failing value divided by average non-failing value. Metabolites reaching a two-tailed t-test p-value below 0.05 (calculated in Excel using the 't.test' parametric function) were considered significantly different between failing and non-failing samples.

PCA plots were generated using the 'ggfortify' package in R.
Polar plots were generated using the 'tidyverse' and 'plotrix' packages in R.
Violin and bar graphs were generated in GraphPad Prism 8.3.0 and 9.1.1.
Sample procurement figure was generated using BioRender.
Volcano plots were generated in Excel.
Similarity matrices were generated using Morpheus.

Western blots and molecular assays

Protein for western blots was extracted from frozen left ventricular powdered tissue as described previously[63]. Thiourea (TU) buffer (8M urea, 2M thiourea, 3% SDS, 75 mM

dithiothreitol, and 50 mM Tris pH 7.5; also requires mixed-bed resin - BioRad: cat #1426425) was added to tissue (40uL TU/mg tissue) and mixed via pipette to rapidly thaw tissue. Mixture was transferred to a small glass tissue homogenizer and homogenized. 40 volumes of glycerol buffer (1:1 glycerol/water + 2x protease inhibitor (complete miniproteinase inhibitor cocktail, Roche)) was added, and a few additional strokes were performed. Lysates were incubated at 37°C for 30 minutes and spun in a centrifuge for 5 minutes at 10°C and 20kg speed. The supernatant was stored at -80°C until use.

Protein lysates were loaded into a 4% to 20% gradient Tris-glycine polyacrylamide gel (Bio-Rad) and electrophoresed. Samples were transferred to PVDF membrane (MilliporeSigma) and incubated for 15 minutes with Ponceau S solution (Sigma P7170) to expose total protein. The membrane was washed twice with ddH2O for five minutes each and imaged. Protein for each well was quantified using Fiji imaging software, and relative values were used to normalize protein loading for future blots. Subsequent westerns were performed as described above, including ponceau staining and imaging. Membranes were then blocked with 5% in TBS-T for 30 minutes and incubated overnight in primary antibodies at 4°C. The next day, membranes were washed with TBS-T and incubated with species-appropriate HRP-conjugated secondary antibodies in 5% milk in TBS-T for one hour. Membranes were imaged using the ImageQuant LAS 4000 (GE Healthcare Life Sciences) and quantified using fiji software. Blots were normalized to total protein via ponceau stain.

The following primary antibodies were used: Acsl1 (Cell Signaling Technology 4047S), PDK4 (Abcam ab 110336), AKR1B1 (Thermo Fisher 15439-1-AP), ME1 (Abcam ab97445), ME3 (Abcam ab172972), HK1 (Cell Signaling Technology 2024S), HK2 (Cell Signaling Technology 2867S), MPC1 (Cell Signaling Technology 14462S), MPC2 (Cell Signaling Technology 46141S), total PDH (Cell Signaling Technology 3205S), phospho-PDH E1 alpha S293 (Abcam ab92696), GLUT1 (Abcam ab 115730), GLUT4 (Abcam ab33780), total AKT (Cell Signaling Technology 4961), phospho-AKT (Cell Signaling Technology 4058S), total S6 (Cell Signaling Technology 2217S), phopsho-S6 Ser235/236 (Cell Signaling Technology 2211).
The following secondary antibodies were used: Anti-mouse IgG HRP-linked (Cell Signaling Technology 7076S), Anti-Rabbit IgG HRP-linked (Cell Signaling Technology 7074S). All primary antibodies were used at a dilution of 1:1000, and all secondary antibodies were used at a dilution of 1:10000.

PDH activity was determined using a colorometric assay kit (Millipore Sigma MAK183) as described in the kit protocol using frozen left ventricular tissue.

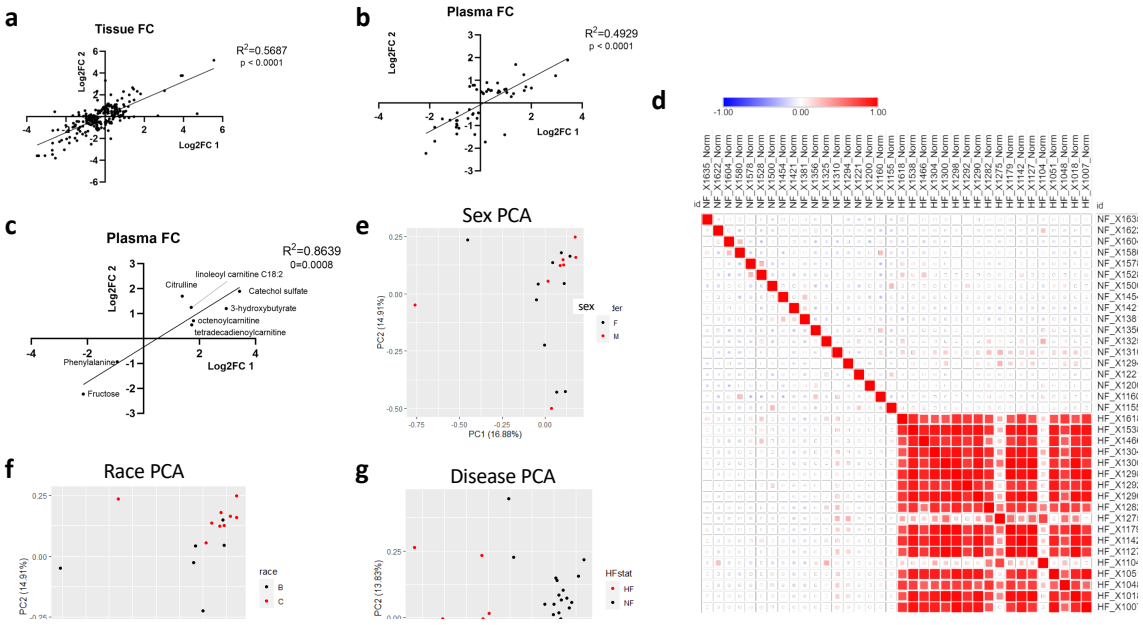
Data Availability

The analyzed metabolomics data are available in the supplementary information files. The raw metabolomics data generated in this study are available from the corresponding author upon reasonable request. The RNA-seq data are publicly available in NCBI GEO repository with accession GSE14190. The proteomics data are available in the ProteomeXchange Consortium the dataset identifier PXD008934.

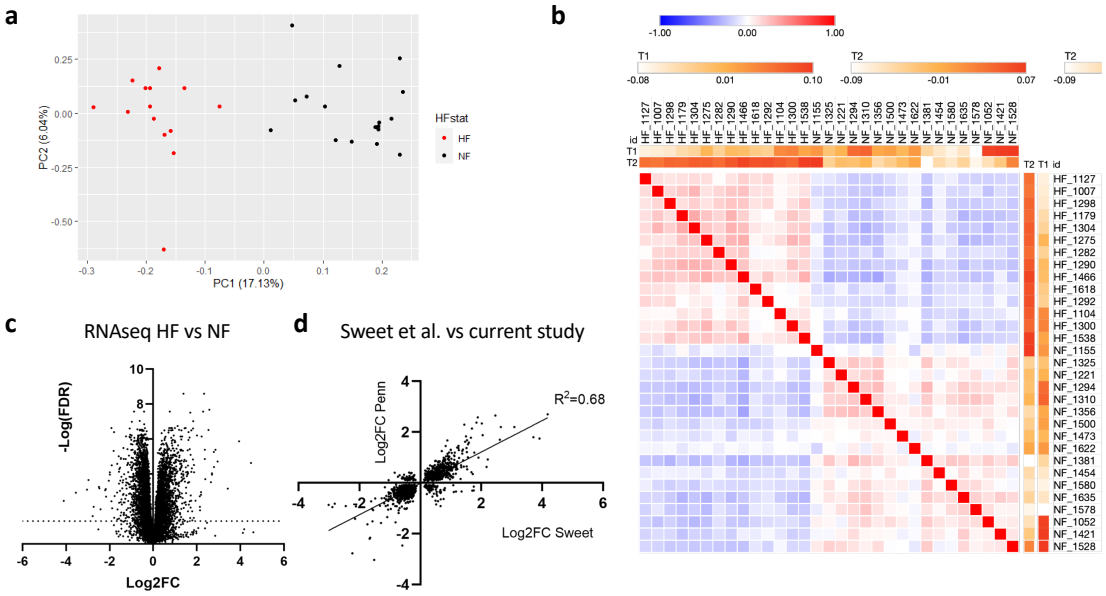
Table S1

	Cohort 1 NF (N=18)	Cohort 1 DCM (N=18)	Cohort 1 p-val	Cohort 2 NF (N=30)	Cohort 2 DCM (N=21)	Cohort 2 p-val	Combined NF (N=48)	Combined DCM (N=39)	Combined p-val
Age (yrs)	51.0 ± 9.7	52.1 ± 10.9	0.76	56.3 ± 11.5	50.9 ± 11.6	0.11	54.3 ± 11.1	51.5 ± 11.3	0.2
Male (%)	8(44)	10(55)		15(48)	12(57)		23(48)	22(56)	
Caucasian (%)	10(56)	9(50)		27(87)	14(67)		37(77)	23(59)	
Weight (kg)	81.8 ± 19.4	80.8 ± 15.0	0.87	82.0 ± 20.0	82.0 ± 19.0	1.0	81.9 ± 19.8	81.5 ± 17.3	0.9
Height (cm)	167.1 ± 9.3	173.3 ± 10.3	0.071	170.9 ± 10.0	169.7 ± 9.3	0.7	169.5 ± 9.9	171.4 ± 10.0	0.4
BMI (kg/m^2)	29.2 ± 6.1	26.8 ± 3.6	0.17	28.2 ± 7.5	28.2 ± 5.0	1.0	28.6 ± 7.0	27.6 ± 4.5	0.4
Heart Weight (g)	377.3 ± 80.8	475.9 ± 106.1	0.0044	365.5 ± 98.0	475.7 ± 139.9	0.009	369.9 ± 91.2	466.1 ± 125.8	0.0001
LV Mass (g)	216.2 ± 46.5	301.8 ± 81.4	0.0072	216.7 ± 51.7	297.6 ± 97.2	0.00044	216.6 ± 50.1	298.8 ± 93.1	8.1E-06
LVMI	107.5 ± 15.6	154.3 ± 43.8	0.0026	110.2 ± 19.8	150.9 ± 39.6	1.9E-05	109.4 ± 18.6	151.8 ± 40.8	9.8E-08
LVEF (%)	65.7 ± 8.2	10.5 ± 3.7	1.15E-23	64.3 ± 7.0	15.9 ± 6.1	1E-29	64.8 ± 7.5	13.4 ± 5.8	5E-52
LVEDD (cm)	4.3 ± 0.2	7.6 ± 1.3	0.0015	4.2 ± 0.6	7.0 ± 1.1	2E-12	4.2 ± 0.6	7.2 ± 1.2	4E-16
LVESD (cm)	2.4 ± 0.4	7.0 ± 1.2	4.5E-05	2.7 ± 0.8	6.4 ± 1.2	2E-14	2.7 ± 0.5	6.6 ± 1.3	1E-20
PW Thickness (cm)	1.1 ± 0.2	0.86 ± 0.2	0.10	1.0 ± 0.2	0.9 ± 0.2	0.01	1.0 ± 0.2	0.9 ± 0.2	0.001
h/o VT/VF (%)	0(0)	11(61)		0(0)	14(67)		0(0)	25(64)	
h/o ChrAfib (%)	0(0)	4(22)		4(13)	15(71)		4(9)	19(49)	
h/o Htn (%)	8(44)	4(22)		14(45)	20(95)		22(46)	24(62)	
Creatinine	1.9 ± 2.3	1.5 ± 0.8	0.60	1.5 ± 1.2	1.0 ± 0.2	0.09	1.6 ± 1.7	1.2 ± 0.5	0.1

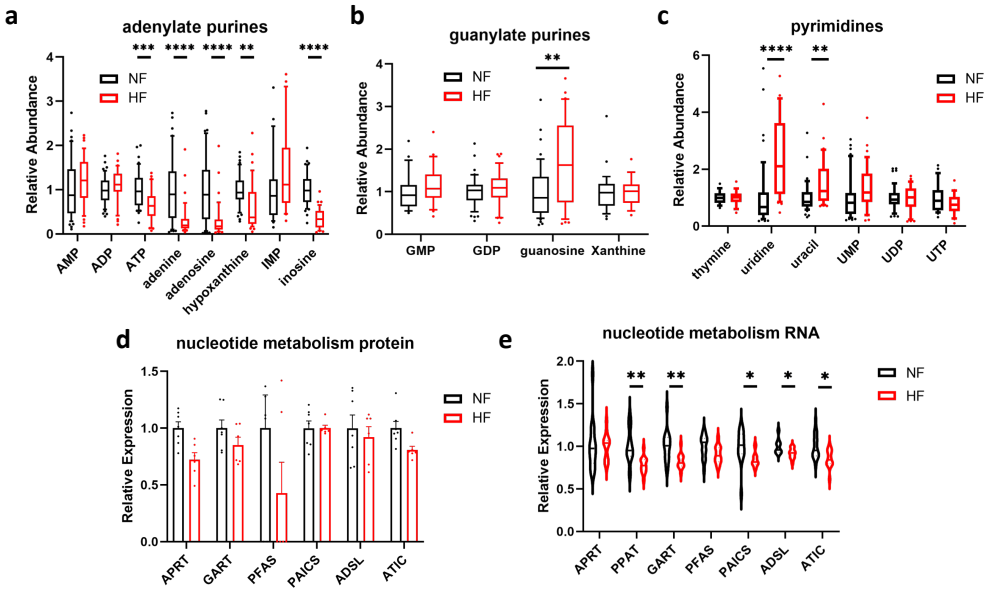
Extended Data Table1: Patient demographics and clinical information for cohort 1 (left), cohort 2 (middle) and combined cohorts (right). P-values determined by two-sided t-test between combined cohort failing and non-failing samples. BMI = body mass index, LV = left ventricle, LVMI = left ventricular mass index, LVEF = left ventricular ejection fraction, LVEDD = left ventricular end-diastolic diameter, LVESD = left ventricular end-systolic diameter, PW = posterior wall, h/o = history of, VT/VF = ventricular tachycardia/ventricular fibrillation, ChrAfib = chronic atrial fibrillation, Htn = hypertension. +/- standard deviations.



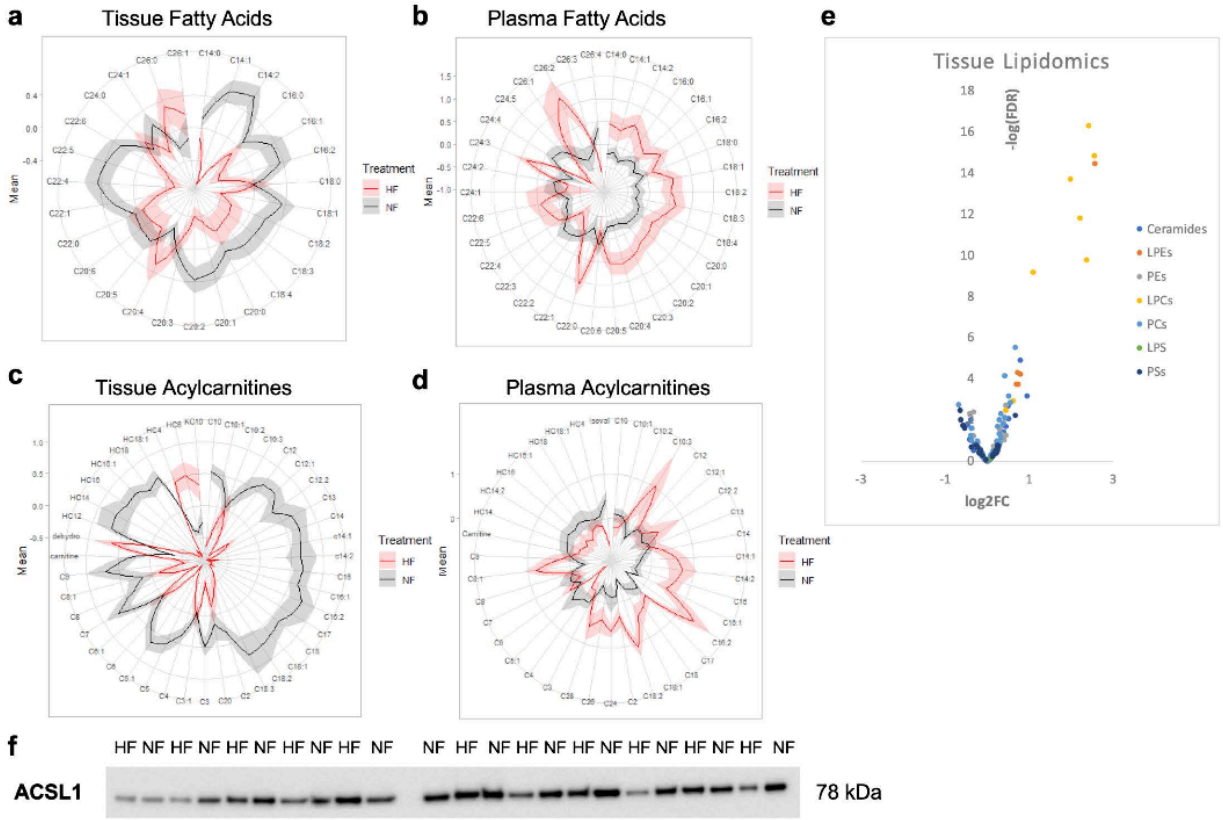
Extended Data Figure 1: a-b) Correlation between cohort 1 and 2 tissue (**a**) or plasma (**b**) of fold-changes (FC) between non-failing and failing samples of metabolites significantly altered (FDR < 0.05) in at least one cohort. One outlier was removed from analysis in figure **b**. **c**) Correlation between cohorts 1 and 2 of plasma fold-change of metabolites significantly altered in both cohorts (FDR <0.05). **d**) Principal component analysis (PCA) of non-failing tissue samples from cohort 1. Data points represent patients and are pseudo-colored to reflect sex of donor. **e**) PCA of non-failing tissue samples from cohort 1. Data points represent patients and are pseudo-colored to reflect race of donor. **f**) PCA plot of all tissue samples from cohort 1. Data points represent patients and are pseudo-colored to reflect heart failure (HF) vs nonfailing (NF). **g**) Similarity matrix of samples from cohort 1 based on metabolomics data. Size of square is proportional to Pearson correlation coefficient.



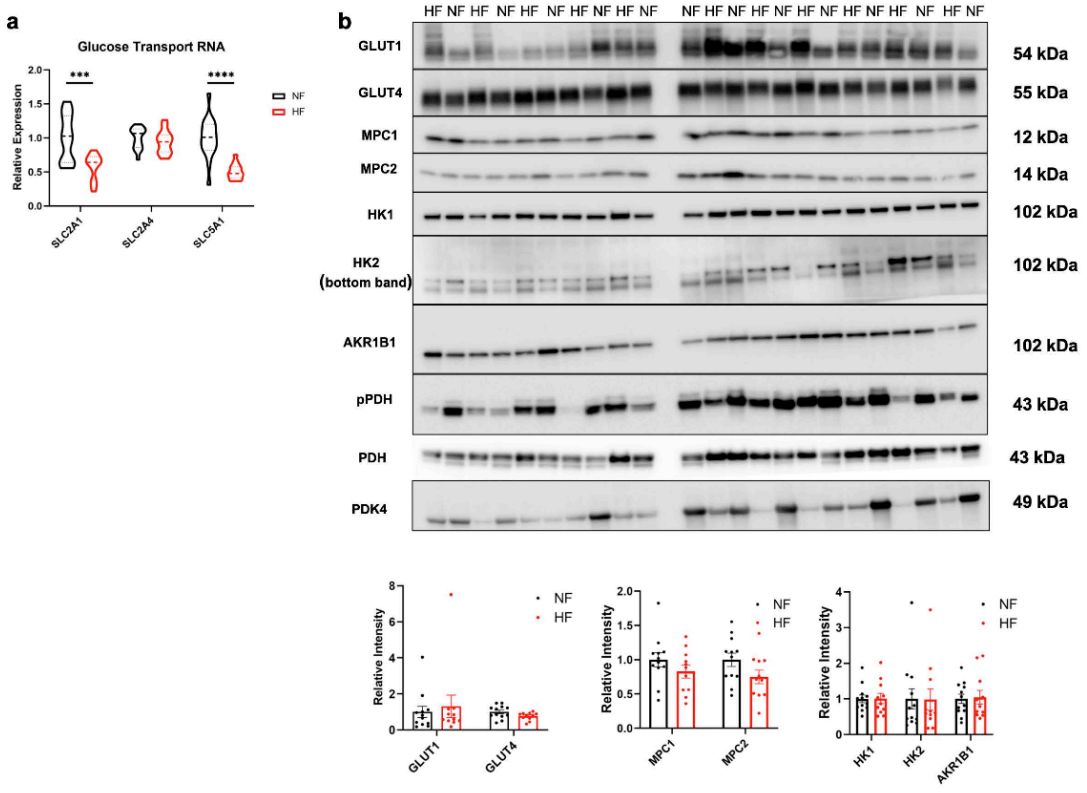
Extended Data Figure 2: a) PCA plot of mRNA expression from all tissue samples. Data points represent patients and are pseudo-colored to reflect heart failure (HF) vs nonfailing (NF). **b**) Similarity matrix of RNA-seq data between individual samples. **c**) Volcano plot of RNA-seq data from tissue samples. **d**) Correlation between the current cohort (combined 1 and 2) and a previously published data set (Sweet et al.) of significant fold-changes (FC; nominal p-val < 0.05 by two-sided t-test) in mRNA expression between non-failing vs failing cardiac samples.



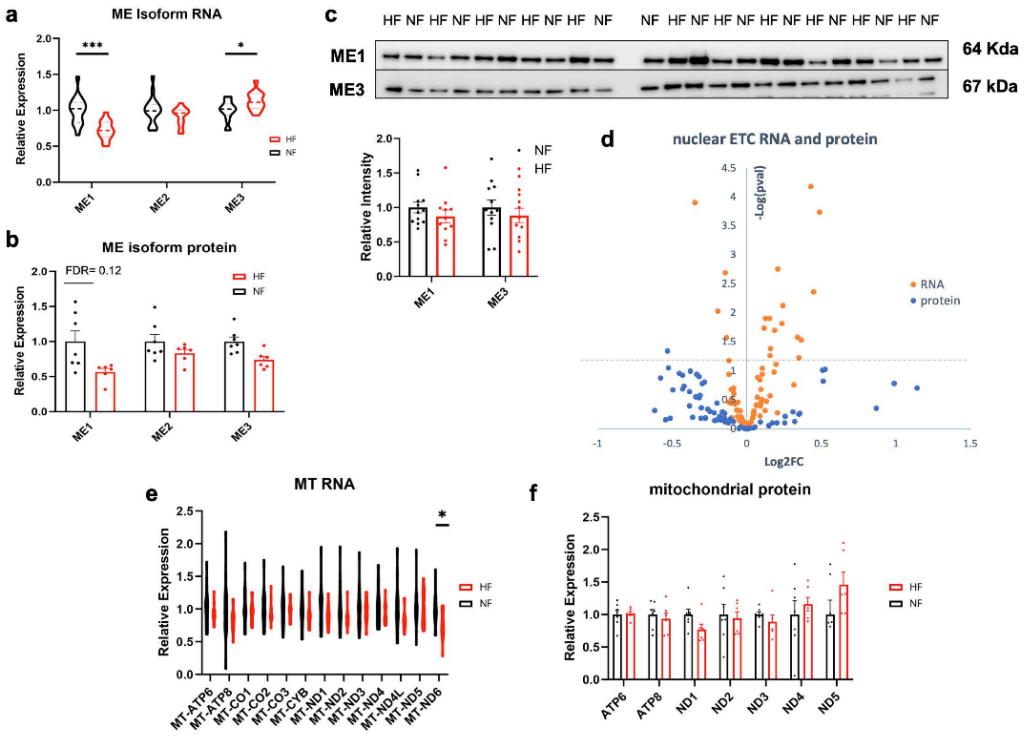
Extended Data Figure 3: a-c) Relative abundance of metabolites involved in adenylate (**a**), guanylate (**b**), or pyrimidine (**c**) metabolism in cardiac tissue from nonfailing donors (NF) or subjects with heart failure (HF). Whiskers represent 10th and 90th percentiles, midline represents median, edges of boxes represent first and third quartiles, and points represent data points outside the 10th-90th percentile range. N = 48 NF and N = 39 HF samples. ATP FDR = 0.000904; adenine FDR = 1.09E-05; adenosine FDR = 1.4E-05; hypoxanthine FDR = 0.00126; inosine FDR = 3.02E-13; guanosine FDR = 0.00227; uracil FDR = 0.00202; uridine FDR = 1.09E-05. **d-e**) Relative protein (**d**) and mRNA (**e**) expression of enzymes involved in nucleotide metabolism. Bars represent mean and standard error (N = 7 NF and N = 6 HF). RNA: PPAT FDR = 0.00425; GART FDR = 0.00967; PAICS FDR = 0.0292; ADSL FDR = 0.0204; ATIC FDR = 0.0144. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. P-values were determined by FDR-corrected two-tailed t-test.



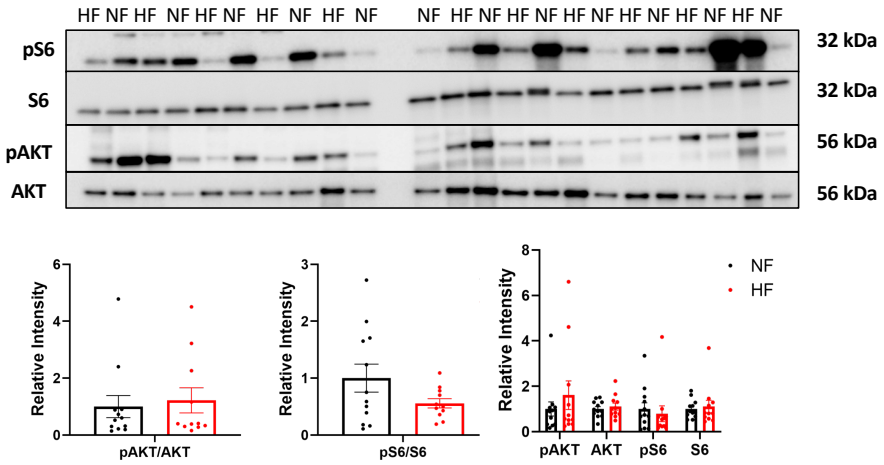
Extended Data Figure 4: a-b) Polar plots showing average relative abundance of tissue (**a**) and plasma (**b**) fatty acids between failing and non-failing subjects. Fold-change increases with distance from the origin, and shaded extended borders indicate standard error. **c-d**) Polar plots showing average relative abundance of carnitine species in tissue (**c**) and plasma (**d**) between failing and non-failing subjects. **e**) Volcano plots of differences in metabolite abundance in cardiac tissue after lipid extraction. **f**) Full western blot of ACSL1 protein (see Fig 3 for quantification).



Extended Data Figure 5: a) Relative mRNA expression of glucose transport genes. SLC2A1 FDR = 0.00196; SLC51A FDR = 5.88E-05. **b**) Western blots and quantification of various proteins from failing and non-failing tissues. Bars represent mean and standard error (N = 12 NF and N = 11 HF). ***P<0.001, ****P<0.0001. P-values were determined by FDR-corrected two-tailed t-test.



Extended Data Figure 6: a-b) Relative mRNA (**a**) and protein (**b**) expression of malic enzyme isoforms. RNA: ME1 FDR = 0.000973; ME3 FDR = 0.0324. Bars represent mean and standard error (N = 7 NF and N = 6 HF). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. P-values were determined by FDR-corrected two-tailed t-test. **c**) Western blots and quantification of ME1 and ME3 protein in failing and non-failing tissue. Bars represent mean and standard error (N = 12 NF and N = 11 HF). **d**) Volcano plot of differences in nuclear-encoded mRNAs (orange) and proteins (blue) composing the electron transport chain in cardiac tissue, comparing non-failing to failing samples. Y-axis represents FDR-corrected two-sided t-test between failing and non-failing samples. **e-f**) Relative expression of RNA (**e**) and protein (**f**) encoded by the mitochondrial genome. Bars represent mean and standard error (N = 7 NF and N = 6 HF). RNA: MT-ND6 FDR = 0.0296.



Extended Data Figure 7: a) Western blots and quantification below of mTOR-related proteins from failing and non-failing tissues. Bars represent mean and standard error (N = 12 NF and N = 11 HF).